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The two step biosynthesis of cyclic peptides from linear precursors in a member of the plant family Caryophyllaceae involves cyclization by a serine protease-like enzyme.

Carla J.S. Barber1, Pareshkumar T. Pujara1, Darwin W. Reed1, Shiela Chiwocha1, Haixia Zhang1 and Patrick S. Covello1*

1From the National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK, Canada S7N 0W9

*Running Title: Caryophyllaceae cyclic peptide biosynthesis

To whom correspondence should be addressed: Patrick Covello, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK Canada S7N 0W9; Tel.: (306) 975-5269; Fax: (306) 975-4839; E-mail: patrick.covello@nrc-cnrc.gc.ca

*The cDNA sequence reported in this paper for Pcy1 has been submitted to the Genbank™/EBI Data Bank with accession number KC588970; nucleotide sequences for two Sanger sequencing-based EST collections from S. vaccaria referenced in this paper have been submitted to Genbank™/EBI Data Bank with accession numbers LIBEST_028081 and LIBEST_028082; nucleotide sequences for two additional S. vaccaria EST collections have been submitted to the short read archive at NCBI with accession numbers SRX202186 and SRX202889.

Keywords: Peptidases; Peptide biosynthesis; Plant biochemistry; Plant molecular biology; Protease; Caryophyllaceae; Saponaria vaccaria; cyclic peptide; peptide cyclase; orbitide

Background: In the Caryophyllaceae, cyclic peptides (CP) are biosynthesized from linear precursors via an unknown pathway. Two protease-like enzymes are involved in precursor processing. A serine protease-like enzyme was recruited for the cyclization step in CP biosynthesis. This represents a very significant advance in our understanding of the mode and evolution of CP biosynthesis in plants.

SUMMARY

Caryophyllaceae-type cyclic peptides (CP) of 5-12 proteinogenic amino acids occur in ten plant families. In Saponaria vaccaria (Caryophyllaceae), these have been shown to be formed from linear peptide precursors derived from ribosomal translation. There is also evidence for such precursors in others members of the Caryophyllaceae, Rutaceae and Linaceae. The biosynthesis of CP in the developing seeds of S. vaccaria was investigated with respect to the enzymes involved in precursor processing. Through biochemical assays with seed extracts and synthetic peptides, an enzyme activity named Oligopeptidase 1 (OLP1) was found which catalyzes the cleavage of intermediates at the N-terminus of the incipient CP. A second enzyme, Peptide Cyclase 1 (PCY1), which was separated chromatographically from OLP1 was found to act on the product of OLP1 giving rise to a cyclic peptide and concomitant removal of a C-terminal flanking sequence. PCY1 was partially purified and, using the methods of proteomics, a full length cDNA clone encoding an enzyme matching the properties of PCY1 was obtained. The substrate specificity of purified recombinant PCY1, believed to be the first cloned plant enzyme whose function is peptide cyclization, was tested with synthetic peptides. The results are discussed in the light of CP biosynthetic systems of other organisms.

In 1959, Kaufmann and Tobschirbel (1) reported the isolation of a cyclic nonapeptide, cyclolinopeptide A, from flax (Linum...
usitatissimum) oil with the formula cyclo[ILVPPFFLI]. This is the first of the class which came to be called Caryophyllaceae-type CP. CP have been discovered in a wide range of taxa, including eubacteria, fungi, plants and animals (2-8). Recently, the name orbitide has been suggested for the Caryophyllaceae-type CP (9). Many CP are known to have interesting biological activities (4,10). For example segetalin F has been reported to have vasorelaxant activity (11). CP can be formed with a variety of chemical linkages beyond the peptide bond. In plants, however, CP tend to be homodetic, in the sense that at least one ring of amino acids involves peptide bond linkages exclusively. There is also a tendency in plants for CP to include only proteinogenic amino acids, or simple derivatives of them. The four major classes of CP in plants include the Caryophyllaceae (orbitide), kalata, PawS and knottin types (9). These differ in typical size, and degree of disulfide bridging, for example.

Given the physiological roles and technological and pharmaceutical interest in CP (12-14), understanding the natural and engineered processes for CP production is currently an active area of research. For many CP in eubacteria and fungi, their biosynthesis involves the linkage and modification of amino acids and other compounds with the help of non-ribosomal peptide synthetases (15,16). More recently, it has become clear that in cyanobacteria, fungi and especially in plants, CP biosynthesis can occur via the processing of ribosomally-produced precursors (17-26). Indeed, for the case of plant CP, in essentially all cases for which there is sufficient evidence, ribosome-derived precursors are involved in CP biosynthesis. As well, there are examples from cyanobacteria and fungi in which CP are formed from ribosome-derived precursors (24,26).

The Caryophyllaceae-type CP occur in members of the Caryophyllaceae, Linaceae, Rutaceae and seven other plant families. They typically consist of 5 to 12 proteinogenic amino acids in a single ring formed from peptide bonds. In some cases, nonproteinogenic amino acids are included in the structure. Within the Caryophyllaceae, Saponaria vaccaria (syn. Vaccaria hispanica) has been investigated and 8 CP called segetalins (A to H) have been isolated (10,27). More recently, evidence for two additional segetalins (J and K) has been reported (17).

It appears that these segetalins are biosynthesized via precursors of ~35 amino acids which have conserved N- and C-terminal flanking regions and a variable region representing the sequence of the mature segetalin (see Fig. 1) (17). Expression of cDNAs encoding the presegetalins in transformed roots of S. vaccaria gives rise to CP. In this paper, we describe efforts to understand the process by which precursors of Caryophyllaceae-type CP, such as presegetalins, are processed to CP. In an important advance in our understanding of CP biosynthesis, a combination of partial enzyme purification, mass spectrometry and bioinformatics has led to the elucidation of a biosynthetic process involving two peptidase-like enzymes and the molecular cloning and preliminary characterization of the enzyme which catalyzes the cyclization step.

**EXPERIMENTAL PROCEDURES**

**Chemicals**-Presegetalin A1 (purity ≥ 75%; see Table 1) and presegetalin A1[14,32] (purity > 75%) were chemically synthesized at the Sheldon Biotechnology Centre, McGill University, Montreal, PQ, Canada. The presegetalin A1 was further purified by HPLC fractionation with a C18 column using a water/acetonitrile gradient (with trifluoroacetic acid as a modifier). All other linear peptides (purity, >90%; see Table 1) were chemically synthesized at Bio Basic Canada Inc. (Markham, ON, Canada). Segetalin A isolated from S. vaccaria seed by the method of Morita (28) was obtained from John Balsevich (National Research Council of Canada, Saskatoon, Canada).

**Plant material and RNA isolation**-Seeds of S. vaccaria ‘White Beauty’ were obtained from CN Seeds Ltd (United Kingdom). Plants were grown under greenhouse conditions with a daily regime of 12 h light and 12 h dark at 23°C. S. vaccaria developing seeds at stages 1 and 2 were harvested according to the following scheme: Stage 1, seed white, pod green; Stage 2, seed tan, pod green; Stage 3, seed copper, pod partially dessicated; Stage 4, seed dark brown, pod dessicated.

For total RNA isolation from S. vaccaria developing seeds, the protocol of Gambino et al. (29) was modified. For the rapid CTAB-based procedure, 0.6 mL of extraction buffer, containing...
2% cetyltrimethylammonium bromide (CTAB), 2.5% polyvinylpyrrolidone (Mző = 40,000), 2 M NaCl, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA and 2% of β-mercaptoethanol (added just before use), was heated to 65 °C in a microcentrifuge tube. One hundred and fifty mg of developing seeds were ground in liquid nitrogen and added to the extraction buffer and the tube was incubated at 65 C for 10 min. The sample was extracted two times with chloroform/isoamyl alcohol (24:1 v/v) and 0.25 volumes of 3 M LiCl was added. The mixture was kept on ice for 30 min and centrifuged at 20,000 g for 20 min at 4 °C. The pellet was dissolved in 0.5 mL of SSTE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 1 M NaCl), extracted with 0.5 mL of chloroform/isoamyl alcohol (24:1, v/v), followed by precipitation with isopropanol.

cDNA sequencing—A collection of S. vaccaria developing seed ESTs based on Roche 454 sequencing technology was developed as follows. First-strand cDNA was synthesized from S. vaccaria Stage 1 developing seed total RNA using the Omniscript® Reverse Transcription Kit (Qiagen, Mississauga, Canada) according to the manufacturer’s instructions. ESTs were generated from cDNA prepared from the isolated RNA using Roche (Indianapolis, IN, USA) GS-FLX Titanium Technology at the McGill and Genome Quebec Innovation Centre (Montreal, Canada) according to the manufacturer’s instructions. Within the MAGPIE software system (30), sequences were assembled using MIRA (31) and contigs were annotated based on BLASTX searches of Genbank.

Protein assay—For enzyme assays and purification, protein was measured using a modified micro BCA protein assay (Pierce, http://www.piercenet.com). Ten µL samples were mixed with 100 µL BCA Working Reagent and incubated at 60 °C for 30 min and after which optical density at 562 nm was recorded. Oligopeptidase 1 (OLP1) assay—The cleavage of presegetalin A1 (Table 1) by plant extracts was tested using the assay conditions reported by Condie et al. (17). The formation of presegetalin A1[14,32] was determined by ion trap LC/MS (see below). Peptide Cyclase 1 (PCY1) assay of plant extracts—The formation of segetalin A [cyclo(GVPVWA)] and linear segetalin A (GVPVWA) from presegetalin A1[14,32] (Table 1) by plant extracts was tested as described by Condie et al. (17) except that 15 µg/ml presegetalin A1[14,32] was used as the substrate.

Assay of recombinant PCY1—Unless otherwise noted, the activity of recombinant PCY1 was determined in an optimized assay as follows. The assay contained 20 mM Tris (pH 8.5), 100 mM NaCl, 5 mM DTT, 0.2 mg BSA, and 15 µg/ml of presegetalin A1[14,32] or other synthetic peptides. The reaction was initiated by the addition of 0.3 µg of recombinant PCY1, in a total reaction volume of 100 µL. The assay was incubated at 30°C for up to 1 h and stopped by the addition of 0.9 ml of methanol. Samples were centrifuged and the supernatants were evaporated and resuspended in 50% (v/v) methanol in water. The samples were then analyzed by ion trap LC/MS (see below). Controls were performed with either omission of enzyme or stopping the reaction at 0 h. Where necessary, 0 h peak areas were subtract from 1 h peak areas for the linear product.

Enzyme Purification—Eight grams of Stage 2 developing seeds from S. vaccaria (var. White Beauty) were homogenized manually with a plastic pestle in 1.5 mL low protein binding microcentrifuge tubes. The seeds were ground for 2 min in 20 aliquots of 500 µL 20 mM Tris buffer (pH 8) on ice followed by centrifugation at 13,000 g for 10 min. The supernatants were removed and additional 250 µL aliquots of buffer were added and the grinding and centrifugation was repeated. The supernatant fractions were pooled to a total volume of 17 ml, and filtered (0.2 µm 25 mm cellulose acetate membrane, VWR International, Mississauga, Canada). The resulting crude filtrate was used for enzyme assays and chromatographic purification.

Three separate applications of 5 mL each of the crude filtrate (see above) were made to an anion exchange column (Mono Q 10/100, GE Healthcare Life Sciences, Mississauga, Canada) connected to an Agilent 1100 series HPLC equipped with an auto injector, diode array detector and fraction collector cooled to 4°C. The column was held at 4 °C and pre-equilibrated with 20 mM Tris-HCl (pH 8.0). The column was eluted with 180 mL of a linear gradient of NaCl (0-1 M) in 20 mM Tris-HCl (pH 8.0) at a flow rate of 3 mL/min. Six mL fractions were collected, desalted with Sephadex G-25 M PD-10 columns (GE Healthc
Healthcare Life Sciences, Mississauga, Canada) and then concentrated to approximately 200 µL in Amicon Ultra centrifugal filters (Ultracel 30K cellulose, 30 MWCO; Millipore, Bellerica, MA, USA). These fractions were assayed for both OLP1 as well as PCY1 activities. Fractions containing PCY1 activity were combined and concentrated to 100 µL and applied to a Superose™ 6 10/300 Gel Filtration column (GE Healthcare Life Sciences, Mississauga, Canada) pre-equilibrated with 3 M ammonium sulfate in 20 mM Tris-HCl (pH 8.0) and applied to a hydrophobic interaction perfusion chromatography column (PerSeptive POROS® 20 HP2, Bio-Rad Laboratories Ltd, Mississauga, Canada) pre-equilibrated with 3 M ammonium sulfate in 20 mM Tris-HCl (pH 8.0). The column was eluted with a 60 mL decreasing linear gradient of ammonium sulfate (3-0 M in 20 mM Tris-HCl, pH 8.0) at a flow rate of 4 mL/min. Four mL fractions were collected, desalted with Sephadex G-25 M PD-10 columns and then concentrated to approximately 200 µL in Amicon Ultra centrifugal filters and assayed for PCY1 activity. Fractions containing PCY1 activity were combined and concentrated to 100 µL and applied to a Superose™ 6 10/300 Gel Filtration column (GE Healthcare Life Sciences, Mississauga, Canada) which had been pre-equilibrated with 20 mM Tris-HCl, 100 mM NaCl (pH 8.0). Proteins were eluted with 20 mM Tris-HCl, 100 mM NaCl (pH 8.0) over 30 ml at a flow rate of 0.2 mL/min. One mL fractions were collected and concentrated to ~200 µL with Amicon® Ultracel-10K membrane centrifugal filter units (Millipore, Bellerica, MA, USA) and assayed for PCY1 activity.

The retention times of standard proteins [thyroglobulin (M_r = 669,000), ferritin (M_r = 440,000), catalase (M_r = 232,000), aldolase (M_r = 158,000), BSA (M_r = 67,000), ovalbumin (M_r = 43,000), chymotrypsinogen (M_r = 25,000) and ribonuclease A (M_r = 14,000); GE Healthcare Life Sciences, Mississauga, Canada] were measured in a separate chromatography experiment under identical conditions. The size exclusion chromatography indicated that the relative molecular mass of the peptide cyclase was approximately 90,000 (data not shown).

Ion trap LC/MS-For ion trap LC/MS analysis of enzyme assays, an Agilent 6320 Ion Trap LC/MS system was used under default Smart Parameter settings. The analyzer and ion optics were adjusted to achieve optimal resolution (Agilent Installation Guide #G2440-90105) using the ESI Tuning Mix (Agilent #G2431A). The mass spectrometer was scanned in the m/z range of 50 to 2200 at 8100 mass units/s with an expected peak width of ≤0.35 mass units. For automated MS/MS, the trap isolation width was 4 atomic mass units. The associated Agilent 1200 LC was fitted with a Zorbax 300 EXTEND-C18 column (150 X 2.1 mm, 3.5 µm particle size) maintained at 35 C. The binary solvent system consisted of 90:10 v/v water/acetonitrile containing 0.1% formic acid and 0.1% ammonium formate (solvent A) and 10:90 v/v water/acetonitrile containing 0.1% formic acid and 0.1% ammonium formate (solvent B). The separation gradient was 90:10 A/B to 50:50 A/B in 3 mL over 20 min. The detection of segetalin A in assay samples is described previously (17).

MALDI-TOF MS-MALDI-TOF MS of enzyme assays was performed on samples which were purified by adsorption onto and elution from C18 Empore High Performance Disk material (3M, Minneapolis, MN, USA) using the “Stage tip” method (32). Stage tips were prepared by removing the beveled tip from a 20 gauge syringe needle with a tubing cutter. Empore disk material was then cut, cookie cutter style, with this needle and packed into the narrow end of a 10 µL disposable pipette tip with a piece of fused silica tubing. Methanol (10 µL) was applied to the tip and expelled slowly with a 1.25 mL syringe. Aqueous trifluoroacetic acid (TFA; 0.1%) was then passed through the tip, followed by assay sample (20 µL). The disk material was washed with 20 µL 0.1% TFA and peptides were then eluted with 20 µL acetonitrile and peptides were then lyophilized on a vacuum concentrator. Peptide samples were then eluted with 20 µL acetonitrile:aqueous 0.1% TFA (1:1). Analysis of the peptides was carried out using an AB Sciex 4800 MALDI TOF-TOF™ Analyzer (Applied Biosystems, LLC., Frederick, MD, USA). The mass spectrometer was operated in positive ion reflectron mode scanning m/z values from 500 to 4000. The default calibration was updated with a standard mixture of peptides containing des-Arg^1 bradykinin (m/z 904.468), Gu^1 fibrinopeptide B (m/z 1570.677), and three ACTH fragments corresponding to amino acids 1-17 (m/z 2093.087), 18-39 (m/z 2465.199), and 7-38 (m/z 3657.929). All samples and calibrants (0.5 µL) were mixed on the MALDI plate with the matrix α-cyano-4-hydroxycinnamic acid (0.5 µL). Data were collected and averaged from 800 laser irradiation events. Monoisotopic mass lists were...
generated with Data Explorer (Applied Biosystems) and copied into the BioLynx program in MassLynx 4.0 (Waters). Matches to subsequences of presegetalin A1 were investigated using the Find Mass program with an allowed mass deviation of 0.5 Da. Masses within 0.2 Da were considered to be matching.

Q-TOF LC/MS-Q-TOF LC/MS analysis of tryptic peptides was performed using a Quadrupole Time-Of-Flight (Q-TOF) Global Ultima mass spectrometer (Micromass, Manchester, UK) equipped with a nano-electrospray (ESI) source and a nanoACQUITY UPLC solvent delivery system (Waters, Milford, MA, USA). The mobile phase was composed from a binary solvent system of C, 0.2% formic acid and 3% acetonitrile and D, 0.2% formic acid and 95% acetonitrile. Peptides were desalted with an in-line solid-phase trap column (180 µm x 20 mm) packed with 5 µm resin (Symmetry C18, Waters) and separated on a capillary column (100 µm x 100 mm, Waters) packed with BEH130 C18 resin (1.7 µm, Waters) using a column temperature of 35 °C. Five µL samples were introduced into the trap column at a flow rate of 15 µL/min for 3 min, using C:D 99:1 and flow was diverted to waste. After desalting, the flow was routed through the trap column to the analytical column with a linear gradient of 1-10% solvent D (400 nL/min, 16 min), followed by a linear gradient of 10-45% solvent D (400 nL/min, 30 min). Unless otherwise stated, Q-TOF parameter settings consisted of a capillary voltage of 3,850 V, a cone voltage of 120 V and a source temperature of 80 °C.

Samples were analyzed using Data Dependant Acquisition (DDA), which consisted of the detection of multiply charged positive ions (z = 2-4) from an MS survey scan. The scan range was from m/z values of 400 to 1900, with a scan time of 1 s. Up to three MS/MS scans were triggered (collision energy ranged from 20 to 80 eV, depending on charge state and precursor m/z) from each MS scan event with a peak detection window of 4 m/z units (signal intensity threshold was 16 counts/s). In MS/MS experiments, data was acquired in continuum mode with a scan time of 1.9 s and dynamic exclusion of previously detected precursors was set at 2 min. Peptide signals corresponding to trypsin and keratin were also excluded from MS/MS data collection. To obtain high mass accuracy, the reference compound leucine enkephalin (80 nM in 1:1 acetonitrile:0.2% aqueous formic acid, Environmental Resource Associates, Arvada, CO, USA; m/z = 556.2771) was continuously introduced to a second ESI source and used for the mass calibration.

Data was processed with ProteinLynx Global Server 2.4 (PLGS 2.4, Waters) using RAW files from Q-TOF LC/MS. The resulting PKL files were submitted to MASCOT (version 2.3.02; Matrix Science Ltd., London, UK) for peptide searches against the NCBI nr database (version 011110) hosted by National Research Council of Canada and local databases containing the sequence information from Sanger (33) and 454 (this work) sequencing of S. vaccaria developing seed cDNA. MASCOT search parameters allowed for a maximum of 1 miscleavage for tryptic digestion and a mass tolerance for precursor peptide ions of ±50 ppm and for fragment ions up to ±0.2 Da. Carbamidomethylation of cysteine was selected as a fixed modification and oxidation of methionine was used as a variable modification.

Isolation of a full-length Pcy1 cDNA clone from S. vaccaria-Fractions which catalyzed the formation of segetalin A from presegetalin A1[14,32] from the various stages of chromatography were mixed 1:1 with SDS-PAGE Laemmli sample buffer (200 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 200 mM dithiothreitol, 40% glycerol) and heated at 99 °C for 5 min. The samples were subjected to SDS-PAGE under denaturing conditions using Electrophoresis Buffer (25 mM Tris-HCl, pH 7.5 , 250 mM glycine, 0.1% SDS) and a 10% Ready Gel® pre-cast polyacrylamide mini-gel (Bio-Rad Laboratories Ltd, Mississauga Canada). Precision Plus Protein™ molecular weight standards (Bio-Rad) were loaded on the same gel. The gel was stained with Oriole™ Fluorescent Gel Stain (Bio-Rad Laboratories Ltd, Mississauga Canada) for 15 h. Protein bands were visualized by UV illumination and the most prominent bands, corresponding to the last chromatographic step, were excised from the gel and digested with trypsin using the MassPrep II Proteomics Workstation (Micromass, UK) following a procedure described previously (34). Tryptic peptides were analyzed by Q-TOF LC/MS and the resulting data was used for MASCOT searches of sequence databases (see above).
A DNA plasmid clone containing the full length open reading frame of contig c272 (from Roche 454 sequencing; NCBI accession SRX202186), identified by MASCOT searches as representing a peptide cyclase candidate cDNA, was obtained as follows. First-strand cDNA was synthesized from S. vaccaria developing seed Stage 1 total RNA using the Omniscript® Reverse Transcription Kit (Qiagen, Mississauga, Canada) according to the manufacturer’s instructions. The contig c272 ORF was amplified by PCR (denaturation at 95°C for 4 min; 35 cycles of 95°C for 20 s, annealing at 54°C for 30 s and extension at 72°C for 2.3 min; followed by 10 min at 72°C) using forward (ATG GCG ACT TCA GGA TCG) and reverse (TCA GTC TAT CCA AGG AGC TTC AAG C) oligonucleotide primers (named pCB006) containing the contig c272 ORF was identified by colony PCR and DNA sequencing. The corresponding gene was named Pcy1. To allow E. coli expression of a N-terminal His₆-tagged version of PCY1, the PCY1 ORF of pCB006 was recombined with the Gateway expression vector pDEST17 (Invitrogen-Life Technologies, Mississauga, Canada) using spectinomycin for selection, a plasmid clone (named pCB008) containing the contig c272 ORF was identified by colony PCR and DNA sequencing. The resulting PCR product was gel-purified and recombined with pCR8/GW/TOPO® using a TA Cloning® Kit (Invitrogen, Life Technologies, Mississauga, Canada). After transformation of ONE SHOT ®TOP 10 competent E. coli cells (Invitrogen, Life Technologies, Mississauga, Canada) using spectinomycin for selection, a plasmid clone (named pCB008) containing the contig c272 ORF was amplified by PCR (denaturation at 95°C for 4 min; 35 cycles of 95°C for 20 s, annealing at 54°C for 30 s and extension at 72°C for 2.3 min; followed by 10 min at 72°C) using forward (ATG GCG ACT TCA GGA TCG) and reverse (TCA GTC TAT CCA AGG AGC TTC AAG C) oligonucleotide primers and Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Life Technologies, Mississauga, Canada). The resulting PCR product was gel-purified and recombined with pCR8/GW/TOPO® using a TA Cloning® Kit (Invitrogen, Life Technologies, Mississauga, Canada). After transformation of ONE SHOT ®TOP 10 competent E. coli cells (Invitrogen, Life Technologies, Mississauga, Canada) using spectinomycin for selection, a plasmid clone (named pCB008) containing the contig c272 ORF was identified by colony PCR and DNA sequencing. The corresponding gene was named Pcy1. To allow E. coli expression of a N-terminal His₆-tagged version of PCY1, the PCY1 ORF of pCB008 was recombined with the Gateway expression vector pDEST17 (Invitrogen-Life Technologies, Carlsbad, CA, USA) and used to transform competent E. coli BL21-AI™. The resulting plasmid and E. coli strain were called pCB008 and pCB008/BL21-AI, respectively.

E. coli Expression and Purification of PCY1-An overnight 1 mL LB culture of pCB008/BL21-AI was used to inoculate 100 mL of Overnight Autoinduction Medium (35) which was incubated at 37°C with shaking until an OD₆₀₀ of 0.4 was reached. Arabinose was then added to the a concentration of 0.2% (w/v) and culture growth was continued at 16°C with agitation overnight. The cultures were centrifuged at 2,000 g at 4°C for 10 min and the resulting cell pellets were frozen at -20°C. The pellets were resuspended in chilled 500 µL of B-Per ®Bacterial Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA), then transferred to two 1.5 mL Eppendorf tubes for cell lysis at room temperature for 20 min. Lysis was promoted with ultrasonication. The lysates were then centrifuged (12,000 g, 4°C; 8 min) and the supernatants were mixed with an equal volumes of resin binding Equilibration/Wash Buffer and added to 250 µL HisPur™ Cobalt Resin (Pierce Biotechnology, Rockford, IL, USA) for purification of PCY1 by batch adsorption and elution at 150 mM and 300 mM imidazole, respectively, according to the manufacturer’s recommendations. Each eluate was concentrated to 150 µL and desalted by spin dialysis (Amicon Ultra–15 devices; Millipore, Bellerica, MA) following the manufacturer’s protocol and subsequently assayed for peptide cyclase activity. The purity of recombinant PCY1 was judged by SDS-PAGE to be ~90%.

RESULTS

Detection of separate oligopeptidase and peptide cyclase activities involved in cyclic peptide biosynthesis – In previous work (17), it was shown that extracts of the developing seeds of S. vaccaria were capable of catalyzing the conversion of the 32 amino acid linear precursor, presegetalin A1 to the six-membered CP, segetalin A (see Fig. 1, overall reaction). In a further effort to understand the enzyme or enzymes involved in cyclic peptide formation in the Caryophyllaceae, S. vaccaria seed extracts were subjected to separation by ion exchange chromatography. The products of assays (see Experimental Procedures, Fig. 2 and (17)) using presegetalin A1 as a substrate were then analyzed by MALDI-TOF MS (see Experimental Procedures and Fig. 3). Assay of fractions eluting at ~0.35M NaCl (see Fig. 2) showed significant loss of substrate and formation of prominent MS peaks corresponding to peptide masses of 1434.8 and 1984.0 which, in turn, correspond to linear peptides with the sequences MSPILAHDVVKPQ and GVPVWAFQAKDVENASAPV, respectively (see Fig. 3). Daughter ion analysis of the peaks was consistent with the above cleavage products (Fig. S0). This suggests that cleavage of the QG peptide bond in presegetalin A1 is an important reaction in the biosynthesis of segetalin A. The formation of MSPILAHDVVKPQ and GVPVWAFQAKDVENASAPV from presegetalin A1 was confirmed by ion trap LC/MS (Fig. 4a and b). Thus, the data are consistent with a peptide
with the sequence GVPVWAFQAKDVENASAPV (called presegetalin A1[14,32]) being an intermediate in segetalin A biosynthesis. Consequently, the pathway from presegetalin A1 to segetalin A shown in Fig. 1 is hypothesized. Presegetalin A1 is suggested to be cleaved initially after position 13, giving rise to presegetalin A1[1,13] and presegetalin A1[14,32]. The latter is then processed, giving rise to segetalin A.

Given the above results, fractions obtained from ion exchange chromatography were tested with synthetic presegetalin A1[14,32] and indeed, specific fractions were found to catalyze the formation of segetalin A, as well as the linear form of segetalin A (Fig. 1 is hypothesized. Presegetalin A1 is suggested to be cleaved initially after position 13, giving rise to presegetalin A1[1,13] and presegetalin A1[14,32]). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d). This activity was distinguishable from that giving rise to segetalin A (Fig. 1, 2 and 4c and d).
catalytic triad which shows excellent three dimensional alignment with those of porcine muscle POP (see Fig. 6). The putative catalytic triad faces the β-propeller domain. S562 occurs within the sequence GGSNGG, which is conserved among γ/β hydrolases and likely forms a ‘nucleophilic elbow’ (43). Despite the alignment of catalytic amino acids, there is a notable single amino acid insertion/deletion near H695 (Figure S2).

Recombinant PCY1 shows peptide cyclase activity – Given that serine peptidases have a well documented propensity to catalyze transpeptidation and cyclization reactions (37,38) and the similarity of PCY1 to oligopeptidases, recombinant PCY1 was investigated as a candidate for involvement in CP biosynthesis in S. vaccaria. For functional characterization of PCY1, the recombinant enzyme was purified from E. coli cells harbouring the plasmid pCB008 which consists of the Pcy1 ORF fused with an N-terminal His tag. The activity of immobilized metal ion affinity chromatography-purified PCY1 was assayed with presegetalin A1[14,32] using ion trap LC/MS. As did partially purified plant extracts, purified recombinant PCY1 showed the formation of segetalin A and smaller amounts of linear segetalin A in the presence of presegetalin A1[14,32] (Fig. 4c, d and e). Evidence for the formation of the C-terminal fragment presegetalin A1[20,32] was also found by ion trap LC/MS/MS (data not shown). The broad pH optimum of PCY1 was centered between 8.5 and 9.0 (Fig. S3).

Optimal production of segetalin A was dependent on the presence of DTT (see Fig. S4). On the other hand the production of linear segetalin A was significant in the absence of DTT. The estimated turnover number with presegetalin A1[14,32] is notably low at ~1 h⁻¹.

Substrate specificity of PCY1 – A variety of synthetic linear peptides were tested as substrates for PCY1. First, putative native peptide cyclase substrates from S. vaccaria and D. caryophyllus (carnation) were tested (Table 1). Evidence for the expected CP formation was found for the putative A-class precursors for segetalins B, D, G, H and L, the F-class precursors of segetalins F and J and for the putative D. caryophyllus precursor GYKDCCVQAKDLENAAVPV (peptide #11) was found. Thus, PCY1 appears to be able to catalyze the production of a number of native S. vaccaria CP. On the other hand, the data also suggest important differences among peptide cyclases within the Caryophyllaceae. Interestingly, a P20Q substitution in presegetalin D1[14,31] led to formation of an alternate truncated product, suggesting that position 20 may be important in determining the amino acid positions to be included in the CP product.

Variants of presegetalin A1[14,32] differing in both the C-terminal region and that of the incipient cyclic peptide were tested as substrates with recombinant PCY1. Variants representing C-terminal deletions of 1, 2, 4, 8, 12 and 13 (linear segetalin A) amino acids did not give rise to detectable levels of segetalin A or linear segetalin A in the presence of PCY1 (see Table S1). This suggests that the majority of the C-terminal amino acid sequence of presegetalin A1[14,32] is important for segetalin A production. This was investigated further in what is essentially an alanine scanning study of the substrate specificity of PCY1 with respect to the C-terminus (substitution with V was made for two positions which are A in the wild type substrate; see Table S1 and Fig. 7). All except three of the variants show a very significant drop in production of segetalin A when assayed with PCY1. On the other hand, a conservative substitution of V with A at position 25 resulted in a more than two-fold enhancement of the production of both cyclic and linear segetalin A. The substitutions E26A and S29A both had relatively little effect on both cyclic and linear product formation. With these results in hand, deletion of the amino acids at positions 25-26 in presegetalin A1[14,32] (peptide #18 in Table 1) was tested and found to allow the formation of segetalin A at a rate ~170-fold lower than for the wild type substrate.

Alanine scanning was also performed for the sequence of the incipient cyclic peptide in presegetalin A1[14,32] (the A19 was substituted with V). Given low turnover number of PCY1 and the difficulties in obtaining multiple cyclic peptide standards, the qualitative ability of PCY1 to form linear and cyclic products was simply scored based on the presence of relevant mass spectrometric signals (see Table S2). In all but the case of the A19V substitution, it was possible to
detect such signals suggesting that such substitutions are tolerated, at least qualitatively, in the incipient CP sequence.

Given the pharmaceutical (44) and nanotechnological (45,46) applications for cyclic peptides, it was of interest to further test the range of possible CP which could be produced by PCY1. Two presegetalin A1[14,32] variants representing insertions of A and AAA in the incipient CP sequence were tested (see Table 1). Both of these variants were found to act as substrates. Also, a “D-amino acid scan” of the segetalin A sequence was performed by testing variant presegetalin A1[14,32]’s with single D-amino acid substitutions. These were tolerated by the PCY1 at all chiral positions except for the C-terminal Ala (Table 1). Given this, the single and double substitutions were made in presegetalin A1[14,32] in order to test the production of cyclo[Gly-Val-Pro-Val-D-Ala-Ala], cyclo[Gly-Val-D-Pro-Val-D-Ala-Ala], and cyclo[Gly-Val-D-Ala-Val-D-Ala-Ala]. Indeed, all three products were detectable by MS of enzyme assay samples (Table 1). This is particularly significant in that even numbered CP with alternating D-amino acids are known to form nanotubes (45).

DISCUSSION

Prior to 2011, very little was known about the biosynthesis of Caryophyllaceae-type CP. There had been a preliminary report that the linear form of heterophyllin B could undergo cyclization in the presence of plant extracts (47), however this does not appear to have been followed up. Thus it was still unclear whether ribosomes were involved in Caryophyllaceae-type CP. In a previous report (17), we provided evidence for the existence in the Caryophyllaceae and Rutaceae of genes encoding CP precursors which are formed by translation on ribosomes and processed to shorter CP. In this paper, we have obtained biochemical evidence allowing the elaboration of the process by which CP precursors are converted to CP in S. vaccaria. This appears to be a two step process involving two separate enzymes. The first enzyme, which we have called OLP1, is essentially an oligopeptidase which recognizes and cleaves presegetalin A1 at the KPQ-GVP sequence corresponding to the junction between the N-terminal region and the incipient CP sequence. Further work is required to clone and characterize OLP1.

The second enzyme involved in CP precursor processing in the S. vaccaria is PCY1, which we have cloned and characterized. Briefly, PCY1 is a serine protease-like enzyme which recognizes the highly conserved C-terminal region of a number of CP intermediates and catalyzes the excision and cyclization of the incipient CP sequence.

It is interesting, particularly from an evolutionary point of view, to compare and contrast CP biosynthesis in the Caryophyllaceae, with other organisms. Within plants, the biosynthesis of the other three classes of CP, the kalata, PawS and cyclic knottin types, show some common features. All appear to be biosynthesized from ribosome-derived precursors which differ greatly in arrangement and sequence. In many cases where evidence is available ribosome-derived biosynthesis of CP in plants, the general pattern appears to be cleavage at the N-terminus of the incipient CP sequence follow by cleavage and cyclization (2,20,48). For the kalata, PawS and cyclic knottin types, the precursor sequences generally share certain features including Gly and Asx at the N- and C-termini of the incipient CP sequence (48). Thus the evidence suggests an asparaginyl endopeptidase is responsible for the final cyclization step for these CP types. In S. vaccaria, there is also a tendency for Gly at the N-terminus of the incipient CP sequence. This appears to extend to carnation (17). However, the so-called F class precursors in S. vaccaria do not follow this rule, having the rather bulky Phe at that position. In flax (Linum usitatissimum), recently reported multidomain precursors of Caryophyllaceae-type CP show a strong tendency for Met at the N-terminus of the incipient CP sequence (49). At the C-terminal position of incipient CP sequences of S. vaccaria, Citrus spp. and L. usitatissimum (17,49), the amino acid is not strictly conserved. Consequently, asparaginyl endopeptidases are not implicated in the processing of Caryophyllaceae-type CP precursors.

Outside of plants, both ribosome-dependent and ribosome-independent modes of CP biosynthesis are prevalent (5,26). In mammals, cyclic defensins appear to be produced by the ligation of two linear peptides (8). In cyanobacteria and fungi there are examples of CP produced from ribosome derived precursors.
Among the fungi, *Amanita* mushrooms produce precursors to amanita toxins. These have a conserved Pro upstream of the C-terminus of the incipient CP and at the N-terminus of it. Although a prolyl oligopeptidase has been implicated in the processing of the precursors, the mechanism of cyclization remains unclear (26). For cyanobacteria, the biosynthesis of patellamides and related CP have been well-studied (19,50). Indeed, the biosynthetic pathway seems to be very similar to the biosynthesis of segetalins in *S. vaccaria*. Precursors containing one or more CP sequences are flanked by conserved regions. A protease cleaves at the “N-terminus” of the incipient CP sequences and a separate protease-like enzyme cyclizes the peptide with removal of C-terminal sequence. For *Prochloron* spp., the latter enzyme is encoded by *patG* (51) and is notably in the S8 family of serine proteases and not closely related to PCY1. It is notable that the products of *patG* and its homologues and that of *Pcy1* are the only cloned enzymes whose main function appears to be peptide cyclization. Thus, among a wide range of taxa there are some commonalities in the mode of biosynthesis of ribosome-derived CP. For systems in which the pathway is reasonably well-characterized, it appears to be common for a precursor with N- and C-terminal flanking regions to be first processed to remove the N-terminus. This is followed by a combined cleavage and cyclization reaction catalyzed by an acyl-intermediate-forming (cysteine or serine type) protease homologue. This is consistent with the known transpeptidation reactions which cysteine and serine proteases are capable of catalyzing (37,38). This certainly suggests a pattern of convergent evolution in which different proteases were recruited in different lineages, giving rise to diverse CP. Further work is required to determine whether the evolution of the cyclases in question was minimal relative to the ancestral proteases, or whether there was significant specialization involved.

The comparison of PCY1 with porcine muscle POP offers some insights into the structure and function of PCY1. Porcine muscle POP is a serine oligopeptidase with an unusual β-propeller domain. This domain is thought to limit access of larger polypeptides to the active site and this is consistent with porcine muscle POP acting on oligopeptide substrates. Indeed, an engineered disulfide “latch” on the β-propeller lid abolishes even oligopeptidase activity (52). This is consistent with the notion that PCY1 also acts on oligopeptides as shown in this paper, although further work is required to determine the limits of its substrate size. The close agreement of the PCY1 with the porcine muscle POP structure suggests that the structural differences which affect the function of the two enzymes may be quite subtle. On the other hand, the amino acid insertion/deletion near the active site histidine may prove to be important. Clarification of this will likely require a high resolution experimental structure to understand. This is in contrast with the cyanobacterial patG homologues whose products have distinctive “capping helices” (reminiscent of the β-propeller lid), which are not found in related proteases (53,54).

As discussed above, the identity of PCY1 as a serine protease homologue is relevant to the mechanism of the cyclization reaction. Serine proteases are known to catalyze proteolysis in a multistep reaction which includes the early formation of an acylserine intermediate formed by nucleophilic attack of the serine at the carbonyl of the scissile peptide bond, with release of the C-terminus of the substrate. This is typically followed by hydrolysis of the acyl intermediate, freeing the N-terminus of the substrate and regenerating the unmodified form of the enzyme. However, in some enzymes or under some conditions aminolysis of the acylserine intermediate can compete with hydrolysis. Where the amine in question is at the N-terminus of a separate peptide, transpeptidation results. If the amine is at the distal end of the acyl group of the intermediate, then cyclization results. Furthermore, Berkers et al. (38) point out that “if protein complexation or conformation positions the amine-donating component involved in transpeptidation in close proximity to the acyl ester component, this will facilitate its nucleophilic attack on the acyl-enzyme intermediate and hence favor aminolysis”. Given this last point it is difficult to imagine how this might occur for segetalin formation. The acyl putative intermediates expected for the reaction represented in Table 1 are short, but quite variable in sequence and length. On the other hand, the C-terminus of the PCY1 substrates is quite highly conserved and amino acid substitutions can have a
strong negative effect on cyclization. In principle, the C-terminus of the substrate could be released from the enzyme prior to cyclization, the data presented suggest the possibility that the C-terminus remains bound to the enzyme even after acyl-intermediate formation and somehow directs the aminolysis (cyclization). Further work is required to investigate this. It is notable, however, that Agarwal et al. (54), propose a similar retention of the C-terminal fragment for patG homologues.

While the cloning and characterization of PCY1 offers insights into the biosynthesis of CP in plants, the enzyme may also have applications in biotechnology. There is considerable interest in cyclic peptide drugs and vaccines (for example see (55,56)). Biotechnological production of CP allows both the generation of CP libraries for screening as well as the production of individual CP for testing or production. The relative relaxed substrate specificity of PCY1 may be useful in this regard and further investigation of the applications of PCY1 and related enzymes is warranted.
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FOOTNOTES

1To whom correspondence may be addressed: Patrick Covello, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK Canada S7N 0W9; Tel.: (306) 975-5269; Fax: (306) 975-4839; E-mail: patrick.covello@nrc-cnrc.gc.ca

2The abbreviations used are: CP, cyclic peptide; PCY1, Peptide Cyclase 1; OLP1, Oligopeptidase 1; EST, expressed sequence tag; POP, prolyl oligopeptidase

FIGURE LEGENDS

FIGURE 1. Proposed pathway for the processing of presegetalin A1 to segetalin A in S. vaccaria. Presegetalin A1 is derived from a monocistronic mRNA by translation on ribosomes (17). An oligopeptidase activity (OLP1) and a peptide cyclase activity (PCY1) are involved in removal of the N-terminus and cyclization, respectively.

FIGURE 2. Separation of Oligopeptidase 1 and Peptide Cyclase 1 activities in S. vaccaria extracts. Panel a shows a Mono-Q ion exchange chromatogram of stage 2 developing S. vaccaria seed proteins using a NaCl gradient. Two minute fractions were collected and assayed for OLP1 activity (b; presegetalin A1[14,32] formation) and PCY1 activity (c; segetalin A formation from presegetalin A1[14,32]; see Experimental Procedures).

FIGURE 3. Products of Oligopeptidase 1 activity in developing seeds of S. vaccaria. An ion exchange fraction derived from stage 2 developing seeds of S. vaccaria and eluting at ~0.35 M NaCl was assayed using presegetalin A1 (see Experimental Procedures). MALDI-TOF MS analysis provided evidence of the residual substrate as well as its cleavage products, as indicated.

FIGURE 4. Assay of OLP1 and PCY1 activity by ion trap LC/MS. Ion chromatograms are shown for OLP1 assays using presegetalin A1 as a substrate without (a) and with (b) partially purified OLP1, and assays using presegetalin A1[14,32] as a substrate without enzyme (c), and with partially purified plant PCY1 fraction (d) and recombinant PCY1 (e). Products were not detected at significant levels in assays with no incubation time or no peptide substrates (data not shown). Chromatograms consist of the sum of molecular ions for presegetalin A1 (m/z =851.5, z =4; m/z = 681.5, z=5), presegetalin A1[1,13] (m/z = 718.8, z=2; m/z = 729.1, monosodium adduct, z=2; m/z = 740.0, disodium adduct, z=2), presegetalin A1[14,32] (m/z = 993.5, z=2; m/z = 662.7, z=3), linear segetalin A (m/z = 628.5, z=1; m/z = 650.5, sodium adduct, z=1) and segetalin A (m/z = 610.5, z=1; m/z = 632.5, sodium adduct, z=1).

FIGURE 5. Electrophoretic analysis of partially purified peptide cyclase (PCY1) from S. vaccaria. Lane 1, crude filtrate from S. vaccaria developing seed; lane 2, active fraction from anion exchange chromatography; lane 3, active fraction from hydrophobic interaction chromatography; lane 4, active fraction from gel filtration chromatography. The mobility of relative molecular mass standards of 25,000 and 75,000 are shown on the left. The arrow indicates a band corresponding to a major protein with M, of approximately 83,000.
**FIGURE 6.** The predicted structure of PCY1 based on homology modeling using porcine muscle POP. (a) Structural alignment of a homology model of PCY1 (yellow) and porcine muscle prolyl oligopeptidase (blue). The top half of the structure represents the catalytic domain including the putative catalytic triad shown in stick form. The bottom half of the structure is a β-propeller domain which may be involved in controlling access to the active site. (b) Superimposition of the putative catalytic triad (S562, D653, H695) of PCY1 (green) and that of porcine muscle prolyl oligopeptidase (red).

**FIGURE 7.** Effects of mutations in the C-terminal flanking region of presegetalin A1 on production of linear and cyclic segetalin A production by recombinant PCY1. Wild type (WT) and variants of presegetalin A1[14,32] are indicated. Means and standard deviations (n=3) of 1 h assays are indicated.
Table 1. Substrate specificity of PCY1. The synthetic linear peptides indicated were used in 1 h assays with PCY1 (see Experimental Procedures). CP and linear peptide production was determined by ion trap LC/MS and confirmed by ion trap LC/MS/MS (see Table S2). Expected CP sequences are underlined. Variant amino acids are indicated in reverse type. D. caryophyllus precursor sequences are derived from Genbank accession nos. AW697819 and CF259478 (17). Quantitation of segetalin A and linear segetalin A production from selected substrates is shown in Fig. 7. Lower case letters denote D-amino acids.
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| Substrate Category | Peptide No.   | Peptide Name       | Sequence                | PCY1 Product |
|--------------------|---------------|--------------------|-------------------------|--------------|
| native plant substrates |               |                    |                         | Cyclic Linear |
| 1                  | Presegetalin A1[14,32] | GVPVWA             | FQAKDVENASAPV           | + +          |
| 2                  | Presegetalin B1[14,31] | GVAWA              | FQAKDVENASAPV           | + -          |
| 3                  | Presegetalin D1[14,31] | GLSFAFP            | AKDAENASSPVP            | + +          |
| 4                  | Presegetalin G1[14,31] | GVKYA              | FQPKDSENASAPV           | + -          |
| 5                  | Presegetalin H1[14,31] | GYRFSD             | FQAKDAENASAPV           | + -          |
| 6                  | Presegetalin K1[14,31] | GRVKA              | FQAKDAENASAPV           | + -          |
| 7                  | Presegetalin L1[14,32] | GPLGPW             | FQAKDVENASAPV           | + -          |
| 8                  | Presegetalin F1[14,38] | FSASYSSKF          | IQTQVSNGMDNASAPV        | + -          |
| 9                  | Presegetalin J1[14,36] | FGTHGLPAP          | IQVPNGMDDACAPM          | + -          |
| 10                 | D. caryophyllus Precursor 1 | GPIPFYG          | FQAKDAENASVPV           | + -          |
| 11                 | D. caryophyllus Precursor 2 | GYKDCC          | VQAKDLENAAVPV           | - -          |
| C-terminal presegetalin A1[14,32] variants |               |                    |                         |              |
| 12                 | Presegetalin A1[14,31] | GVPVWA             | FQAKDVENASAP            | - -          |
| 13                 | Presegetalin A1[14,30] | GVPVWA             | FQAKDVENASA             | - -          |
| 14                 | Presegetalin A1[14,28] | GVPVWA             | FQAKDVENA               | - -          |
| 15                 | Presegetalin A1[14,24] | GVPVWA             | FQAKD                 | - -          |
| 16                 | Presegetalin A1[14,20] | GVPVWA             | F                    | - -          |
| 17                 | Presegetalin A1[14,19] | GVPVWA             | - n/a                  |              |
| 18                 | Presegetalin A1[14,32]Δ25-26 | GVPVWA          | FQAKD NASAPV           | + +          |
| 19                 | Presegetalin A1[14,32] F20A | GVPVWA          | FQAKDVENASAPV           | + +          |
| 20                 | Presegetalin A1[14,32] Q21A | GVPVWA            | FQAKDVENASAPV           | + +          |
| 21                 | Presegetalin A1[14,32] A22V | GVPVWA            | FQAKDVENASAPV           | + +          |
| 22                 | Presegetalin A1[14,32] K23A | GVPVWA            | FQAKDVENASAPV           | + +          |
|    | Variants of incipient segetalin sequences |    |    |    |    |
|----|-----------------------------------------|----|----|----|----|
| 23 | Presegetalin A1[14,32] D24A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 24 | Presegetalin A1[14,32] V25A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 25 | Presegetalin A1[14,32] E26A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 26 | Presegetalin A1[14,32] N27A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 27 | Presegetalin A1[14,32] A28V             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 28 | Presegetalin A1[14,32] S29A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 29 | Presegetalin A1[14,32] A30V             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 30 | Presegetalin A1[14,32] P31A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 31 | Presegetalin A1[14,32] V32A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 32 | Presegetalin D1[14,31] P20Q             | GLSFA | FQAKDAENASSPV | +  | +  |
|    |                                          |    |    |    |    |
| 33 | Presegetalin A1[14,32] G14A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 34 | Presegetalin A1[14,32] V15A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 35 | Presegetalin A1[14,32] P16A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 36 | Presegetalin A1[14,32] V17A             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 37 | Presegetalin A1[14,32] W18A             | GVPVWA | FQAKDVENASAPV | +  | -  |
| 38 | Presegetalin A1[14,32] A19V             | GVPVWA | FQAKDVENASAPV | -  | -  |
|    |                                          |    |    |    |    |
| 39 | Presegetalin A1[14,32] V15v             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 40 | Presegetalin A1[14,32] P16p             | GVPVWA | FQAKDVENASAPV | +  | -  |
| 41 | Presegetalin A1[14,32] V17v             | GVPVWA | FQAKDVENASAPV | +  | -  |
| 42 | Presegetalin A1[14,32] W18w             | GVPVWA | FQAKDVENASAPV | +  | +  |
| 43 | Presegetalin A1[14,32] A19a             | GVPVWA | FQAKDVENASAPV | -  | -  |
|    |                                          |    |    |    |    |
| 44 | Presegetalin A1[14,32] ins 16A17        | GVPVWA | FQAKDVENASAPV | +  | +  |
| 45 | Presegetalin A1[14,33] ins 16AAA17      | GVPVWA | FQAKDVENASAPV | +  | +  |
Figure 1

Caryophyllaceae cyclic peptide biosynthesis
Figure 2
Figure 3
Figure 4

Caryophyllaceae cyclic peptide biosynthesis
Figure 5
Figure 6
Figure 7