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Identification and Quantification of Cyclolinopeptides in Five Flaxseed Cultivars

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Supporting Information

ABSTRACT: Cyclolinopeptides are a group of naturally occurring hydrophobic cyclic peptides found in flaxseed and flax oil that have immunosuppressive activity. This study describes the measurement of flaxseed cyclolinopeptide concentrations using an internal standard HPLC method. In addition, the concentration of cyclolinopeptides in the seed of Canadian flax cultivars grown at two locations over two years is reported. The data are consistent with the formation of flaxseed cyclolinopeptides from two ribosome-derived precursors. Each precursor protein includes the sequences corresponding to three cyclolinopeptides from which those cyclolinopeptides are presumably derived by precursor processing. The concentrations of cyclolinopeptides C and E, which are encoded by the same gene sequence, are highly correlated, and the concentrations of cyclolinopeptides D, F, and G, which are encoded by a second gene sequence, are also highly correlated. The strong correlation between the cyclolinopeptides arising from the same gene may prove to be important in understanding how peptide concentration is controlled. Additional research may lead to approaches to improve flax either as a platform for peptide production or as a source of oil with improved drying properties and flavor.

KEYWORDS: *cyclolinopeptide, HPLC quantification, flaxseed cultivars, genotype and environment interaction*

INTRODUCTION

Cyclolinopeptides are a group of naturally occurring hydrophobic cyclic peptides derived from the seed and root of flax (*Linum usitatissimum* L.).[1] Cyclolinopeptides comprise eight or nine amino acid residues resulting in molecular masses of approximately 1 kDa. Cyclolinopeptide A (**1**) was the first cyclolinopeptide identified after isolation from sediments in crude flaxseed oil.[1] Subsequently, cyclic peptides from flaxseed were named according to the date of their discovery with each newly discovered peptide being ascribed the next letter in the alphabet. In 1968, Weygand discovered a similar cyclic nonapeptide, cyclolinopeptide B (**2**).[2] Between 1997 and 2001, seven additional naturally occurring cyclolinopeptides C–I (**3**–**9**) were identified.[3−5] Additionally, a cyclic peptide containing the nonproteinogenic amino acid *N*-methyl-4-aminoproline was isolated and characterized.[6] The structures of cyclolinopeptides A–I (**1**–**9**) are shown in Table 1 and Figure 1, respectively. The natural function of these peptides in planta is unknown. In vitro studies of cyclolinopeptide biological activity have been described in the literature. Cyclolinopeptide **1** was shown to inhibit cholate uptake into hepatocytes, potentially protecting the liver against poisoning.[7,8] Cyclolinopeptide **1** was also demonstrated to suppress the calcium-dependent activation and proliferation of T-lymphocytes by cyclophilin-dependent calcineurin inactivation.[9,10] The immunosuppressive activity of cyclolinopeptide **1**, described by others, may be partially or wholly explained by this observation.[11,12]

Brühl et al.[13] investigated the level of cyclolinopeptide **5** in pressed flaxseed oil, but detailed analysis of other cyclolinopeptides in flaxseed cultivars has not been reported previously. Flaxseed oil is still widely used as a drying oil. Reaney et al.[14] reported a treatment of flaxseed oil that removed the cyclolinopeptides also accelerated an increase in viscosity of the oil. Therefore, it is possible that cyclolinopeptides are not desirable in either the food market, as they contribute a bitter flavor to flaxseed oil, or the drying oil market, as they may slow drying. Conversely, due to the remarkable biological activity of cyclolinopeptides, isolation of these compounds from an industrial stream may be desirable. Recently, we prepared fractions from flaxseed using industrial separation processes and reported the concentration of cyclolinopeptides in these fractions.[15] This research showed that the cyclolinopeptides were substantially removed from flaxseed oil by acid degumming, whereas alkali refining lowered cyclolinopeptide contents. The overall goal of this research was to determine the concentration of cyclolinopeptides in the seed of widely grown Canadian flaxseed cultivars (genotypes) grown at multiple locations over several years. This information may prove useful in utilizing cyclolinopeptides for their biological activity[7−12] or quality control of flaxseed oil.

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Table 1. Amino Acid Sequences and Chemical Formulas of Cyclopeptides

cyclopeptide (code)	amino acid sequence[a]	molecular formula	protonated ion mass (m/z)[b]
Seg-A	*cyclo*-(Gly-Val-Pro-Val-Trp-Ala)	$C_{31}H_{43}N_7O_6$	610.32[20]
cyclolinopeptide A (**1**)	*cyclo*-(Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile)	$C_{57}H_{85}N_9O_9$	1040.65[1]
cyclolinopeptide B (**2**)	*cyclo*-(Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	$C_{56}H_{83}N_9O_9S$	1058.61[4]
cyclolinopeptide C (**3**)	*cyclo*-(Mso-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile)	$C_{56}H_{83}N_9O_{10}S$	1074.56[4]
cyclolinopeptide D (**4**)	*cyclo*-(Mso-Leu-Leu-Pro-Phe-Phe-Trp-Ile)	$C_{57}H_{77}N_9O_9S$	1064.54[4]
cyclolinopeptide E (**5**)	*cyclo*-(Mso-Leu-Val-Phe-Pro-Leu-Phe-Ile)	$C_{51}H_{76}N_8O_9S$	977.52[4]
cyclolinopeptide F (**6**)	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Val)	$C_{55}H_{73}N_9O_{10}S_2$	1084.47[5]
cyclolinopeptide G (**7**)	*cyclo*-(Mso-Leu-Mso-Pro-Phe-Phe-Trp-Ile)	$C_{56}H_{75}N_9O_{10}S_2$	1098.50[5]
cyclolinopeptide H (**8**)	*cyclo*-(Mso-Leu-Met-Pro-Phe-Phe-Trp-Ile)	$C_{56}H_{75}N_9O_9S_2$	1082.38[5]
cyclolinopeptide I (**9**)	*cyclo*-(Met-Leu-Mso-Pro-Phe-Phe-Trp-Val)	$C_{55}H_{73}N_9O_9S_2$	1068.35[5]

[a]The first and third positions of amino acid sequences are highlighted in Figure 1. Abbreviations: Met, methionine; Mso, methionine sulfoxide. [b]From reference and ESI-MS data obtained as described in the Supporting Information, File S1.

MATERIALS AND METHODS

Materials. Silica gel 60 (particle size = 0.040−0.063 mm, 230−400 mesh) was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). All solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA) unless otherwise noted. A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water for all mobile phases.

Flaxseed Preparation. The five cultivars of flaxseed used in cyclolinopeptide quantification, CDC Bethune, CDC Valour, Flanders, Somme, and Vimy, were grown in field plots at two locations in Saskatchewan (Saskatoon and Floral) in 2006 and 2008. The trials were standardized as a randomized complete block design (RCBD) with two replications. Each plot contained six rows, 0.30 m apart and 3.66 m long. The seed was a provided by Dr. Gordon G. Rowland, Crop Development Centre, University of Saskatchewan (Saskatoon, SK, Canada).

Isolation of Cyclolinopeptides from Different Flaxseed Cultivars. *Oil Extraction from Flaxseed.* Flaxseed was ground in a coffee grinder for 30 s to pass through a 1.18 mm test sieve (12 in., VWR, West Chester, PA, USA) and dried for 48 h at 55 °C to determine dry matter content. Ground material (5 g) was weighed, wrapped in filter paper (Whatman no. 4, Whatman Inc., Piscataway, NJ, USA), and folded to fit into a cellulose extraction thimble (25 × 80 mm, Whatman Inc.). The thimbles were placed in glass sleeves, clamped into a Goldfisch extractor (Labconco Co., Kansas City, MO, USA), and extracted according to a modified version of the Association of Official Analytical Chemists (AOAC) method 960.39.[16] To ensure the extraction of more polar peptides, acetone (50 mL) was added to the oil extraction beaker, instead of hexane, and attached to the lipid extractor. The extraction was carried out for 5 h. After extraction, acetone in the oil samples was recovered in a solvent recovery tube. Subsequently, contents of the beaker were placed in a fume hood for 1 h to allow for solvent evaporation. The beakers were heated in a vacuum oven at 70 °C for 1 h to remove traces of solvent, then purged with nitrogen, and placed in a desiccator to cool. The mass of oil present in the beaker was used to calculate the oil content according to eq 1. Three separate oil extractions were performed for each flaxseed sample from each plot.

$$\text{oil (\%)} = \frac{(\text{wt of beaker} + \text{oil}) - \text{wt of beaker}}{\text{wt of sample}} \times 100 \quad (1)$$

Cyclolinopeptide Isolation from Acetone Extracts. Silica gel 60 solid phase extraction (SPE) was used to separate cyclolinopeptides and other polar compounds such as pigments, phospholipids, and waxes from acetone-extracted samples obtained as described in Figure S1 of the Supporting Information. SPE silica gel columns (vertical) were prepared as follows: a cotton ball was placed in the bottom of a 3 mL plastic syringe with sand (50−70 mesh; 1 cm height) added on top for support. Silica gel (0.5 g) was slurried in hexane (1 mL), poured onto the sand, and then covered by another layer of sand (50−70 mesh, 0.5 cm height). The plastic syringes were inserted into the Luer-Lok fittings (Becton Dickinson, Sparks, MD, USA) of a 12-port Visiprep SPE vacuum manifold (Supelco Inc., Bellefonte, PA, USA). The silica gel columns were equilibrated with 2 mL of hexane for 2 min before a mixture of oil sample (1 mL) and hexane (1 mL) was loaded onto the gel. The column was then eluted by various solvents (10 mL each) of increasing polarity under vacuum pressure (7.1×10^4 Pa): 100% hexane, 20% (v/v) ethyl acetate (EtOAc) in hexane, 50% (v/v) EtOAc in hexane, 100% EtOAc, and 10% (v/v) methanol (MeOH) in dichloromethane (DCM). Cyclolinopeptides are known to elute with 100% EtOAc and 10% MeOH in DCM wash solvents.[14] The two peptide-enriched fractions were combined and solvent was removed by a rotary vacuum evaporator at 40 °C (Brinkmann, Westbury, NY, USA). The residue was collected for further analysis. The fractions from SPE were analyzed by reversed-phase high-performance liquid chromatography (HPLC).

Calibration Curves of Cyclolinopeptides with Internal Standard. Standards of flaxseed peptides were prepared by extraction from flax oil followed by separation using preparative reversed phase chromatography as described previously.[14] Standards of **4**, **5**, and **7** were used without further purification. Standards of **1**−**3** were crystallized as described.[17−19] Segetalin-A (Seg-A) was prepared according to the method of Balsevich et al.[20] Finally, to produce calibration curves for determining linearity and enabling the determination of the cyclolinopeptide concentration, cyclolinopeptide standards and Seg-A were weighed and dissolved in 100% MeOH to make stock solutions of 2.0 mg/mL. A mixture of **1**, **2**, **3**, **4**, **5**, **7** (0.2 mg/mL, each), and Seg-A (0.1 mg/mL) was filtered through a 0.45 μM PTFE syringe filter (Whatman Inc.). The sampling rate was set at 10 (Hz) for the wavelengths of 214 and 244 nm (bandwidth of 10 nm). These wavelengths were recorded with a reference wavelength of 300 nm (bandwidth of 10 nm). The wavelength of 280 nm (bandwidth of 10 nm) was recorded with a reference wavelength of 360 nm (bandwidth of 100 nm). UV spectra of the cyclolinopeptides were recorded at the peak center of each compound in the chromatogram (Figure 2) and in water containing 5% (v/v) dimethyl sulfoxide (data not shown). Calibration curves for cyclolinopeptide quantification were established between 10 and 500 μg/mL in the presence of Seg-A at a constant concentration of 50 μg/mL. The concentrations of cyclolinopeptide (x-axis) and the area ratio of the peaks of cyclolinopeptide to Seg-A (y-axis) were plotted as a standard curve. Cyclolinopeptide concentration was determined by calculating peak area relative to the internal standard, Seg-A, using the standard curves. Three samples were prepared at each concentration of cyclolinopeptide and injected on each day to establish the calibration curve. No standard was available for **6**. The concentration of **6** was estimated on the basis of that of **7**. This assumption is based on the highly similar polarity, molecular weight (MW), and chromophore composition shared by the two compounds (Figure 3). Standards were not available for compounds **8** and **9**.

A recovery test was run on control solutions (blind samples, cyclolinopeptides) to validate the analytical method. Stock solutions were diluted to obtain four different concentrations: 50, 125, 200, and 500 μg/mL. The accuracy of the HPLC method in measurement of

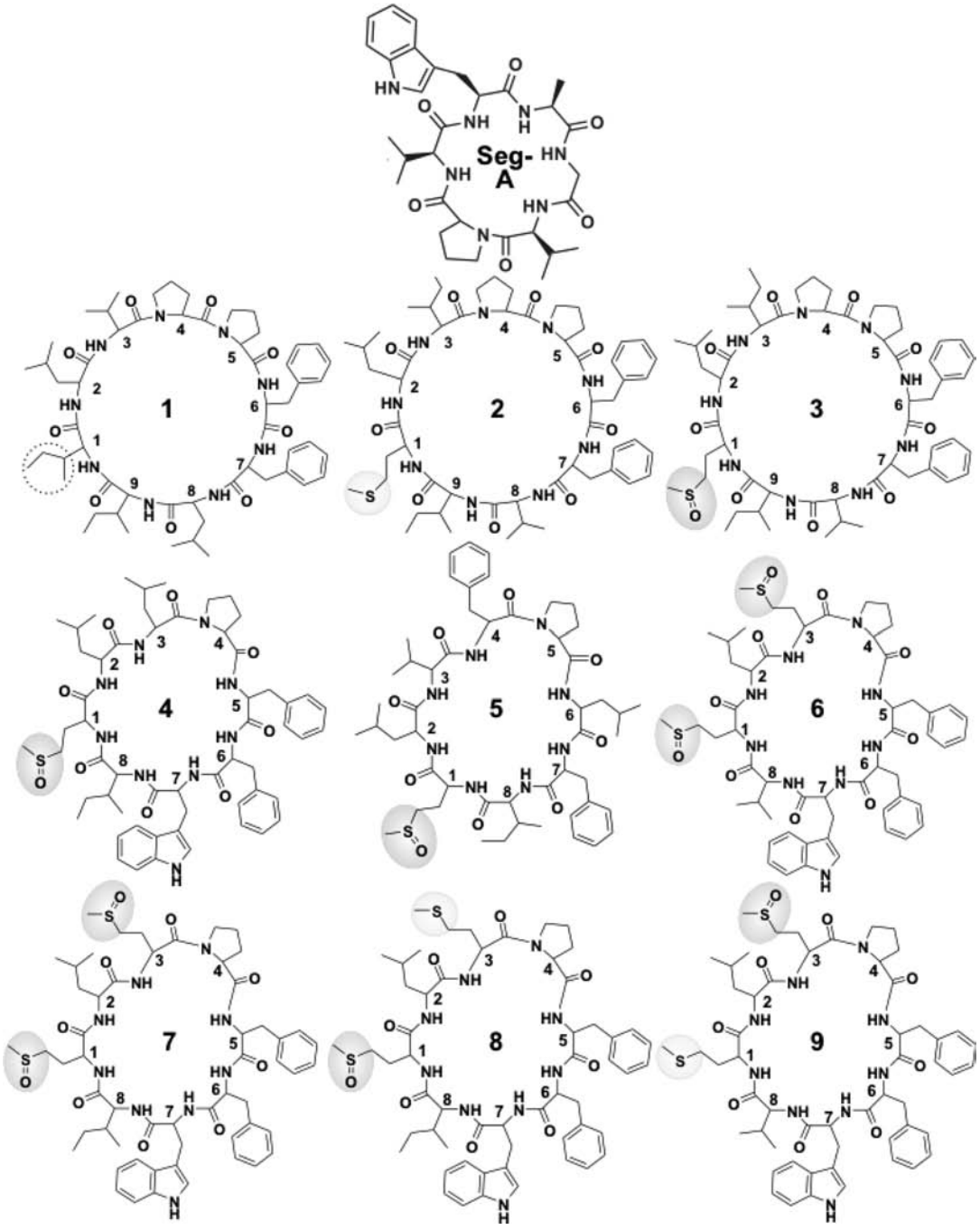

Figure 1. Structures of cyclopeptides. The first and third positions of amino acid sequences are highlighted in cyclolinopeptides: methionine and methionine sulfoxide.

the concentration of these solutions was determined using the calibration curves (n = 3). Recovery was calculated according to eq 2:

$$\text{recovery (\%)} = \frac{\text{calcd concn}}{\text{actual concn}} \times 100 \quad (2)$$

Sample Preparation for Cyclolinopeptide Quantification. Flaxseed oil and cyclolinopeptide were extracted by acetone from ground flaxseed as described above. After evaporation of the acetone, 1 g of each sample was then weighed in a 10 mL beaker. Internal standard, Seg-A, solution (25 μL, 2 mg/mL) and hexane (1 mL) were added to the oil. The solution was swirled by hand before it was loaded onto a silica gel SPE column. After elution from the column, as described previously, the peptide fraction was taken to dryness and dissolved in 1 mL of MeOH. The dissolved sample was filtered through a 0.45 μM PTFE syringe filter prior to HPLC analysis. The area of each eluting peptide peak observed in the chromatograms was recorded, and the concentration of cyclolinopeptide in flaxseed oil was calculated using calibration curves as described above. The concentrations of cyclolinopeptides in flaxseed oil were calculated using eq 3:

$$\text{concn of cyclolinopeptide} = \frac{\text{calcd concn in oil}}{\rho} \times \text{oil content} \quad (3)$$

The calculated concentration in oil (μg/g) was obtained from the calibration curve, ρ (g/mL) was the density of the flaxseed oil, and oil content (%) was obtained from eq 1.

HPLC Analysis. An Agilent 1200 series HPLC system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a

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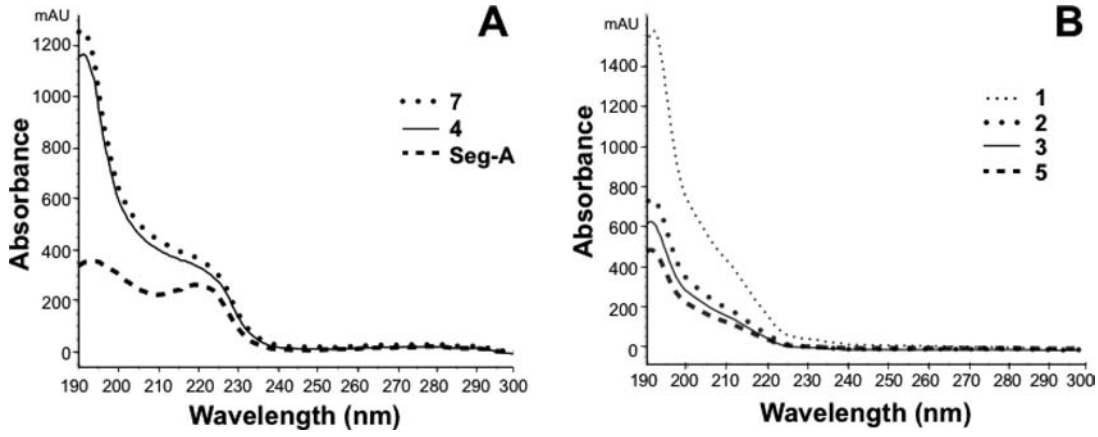

Figure 2. Comparison of UV spectra of (A) **4**, **7**, and Seg-A (tryptophan-containing peptides) and (B) **1**−**3** and **5** (tryptophan-free peptides).

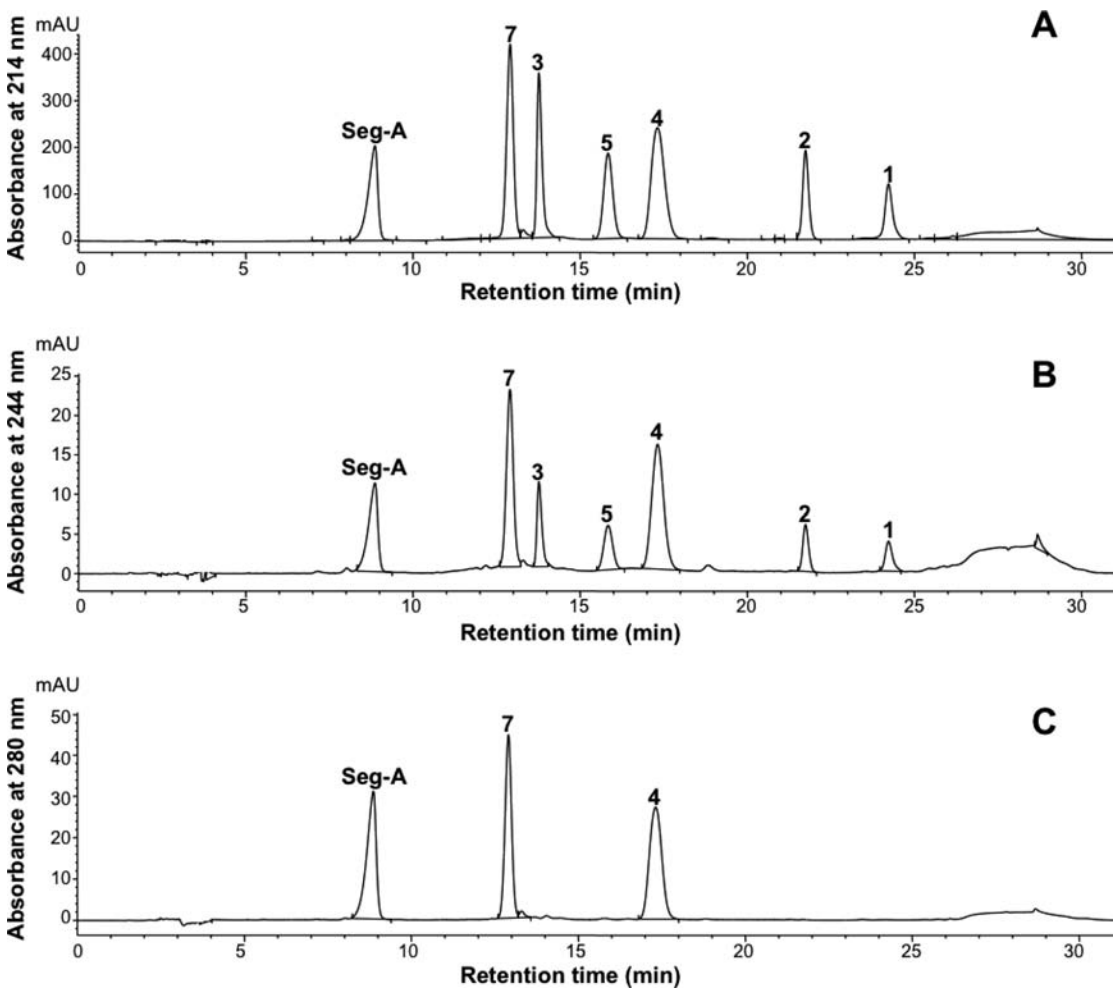

Figure 3. HPLC chromatograms of **1**−**5** and **7** (0.2 mg/mL, each) and Seg-A (0.1 mg/mL) at wavelengths of (A) 214, (B) 244, and (C) 280 nm with a bandwidth of 10 nm in each case. Defaulted reference signals were used (300 nm with a bandwidth of 10 nm for 214 and 244 nm, respectively, and 360 nm with a bandwidth of 100 nm for 280 nm).

quaternary pump, a variable-wavelength diode array detector (DAD, 190−300 nm), and an autosampler was used with a column compartment temperature of 23 °C. The samples were separated on a ZORBAX Eclipse XDB-C18 column (150 × 4.6 mm i.d., 5 μm, Agilent Technologies Canada Inc.). The mobile phase consisted of acetonitrile (A) and water (B). The elution gradient varied as follows: 0 min to 30% A at 0.5 mL/min, 3 min to 40% A at 0.5 mL/min, 6 min to 45% A at 0.5 mL/min, 7 min to 65% A at 0.5 mL/min, 19 min hold at 65% A at 0.5 mL/min, 22 min to 66% A at 0.5 mL/min, 23 min to 70% A at 1.0 mL/min, 24 min to 100% A at 1.0 mL/min, 26 min at 100% A, and 31 min to 30% A. The column was equilibrated with 30% A for 10 min at the end of each gradient (Table S1 of the Supporting Information). Each sample (15 μL) was injected onto the HPLC column and analyzed in triplicate. Data analyses were conducted using ChemStation computer software for LC 3D system (Agilent

Technologies Canada Inc.). Area integration of eluting peaks was obtained at 214 nm.

Identification of Cyclolinopeptide Encoding Genes. A database search of a developing flaxseed expressed sequence tag (EST) library was screened, with putative transcripts containing **4**, **6**, and **7** identified.[21] The EST sequence was used to query a BLASTx 2.2.22+ search of a draft flax genome.[22] The genomic DNA sequences of two genes containing **4**, **6**/**9**, and **7**/**8** (AFSQ01025165.1) and **1**, **3**/**2**, and **5** (AFSQ01016651.1) were identified.[23]

Statistical Analysis. The EST and whole genome sequence of flax have been reported during the course of this study.[23,24] Searches of this database revealed that peptides **1**, **2**, **3**, and **5** are encoded by a single gene sequence encoding a precursor protein;[23] additionally, **4**, **6**, **7**, **8**, and **9** are encoded by a second gene sequence encoding a precursor protein.[23,24] The reliability of cyclolinopeptide quantification was improved by grouping peptides that were products of a single precursor protein. Products of AFSQ01016651.1 (**1**−**3** and **5**) are, hereafter, referred to as cyclolinopeptide-51 (total cyclolinopeptides expressed by gene AFSQ01016651.1), and cyclolinopeptide-65 includes all cyclolinopeptides expressed by gene AFSQ01025165.1 (**4** and **6**−**9**). These groupings were used in subsequent statistical analysis. All statistical analyses were conducted using the Statistical Analysis System (SAS for Windows, release 9.2, SAS Institute Inc., Cary, NC, USA). One-way ANOVA was used to analyze the effect of cultivar on the level of cyclolinopeptides in flaxseed. Differences were considered to be significant at $p < 0.05$. To analyze the effects of cultivar, year, location, and their interactions on the expression of cyclolinopeptides, analysis of variance by PROC MIXED procedure, Pearson correlation (to estimate the linear relationships between the expressions of group cyclolinopeptide-51 and cyclolinopeptide-65), and variance components using the PROC VARCOMP procedure were performed according to SAS methods. Post hoc multiple-comparison test was Tukey's test. Differences were considered to be significant at $p < 0.05$. All results were expressed as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Oil Content of Flaxseed. The oil content of flaxseed from different cultivars varied from 38.0 ± 1.4 to 42.4 ± 0.7% (w/w, data not shown). The variance may be caused by sampling errors or genetic or environmental factors (Tables S2−S4 of the Supporting Information). It is noteworthy that these values are lower than the oil contents of the same cultivars published by the Flax Council of Canada (42.5−45.7%).[25] The difference could be due to the use of acetone instead of hexane as solvent polarity may affect recovery.

Cyclolinopeptide Isolation. Silica gel was used by Brühl et al.[26] to isolate cyclolinopeptide from pressed flaxseed oil. In this project, silica gel columns were also used for cyclolinopeptide isolation from solvent-extracted flaxseed oil at the ratio of 1:2 (w/v, silica to oil). Less polar solvent washes (such as hexane, 20% EtOAc in hexane, 50% EtOAc in hexane) were used to elute low-polarity neutral compounds of flaxseed oil (e.g., triacylglycerols, wax, and pigments), whereas 100% EtOAc and 10% MeOH in DCM eluted cyclolinopeptide from the silica gel.

Cyclolinopeptide Identification and Quantification. Brühl et al.[13] determined the **5** concentration in flaxseed oil using an external standard method. In their study, an external calibration curve was established from 3 to 900 μg/mL **5** with a coefficient of determination of 0.998. However, the calibration curve or equation was not included in their publication. Cyclolinopeptide standards were not available to previous researchers. Therefore, there is no information available on the cyclolinopeptide concentration in flaxseed tissues. In the current study, a HPLC method for cyclolinopeptide detection and quantification was developed using Seg-A as an internal standard. Seven peaks were observed in HPLC chromatograms of flaxseed oil extracts after the addition of the standard that included Seg-A, **1**−**5**, and **7**, respectively (Figure 3). These compounds have chromophores that include peptide bonds (214 nm; Figure 3A), phenylalanine (244 nm; Figure 3B), and tryptophan (280 nm; Figure 3C), which are useful for peptide detection, characterization, and quantification.[27,28] In HPLC chromatograms, all cyclolinopeptide peaks except for peak 1, the internal standard, at 0.2 mg/mL presented useful absorbances (>150 mAU) at 214 nm due to UV absorption by peptide bonds and conjugated double bonds in aromatic amino acids. Comparatively weaker absorbance (<25 mAU) at 244 nm was found in all peptides. Three standards (Seg-A, **4**, and **7**) all had weak absorbance (approximately 40 mAU) at 280 nm due to the presence of the indole group of tryptophan (Trp) in their structure. The complete UV spectra of Trp-containing peptides (190−300 nm, Figure 2) confirmed this observation.

In a previous report of chromatographic conditions for separation of cyclolinopeptides, Brühl et al.[26] separated five peptides (**1**, **3**, and **5**−**7**) with elution times of 20−30 min. They reported that crude extracts (4.4 mg) were dissolved in 1.5 mL of water/ethanol (1:1, v/v) and 100 μL of the aliquots were injected into a LiChrospher 100 RP-18 column (250 × 4.0 mm, 5 μm). Chromatography was performed using a mixture of MeOH/water within 25 min (from 75:25 to 100:0, v/v). In the current study, a shorter column (150 × 4.6 mm, 5 μm) and a different solvent system (acetonitrile/water) were employed. The same cyclolinopeptides were more evenly distributed throughout the chromatogram, eluting between 13 and 24 min (Table 2). The coefficient of variation (CV) of HPLC elution time was <3% for all cyclolinopeptide measurements, which indicated good reproducibility of the HPLC method. The equations extracted from calibration curves were used to calculate the concentrations of cyclolinopeptides of unknown samples (Table 3). Solutions of cyclolinopeptides were made by another analyst in the laboratory to test the accuracy of HPLC for determination of cyclolinopeptide concentration. The recovery of cyclolinopeptides ranged from 92 to 115% for a concentration of 50 μg/mL and from 95 to 118% at 125 μg/mL. The apparent high recovery of 115−118% could be explained by human and systematic errors when dilute samples were handled. At higher concentrations, the CV of recovery was reduced and the range of results was consistent with 100% recovery; 94−104% at 200 μg/mL and 95−103% at 500 μg/mL (data not shown).

Table 2. Retention Times of Cyclopeptides

standard	retention time[a] (min)	CV[b] (%)	area[c] (mAU × s)	CV[b] (%)
Seg-A	8.79	1.41	4203	1.49
7	12.83	0.46	5945	1.00
3	13.61	1.95	3875	1.43
5	15.72	0.77	3507	2.72
4	17.22	0.99	6705	1.32
2	21.55	1.02	2453	2.34
1	24.05	0.84	2080	2.74

[a]Mean of retention time for 12 runs (3 runs/day for 4 days). [b]Coefficient of variation = SD/mean × 100%. [c]Mean of peak area for 12 runs (3 runs/day for 4 days).

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Table 3. Quantification Equations for Cyclolinopeptides Using Seg-A as Internal Standard

cyclolinopeptide	equation[a]	correl coeff
1	$Y = (X + 0.0398)/5.69$	0.9984
2	$Y = (X - 0.0104)/5.92$	0.9998
3	$Y = (X + 0.0015)/9.67$	0.9999
4	$Y = (X - 0.0125)/15.67$	0.9997
5	$Y = (X + 0.0477)/9.85$	0.9990
7	$Y = (X + 0.0349)/16.31$	0.9993

[a] Y expresses the concentration of cyclolinopeptide (mg/mL) in analyzed sample and X the area ratio of cyclolinopeptide/Seg-A from the HPLC chromatogram.

Cyclolinopeptide Distribution of Flaxseed from Different Cultivars. The only reports of the concentration of a cyclolinopeptide in flaxseed oil are by Brühl et al.[13,26] Brühl et al.[13] determined the effect of storage on cyclolinopeptide **5** concentration in pressed flaxseed oil derived from flaxseed grown in Switzerland. This study provided analysis of single samples of each flaxseed cultivar and did not measure the amount of peptide remaining in the flaxseed meal. Therefore, the total amount of peptide was not determined. A study involving repeated sampling of the same cultivars grown in plots might overcome the limitations of the previous study and determine the range of flaxseed peptide content. In the current research, flaxseed samples of five cultivars grown at two locations in two growing seasons were analyzed to study the possible effects of both genotype and environment on the concentration of cyclolinopeptides in flaxseed. The cyclolinopeptide content of flaxseed differed significantly among cultivars (Table 4). The one-way ANOVA analyses showed there were significant differences in single and overall cyclolinopeptide levels among the five cultivars. The Somme cultivar had higher levels of **1** (65.9 ± 5.5 μg/g) than the Flanders cultivar (53.5 ± 4.9 μg/g), higher levels of **4** (42.5 ± 5.0 μg/g) than all other cultivars, higher levels of **6** (16.6 ± 2.0 μg/g) than Bethune Valor and Flanders, higher levels of **7** (51.0 ± 4.3 μg/g) than Bethune Valor and Flanders, and higher levels of overall cyclolinopeptides (302.8 ± 27.4 μg/g) than Flanders (188.6 ± 17.8). The levels of **1** in CDC Bethune and Somme were similar. The concentrations of **2**–**9** were reported previously by others for flaxseed.[4,29] With the exception of **2**, **8**, and **9**, concentrations of these peptides (**3**, 37 μg/g; **4**, 15 μg/g; **5**, 58 μg/g; **6**, 8 μg/g; and **7**, 24 μg/g) were lower than the average concentrations we determined but similar to lower concentrations measured.[4,29] We did not observe appreciable concentrations of **2**. The concentration of **4** reported in the literature was within the variation determined in this study (15 vs 12–43 μg/g).[4] The concentration of **5** also showed significant variability, where CDC Valour shared similar results with the literature report (56 vs 58 μg/g), whereas other varieties had higher levels of **5**.[4] Equal or higher concentrations of **6** and **7** were also found in this study than in previous literature (8–17 vs 8 μg/g and 24–51 vs 24 μg/g, respectively).[29] The levels of **3**, **6**, and **7** in all of the analyzed samples were higher than those published previously. This observation may be due to methionine oxidation of **2**, **8**, and **9** or genetic and/or environmental differences among different flaxseed cultivars.

Analysis of cyclolinopeptides was complicated by methionine oxidation. According to the literature, methionine can be transformed to oxidized forms (methionine sulfoxide and methionine sulfone) by chemical and biological means.[30,31] Hydrogen peroxide proved to be effective in oxidizing methionine in an acid environment.[31] The superoxide anions produced in oxidative metabolism in biological systems could oxidize methionine to the sulfoxide.[32] Brühl et al.[13] reported an increase in the mean content of **5** in flaxseed oil recovered from 21 cultivars stored over 150 days due to oxidation. The mean value of **5** increased from 24 to 760 mg/kg (mean of 21 cultivars) over the storage period. In all of the samples analyzed, the methionine-containing peptides, including **2**, **8**, and **9**, were not observed. Using standard **2** we found that the extraction procedure did not oxidize methionine in this peptide (data not shown). It is likely that **3**, **6**, and **7**, were produced before oil extraction.

High intraspecific variation of secondary metabolites might be the other reason for the difficulty in measuring cyclolinopeptide levels in multiple samples from the same flaxseed cultivar. Unlike primary metabolites (such as protein, carbohydrate, and lipid) that are indispensable, uniform, and conserved for plant growth and development, secondary metabolites (such as flavonoids, lignans, cyclolinopeptides) are often unique, diverse, and adaptive to their environment.[33] The variance in cyclolinopeptide concentration in different flaxseed cultivars has not been thoroughly studied, except for the study of **5** changes in oil during storage.[13] The levels of **5** in that study showed great variance from 0 to 53 mg/kg among flaxseed cultivars at the beginning of the study. After a 150 day storage period, the levels of **5** increased in 21 cultivars to between 485 and 925 mg/kg without other obvious changes noted in the flaxseed oil.[13] The large intraspecific variance of **5** content at the beginning of the study and the corresponding increase in **5** in the sample over time indicated uncertainty in measuring cyclolinopeptide in flaxseed oils, especially where single samples and measurements were considered.

Translation of Cyclolinopeptide from Two Genes. A whole genome shotgun assembly of *Linum usitatissimum* L. (var. CDC Bethune) has been published online by Wang et

Table 4. Cyclolinopeptide Levels in Field-Grown Seed from Different Flaxseed Cultivars

	concn of cyclolinopeptides[a] (μg/g)						
cultivar ($n = 4$)[b]	**1**	**3**	**4**	**5**	**6**	**7**	total
CDC Bethune	62.3 ± 7.9ab	68.4 ± 8.8b	23.5 ± 3.1b	70.1 ± 7.2a	12.4 ± 4.3bc	36.2 ± 3.6bc	272.9 ± 32.9ab
CDC Valour	49.2 ± 8.2ab	61.9 ± 8.9ab	12.8 ± 4.9b	56.0 ± 5.2ab	10.7 ± 3.7bc	30.5 ± 2.5bc	221.1 ± 32.4ab
Flanders	44.0 ± 3.2b	53.5 ± 4.9b	12.4 ± 3.7b	46.4 ± 3.9b	8.3 ± 0.6c	24.0 ± 1.5c	188.6 ± 17.8b
Somme	65.9 ± 5.5a	65.2 ± 6.5ab	42.5 ± 5.0a	61.6 ± 5.1ab	16.6 ± 2.0a	51.0 ± 4.3a	302.8 ± 27.4a
Vimy	54.1 ± 5.2ab	79.7 ± 8.1a	21.1 ± 9.2b	71.3 ± 8.6a	14.5 ± 2.2ab	41.3 ± 5.1ab	282.0 ± 39.4a

[a] Cyclolinopeptide **2** was not detected by HPLC. Values in a column followed by the same letters are not significantly different at $p < 0.05$. [b] Seed samples from two years and two locations (2 × 2) were analyzed.

Table 5. Cyclolinopeptide Content[a] of Flaxseed Cultivars Grown at Two Locations for Two Years

cultivar (n = 4)	mean of cyclolinopeptide-51[b] (μg/g)	range (μg/g)	mean of cyclolinopeptide-65[c] (μg/g)	range (μg/g)
CDC Bethune	200.8a	123.6−257.9	72.11b	59.4−109.2
CDC Valour	167.1b	113.2−235.2	54.05c	35.6−75.3
Flanders	152.0c	139.3−208.8	50.45c	38.2−91.1
Somme	192.7a	158.9−243.1	99.15a	91.3−124.4
Vimy	205.1a	142.3−303.5	76.85b	62.6−140.3

[a]Values in a column followed by the same letters are not significantly different at $p < 0.05$. [b]Cyclolinopeptide-51 was the total cyclolinopeptide production (**1**−**3** and **5**) by gene AFSQ01016651.1. [c]Cyclolinopeptide-65 was the total cyclolinopeptide production (**4** and **6**−**9**) by gene AFSQ01025165.1.

al.[23] Searches of the annotated database generated from the sequences have revealed that **1**, **3**, and **5** are included in a predicted precursor protein encoded by a single gene (cyclolinopeptide-51; Supporting Information, Table S5). Similarly, **4**, **6**, and **7** amino acid sequences occur in another predicted precursor protein encoded by another gene (cyclolinopeptide-65; Supporting Information, Table S6) present in the flax genome.[23,24] There is just one copy of the sequence for each of **1**, **3**, and **5** in sequence cyclolinopeptide-51. The gene that includes the motifs for **4**, **6**, and **7** includes one copy of **4** and **6** and three copies of the motif that encodes the **7** amino acid sequence. The amino acid sequences are provided in the order they occur in the peptide gene. This is reminiscent of the situation in *Saponaria vaccaria* L. (Caryophyllaceae) in which similar precursors are encoded, although each precursor contains only a single cyclic peptide sequence in that species.[34] Interestingly, larger cyclic peptides produced by *Oldenlandia affinis* also arise from a peptide that contains multiple precursor sequences.[35]

It was recently discovered that cyclolinopeptides are encoded in individual genes and that several peptides originate from the corresponding preproteins produced from these coding sequences.[21] It is proposed that **1**−**3** and **5** be grouped (cyclolinopeptide-51) for statistical analysis as the products expressed by a single gene sequence, whereas **4** and **6**−**9** are also grouped as the products of gene cyclolinopeptide-65. The SD obtained from Table 4 in the levels of cyclolinopeptides and the wide ranges of cyclolinopeptide-51 and cyclolinopeptide-65 (Table 5) in the same flaxseed cultivars indicated that both genotype and environment played a role in the concentration of cyclolinopeptides observed in flaxseed. Analysis of variance was conducted to determine the effect of cultivar (C), year (Y), location (L), and their interaction on the concentrations of cyclolinopeptide-51 and cyclolinopeptide-65 in flaxseed grown at two locations for two years (Table S7, Supporting Information). Whereas there was no impact of year alone on the concentration of cyclolinopeptide-51 ($p < 0.68$), other effects were all significant ($p < 0.0001$) in contributing to variation. The interaction of C × Y × L (95.97%) suggested that the concentration of peptides in the cultivars responded differently to year for each location. The effects of cultivar, location, year, and their interaction all were significant to the expression of cyclolinopeptide-65. Variance of cyclolinopeptide-51 was mostly caused by C × Y × L interactions, whereas the variance observed in cyclolinopeptide-65 content was contributed by C, L, C × Y, L × Y, and C × Y × L, respectively. The interaction of cultivar and environment on cyclolinopeptide levels is complex, with no obvious pattern between environmental effects such as growth location and climate and the production of cyclolinopeptides in different flaxseed cultivars (data not shown).

Correlation between Cyclolinopeptides. The correlation among cyclolinopeptide concentrations found in flaxseed was evaluated using the data from the study of genotype by environment (n = 20) on peptide levels. These data may be used to determine the relationship of the expression level of cyclolinopeptide in flaxseed. Pearson's product momentum correlation coefficients (r, p) between cyclolinopeptides are listed in Table S8 of the Supporting Information. It is noteworthy that the relationship between individual and total cyclolinopeptides was highly significant ($p < 0.0001$), meaning there was a linear relationship between these compounds. The highest correlations between individual cyclolinopeptides occurred among peptides in the same gene; however, the correlations between **2** and the other peptides were weaker [**1** with **3** (r = 0.6575, $p < 0.0001$); **1** with **5** (r = 0.7019, $p < 0.0001$)]. The correlations of cyclolinopeptides within cyclolinopeptide-65 were high [**6** and **7** (r = 0.9959); **4** and **6** (r = 0.8509); and **4** and **7** (r = 0.8694)] and significant (at $p < 0.0001$). In comparison, the relationship between the cyclolinopeptides on cyclolinopeptide-65 and cyclolinopeptide-51 was weaker [**7** with **2** (r = 0.4847, $p < 0.0015$); **7** with **1** (r = 0.5176, $p < 0.0006$); **4** with **1** (r = 0.3999, $p < 0.0106$); **4** with **3** (r = 0.5180, $p < 0.0006$)]. For **3** and **5** in cyclolinopeptide-51 a strong correlation was observed (r = 0.9634, $p < 0.0001$). The observed lower correlations between peptide **1** levels and other cyclolinopeptides may be due to incomplete recovery of **1**, random error, or a real difference in the expression of this compound. The correlation between cyclolinopeptide-51 (**1**−**3** and **5**) and cyclolinopeptide-65 (**4** and **6**−**9**) was significant (r = 0.7439, $p < 0.0001$).

It is generally accepted that plant primary and secondary metabolites arise as products of a multitude of enzymes involved in metabolism. The concentration of any individual metabolite is controlled by a number of factors related to metabolic processes.[36,37] These metabolites make up the metabolome. Enzymes and other protein products are produced as the result of ribosomal translation of mRNA. These compounds would be seen as belonging to the proteome. The concentration of a cyclolinopeptide is determined by transcription, translation, and post-translational modification, typical of components of the proteome.[34] The structure of cyclolinopeptide genes could lead to as many as three peptides being produced by the post-translational cyclization of the prepeptide protein cyclolinopeptide-51 (Table S5 of the Supporting Information) and three peptides being produced from the cyclization of cyclolinopeptide-65 (Table S6 of the Supporting Information). Oxidation of methionine in these peptides will increase the number of peptides produced. Although there is no reason to assume that each translated cyclolinopeptide-51 or -65 would produce three cyclolinopeptides, respectively, lower numbers of cyclolinopep-

tides arising from sequence post-translational modification are possible. Nevertheless, the strong correlation between the cyclolinopeptides arising from the same gene may prove to be important in understanding how peptide concentration is controlled. Additional research may shed light on the post-translational modification of peptides and lead to approaches to improve flaxseed either as a platform for peptide production or as a source of oil with improved drying properties and flavor.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental tables: solvent program for cyclolinopeptide identification and quantification by HPLC (Table S1); climatic conditions from the Saskatoon airport (nearest weather station to plots) in 2006 (Table S2); climatic conditions from the Saskatoon airport (nearest weather station to plots) in 2008 (Table S3); long-term average climatic conditions from the Saskatoon airport (nearest weather station to plots) (Table S4); nucleotide and protein sequence of cyclolinopeptide-51 embedded with **1** (ILVPPFFLI), **3** (MLIPPFFVI), and **5** (MLVFPLFI) (Table S5); nucleotide and protein sequence of cyclolinopeptide-65 embedded with **4** (MLLPFFWI), **6** (MLMPFFWV), and **7** (MLMPFFWI) (Table S6); analysis of variance for concentration of cyclolinopeptide-51 and cyclolinopeptide-65 of flaxseed grown at two locations for two years (Table S7); and correlation coefficients between cyclolinopeptides in flaxseeds (Table S8). Supplemental figure: schematic diagram of cyclolinopeptide isolation by solid phase extraction on silica gel (Figure S1). Supplemental file: electrospray ionization mass spectrometry spectra of standard cyclopeptides (File S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

kDa, kilodalton; Seg-A, segetalin-A; SPE, solid phase extraction; EtOAc, ethyl acetate; MeOH, methanol; DCM, dichloromethane; EST, expressed sequence tag; SD, standard deviation; HPLC, high-performance liquid chromatography; CV, coefficient of variation.

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