Hevein-like peptides in plants : discovery, characterization and molecular diversity

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**HEVEIN-LIKE PEPTIDES IN PLANTS:
DISCOVERY, CHARACTERIZATION AND
MOLECULAR DIVERSITY**

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SCHOOL OF BIOLOGICAL SCIENCES

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Hevein-like Peptides in Plants: Discovery, Characterization and Molecular Diversity

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Dedicated to Amma and Annu

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Abbreviations

<i>A. alternata</i>	<i>Alternaria alternata</i>
<i>A. brassiciola</i>	<i>Alternaria brassiciola</i>
<i>A. niger</i>	<i>Aspergillus niger</i>
<i>A. sessilis</i>	<i>Alternanthera sessilis</i>
ACN	Acetonitrile
AEP	asparaginyl endopeptidase
AMP	antimicrobial peptide
BLAST	basic local alignment search tool
<i>C. lunata</i>	<i>Curvularia lunata</i>
cDNA	complementary deoxyribonucleic acid
COSY	correlation spectroscopy
CRP	cysteine-rich peptide
C-tail	C-terminal tail
D2O	deuterium oxide
DCM	Dichloromethane
DTT	dithiothreitol
DTT	Dithiothreitol
EDTA	ethylenediaminetetracetic acid
ER	endoplasmic reticulum (signal peptide)
ER	endoplasmic reticulum
EST	expressed sequence tags
EtOH	Ethanol
<i>F. oxysporum</i>	<i>Fusarium oxysporum</i>
FA	Formic acid
GlcNAc	N-acetylglucosamine
HPLC	high-performance liquid chromatography
IAA	Iodoacetamide
IC ₅₀	half maximal inhibitory concentration

K_D	dissociation constant
kDa	kilo Dalton
<i>M. oleifera</i>	<i>Moringa oleifera</i>
MALDI-TOF MS	matrix-assisted laser desorption/ionization time of flight mass spectrometry
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NH ₄ HCO ₃	Ammonium bicarbonate
NMR	Nuclear Magnetic Resonance
NOESY	nuclear overhauser effect spectroscopy
ORF	open reading frame
PCR	polymerase chain reaction
<i>R. solani</i>	<i>Rhizoctonia solani</i>
RACE	rapid amplification of cDNA ends
RP	reversed-phase
SCX	strong cation exchange
tblastn	translated nucleotide BLAST
TFA	Trifluoroacetic acid
UPLC	Ultra-performance liquid chromatography
<i>V. dahliae</i>	<i>Verticillum dahliae</i>

Abstract

Hevein-like peptides belong to a family of chitin-binding, cystine-knotted peptides. Based on the number of cysteine residues in hevein-like peptides they can be divided into three sub-classes, namely, 6C-, 8C- and 10C- hevein-like peptides. The objectives of my thesis are to isolate and characterize novel hevein-like peptides and to study their distribution and evolution in plants. From two herbs, *Alternanthera sessilis* and *Moringa oleifera*, six novel 6C-hevein-like peptides and three novel 8C-hevein-like peptides were isolated and purified. In addition, we gained insights into their chitin-binding interaction, anti-fungal activity and biosynthesis pathway.

Using an *in silico* approach, a total of 385 novel hevein-like peptides were found in 124 plant families including bryophytes and gymnosperms. In conclusion, my thesis expands the existing knowledge of hevein-like peptides regarding their structure; function and biosynthesis which may help to develop robust anti-fungal agents and transgenic plants with increased specificity and resistance to fungal infections.

Chapter 1

Introduction

1. Cysteine-rich peptides in plant defense

Plants are highly susceptible to microbial invasions and pathogenic attacks. The first line of defense to combat these attacks constitutes the thick cell wall [1]. Pathogens crossing this barrier are tackled by the cellular innate immune system comprised of defense-related chemical compounds such as phytoalexins [2], pathogenesis-related proteins [3], reactive oxygen species and enzyme inhibitors [4]. Inherently expressed plant defense compounds are also known to be beneficial for humans. Since prehistoric times, plants have been used for treatment of a number of ailments and diseases [5]. Long-standing proofs of this principle are traditional folk medicine practices like traditional Chinese medicine (TCM) and Ayurveda. Today, drugs of herbal origin constitute 25% of prescribed drugs worldwide [6]. In fact, out of approximately 120 herbal drugs used today, 96 have a usage identical to their ethnomedicinal use [5]. However, their mechanism of action is still an unsolved mystery.

Efforts have been made to isolate and characterize active principles in medicinal plants which are responsible for their therapeutic effects. Most of the isolated compounds are plant secondary metabolites like tannins, flavonoids and alkaloids which have a molecular weight less than 500 Dalton (Da). Some such popular herbal-small-molecule drugs include aspirin, isolated from the bark of *Salix alba* (willow tree) and *Spiraea salicifolia* and used as an anti-pyretic and analgesic [7]; morphine, an alkaloid from *Papaver somniferum* (opium) widely

used as an analgesic [8] and quinine, isolated from *Cinchona officinalis* trees, used as an anti-malarial drug [9]. Pharmaceutical industries prefer small-molecule drugs as their production is cost-effective and they have high oral bioavailability. However, a major drawback of these drugs is that their small size results in smaller footprint and hence reduced specificity, increased off-target effects and toxicity [10]. Therefore, recent focus has shifted from small molecules to macromolecular therapeutics like peptide biologics [11]. Peptide biologics are defined as proteinaceous compounds with therapeutic potential and a molecular weight above 500 Da [10]. Their large molecular weight confers them a large footprint resulting in high specificity and reduced off-target effects. However, the use of plant biologics as drugs is still debated due to the common notion that proteins and peptides are easily denatured on heating, are readily digested by proteolytic enzymes in the digestive tract and are poorly absorbed through the gut resulting in low bioavailability [12].

Advances in understanding of cysteine-rich peptides (CRPs) in recent years have helped to disprove the above misconceptions. CRPs are present throughout the plant kingdom and can be defined as peptides with a molecular weight between 2 kDa to 6 kDa containing a high number of cysteine residues [13]. CRPs contain an even number of cysteine residues that form intramolecular disulfide bonds in different configurations (Table 1.1) [14]. These intramolecular disulfide bonds render CRPs highly stable against thermal and enzymatic degradation [15, 16].

Table 1.1. Classification of plant cysteine-rich peptides.

Peptide family	Peptide name	Plant species	No. of residues	Cysteine pattern
Plant defensin	Rs-AFP2	<i>Raphanus sativus</i>	45 - 57	[1-9] - C - [8-11] - C - [3-5] - C - [3] - C - [9-11] - C - [4-8] - C - [1] - C - [3] - C
Thionin	α - purothionin	<i>Hordeum vulgare</i>	42 - 47	[2] - CC - [7] - C - [3] - C - [8-10] - C - [3] - C - [1] - C [7] - C - [3-6]
Knottin-type peptides	Mj-AMP1	<i>Mirabilis jalapa</i>	36 - 38	[0-2] - C - [6] - C - [8] - CC- [3] - C - [10] - C - [3]
Cyclotide	Kalata B1	<i>Odenlandia affinis</i>	28 - 37	[3-5] - C - [3] - C - [4-5] - C- [4-7] - C - [1] - C - [4-7] - C - [2-4]
Impatiens	Ib-AMP1	<i>Impatiens balsamina</i>	20	[5] - CC- [8] - C - [3] - C
Hevein-like peptides	hevein	<i>Hevea brasiliensis</i>	29 - 45	[2-6] - C - [1-9] - C - [4-5] - CC- [5] - C - [3-4] - C - [1-5] - C - [2-6] - C - [2-6]

The consensus sequence and cysteine connectivity of each family are summarized from the PhytAMP database [17]. Numbers within brackets indicate the number of non-cysteine amino acid residues in the backbone. The disulfide connectivity is shown by solid lines while the cyclic backbone of cyclotides is indicated by a dotted line.

CRPs are known to have diverse biological roles and are an integral part of the plant defense system. They are expressed in peripheral tissues like leaves and flowers to combat pathogenic attacks. Some CRPs play non-defensive roles like regulation of reproduction, growth and development while others are toxic to insect-larvae [18], inhibit α -amylase [19] or are toxic to mammalian cells [20]. Some examples of CRPs include plant defensins [21], thionins [22] knottin-type peptides [23], cyclotides [24], and impatiens [25]. As a consequence of their diverse pharmacological profiles along with their enhanced resistance to thermal and enzymatic degradation, we hypothesize that CRPs are the active principles responsible for the therapeutic properties of medicinal herbs.

A class of CRPs called hevein-like peptides, comprising 29-45 amino acid residues are rich in glycine and cysteine residues [26]. Hevein-like peptides are known for their ability to bind chitin, a polymer of repeating N-acetylglucosamine units (GlcNAc), linked by β -(1-4)-glycosidic bonds and the primary constituent of fungal cell walls. As a consequence of their chitin-binding property, hevein-like peptides are potent anti-fungal agents against a number of chitin-containing fungi. These hevein-like peptides are the subject of this thesis.

2. Hevein-like peptides

2.1. Discovery of hevein-like peptides

The rubber tree (*Hevea brasiliensis*) is the source of the world's natural rubber with a global production of 12 million tons in 2014 (Fig. 1.1). Rubber is obtained from the latex of this plant which constitutes rubber particles, proteins, lipids and non-rubber particles such as alkaloids, tannins and starch. In 1953, Cook and Sekhar showed that latex was fractionated into three layers on centrifugation. A creamy rubber top layer, a C-serum intermediate layer and a bottom layer comprised of lutoids [27, 28]. Lutoids are vacuolar organelles that make up 12% of the latex and comprise proteins that play an integral role in stress-response, chitin degradation and plant defense [29, 30].

In the early 1950s Archer *et al.* conducted a study to elucidate the protein components of the bottom layer of rubber latex and reported that it comprised of several electrophoretically distinct protein components [20, 27, 28]. In 1960, Archer and his group isolated a 10 kDa anionic non-glycosylated protein from the 'bottom layer' of fractionated latex and reported that it was rich in cysteine, aspartic acid and serine [31]. They named this protein hevein which stands for a protein "ein" isolated from *Hevea brasiliensis* "hev". Later in 1975, Walujono *et al.* sequenced hevein and presented its primary sequence in the International Rubber Conference held in Malaysia [32].

Figure 1.1. Natural rubber tapping from *Hevea brasiliensis* latex

The rubber tree is the major source of the world's natural rubber with a global production of 12 million tons in 2014. Picture adapted from <https://goo.gl/images/hmYqIN>.

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Walujono and group showed that the hevein “protein” isolated by Archer was a dimer of a glycine and cysteine-rich peptide which was 43 amino acid residues in length [32]. The sequence of hevein was homologous to the cysteine/glycine-rich domain of chitin-binding proteins like basic chitinases from cucumber [33], tobacco [34], chitin-binding lectins from wheat [35] and stinging nettle lectin [36]. In 1990, Broekaert *et al.* isolated the cDNA clone (HEV1) of hevein from the latex of *Hevea brasiliensis* [37]. The hevein precursor was encoded by an open reading frame of 1080 bp and was 204 amino acid residues long. The hevein precursor comprised a 17 amino-acid-long signal peptide followed by a 43 amino acid long mature domain encoding for hevein and a long C-terminal tail which was 144 amino acid residues in length. Hevein was found to be homologous to *Urtica dioica* agglutinin (UDA), a lectin that bound specifically to sugars like chitin that contained repeating N-acetylglucosamine units (GlcNAc) and displayed an inhibitory effect against a myriad of chitin-containing fungi *in vitro* [36]. Due to sequence homology, anti-fungal activity of hevein was tested against chitin-containing fungal strains *in vitro* and it was observed that treatment with hevein resulted in inhibition of fungal growth [38]. The anti-fungal activity of hevein was attributed to the presence of a chitin-binding domain distinguishing it from other CRPs like thionins [20], defensins [39] and cyclotides [40].

After its discovery in 1960, the structure of hevein was solved by X-ray crystallography at 2.8 Å and 1.5 Å in 1991 [41] and 2004 [42] including NMR studies in 1993 [43]. These structural analyses showed that the first three disulfide bonds in hevein formed a stable cystine-knot motif and the fourth bond

flanked the C-terminal. The small size and chitin-binding property of hevein made it an interesting candidate to study peptide-carbohydrate interactions. Asensio *et al.* first reported the NMR structure of the complex between hevein and N, N'-diacetylchitobiose (GlcNAc)₂ in 1995. They showed that hevein bound to (GlcNAc)₂ with millimolar affinity and amino acid residues Ser-19, Trp-21, Trp-23 and Tyr-30 were involved in the binding interaction. This binding motif was later termed the chitin-binding domain [44].

Since its discovery in 1960, hevein was the only member of this CRP family for 32 years (Fig. 1.2). Later, from 1992-1997, there was a rapid increase in the discovery of peptides like Ac-AMPs and IWF-4 which comprised six cysteine residues and were homologous to cysteine/glycine rich domains of chitin-binding proteins and hevein [45, 46]. These peptides are 29-30 residues in length, bound to chitin in a reversible manner and inhibited the growth of phytopathogenic fungi with a higher potency than hevein and other chitin-binding proteins. These peptides were considered “truncated” variants of hevein since they lacked the fourth disulfide bond at the C-terminal.

Figure 1.2. Timeline of major milestones in the hevein-like peptide family. Hevein, the prototypic member of the family, was first discovered in *Hevea brasiliensis* in 1960 and was sequenced in 1975. The cDNA of hevein was cloned in 1990 shortly after which the 3D-structure was solved by X-ray crystallography in 1991. Hevein remained the sole member of this family for 32 years after its discovery in 1960. In 1992, Ac-AMPs were discovered in *A. caudatus* and were considered "truncated" variants of hevein as they comprised six cysteine residues. The term "hevein-like peptide" was first coined in 1998 when Pn-AMPs were isolated from *Pharbitis nil.* In 2002, EAFPs, the first 10C-hevein-like peptides, were isolated from *Eucommia ulmoides*. In a span of 10 years from 2005-2015, a total of 15 new CRPs were discovered and the classification of this family into three sub-classes, 6C-, 8C- and 10C-hevein-like peptide was suggested.

The term “hevein-like peptide” was first coined in 1998 by Koo *et al.* who isolated two eight cysteine hevein homologs called Pn-AMPs from *Pharbitis nil* [47]. In 2002, natural variants of hevein that comprise ten cysteine residues were isolated from *Eucommia ulmoides* and named EAFP-1 and EAFP-2 [48]. NMR analysis of EAFP1 showed that it comprised similar cysteine connectivity as hevein with an additional disulfide bond connecting the C-terminal to the cysteine core. Thus, the hevein-like peptide family is a highly diverse family in terms of its cysteine connectivity. Since 2003 the library of hevein-like peptides was expanded by the addition of 12 novel peptides. In a recent review published by our laboratory, we have classified hevein-like peptide into three sub-classes, namely 6C-, 8C- and 10C-hevein-like peptides based on their cysteine content [49]. Thus far there are a total of ten 6C-hevein-like peptides, five 8C-hevein-like peptides and five 10C-hevein-like peptides reported. Due to the diversity and widespread distribution of hevein-like peptides we hypothesize that they are potentially one of the chemical constituents that are constitutively expressed in plants.

2.2. Sequence diversity of hevein-like peptides

Hevein-like peptides contain 29-45 amino acid residues with strictly conserved cysteine and glycine residues. Thus far, four 6C-hevein-like peptides have been isolated and characterized, namely, two Ac-AMPs from *Amaranthus caudatus* [45], one IWF-4 from *Beta vulgaris* [46] and one Ar-AMP from *Amaranthus retroflexus* [50]. 6C-hevein-like peptides contain 29-30 amino acid residues and six cysteine residues.

The cysteine-spacing pattern of 6C-hevein-like peptides is $X_3\mathbf{C}\text{-}X_4\mathbf{C}\text{-}X_4\mathbf{CC}\text{-}X_5\mathbf{C}\text{-}X_6\mathbf{C}\text{-}X_{1-2}$, where X represents non-cysteine amino acid residues and the number represents the number of amino acid residues. The residues between two cysteines are termed a “loop” and named according to the preceding cysteine. The majority of hevein-like peptides including hevein contain eight cysteine residues. Currently, five 8C-hevein-like peptides reported are hevein, two Pn-AMPs from *Pharbitis nil* [47] and two Fa-AMPs from *Fagopyrum esculentum* (Buckwheat) [51]. These hevein-like peptides contain 37-44 amino acid residues with a cysteine-spacing pattern of $iX_{2-6}\mathbf{C}\text{-}X_{1-9}\mathbf{C}\text{-}X_{4-5}\mathbf{CC}\text{-}X_5\mathbf{C}\text{-}X_6\mathbf{C}\text{-}X_{1-5}\mathbf{C}\text{-}X_{2-6}\mathbf{C}\text{-}X_{2-10}$.

In 10C-hevein-like peptides, modifications in the position of cysteine residues result in three distinct cysteine-spacing patterns which we have designated Type I, Type II and Type III. Type I, $X_2\mathbf{C}\text{-}X_3\mathbf{C}\text{-}X_3\mathbf{C}\text{-}X_4\mathbf{CC}\text{-}X_5\mathbf{C}\text{-}X_6\mathbf{C}\text{-}X_4\mathbf{C}\text{-}X_1\mathbf{C}\text{-}X_1\mathbf{C}\text{-}X_2$ was observed in Ee-CBPs from *Euonymus europaeus* [52]. Type II, $X_2\mathbf{C}\text{-}X_8\mathbf{C}\text{-}X_4\mathbf{CC}\text{-}X_5\mathbf{C}\text{-}X_6\mathbf{CC}\text{-}X_4\mathbf{C}\text{-}X_3\mathbf{C}\text{-}X_2\mathbf{C}\text{-}X_1$ and Type III, $X_{2-3}\mathbf{C}\text{-}X_{4-8}\mathbf{C}\text{-}X_2\mathbf{C}\text{-}X_1\mathbf{CC}\text{-}X_5\mathbf{C}\text{-}X_6\mathbf{C}\text{-}X_{3-4}\mathbf{C}\text{-}X_3\mathbf{C}\text{-}X_{2-3}\mathbf{C}\text{-}X_{2-5}$ were previously reported in WAMPs isolated from *Triticum kiharae* [53] and EAFPs isolated from *Eucommia ulmoides* [48], respectively. This promiscuity of the cysteine residues in 10C-hevein-like peptides may lead to functional diversity among the 10C-hevein-like peptide family. Similar observations have been made in defensins and conotoxins where minor modifications in the primary sequence result in alterations of defensin activity and specificity of conotoxins for distinct ion channels, respectively [54, 55].

Based on the cysteine spacing pattern of 6C-, 8C- and 10C-hevein-like peptides they can be divided into four, six and seven loops, respectively (Table 1.2). In

6C-hevein-like peptides each loop is four to five residues in length. Sequence comparison shows that Asn and Arg in loop 1, Pro in loop 2, Ser, Phe, in loops 3 and Tyr in loops 3 and 4 along with all Gly residues are absolutely conserved. The C-terminal comprises a conserved Gly and Arg residue while the N-terminal shows greater sequence diversity. In 8C-hevein-like peptides, loop 1 is the longest loop and is eight residues in length while the length of loop 5 is highly variable (3-5 residues). A conserved Glu residue is present in loop 1 which may play a role in stabilizing the framework of 8C-hevein-like peptides by forming hydrogen bonds with neighboring residues. A Leu residue in loop 2 and Ser, Glu, Trp and Gly residues in loop 3 are absolutely conserved. The sequence of loop 4 differs considerably from 6C-hevein-like peptides but comprises a conserved Tyr residue that is known to be crucial for chitin-binding activity. Loop 5 is the most variable while loop 6 shows higher sequence conservation and comprises a consensus sequence CQS(N/Q)C. The 10C-hevein-like peptides show the highest sequence variation and comprise a conserved Leu and Glu in loop 2 and loop 6, respectively (named according to Ee-CBP) (Table 1.2).

A striking feature that distinguishes the hevein-like peptide family from other CRPs is the presence of a hevein domain [56] or chitin-binding domain [57] between loops 3 and 4. Sequence alignment of the reported 6C-, 8C- and 10C-hevein-like peptides shows that the binding motif is conserved throughout the hevein-like peptide family (Table 1.2).

Table 1.2. Consensus sequences of reported hevein-like peptides.

Peptide	Peptide Sequence						
Loop	1	2	3	4	5	6	7
			* * *		*		
<i>Ac-AMP2</i>	VGEC	-VRG---	RCPSGMCC	SQFGY	CGKGPKY	CG	-----
<i>Ar-AMP</i>	AGEC	-VQG---	RCPSGMCC	SQFGY	CGRGPKY	CGR	-----
<i>IWF-4</i>	SGEC	NMYG---	RCPPGYCC	SKFGY	CGVGRAY	CG	-----
<i>Hevein</i>	-EQC	GRQAGGKL	CPNNLCC	SQWGW	CGSTDEY	CSPDHNC	QSNCKD--
<i>Fa-AMP</i>	-AQC	GAQGGGAT	CPGGLCC	SQWGW	CGSTPKY	CG--AGC	QSNCK---
<i>Pn-AMP</i>	-QQC	GRQASGRL	CGNRLCC	SQWGY	CGSTASY	CG--AGC	QSQCRC--
<i>EAFP-1</i>	-QTC	ASRCP-RPC	NAGLCC	SIYGY	CGSGNAY	CG-AGNC	RCQCRG--
<i>Ee-CBP</i>	-QQC	GRQAGNRR	CANNLCC	SQYGY	CGRTNEY	CCTSQGC	QSQCRRCG
<i>Wamp-1a</i>	AQRC	GDQARGAK	CPNCLCC	GKYGF	CGSGDAY	CG-AGSC	QSQCRCG-

Sequences of reported hevein-like peptides are aligned and they cysteine residues are highlighted in yellow. Amino acid residues between two cysteine residues are termed a loop and numbered according to the first cysteine residue in each loop. The conserved chitin-binding domain is indicated by asterisk.

The chitin-binding domain is a motif comprising a conserved serine residue, one glycine residue and three aromatic residues present in the sequence S-X-(F/W/Y)-X-(F/W/Y)-C-G-X₄-Y organized around a stable core of three to five disulfide bonds [26, 58]. Multiple repeats of the chitin-binding domain are found in proteins like wheat germ agglutinin (WGA) [59], *Urtica dioica* agglutinin (UDA) [36] and several plant chitinases where the chitin-binding domain is fused to an unrelated catalytic domain [60]. It was speculated that chitinases bound to chitin-rich fungal cell walls by virtue of the chitin-binding domain, eliciting anti-fungal effect. However, peptides like hevein [31], its natural variant, pseudohevein, with six amino acid alterations [61] as well as all 6C-, 8C- and 10C-hevein like peptides comprise a single chitin-binding domain which is not fused to a catalytic domain and are more potent anti-fungal agents than their protein counter-parts (Fig. 1.3). The chitin-binding domain is also of medical interest as it is the major allergen involved in the latex-fruit syndrome [62]. The latex-fruit syndrome is an increased sensitivity to fresh fruits like banana, avocado and water chestnuts in individuals who are allergic to natural rubber latex. It is hypothesized that cross-reactivity of allergens occurs as IgEs recognize the chitin-binding domain of Class I chitinases in fruits due to its similarity to hevein found in latex [63]. Thus, studies have been done to understand the structural features of the chitin-binding domain to gain insight into the distinct conformational epitope responsible for allergic reactions [64, 65].

Figure 1.3. Schematic representation of primary structure of hevein-like peptides, Class I chitinases and *Urtica dioica* agglutinin (UDA). Hevein-like peptides comprise a single chitin-binding domain, class I chitinases comprise a single chitin-binding domain fused to a catalytic chitinase domain and UDA consists of multiple repeats of the chitin-binding domain linked to a catalytic domain.

2.3. Structure of hevein-like peptides

The secondary structure of hevein-like peptides is comprised of a coil- β 1- β 2-coil- β 3 motif which varies in the number of short turns in the two long coiled regions [49]. This arrangement differs from other CRPs like defensins and thionins which comprise a CS $\alpha\beta$ and γ -fold structural motifs, respectively [49]. The C-terminal of hevein-like peptides generally comprises a short helical segment [66]. The central β -sheet of the hevein motif is formed by two anti-parallel β -strands and the core is stabilized by disulfide bonds [49]. The conserved cysteine residues form a highly stable knotted topology called the cystine knot motif [67]. The first two disulfides (Cys I-IV and Cys II-V) form a ring through which the third bond (Cys III-VI) penetrates (Fig. 1.4). Interestingly, this cystine-knot motif is widespread in a variety of phyla including animals, plants and fungi and is highly prevalent in venoms of spiders, scorpions and cone snails [68].

The 6C-hevein-like peptides are the smallest hevein-like peptides that can elicit anti-fungal effects on a myriad of chitin-containing fungi. The structure of these peptides contains two anti-parallel β -strands which form the main central β -sheet, an N-terminal coil region and a C-terminal helical coil. Two disulfide bonds link the N-terminal coil region to the two central β -strands while the third disulfide bond connects the C-terminal coil to the first β -strand (Fig. 1.5 A). This structural framework allows the residues involved in chitin-binding to be exposed on the surface [69].

Figure 1.4. Cystine-knot motif. The motif has an embedded ring formed by two disulfide bonds between Cys I- Cys IV and Cys II- Cys V. A third disulfide bond between Cys III- Cys VI penetrates through this ring resulting in a knotted motif. The disulfide bonds are indicated by thick lines while dotted lines represent the peptide backbone. 'X' represents any non-cysteine amino acid

Comparison with 6C-hevein-like peptides shows that 8C-hevein-like peptides contain an additional loop at the C-terminal formed by the fourth disulfide bond. This loop constitutes the third β -strand in the antiparallel β -sheet formed in 8C-hevein-like peptides and is parallel to the fourth disulfide bond. Therefore the structure of 8C-hevein-like peptides is comprised of a central β -sheet formed by three antiparallel β -strands. (Fig. 1.5 B).

The 10C-hevein-like peptides contain five disulfide bonds but vary in the position of the fifth disulfide bond. Examples include EAFP1 and EAFP2 isolated from the bark of *Eucommia ulmoides* Oliv. [48], WAMP-1a and WAMP-1b isolated from *Triticum kiharae* seeds [53] and Ee-CBP from *Euonymus europaeus* [70]. A striking feature that distinguishes 10C-hevein-like peptides from 8C-hevein-like peptides is the presence of an additional disulfide bond within the molecule. The 10C-hevein-like peptides are the most structurally diverse sub-class of hevein-like peptides as their 3D-structure varies depending on the position of the fifth disulfide bond. NMR and X-ray crystallography of EAFP2 revealed that it contains a 3_{10} helix (Cys3-Arg6), an α -helix (Ala27-Cys31) and three anti-parallel β -sheets (Cys16-Ser18, Cys23-Ser25 and Cys35-Cys37). Residues 11-30 of EAFP1 constitute a hydrophobic patch in the structure and include the chitin-binding domain.

Figure 1.5. NMR structures and scheme of binding interaction. Tertiary structures of (A) Ac-AMP2 (63), (B) hevein (34), (C) EAFP-2 (66) and (D) WAMP-1a (68). The secondary structure is depicted by different colors, cyan- α -helix, magenta- β -sheet, yellow- disulfide bonds. Figure adapted from J.P Tam *et al.* (40).

Disulfide mapping indicated that the first four disulfide bonds of EAFPs are similar to hevein and the fifth disulfide bond connects N-terminal Cys 7 with Cys 37 at the C-terminal [71]. This connection of the N- and C-termini results in the rigidification of the conformation with the presence of a cationic surface. The amphiphilic nature of EAFP1 can be attributed to the presence of the cationic and hydrophobic patches in the structure. Structural analysis of EAFP2 revealed that the three Tyr residues of the chitin-binding domain are exposed to the solvent resulting in the formation of a hydrophobic pocket around the Ser residue. Hydrogen bonding between this Ser residue and the sugar further stabilize the orientation of the Ser for efficient binding to chitin oligosaccharides [72] (Fig. 1.5 C). The fifth disulfide bond of Wamp-1a connects the C-terminal to loop 1 of the structure resulting in a cysteine motif similar to chitinases [73] (Fig. 1.5 D). Similar to EAFP1 the fifth disulfide bond brings the positively charged residues of the N- and C-termini in close proximity resulting in a cationic surface [53].

2.4. The Hevein-Chito-Oligosaccharide complex

2.4.1. Active site involved in the binding interaction

The small size and easy availability of hevein and hevein-like peptides using isolation and purification [31], molecular biology [65] and chemical synthesis [74] makes them ideal candidates to study their chitin-binding interaction. Structural and thermodynamic data of the complex between chitin oligosaccharides (GlcNAc)₁₋₅ and hevein [75], pseudohevein [76], WGA [77], Ac-AMP2 [78], Hev32 (a truncated hevein mutant) [79] and Hev32S19D (a hevein-32 mutant

comprising a Ser19 to Asp19 mutation) [80] have been obtained using techniques like isothermal titration calorimetry (ITC) [66, 81], NMR spectroscopy [44, 66], X-ray crystallography [42] and ultracentrifugation [58]. These studies have unequivocally shown that aromatic residues occupying the chitin-binding domain stabilize the complex with chitin through CH- π stacking interactions and van der Waals contacts. A sequence logo was obtained by aligning the chitin-binding domains of 6C-, 8C- and 10C-hevein-like peptides to illustrate the sequence conservation and relative frequency of appearance of each amino acid at a specific position. Amino acid residues occupying the chitin-binding domain were numbered from I to V, based on their position in the sequence logo (Fig. 1.6 A). The serine residue at position I is conserved in all hevein-like peptides except WAMP-1b, where serine is replaced by glycine. In the second position, an aromatic amino acid is preferred with higher occurrence of Phe in 6C-hevein-like peptides, Trp in 8C-hevein-like peptides and Tyr in 10C-hevein-like peptides. Any aromatic amino acid is favored at position III while glycine and tyrosine at positions IV and V, respectively are absolutely conserved in all hevein-like peptides. X-ray and NMR studies on complexes of WGA, hevein and Ac-AMP2 with chitin oligosaccharides have clearly indicated the presence of two binding subsites, namely subsite +1 and subsite +2 at the structural level. The aromatic residues at position III and position II of the chitin-binding domain comprise subsite +1 and subsite +2, respectively (Fig. 1.6 A). Hydrogen bonding between Ser I, named according to the Weblogo of the chitin-binding domain, and an acetamide moiety of one GlcNAc residue stabilizes the complex.

Figure 1.6. Sequence logo of the chitin-binding domain and scheme of binding interaction. (A) Amino acid residues in positions labelled I to V are involved in the binding interaction with chitin oligosaccharides. Residues at position I, III and V are part of subsite +1 and position II is a part of subsite +2. (B) A hydrogen bond between Ser-19 of HEV32 and the acetamide moiety and Tyr-30 with OH-3 of GlcNAc stabilize the binding interaction. A stacking interaction between the aromatic ring of Tyr-30 and the acetamide moiety further stabilizes the complex. Scheme adapted from M. Chavez *et al.* (63)

Another hydrogen bond between conserved Tyr V of the chitin-binding domain and OH-3 of the same GlcNAc unit was found to be important in the complex between WGA and chitin oligosaccharides [80, 82-84] (Fig. 1.6 B). Ser I and Tyr V of the chitin-binding domain are also part of subsite +1 and are important for binding. Thermodynamic data from ITC experiments have revealed that hevein binds chitin oligosaccharides with a K_a of $8,500 \text{ M}^{-1}$ at 303K and the binding process is enthalpically driven (Table 1.3). The binding interaction was not calcium-dependent and the negative free energy (ΔG^0) and enthalpy (ΔH^0) values are indicative of van der Waals forces and hydrogen bond interactions [44].

2.4.2. Effect of mutations on carbohydrate binding

A number of studies have been done to understand the effect of mutation on the binding of hevein-like peptides to chitin oligosaccharides. To gain an insight into the role of the C-terminal of hevein in binding, Hev32, a hevein mutant comprising a C-terminal truncation of 10 residues was chemically synthesized by Aboitiz *et al.* Comparison of ΔG^0 values from calorimetry experiments of hevein and Hev32 with $(\text{GlcNAc})_6$ showed that C-terminal truncation of hevein leads to a loss of $3.6 \pm 0.7 \text{ kJ/mol}$ in ΔG^0 . This data suggests that there is no significant loss in binding affinity when the C-terminal of hevein is truncated. However, vant Hoff plots show that Hev32 has a larger ΔH^0 value compensated for by increased change in entropy (ΔS^0).

Table 1.3. Association constants (K_a) and thermodynamic parameters of the binding interaction of hevein-like peptide and chitin oligosaccharides

	K_a [M^{-1}]				Thermodynamic Parameters		
					ΔG	ΔH	ΔS
	T = 298 K	T = 303 K	T = 308 K	T = 313 K	[$kJmol^{-1}$]	[$kJmol^{-1}$]	[$kJmol^{-1}$]
Hevein*(ITC)		8,500			-22.6	-34.7	-41.4
Hevein*(NMR)	11,500	8,700	6,900	5,700	-23.0	-34.7	-44.7
Hevein**(NMR)	-	474,000	-	-	-32.6	-46.1	-45.0
Hev32*(NMR)	7,700	4,200	3,400	2,200	-21.8	-62.6	-136.0
Hev32S19D**(NMR)	2,800	2,400	2,200	1,400	-19.7	-33.7	-46.4
Hev32S19D*(NMR)	-	2,400					
Hev32S19D*** (NMR)	150	110	90	80	-11.8	-26.2	-47.6
Ac-AMP2*(NMR)				1,000			

K_a and thermodynamic parameters between hevein, Hev32, Hev32S19D, Ac-AMP2 with (GlcNAc)₃ (*), (GlcNAc)₅ (**) and GlcNH₂β(1-4) (GlcNAc)₄ (***) by NMR and ITC. K_a and thermodynamic parameters adapted from Chavez *et al.* and Verheyden *et al.* (73, 75).

This increased ΔS^0 value could be attributed to a reduced flexibility of the side chains of amino acid residues upon binding, due to the C-terminal truncation. Thus, Aboitiz *et al.* named the N-terminal 32 residues as the 'minimum hevein domain' required for effective binding to the chitin oligosaccharides [79]. Since Ser and aromatic residues occupying the chitin-binding domain are primarily involved in binding to chitin oligosaccharides, effect of mutations in each of these positions on chitin-binding was studied. To quantify the energy value of the hydrogen bond between Ser I and the acetamide moiety of the sugar, Chavez *et al.* mutated the Ser residue of Hev32 to Asp (Hev32S19D), as it is of the same chain length as Ser but lacks the side-chain hydrogen bond donor group (Fig. 1.7). Calorimetry experiments showed that there was a drastic decrease in the binding affinity of Hev32S19D with (GlcNAc)₃ as compared to Hev32. This decrease could be attributed to the elimination of the hydrogen bond between the peptide and the acetamide moiety of the sugar. Due to the loss of the hydrogen bond, there is not enough driving force for the rearrangement of the Trp II residue for efficient CH- π stacking interactions. Thus, it can be concluded that the intermolecular hydrogen bond between Ser I and the carbonyl of the acetamide group on the sugar is crucial for efficient binding interactions [80].

The relevance of Trp II in the chitin-binding domain of hevein, was analyzed by Asesnio *et al.* who performed ¹H-NMR titration and ITC experiments of pseudohevein, a natural mutant of hevein comprising a Tyr residue at position II of the chitin binding domain instead of Trp [76].

Figure 1.7. Effect of mutation on chitin-binding interaction. Mutation of the Ser-19 residue to Asp-19 prevents the formation of a hydrogen bond with the acetamide moiety, thus drastically affecting the binding affinity with GlcNAc. Scheme adapted from M. Chavez *et al.* (63)

Significant changes in chemical shift of a number of peaks in ^1H -NMR spectra indicated the formation of complexes between the sugar and pseudohevein. Comparison of ITC data for hevein and pseudohevein titrated with $(\text{GlcNAc})_3$ revealed a difference of $<1\text{kJ/mol}$ in ΔH^0 values. This data suggests that there is no substantial change in binding affinity when Tyr replaces Trp II of the chitin-binding domain. In Ac-AMP2, position II is occupied by a phenylalanine residue.

Muraki *et al.* replaced Phe II with non-natural amino acid residues like 18- β -(cyclohexyl)alanine (Cha18), 18- β -(p-nitrophenyl) alanine (pNO2-Phe18) and 18- β -(pentafluorophenyl) alanine (F5-Phe18) which resulted in enhanced, unchanged and reduced binding affinity, respectively when compared with wild type Ac-AMP2 [74]. Replacement of each of the aromatic amino acid residues occupying the chitin-binding domain with an Ala residue resulted in reduction of affinity in the order Tyr III>Tyr IV>Phe II [74]. This data further validates that the stacking interaction between the aromatic side-chain and the apolar carbohydrate surface is essential for efficient binding to occur [85].

2.4.3. Multivalent nature of the binding interaction

From NMR and ITC experiments it was established that hevein-like peptides bind to chitin oligosaccharides with millimolar affinity. Initial modeling experiments on WGA showed that GlcNAc residues occupying the center of the polymer chain of chitin oligosaccharides would be sterically hindered and not be involved in binding [80, 82-84]. It was therefore suggested that hevein-like peptides bound chitin oligosaccharides only at the terminal non-reducing end of the sugar in a 1:1

stoichiometry. Computer modelling of the complex between hevein and (GlcNAc)₄ by Asensio *et al.* showed that the reducing end of the sugar is not bound by the peptide and is completely exposed to the solvent [58]. However, this hypothesis could not be used to explain the efficient recognition and binding of hevein-like peptides to the long chitin chain. Asensio *et al.* reported that the use of longer chitin chains (GlcNAc)_n, where $n > 2$ are more appropriate models to gain insight on the location of the sugar in the binding site. They reported that from $n=1$ to $n=3$ a one-order-of-magnitude increase in binding affinity was observed [58]. This increase could be attributed to the fact that longer chitin chains provide more number of binding sites. Thus, this data suggests that more than one hevein molecule can bind to the sugar, thereby refuting the 1:1 stoichiometry hypothesis. This result was further validated by assessing the molecular weights of hevein-chitin oligosaccharide complexes with different peptide:ligand ratios by analytical ultracentrifugation. For chitin oligosaccharides upto $n=4$, the chain length is too short to allow multiple binding sites, therefore 1:1 complexes were observed. However, when (GlcNAc)₅ and (GlcNAc)₈ were used, 2:1 and to an extent 3:1 peptide:sugar complexes were observed. To further validate these results, titration experiments of hevein with LacNAc were performed. Despite the presence of a terminal GalNAc residue instead of GlcNAc at the non-reducing end, changes in chemical shift were observed indicating that binding can occur at residues other than the non-reducing end of the sugar. Thus, it can be concluded that hevein binds chitin in a multivalent fashion, the binding process is enthalpically driven and is mediated by hydrogen bond interactions

and van der Waal's contacts between the sugar and the residues of the chitin-binding domain [77].

2.5. Biosynthesis of hevein-like peptides

In plants, hevein-like peptides are ribosomally synthesized peptides encoded by dedicated genes as linear precursors. These precursors are then processed to produce the mature hevein-like peptides. Insight into the distribution, evolution and precursor organization of hevein-like peptides has been obtained by genomic and transcriptome data analysis. Sequence alignment of precursors obtained by cDNA cloning and transcriptome data mining of 6C-, 8C- and 10C-hevein-like peptides reveal that the overall precursor organization is conserved (Fig. 1.8 A) [46, 47, 50, 70, 86-89]. The precursors are comprised of three domains, an N-terminal signal peptide domain, followed by a mature CRP domain and a C-terminal tail. This precursor organization suggests that hevein-like peptides are ribosomally synthesized and their biosynthesis involves the secretory pathway [90, 91]. In the secretory pathway, the N-terminal signal peptide directs the precursor peptide to the endoplasmic reticulum where the signal peptide is cleaved by SPaseI followed by cleavage of the C-terminal tail by an endopeptidase and subsequent release of the mature peptide (Fig.1.8 B). Hevein-like peptide precursors differ from CRPs like cyclotides that comprise an N-terminal pro-domain before the mature peptide domain [92-94].

An interesting feature of hevein-like peptide precursors is the presence of a C-terminal tail. The cDNA of hevein was cloned in 1990 and comprised a 144-

amino-acid-long C-terminal tail homologous to the Barley wound-inducing protein (Barwin) [88]. In 1993, the gene for Ac-AMP2 was cloned by De Bolle *et al.* who found that the C-terminal tail of the Ac-AMP2 precursor showed no sequence homology to any known proteins in the Swiss-Prot databank [86]. It was found that the C-terminal tail of Ac-AMP2 showed similar features as the carboxyl-terminal of Gramineae lectins and tobacco glucanase [86, 95]. These features include the presence of an N-glycosylation site [45], amino acid residues with short hydrophobic side chains and a valine residue eight amino acid residues ahead of the glycosylation site [96]. The carboxyl terminal of Gramineae lectins is known to be involved in the sorting of peptides to be transported to the vacuoles, thus it was speculated that the C-tail of Ac-AMP2 may play a similar role [86]. However this hypothesis was later disproved by De Bolle and group who studied the processing, sorting and intracellular localization of recombinant Ac-AMP2 in transgenic tobacco leaves [97]. Similarly, precursors of 6C- and 8C-hevein-like peptides, Ar-AMP, IWF-4 and Pn-AMPs, respectively, comprised C-terminal tails bearing no homology to any known proteins domains [46, 50, 98]. Cloning of the cDNA of Ee-CBPs, 10C-hevein-like peptides from *Euonymus europaeus* revealed that they are expressed as class-I-chitinase-like precursors and post-translational cleavage of the C-terminal chitinase domain yields the mature Ee-CBP peptide [52, 70]. However, in WAMPs, 10C-hevein-like peptides from *Triticum kiharae*, the C-terminal type-I chitinase domain observed in Ee-CBPs is absent and the C-terminal tail bears no homology to any known conserved domains.

Figure 1.8. Biosynthesis of hevein-like peptides. (A) Sequence alignment of precursors of 6C-, 8C- and 10C-hevein-like peptides showed that the precursor organization is conserved and comprises a signal peptide, followed by a mature domain and a C-terminal tail. (B) Putative biosynthesis pathway of hevein-like peptides. ER- endoplasmic reticulum, ECM- extra-cellular matrix.

The promiscuity of the precursors of hevein-like peptides suggests that they are a highly diverse class of CRPs and further analysis of their precursor organization and biosynthesis may be useful in predicting their evolutionary relationship.

2.6. Anti-microbial activity of hevein-like peptides

The anti-fungal activity of hevein was studied by Van Parijs *et al.* due to its sequence homology with UDA, a carbohydrate binding protein that could inhibit the growth of chitin-containing fungi. Although hevein lacks the chitinase domain, it could inhibit the growth of eight chitin-containing fungi with IC₅₀ in the range of 90-1250 µg/ml. This anti-fungal activity of hevein was retained upon heat treatment and resulted in morphological changes like the formation of thick hyphae with buds [38]. 6C-hevein-like peptides, Ac-AMP2, IWF-4 and Ar-AMP are relatively more potent inhibitors of fungal growth when compared to hevein (Table 1.4). Treatment with IWF-4 resulted in the formation of shorter, more branched hyphae as compared to the control cultures [46]. The anti-fungal property of Ac-AMP2 and IWF-4 was resistant to heat treatment but was antagonized in the presence of calcium ions [45, 46]. Ar-AMP (4-32 µM) had a protective effect on barley seedlings inoculated with *Helminthosporium sativum*, a fungus responsible for root rot [50].

Interestingly, 8C-hevein-like peptides like Pn-AMPs isolated from *Pharbitis nil* could inhibit the growth of chitin-containing fungi, *oomycetes* (chitin-free fungi) and gram-positive bacteria [47]. Similarly, Fa-AMPs isolated from *Fagopyrum*

esculentum could inhibit the growth of gram-positive and gram-negative bacteria apart from chitin-containing fungi [51].

The 10C-hevein-like peptides, EAFPs from *Eucommia ulmoides* Oliv., could inhibit the growth of chitin-containing fungi with IC₅₀ in the range of 18-155 µg/ml [48] while Ee-CBPs from *Euonymus europaeus* and WAMPs from *Triticum kiharae* were more potent than Ac-AMP2 in inhibiting phytopathogenic fungi and could also inhibit gram-positive bacteria [53, 99]. The anti-fungal activity of Ee-CBPs was similar to hevein and was inhibited in the presence of cations but was resistant to heat and resulted in morphological changes like bulbing, branching and stunted growth of hyphae accompanied by spore release.

Although the fungal growth inhibition property of hevein-like peptides is known, the mechanism of action is still not clear. Currently, three proposed mechanisms of action have been suggested. Initially, Van Parijs *et al.* speculated that since hevein is localized in the vacuole-derived luteoid bodies of the plant cells, it might be secreted with a cascade of lytic enzymes if a fungal pathogen breaks through the plasma membrane and enters the cell. As a consequence of its small size and ability to bind to chitin, hevein could then penetrate through fungal cell walls and inhibit growth by binding to nascent chitin chains and interfering with the “steady state” of hyphal growth [38, 100]. Subsequently, hevein’s chitin-binding property could also create an imbalance between chitin-synthesis and hydrolysis of pre-formed chitin chains, which is essential for growth of hyphae. However, this mechanism is not sufficient to explain the inhibitory activity of Pn-AMPs and other hevein-like peptides against non-chitin-containing fungi.

Table 1.4. Summary of anti-fungal activity (IC₅₀) of hevein-like peptides.

Fungi Peptide	IC ₅₀ (µg/ml)							
	Ac-AMP2	IWF-4	Hevein	Pn-AMP1	Fa-AMP1	Ee-CBP	EAFP1	WAMP1a
<i>Alternaria brassicola</i>	4	-	-	-	-	3	-	-
<i>Ascochyta lycopersici</i>	-	-	-	-	-	-	155	-
<i>Ascochyta pisi</i>	8	-	-	-	-	-	-	-
<i>Bipolaris sorokiniana</i>	-	-	-	-	-	-	-	5
<i>Bortrytis cinerea</i>	8	-	500	16	-	1	-	20
<i>Circospora beticola</i>	-	2	-	-	-	-	-	-
<i>Coleotrichum langenarium</i>	-	-	-	10	-	-	-	-
<i>Coleotrichum gossypii</i>	-	-	-	-	-	-	35	-
<i>Fusarium culmorum</i>	2	-	600	-	-	3	-	-
<i>Fusarium oxysporum</i>	-	-	1250	10	19	15	46	5
<i>Fusarium moniliforme</i>	-	-	-	-	-	-	56	-
<i>Fusarium solani</i>	-	-	-	-	-	-	-	5
<i>Geotrichum candidum</i>	-	-	-	-	36	-	-	-
<i>Phycomyces blakeseeleanus</i>	-	-	300	-	-	-	-	-
<i>Phytophthora capsici</i>	-	-	-	5	-	-	-	-
<i>Phytophthora parasitica</i>	-	-	-	3	-	-	-	-
<i>Pyricularia oryzae</i>	-	-	500	-	-	-	-	-
<i>Saccharomyces cerevisiae</i>	-	-	-	14	-	-	-	-
<i>Septoria nodorum</i>	-	-	500	-	-	-	-	-
<i>Rhizoctonia solani</i>	-	-	-	26	-	25	-	-
<i>Trichoderma hamatum</i>	3	-	90	-	-	100	-	-
<i>Verticillium dahliae</i>	8	-	-	-	-	-	-	-

Ac-AMP2 (36), IWF-4 (37), hevein (29), Pn-AMP1 (38), Fa-AMP1 (64), Ee-CBP (42), EAFP-1(39) and WAMP-1a (41). The IC₅₀ is represented in µg/ml and ‘-’ indicates that the peptide has not been tested against the fungal strains.

Koo *et al.*, proposed the second mechanism of action based on confocal studies which showed that Pn-AMPs penetrate through the fungal cell walls at growing tips of hyphae and septa within 15 minutes of incubation and result in breakage of the cytoskeleton and subsequent release of cytoplasmic material [47]. This ability to penetrate through the fungal cell wall could be a consequence of their highly basic pI (12.02) and is a suitable explanation for the broad-spectrum anti-fungal activity of highly basic hevein-like peptides. In a recent study by Slavokhotova *et al.*, on WAMPs, a novel mode of action involving inhibition of fungal metalloproteases was reported. Due to sequence homology of WAMPs with class I/IV chitinases, their susceptibility to cleavage at the Gly-Cys site of the chitin-binding domain by the fungal metalloprotease FV-cmp was tested. Surprisingly, it was found that WAMPs were not only stable to degradation by the enzyme but could also inhibit its activity against chitinases. This inhibitory activity was attributed to the presence of a Ser residue between the Gly-Cys cleavage site in WAMPs that could prevent recognition by the enzyme. It was proposed that WAMPs bind to the fungal metalloprotease, thus preventing its binding to the plant chitinase which can then degrade the fungal cell wall and inhibit fungal growth [101].

Although the mechanism of action against chitin-free fungi is not known, the anti-bacterial activity of hevein-like peptides could be attributed to the composition of the cell wall of gram-positive bacteria. The peptidoglycan layer accounts for approximately 90% of the dry weight of gram-positive bacteria. The composition of peptidoglycan is similar to chitin and comprises repeating units of N-

acetylglucosamine and N-acetylmuraminic acid linked by β -(1-4)-glycosidic bonds interspersed with oligopeptide chains. It has been speculated that the peptidoglycan layer may assist in the docking of anti-microbial peptides on the bacterial cell surface subsequently causing membrane permeabilization [102]. The chitin-binding domain of hevein-like peptides can bind to N-acetylglucosamine of the peptidoglycan layer leading to accumulation of the peptide on the surface subsequently causing loss of membrane integrity and cell death. Together, this data shows that hevein-like peptides have diverse modes of action against microbes based on their sequence, net charge and cell-wall composition of the pathogens. More studies need to be done to better elucidate the mechanism involved in hevein-like peptide defense against fungi to develop potent anti-fungal agents.

3. *Alternanthera sessilis* and *Moringa oleifera*

A program to discover novel CRPs, with a focus on hevein-like peptides was initiated in our laboratory. During our screening process we came across two herbs, *Alternanthera sessilis* and *Moringa oleifera* which have a myriad of health benefits and exhibited an abundance of hevein-like peptides (Fig. 1.9).

A. sessilis is a perennial herb belonging to the Amaranthaceae family and is a rich source of vitamin A, iron, dietary fibre [103], β -carotene [104], palmitic, myristic and linoleic acid [105]. *A. sessilis* has also been used in folk medicine to treat a myriad of ailments. In Ayurveda, *A. sessilis* is used to treat itchy and inflamed skin [106] and in local Taiwanese medicine it is used to treat hepatitis, bronchitis and asthma. A boiled decoction of the leaves and shoots are used as an anti-hypertensive remedy [107]. Kumar *et al.* reported that the aqueous and ethanolic extracts of *A. sessilis* have anti-diabetic effects [108]. Subhashini T *et al.* clinically evaluated the anti-inflammatory effects of the chloroform and petroleum leaf extracts of *A. sessilis* and showed that the chloroform extract at 200 mg/kg was more effective than the petroleum extract [109]. This combination of dietary and medicinal benefits is the reason why *A. sessilis* is an important herb in Indian folk medicine, where 'Food as Medicine' is the major principle [110].

Figure 1.9. Aerial parts of plants selected for the study. (A) *Alternanthera sessilis* var. green (B) *Alternanthera sessilis* var. red (C) *Moringa oleifera*.

Moringa oleifera belongs to the flowering plant family Moringaceae and is commonly known as the drumstick tree or the horse-radish tree [111, 112]. It was once native to the foothills of the Himalayas but is now cultivated globally especially in tropical and sub-tropical areas [113, 114]. *M. oleifera* leaves are a rich source of vitamin C, calcium, potassium, β -carotene and natural antioxidants [111]. The stem-bark, flowers, seeds and roots are edible and nutritious, comprising compounds such as alkaloids, potassium, calcium, ascorbic acid and antioxidants [115, 116]. Since *M. oleifera* is rich in nutrients it is commonly referred to as the “Miracle Tree” to combat malnutrition around the world [117]. Aqueous and ethanol extracts of *M. oleifera* leaves are known to be anti-inflammatory, antimicrobial, anti-diabetic, anti-ulcer and have cholesterol lowering and blood pressure stabilizing effects [118].

The medicinal and nutritional benefits of *A. sessilis* and *M. oleifera* have been attributed to small-molecules thus far. Since the plant extracts were boiled before studying their medicinal properties, we speculate that the bioactive principles in these herbs could be heat-stable proteins or peptides like CRPs that are in high abundance.

4. Hypothesis and Objectives of this study

Hevein-like peptides are a highly diverse class of chitin-binding peptides that play an integral role in plant defense against microbes and insect pathogens. Therefore, insights into their sequence diversity, structure, function and biosynthesis will be beneficial for the development of peptide-based anti-fungal agents.

The aims of my thesis are:

1. To isolate and characterize novel 6C-hevein-like peptides from two varieties of *Alternanthera sessilis*
2. To isolate and characterize novel 8C-hevein-like peptides from *Moringa oleifera*
3. To study the distribution and diversity of hevein-like peptides in plants using EST-based bio-informatics approaches

This work describes the discovery of novel 6C- and 8C-hevein-like peptides from *A. sessilis* and *M. oleifera*, respectively. Genomic and transcriptomic analysis showed that the precursor organization is conserved among hevein-like peptides. It was shown that the binding affinity of 6C-hevein-like peptides to chitin oligosaccharides is comparable to hevein and aromatic residues in the chitin-binding domain are involved in binding. Both 6C- and 8C-hevein-like peptides could inhibit the growth of chitin-containing phytopathogenic fungi. Knowledge of the bioactivity and gene organization may be useful in the development of transgenic plants with enhanced resistance against fungi.

An insight into the distribution of hevein-like peptides in plants was obtained using EST-based data-mining in NCBI and OneKp databases. Interestingly, a total of 385 novel hevein-like peptides were found in 124 different plant families. These peptides were expressed in mosses, the first terrestrial plants on Earth and their expression has been retained in highly evolved angiosperms till date. This data shows that hevein-like peptides could be the largest family of plant CRPs and are widespread across the plant kingdom due to their integral role in defense against fungal pathogens.

Chapter 2

Materials and Methods

1. Materials

1.1. Chemical reagents

All the chemicals and reagents used in this study were of analytical or molecular biology grade and purchased from the following companies:

Acetic acid	Merck
Acetonitrile (ACN)	Fisher
Agarose	Bio-Rad
Ammonium bicarbonate (NH_4HCO_3)	Sigma-Aldrich
C18 media	Grace Davison Discovery Sciences
Dichloromethane (DCM)	Merck
Dithiothreitol (DTT)	Sigma-Aldrich
dNTP nucleotide mix	Fermentas
Ethanol (EtOH)	Merck
Formic acid (FA)	Sigma-Aldrich
Iodoacetamide (IAM)	Sigma-Aldrich
Isopropanol	Fisher
Methanol	Merck
Sodium chloride	Sigma-Aldrich

1.2. Kits

The kits for molecular cloning were purchased from Invitrogen (Life Technologies, USA), Qiagen (USA), Clontech (Takara Bio, Japan), and Promega (USA).

1.3. Plant materials

Alternanthera sessilis (green) and *Alternanthera sessilis* (red) were obtained from Singapore botanic gardens and grown in Herb garden of School of Biological Sciences, NTU, Singapore. *Moringa oleifera* was purchased from local markets in Singapore.

1.4. Fungal strains

Seven phyto-pathogenic fungal strains were acquired from China Center of Industrial Culture Collection, including *Curvularia lunata* (CICC 40301), *Fusarium oxysporum* (CICC 2532), *Aspergillus niger* (CICC 2089), *Verticillium dahliae* (CICC 2534), *Rhizoctonia solani* (CICC 40259), *Alternaria alternata* (CICC 2465) and *Alternaria brassiciola* (CICC 2646).

2. Genomics

2.1. RNA extraction

Plant material was homogenized using liquid nitrogen. TRIzol® Reagent (Life Technologies) was added to the homogenized sample and incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complex. Chloroform (0.2 ml) was added sample was incubated for 3 minutes followed by centrifugation at 12,000 rpm for 15 minutes. Equal volume of

isopropanol and 25% salt solution (1.2 M sodium chloride + 0.8 M sodium acetate) were added to the RNA containing aqueous phase and incubated at room temperature for 10 min to allow precipitation of RNA. The samples were then centrifuged at 12000 rpm for 10 min and the RNA pellet was washed with 75% ethanol (1 ml). The pellet was dried and resuspended in diethylpyrocarbonate (DEPC) water (30 µl). Concentration and purity of extracted RNA was measured using NanoPhotometer™ (Implen, Northstar Scientific, Germany).

2.2. DNA extraction

Samples were homogenized in liquid nitrogen. CTAB buffer (500 µl) was added to 50-60 mg of homogenized sample and incubated in a water bath at 55°C for 1 h with intermittent shaking. After incubation, chloroform (500 µl) was added to the samples followed by centrifugation at 16000 x *g* for 7 minutes. The aqueous layer was collected and 0.08 volumes of cold 7.5 M ammonium acetate and 0.54 volumes of cold isopropanol were added and incubated on ice for 30 minutes. The sample was centrifuged at 16000*g* for 3 minutes and the pellet was washed twice with 75% ethanol. After the pellet was dried it was resuspended in DEPC water (30 µl) and DNA concentration and integrity were determined using NanoPhotometer™ (Implen, Northstar Scientific, Germany) and 1.0% agarose gel electrophoresis.

2.3. Rapid amplification of cDNA ends (RACE) and PCR analysis

Total RNA extract was used as a template to generate the 3'-cDNA library using 3'RACE (rapid amplification of cDNA ends) system (Invitrogen) according to the manufacturer's protocol. Degenerate primers were designed using GeneRunner software and were used with universal adaptor primers (UAP/AUAP) to amplify the desired sequence using a T100 thermal cycler (BioRad). The PCR products were run on a 1% agarose gel and the target bands were excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned with pGEM®-T Easy Vector System (Promega) using JM109 high-efficiency competent cells. Sequencing of insert containing plasmids was performed using 1st Base Company's service. Degenerate primers targeting regions PQC�NHG (5'-CCTGGTCArTGyAAyCAyGG-3') and QGYCGTG (5'-CAAGGTTATTGyGGnACnGG-3') were used in the 3'-RACE polymerase chain reaction (PCR). Specific primers 5'-AAACACATGCCCAACAAAGAG-3' and 5'-AATAATTATAACATACAGTGTTAT-3' were designed against the 3'UTR region of the partial sequences obtained from 3'RACE and primed against the 5'cDNA obtained using SMARTer™ RACE cDNA Amplification Kit (Clontech). The complete protocol from PCR to sequencing was performed to obtain complete sequences of the transcripts of interest.

To determine presence and location of introns, specific primers were designed from obtained cDNA sequences and primed against a DNA template. Forward primers were designed from the 5'UTR or ER signal peptide and reverse primers from 3'UTR. The PCR set up was as follows: initial denaturation at 94°C for 5

min, main amplification for 35 cycles (denaturation at 94°C for 30 sec, annealing at $T_m - 3^\circ\text{C}$ for 30 sec, elongation at 72°C for 45 – 60 sec), final elongation at 72°C for 10 min, and cooling to 4°C. PCR products were tested on a 1.5% agarose electrophoresis, purified, cloned into pGEM-T Easy vector and sequenced as described.

2.4. Sequence analysis

Sequencing results were analyzed using the BioEdit software. ExPasy translate tool (<http://www.expasy.ch/tools/dna.html>) was used to predict the amino acid sequences of the clones. Prediction of endoplasmic reticulum (ER) signal sequences of the precursor sequences was performed using SignalP 4.0 prediction server (<http://www.cbs.dtu.dk/services/SignalP/>) [119, 120]. Logo sequences were built with WebLogo application, version 3 (<http://weblogo.berkeley.edu/>) [121].

3. Proteomics

3.1. HPLC and UPLC analysis

Shimadzu systems were used for high performance liquid chromatography (HPLC). Reverse-phase-HPLC (RP-HPLC) was performed using Phenomenex C18 columns (particle size, 5 μm ; pore size, 300 Å; Hesperia, CA, USA) with dimensions of 250 x 22 mm, 250 x 10 mm, and 250 x 4.6 mm for preparative, semi-preparative and analytical HPLC at a flow rate of 5 mL/min, 3mL/min and 1 mL/min respectively. A polyLC polysulfoethyl A column (250 x 9.4 mm and 250 x 4.6 mm) was used for strong cation exchange (SCX)-HPLC with dimensions of

250 x 9.4 mm and 250 x 4.6 mm run at flow rates of 3 and 1 mL/min flow rates, respectively.

3.2. MALDI-TOF MS and MS/MS

Mass spectrometry analysis was performed using the ABI 4800 MALDI-TOF/TOF system (Applied Biosystems, Framingham, MA, USA). A saturated solution of CHCA in 60% ACN, 0.05% TFA was used as the matrix for matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and MS/MS. Samples were mixed in a 1:1 ratio (v/v) with the matrix and spotted on the target plate.

The reflectron mode with laser intensity set between 3000-4000 was used to scan both MS and MS/MS spectra. Average spectra for MS and MS/MS were obtained from 1000 and 5000 shots with an accelerating voltage of 20 kV and 8 kV respectively.

3.3. Protein extraction and purification

Fresh leaves (5-10 kg) of *Alternanthera sessilis* and *Moringa oleifera* were obtained from the Nanyang Technological University (Singapore) herb garden and homogenized in water (1:1 ratio). Debris was filtered using a muslin cloth and the filtrate was centrifuged at 8000 rpm for 10 min at 4°C. After centrifugation, the supernatant was concentrated, filtered and loaded on a C18-flash column. Increasing concentration of ethanol (20%-70%) was used for elution from the column. Presence of desired peptides in the eluents was confirmed by MALDI-TOF MS and these fractions were pooled and purified

using several dimensions of RP-HPLC and SCX-HPLC. A linear gradient from buffer A (5% ACN, 20 mM KH_2PO_4 ; pH 3) to buffer B (5% ACN, 0.5 M KCl, 20 mM KH_2PO_4 ; pH 3) was used for SCX-HPLC. Fractions from SCX-HPLC that contained desired peptides were pooled and purified by RP-HPLC using buffer A (0.1% TFA in water) and buffer B (0.1% TFA in 100% ACN).

3.4. De novo sequencing with MALDI-TOF MS/MS

Approximately 50 μg of purified peptide was dissolved in 100 mM ammonium bicarbonate (NH_4HCO_3) buffer and was incubated with 50 mM dithiothreitol (DTT) at 37°C for 45 min to reduce disulfide bonds. The reduced disulfides were alkylated with iodoacetamide (IAM) (300 mM) for 2 h at 37°C. Digestion of alkylated samples was done at room temperature for 5 min with trypsin, chymotrypsin, and endo-GluC digestion. *De novo* sequencing of peptides was performed based on both b- and y-ion series in the tandem mass spectrometry (MS/MS) profiles.

3.5 LC-ESI-MS/MS analysis

The reduced-alkylated peptide sample was desalted using Millipore ZipTips and lyophilized. The peptide was re-dissolved in 0.1% formic acid (FA) before MS analysis. A Dionex UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled with an Orbitrap Elite mass spectrometer (Thermo Scientific Inc., Bremen, Germany) was used to perform LC/MS-MS analysis. Elution was performed over a 60 min gradient from eluent A (0.1% FA) to eluent B (90% ACN/0.1% FA). The LTQ Tune Plus software (Thermo Fisher Scientific,

Bremen, Germany) was used to set the mass spectrometer to positive mode for data acquisition using. A Michrom's Thermo CaptiveSpray nanoelectrospray ion source (Bruker-Michrom, Auburn, USA) was used to generate the spray. A Full FT-MS (350-2000 m/z, resolution 60.000, with 1 μ scan per spectrum) was alternated with Full FT-MS and a FT-MS/MS scan applying 27%, 30% and 32% normalized collision energy in high-energy collisional dissociation (110-2000 m/z, resolution 30.000, with 2 μ scan averaged per MS/MS spectrum) for data acquisition where three intense ions with a charge greater than +2 and a mass difference of 3 Da were isolated and fragmented. Source voltage of 1.5 kV and capillary temperature of 250°C were used. Automatic gain control was set to 1×10^6 for full scan-MS and MS/MS. PEAKS studio (version 7.5, Bioinformatics Solutions, Waterloo, Canada) was used to process data from LC-MS/MS analysis with parent error tolerance and a fragment error tolerance of 10 ppm and 0.05 Da respectively.

3.6. Spectrophotometric determination of protein concentration

The Beer-Lambert law was used to calculate concentrations of purified peptides according to the equation

$$A = \epsilon \cdot l \cdot c$$

Where,

A : the absorbance at 280 nm of peptide solution in miliQ water measured on the Nanophotometer (Implemen, Germany)

ϵ : molar absorption coefficient ($M^{-1}cm^{-1}$)

l : cell path length (cm)

The theoretical ϵ value of a protein at 280 nm was calculated as follows [122]:

$$\epsilon_{280} = (5500 \cdot n_{\text{Trp}}) + (1490 \cdot n_{\text{Tyr}}) + (125 \cdot n_{\text{SS}})$$

where n_{Trp} : the number of Trp residues

4. Structural analysis

4.1. NMR spectroscopy

To perform nuclear magnetic resonance (NMR) experiments, samples containing approximately 0.5-0.6 mM peptide were prepared in a 20 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl and 0.01% NaN₃ in D₂O or 10% D₂O. Nuclear Overhauser effect spectroscopy (NOESY) experiments were acquired with 200 and 300 ms mixing times [123, 124]. Total correlation spectroscopy (COSY) [125] data were recorded with a mixing time of 69 or 78 ms using MLEV17 spin lock pulses [126]. Vicinal coupling constants were measured using the double-quantum-filtered (DQF)-COSY [127] and one-dimensional (1D)-¹H-NMR experiments. All 2D-NMR data were recorded in the phase sensitive model using the time-proportional phase increment method [128], with 2048 data points in the t₂ domain and 512 points in the t₁ domain. Slowly-exchanging amide protons were identified by immediate acquisition of a series of 1D-experiments after dissolving the lyophilized peptide in a D₂O solution. The water signal was suppressed using water-gated pulse sequences [129] or excitation sculpting [130] combined with pulsed-field gradients. All NMR data were processed using Bruker TOPSPIN 2.1 or NMRPipe [131] programs on a Linux workstation and

analyzed using Sparky 3.12 software. DQF-COSY spectra were processed on 8192 x 1024 data matrices to obtain a maximum digital resolution for coupling constant measurements. Sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS-d6) was used as internal reference.

4.2. Structure calculations

Solution structures of aSG1 were calculated by hybrid distance geometry and a simulated annealing protocol in torsion angle space with CNS 1.2 [132]. The three disulfide bonds were restrained in accordance with the disulfide bonding patterns based on the observed H β -H β NOEs from NOESY spectra by generation of covalent disulfide linkages during the initial molecular topology file generation stage using a CNS script. A total of 597 distance and 18 torsion angle constraints were used for structure calculations. NOE distance restraints were classified strong (1.8-3.0 Å), medium (1.8-3.5 Å), weak (1.8-5.0 Å), or very weak (1.8-6.0 Å) based on intensities derived from NOESY spectra recorded in 10% D₂O or 100% D₂O. To validate the disulfide bond connectivities, structure calculations & refinements were performed for 15 different disulfide bond patterns. Molecular topology files for 15 different disulfide bond connectivities were generated using molecular topology file generating script of CNS. 200 structures were calculated by distance geometry regularization and simulated annealing protocol out of which the lowest 18-20 structures were selected for each disulfide bond combination and average total energies were compared. Corrections for pseudo-atom representations were used for non-stereospecifically assigned methylene, methyl group, and aromatic ring protons

[128]. Backbone dihedral angle restraints were derived from $3J_{HN\alpha}$ coupling constants in DQF-COSY or $1D\text{-}^1H\text{-NMR}$ spectra in a H_2O solution [133, 134]. Backbone dihedral restraints were $-55^\circ \pm 45^\circ$ ($3J_{HN\alpha} < 6$ Hz) and $-120^\circ \pm 50^\circ$ ($3J_{HN\alpha} > 8$ Hz). Hydrogen bond donors were identified from proton-deuterium exchange experiments, and hydrogen bond acceptors were determined in the preliminary structure calculation stage. The 200 starting structures were generated and refined using a hybrid distance geometry-simulated annealing protocol [135-137] in the CNS 1.2 program. Finally, 18 final structures were selected by their total energy values for display and structural analysis. MOLMOL [138] and PyMOL [139] programs were used for structure visualization and PROCHECK-NMR [140] were used for structure validation.

4.3. NMR titration experiments

The binding affinity of aSG1 and aSR1 towards N, N'-diacetyl chitobiose ($GlcNAc$)₂ and N, N', N''-triacetyl chitotriose ($GlcNAc$)₃ was determined by monitoring chemical shifts in $^1H\text{-NMR}$ spectra from a series of titrations. Samples contained 0.6 mM peptide in 20 mM sodium phosphate buffer (pH 7.4) in D_2O , using 100 μM of DSS-d6 as internal reference. Increasing concentrations of chitobiose (0.036-50.8 mM), chitotriose (0.1-4.8 mM), and chitopentose (0.1-4.8 mM) were then titrated while keeping the peptide concentration constant. Differences in chemical shifts of characteristic protons were plotted against sugar concentrations and fitted using Origin 9.0 according to equation (1) [141]:

$$\Delta\delta_{\text{obs}} = \Delta\delta_{\text{max}} \frac{([P]_t + [L]_t + K_D - \{([P]_t + [L]_t + K_D)^2 - 4[P]_t[L]_t\}^{1/2})}{2[P]_t} \quad (1)$$

where $\Delta\delta_{\text{obs}}$ is the change in the observed shift from the free state, $\Delta\delta_{\text{max}}$ is the maximum shift change upon saturation, $(P)_t$ is the total concentration of the peptide, and $(L)_t$ is the total concentration of the sugar [141].

5. Stability Assays

5.1. Heat stability assay

Purified peptides were incubated at 100°C for 1 h and then analyzed by UPLC. Short linear peptides RLYRRGRLYRRNHV (RV-14) and WV-14 (WRLYRGRLYRRNHV) synthesized in our laboratory served as controls and DALK, a thermostable peptide was used as an internal standard. Peaks from UPLC were analyzed by MALDI-TOF MS.

5.2. Proteolytic enzyme stability assay

Purified peptides were incubated with trypsin, pepsin and chymotrypsin at 37°C for 6 h at a final peptide to enzyme ratio of 20:1(mol/mol). At each time point, 50 μ l of the sample was injected in UPLC to assess degradation. RV-14 and WV-14 were used as controls.

5.3. Serum stability assay

Stability of isolated and purified peptides in human serum was assessed according to the protocol by Jenssen *et al.* [142]. Briefly, 25% human serum (Sigma Aldrich-Singapore) was prepared in PBS and incubated at 37°C for 15

min. Purified peptides (100 µg) were added to the temperature-equilibrated serum and incubated at 37°C. At specified time points, 50 µl aliquots of the sample was added to 100 µl of 95% ethanol and incubated at 4°C for 15 min to precipitate serum proteins. The sample was then centrifuged at 18,000 rpm for 2 min to pellet down the precipitated proteins. The supernatant (50 µl) was injected in UPLC and the chromatographs were analyzed to determine presence and extent of degradation. Linear peptides WV-14 and RV-14 were used as controls.

6. Bioassays

6.1. Disc diffusion assay

Susceptibility of seven fungal strains, *Alternaria alternata*, *Alternaria brassiciola*, *Aspergillus niger*, *Curvularia lunata*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Verticillium dahliae* to purified hevein-like peptides from *A.sessilis* and *M.oleifera* were tested using the disc diffusion assay as described by Ye *et al.* [143]. *A. niger* was grown on malt extract agar plates while the remaining fungal strains were grown on potato dextrose agar plates at 25°C. When sufficient mycelial growth was observed, a hole was punched in the fungal culture and was transferred to a new agar plate and incubated for 48 h-72 h at 25°C till a radial mycelial colony was formed. Paper discs (6 mm) inoculated with 20 µl of peptides dissolved in MilliQ water (17.5 µg- 70 µg) were placed at the growing ends of the mycelia at an equal distance of 1 cm and incubated for 24 h at 25°C. Deionized water was used as a negative control. Formation of crescent-shaped inhibition zones indicated susceptibility of fungi to test peptides.

6.2. Microbroth dilution assay

The half maximal inhibitory concentration (IC_{50}) of peptides was assessed using the microbroth dilution assay [144]. Fungal spores were seeded in half-strength potato dextrose broth or half-strength malt extract at a final density of 2.5×10^3 cells/ml. To 80 μ l of the spore suspension, 20 μ l aliquots of different concentration of peptides were added and incubated at 25°C for 24 h. After incubation, the cells were fixed with 100 % methanol for 30 min followed by staining for 30 min with 1% (w/v) methylene blue in 0.01 M borate buffer. Water was used to wash off excess dye and elution was performed using 1:1 (v/v) ethanol/ 0.1N HCl. Absorbance was read at 650 nm using Infinite@ 200 PRO Tecan microplate reader (Tecan Group Ltd, Germany). Control wells were treated with half-strength media. Percentage inhibition was calculated as 100 times the ratio of absorbance of treated samples to control samples. A dose-response curve using the “Log-inhibitor vs response (variable slope)-four parameters” function was computed using GraphPad Prism 6 for Windows GraphPad Software, San Diego California USA, www.graphpad.com.

6.3. Cytotoxicity assay

PrestoBlue™ Cell Viability Reagent (Invitrogen) was used to test the cytotoxicity of purified peptides. Vero (African green monkey kidney cells) were seeded at a density of 5×10^4 cells/ml in a 96-well plate and incubated with 100 μ M of purified peptides for 24 h at 37°C. After incubation, PrestoBlue™ reagent (10 μ l) was added to the wells followed by incubation for 2 h at 37°C. Fluorescence was

measured as prescribed by the manufacturer. 1% triton-X100 was served as the positive control.

6.4. Hemolysis assay

Fresh type A+ blood was donated by a student volunteer. Erythrocytes from the blood (1 ml) were collected by centrifuging at 700 rpm for 15 min. The pellet was then washed with PBS (1ml) five times and centrifuged at 1000 rpm for 5 min. The centrifuged erythrocytes were diluted 100X in PBS to obtain the stock dispersion. Peptide samples were prepared in PBS and added to the stock dispersion in a 1:1 ratio in a 96-well plate and incubated for 4 h at 37°C with gentle shaking. After incubation, the plate was centrifuged at 1000 rpm for 5 min and the supernatants were transferred to a new plate. Absorbance was read at 415 nm in an Infinite@ 200 PRO Tecan microplate reader. Equal volume of 8% Triton-X100 and PBS were used as positive and negative controls, respectively.

7. EST-Based data mining

7.1. Translated nucleotide based search for putative hevein-like peptides

Database searches were conducted using methods modified from literature [145, 146]. tBLASTn was employed to search for ESTs encoding putative hevein-like peptide precursors in two databases, the National Center for Biotechnology Information (NCBI) [147] and the 1000 Plants Project (OneKP), via queries using all known hevein-like peptide sequences [148]. The retrieved sequences were then used as queries in subsequent tBLASTn searches until no novel sequences

were obtained. The maximum target sequence and expected threshold were set at 1000 and 10, respectively. The accession numbers of all the putative hevein-like peptide sequences are provided in the appendix E. Search results were exported to Microsoft Excel, and sequences with coverage lower than 80% were deleted. The remaining sequences were manually selected based on the following criteria: (1) The open reading frame must contain a stop codon following the C-terminal tail; (2) The translated amino acid sequence must not contain any untranslated amino acid residues, such as X; and (3) The mature peptide must contain six, eight or ten cysteine residues. The sequences were then submitted to SignalP 4.0 [149] for identification of signal peptides and were aligned using ClustalW [150]. Replicate sequences from same plants or different species of the same genus sharing an identical full-length precursor were identified using ClustalW Phylogeny and deleted from the dataset. The elimination of Class I chitinases performed by running an InterPro scan using the BLAST2GO PRO software [151].

7.2. Data analysis

Statistical analyses were performed using GraphPad Prism software version 6.01 (GraphPad Software, CA, USA). The data were analyzed by Student's t-test. The results were expressed as the mean $\pm$ standard error of the mean (SEM), where p-values less than 0.05 were considered statistically significant. Aligned sequences were visualized using Weblogo [121]. The phylogenetic tree was constructed and annotated using the online tool called, Interactive tree of life (iTOL) [152].

Chapter 3

Isolation and Characterization of Novel 6C-Hevein-like Peptides from *Alternanthera sessilis*

1. Introduction

The Amaranthaceae family belongs to the order Caryophyllales of flowering plants and is the one of the most species-rich lineage with 180 species and 2500 genera [153]. This family comprises annual or perennial herbs and shrubs and very rarely vines and trees that grow in tropical climate. Plants of this family are rich in secondary metabolites like isoflavonoids, saponins, triterpenoids and betalain pigments [154]. Some Amaranthaceous plants like *Dysphania ambrosioides* and *Dysphania anthelmintica* are used as medicinal herbs. Species like *Amaranthus* and *Celosia* are commonly found in gardens as ornamental plants.

Here we studied two Amaranthaceae species, *Alternanthera sessilis* var. green and *Alternanthera sessilis* var. red which are perennial herbs commonly found in tropical and sub-tropical areas and make up the staple diet in India and Africa [155]. The herbs comprise prostrate stems that grow upto 0.4 to 1.4 m high and often root at the nodes with broadly elliptical leaves that have petioles about 1-5 mm long petioles [156]. As its flowers are shaped like the eyes of a fish it is commonly called Matsyakashi (fish-eyed). Although the medicinal benefits of *A. sessilis* have been documented, most of the biological activities are attributed to the secondary metabolites in the plant extract. A larger chemical space

comprising peptide biologics has not been studied thus far. Previously, closely related plant species like *Amaranthus caudatus* and *Amaranthus retroflexus* have been reported to comprise 3-4 kDa 6C-hevein-like peptides. These peptides could bind to chitin and inhibit the growth of chitin-containing fungi [45, 50]. Thus, we examined if *A. sessilis* also expresses similar hevein-like peptides and studied their role in plant defense.

This chapter reports the isolation and characterization of six novel 2-4 kDa 6C-hevein-like peptides from red and green varieties of *Alternanthera sessilis*. These newly identified peptides were collectively named altides in accordance with the plant's genus name. In order to distinguish individual altides, they were named using the initial alphabets of the genus and species name followed by the variety and a number indicating the order of discovery. For example, aSG1 and aSR1 are altides from green and red varieties of *A. sessilis*, respectively. ¹H-NMR titration experiments with chitin oligosaccharides provide insight into their binding interaction with chitin. The anti-fungal, cytotoxic and haemolytic activities of altides have also been studied. Gene cloning of the peptide precursors has helped shed light on their biosynthesis. Taken together this work provides insight into the sequence, structure, bioactivity and biosynthesis of altides.

2. Results

2.1. Isolation and sequencing of 6C-hevein-like peptides from *A. sessilis*

Small-scale screening of red and green varieties of *A. sessilis* using MALDI-TOF MS revealed a cluster of peptides between 3 and 4 kDa indicating the presence of CRPs (Fig. 3.1). A large-scale extraction of 5-10 kg of *A. sessilis* leaves was performed to isolate the putative CRPs. The plant material was homogenized in equal volume (v/v) of water and centrifuged to remove plant debris. The filtered supernatant was loaded onto a C₁₈ flash column, which was washed repeatedly with 5% ethanol to partially remove colored pigments and hydrophilic compounds. Elution of bound peptides was performed using 40%-70% ethanol, which were then purified by several rounds of SCX- and RP-HPLC.

Isolated and purified peptides were subjected to disulfide reduction (S-reduction) using dithiothreitol (DTT) to free the cysteine residues followed by alkylation of the free cysteines and subsequent digestion with trypsin, chymotrypsin and endo-GluC. The resulting peptide fragments were sequenced by tandem MS (Fig. 3.2). For example, S-reduced aSG1 had an m/z value of 3010, an increase of +6 Da, indicating the presence of six cysteine residues.

Figure 3.1. MALDI-TOF spectra of crude extracts. (A) *A. sessilis* var. green and (B) *A. sessilis* var. red. Clusters of peaks in the range of 2-3 kDa indicate the presence of putative CRPs.

Figure 3.2. De novo sequencing of aSG1. Tandem MALDI-TOF/TOF MS/MS profiles of two tryptic fragments of aSG1 (m/z 996 and m/z 2374) provide the full aSG1 sequence.

One of the limitations of tandem MS sequencing is the difficulty in differentiating isobaric residues like Leu/Ile and Lys/Gln. Thus, assigned sequences were validated using gene cloning studies where genetic sequence of the precursors helps to unambiguously obtain the complete peptide sequence with the added advantage of providing information about the biosynthesis pathway of the peptide.

A total of six novel peptides, collectively named altides were isolated and sequenced. Table 3.1 summarizes the primary sequences of altides aSG1-aSG3 and aSR1-aSR3 from green and red varieties of *A. sessilis* respectively and previously reported 6C-hevein-like peptides. Altides are 29-31 amino acid residues in length and are rich in cysteine (6 residues), glycine (7 residues) and proline (2-3 residues) accounting for ~60% of the peptide sequence. Altide, aSR1 (29 amino acid residues) is the smallest hevein-like peptide isolated thus far. BLAST analysis revealed that altides belong to a family of hevein-like peptides with a single chitin-binding domain and shared ~80% homology with Ac-AMP2 isolated from *Amaranthus caudatus* [45]. The cysteine-spacing pattern $X_{3-4}\mathbf{C}-X_4\mathbf{C}-X_4\mathbf{CC}-X_5\mathbf{C}-X_6\mathbf{C}-X_{1-2}$ is highly conserved among altides. Apart from the conserved cysteines, all seven glycine residues, Arg-9, Pro-13, Ser-17, Tyr-21 and Tyr-28 (numbered according to aSG1) are absolutely conserved throughout the 6C-hevein-like peptide family. In altides isolated from the same variety, the sequence variability of the loops is minimal with difference in only 1 or 2 residues. Sequence comparison of altides from different varieties shows that loop 1 is the most variable while loop 2 is partially conserved and varies in only 1 or 2 residues.

Table 3.1. Consensus sequences of altides aligned with previously reported 6C-hevein-like peptides.

Peptide/ Loop	Peptide Sequence					
	1	2	3	4		
	I	II	III IV	V	VI	
<i>aSG1</i>	APGQC	-NHGRC	PSGLCC	SQYGY	CGTGPAY	CG-
<i>aSG2</i>	-AGEC	-NHGRC	PSGLCC	SQYGY	CGTGPAY	CG-
<i>aSG3</i>	APGQC	-NHGRC	PSGLCC	SQYGY	CGTGPAY	CGG
<i>aSR1</i>	-VGEC	-VQGRCP	PGLC	SRFGY	CGTGPAY	CG-
<i>aSR2</i>	APGEC	-KHGRC	PPGIC	CSQYGY	CGTGPAY	CG-
<i>aSR3</i>	APGEC	-KHGRC	PPGIC	CSQYGY	CGTGPAY	C--
<i>Ac-AMP1</i>	-VGEC	-VRGRC	PSGMCC	SQFGY	CGKGPKY	CG-
<i>Ac-AMP2</i>	-VGEC	-VRGRC	PSGMCC	SQFGY	CGKGPKY	CGR
<i>Ar-AMP</i>	-AGEC	-VQGRCP	SGMCC	SQFGY	CGRGPKY	CGR
<i>IWF-4</i>	-SGEC	NMYGRC	PPGYCC	SKFGY	CGVGRAY	CG-
<pre> I II III IV V VI +-----+-----+-----+-----+ +-----+-----+-----+-----+ </pre>						

The conserved chitin-binding domain is indicated with an asterisk and the cystine-knot motif is depicted at the bottom of the table.

Loop 3 comprises three residues of the chitin-binding domain and is highly conserved in all altides except aSR1 where Gln-18 and Tyr-19 are replaced by Arg and Phe respectively. Interestingly, loop4 which immediately follows the chitin-binding domain is highly conserved in all altides except aSG2 where Ala27 is replaced by Arg. Overall, the sequence of aSR1 is the most variable as it lacks the N-terminal Ala residue and differs from other altides in 6 positions - Pro-2, Asn-6, His-7, Ser-12, Gln-18 and Tyr-19 are replaced by Val-2, Val-6, Gln-7, Pro-12, Arg-18 and Phe-19 respectively. This data shows that despite being from two distinct varieties, altides show some degree of sequence conservation indicating that they may have evolved from a common ancestor.

2.2. Gene cloning of altides

To obtain the full length cDNA sequence of altide precursors, 3'-RACE PCR was performed by designing a degenerate primer targeting the region QGYCGTG. After cloning products of desired size, 5'-RACE PCR was performed using primers designed to the 3'UTR to obtain the full precursor sequence. Two full-length gene sequences encoding for aSG1 and aSG2 were obtained and designated as *asg1* and *asg2* respectively (Fig. 3.3). To study the location of introns, genomic DNA was used as a template in the PCR reaction with specific primers targeting the untranslated region. Sequence comparison of DNA and RACE PCRs showed that no introns are located in the gene.

The deduced amino acid sequences of the full-length precursors of aSG1 and aSG2 are shown in Figure 3.4 A. The 81-amino acid long precursors contain three domains, a 25-amino acid long ER signal peptide followed by the 30-amino

acid long mature CRP domain and a short 25-amino acid long C-terminal tail. The signal peptide and C-tail sequences are highly conserved among aSG1 and aSG2 and differ by only one amino acid residue; Gly-55 (numbered according to aSG1) is replaced by Ser in aSG2. The precursor of aSR1 shares 70% sequence homology with altides from the green variety with variations at seven positions in the signal peptide, six positions in the mature domain and a highly variable C-tail. The presence of an ER signal sequence indicates that altides are ribosomally synthesized and bioprocessing follows the secretory pathway (Fig. 3.4 B).

asg1

5' untranslated region

actacttaacagcggagttatgactttgtttctaaaata
agctctttgttgggcatgtgtttctttatgttgatcttggtttcttatcgattggatg
gagaattaacgtagttgttatttcttctgttatccaatcatcatgttgtagtactcgtt

atgatgaacatgaagaagtttttgatcgttatggtagtgtagcc
M M N M K K F L I V M V V V A
ctagtgatggtggagccatcaatgggagcaccaggtcaatgcaaccatggacgttgcct
L V M V E P S M G A P G Q C N H G R C P
agtgggtgtgtgtgcagccagtatggttactgtggcactggccccgcttattgtggtggc
S G L C C S Q Y G Y C G T G P A Y C G G
gctgccgagcaacgcgctgctcttcttcagcgcaccggtagtgtcaccgcagacactact
A A E Q R A A L L Q R T G S V T A D T T
gacactactaaagctccatga
D T T K A P *

3' untranslated region

agtgttcaagctagaagataagaaaaaaaaa

asg2

actacttaacagcggagttatgactttgtttctaaaataagctct

atgatgaacatgaagaagtttttgatccttatggta
M M N M K K F L I L M V
gtagttagccctagtgtatggtggagccatcaatgggagcaggcgagtgaaccatggacgg
V V A L V M V E P S M G A G E C N H G R
tgccccagtggtgtgtgtgcagccagtatggttactgtggcactggccccgcttattgt
C P S G L C C S Q Y G Y C G T G P R Y C
ggttagcgctgccgagcaacgcgctgctcttcttcagcgcaccggtagtgtcaccgcagac
G S A A E Q R A A L L Q R T G S V T A D
actactgacactactaaagctccatga
T T D T T K A P *

3' untranslated region

tagagtgttcaagctagaagataagaaaaaaaaacaaaaaaaaa

Figure 3.3. Gene sequences of aSG1 and aSG2. The sequence of the signal peptide is in bold and an inverted triangle depicts the cleavage site of signal peptidase as predicted by Signal P 4.0 server. The mature domain is highlighted in blue and the conserved cysteine residues in yellow. From the sequence it is clear that no intron is present in the gene expressing aSG1. An asterisk is present below the stop codon.

Figure 3.4. Biosynthesis of altides. (A) Aligned full length precursors of altides with Ar-AMP, Ac-AMP2 and IWF4. The precursor organization is conserved among 6C-hevein-like peptides and comprises a signal peptide, mature domain and C-terminal tail. The cleavage site of SPase is indicated with an inverted triangle. (B) Bioprocessing of altides follows the secretory pathway.

2.3. Structural analysis of aSG1

The spin-spin systems of aSG1 and approximately 98% of proton resonances were assigned unambiguously. The solution structure of aSG1 was calculated from a total of 597-NMR derived distance restraints and 18 dihedral angle restraints (Fig. 3.5 B). Root-mean square deviation values relative to the mean of 18 conformers for residues Gly3-Gly 30 were 0.28 Å for backbone atoms and 1.11 Å for heavy atoms respectively. Amide proton exchange experiments were performed to determine the sequential $d_{\alpha N}(i,i+1)$ NOE connectivity, $^3J_{HN\alpha}$ values and hydrogen bond patterns. The secondary structure of aSG1 was found to comprise two extended β -sheets and tight turns. The C-terminal region (Pro-26-Gly-30) also comprised several strong $d_{NN}(i,i+1)$ and medium or weak $d_{\alpha N}(i,i+3)$, $d_{NN}(i,i+2)$, and $d_{NN}(i,i+3)$ NOEs indicating the presence of α -helical structure in this region. Thus, the structure of aSG1 comprise two short, anti-parallel β -sheets ($\beta 1$, Cys-15-Cys-16, and $\beta 2$, Gly-20-Gly-24) and one α -helical segment with several tight turns and loops with a well-defined structure based on medium and long-range NOEs. The disulfide connectivity of aSG1 was determined by calculating the structural energies for all 15 possible disulfide bond patterns. While most disulfide patterns showed high energy values from 200 to 760 kcal/mol, three patterns namely, Cys I-Cys IV/Cys II-Cys V/Cys III-Cys VI, Cys I-Cys V/Cys II-Cys IV/Cys III-Cys VI, and Cys I-Cys II/Cys III-Cys VI/Cys IV-Cys V showed low total energy values (30–66 kcal/ mol). However, the lowest energy value of 29.90 ± 0.87 kcal/mol was observed in pattern Cys I-Cys IV/Cys II-Cys V/Cys III-Cys VI indicating that this disulfide pattern is the most favorable

amongst all 15 patterns tested and is in good agreement with $H\beta$ - $H\beta$ connectivities observed from the NMR spectrum. PROCHECK analysis indicated that all residues were distributed in the allowed region of the Ramachandran map (Fig. 3.5 A). The overall topology of aSG1 was that of a compact globular molecule and seemed to be stabilized by several strong hydrogen bonds and three disulfide bridges (Fig. 3.5 C).

Figure 3.5. NMR structure of aSG1. (A) 2D-NOESY spectra showing the expanded aliphatic regions of aSG1 obtained with a mixing time of 300 ms in D₂O at 25 °C. The correlations between cysteines forming disulfide bridges of aSG1 (Cys-5 H β -Cys-16 H β , Cys-10 H β -Cys-22 H β , and Cys-15 H β -Cys-29 H β) are represented by rectangular boxes. (B) Superposition of the backbone traces from the final 18 ensembles of solution structures and restrained energy minimized (REM) structure of aSG1 determined by NMR spectroscopy. The REM structure of aSG1 is colored red. (C) Ribbon representation of aSG1 REM structure. Three disulfide bridges (Cys I-Cys IV, Cys II-Cys V, and Cys III-Cys VI) are shown as balls and sticks.

2.4. Chitin-binding activity

The chitin-binding activity of altides was assessed by incubating purified altides (4 µg) with chitin beads for 4 h at 4°C in chitin binding buffer. UPLC chromatographs showed that altides, aSG1, aSR1 and aSR2 bound to chitin within 1 h (Fig. 3.6). To elute the peptides, two elution methods were tested. The first method involved the use of chitin elution buffers with increasing salt concentration (up to 1 M NaCl) while the second involved heating the peptide-bound chitin beads at 100°C for 30 min and 1 h in the presence of 0.5 M acetic acid [46]. It was observed that none of the altides could be eluted from the beads when up to 1 M NaCl was used while aSG1, aSR2 and aSR1 were eluted in 30 min and 1 h respectively after heating in acidic conditions (Fig. 3.6). These results indicate that altides bind strongly to chitin and require heating and acidic conditions for elution.

Figure 3.6. Chitin-binding activity of altides. Upon incubation with chitin beads for 1 h at 4°C, altides, (A) aSG1, (B) aSR1 and (C) aSR2 bound within 1 h. Elution of the bound peptides was performed in acidic environment and heating at 100°C. (D) aSG1 and (E) aSR1 eluted in 1 h while (F) aSR2 eluted in 30 min.

2.5. ^1H -NMR titration of altides with GlcNAc oligosaccharides

The binding motif and binding constant (K_D) of the interaction between altides and chitin oligosaccharides was deduced by performing ^1H -NMR titration experiments with chitobiose (GlcNAc_2) and chitotriose (GlcNAc_3). Keeping the peptide concentration constant (0.6 mM), increasing concentrations of chitobiose (0.036-50.8 mM) and chitotriose (0.1-4.8 mM) were titrated into the sample and ^1H -NMR spectra were recorded at each titration point.

^1H -NMR spectra of the titration of altides and chitin oligosaccharides showed significant changes in the chemical shifts of a number of peaks indicating the formation of a complex between altides and the oligosaccharides. When aSG1 and aSR1 were titrated with chitobiose and chitotriose, a difference of chemical shifts of the peaks between 7-8 ppm indicated the involvement of aromatic residues in binding (Fig. 3.7). From the assigned spectra of aSG1, it was deduced that the binding motif comprised Tyr-28, Tyr-19, Tyr-21, and Gln-18, which occupy the conserved chitin-binding domain. Similarly, titration of aSR1 with chitotriose showed shifts in peaks between 7-8 ppm indicating that aromatic residues in the chitin-binding region were involved in the binding interaction. The binding motif was deduced as Arg-17, Phe-19, Tyr-21, and Tyr-27 by overlaying the assigned spectra of aSG1 with aSR1 (Fig. 3.8).

The difference of chemical shift values were plotted against sugar concentration and fitted into equation 1 to calculate the K_D of the binding interaction. Interestingly, the K_D of the interaction between aSG1 and chitobiose was 9.6 ± 0.107 mM while K_D of aSR1 and chitotriose was 97 ± 17.8 μ M (Fig. 3.8). This difference of two orders of magnitude can be due to the increase in number of binding sites when chitotriose is used.

Figure 3.7. ¹H-NMR titration study of altides with chitin oligosaccharides. (A) 0.6 mM aSG1 was titrated with increasing concentration of chitobiose at pH 7.4. Variations in protons of tyrosine residues were followed. Sugar concentration is indicated on the left. (B) Increasing concentration of chitotriose was titrated into 0.6 mM aSR1. Dramatic changes in chemical shift and line broadening of peaks corresponding to aromatic residues were observed. Chitotriose concentration is indicated on the left.

Figure 3.8. ¹H-NMR titration of altides with chitin oligosaccharides. (A) ¹H-NMR titration plot of aSG1 with chitobiose. (B) ¹H-NMR titration plot of aSR1 and chitotriose. (C) Overlay of ¹H-NMR spectra of aSG1 and aSR1 indicating the residues involved in the binding interaction.

2.6. Anti-fungal activity of altides

The susceptibility of seven common phyto-pathogenic fungi (*Alternaria alternata*, *Alternaria brassiciola*, *Aspergillus niger*, *Curvularia lunata*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Verticillum dahliae*) to altides was assessed using the disc diffusion assay. Discs of growing fungal mycelia were inoculated on Potato Dextrose Agar (PDA) or Malt Extract (ME) plates and incubated at 25°C for 48 - 72 h till a radial colony was formed. Paper discs (6 mm) were placed at the growing mycelial ends and impregnated with different concentrations of altides and incubated for 24 h at 25°C.

After incubation with 17.5 to 70 µg of aSG1, crescent-shaped inhibition zones were observed in *A. alternata*, *A. brassiciola*, *F. oxysporum* and *R. solani* indicating that aSG1 had an inhibitory effect on the growth of these fungi (Fig. 3.9 A). Altide aSR1 from the red variety of *A. sessilis* could inhibit the growth of *A. alternata*, *A. brassiciola*, *R. solani* and *C. lunata* (Fig. 3.9 B). Altides aSG1 and aSR1 were ineffective against *A. niger* at the tested concentration. Having established the susceptibility of fungal strains to altides, the half-maximal inhibitory concentration was calculated using the micro-broth dilution assay.

Figure 3.9. Anti-fungal activity of altides (A) aSG1 and (B) aSR1. Formation of arc-shaped inhibition zones in the radial disc diffusion assay indicated susceptibility of fungi against altides. The IC_{50} was calculated from the dose-response curve obtained from the microbroth dilution assay.

To obtain IC_{50} of altides against the susceptible fungi, 2.5×10^3 fungal spores were seeded in 96-well plates and incubated with different concentrations of altides for 24 h at 25°C. After incubation, the cells were fixed, stained with 0.1% methylene blue and absorbance was read at 650 nm. The IC_{50} was calculated from a dose-response curve generated by plotting percentage inhibition against peptide concentration (Fig. 3.9). Altide aSG1 demonstrated high potency against *F. oxysporum* with an IC_{50} 3.5 µg/ml while IC_{50} against *A. alternata*, *A. brassiciola* and *R. solani* ranged 11.39 µg/ml to 13.34 µg/ml. *R. solani* was most sensitive to anti-fungal activity of aSR1 demonstrated by a remarkably low IC_{50} of 1.12 µg/ml.

To gain insight into the putative mechanism of anti-fungal activity of altides, 1×10^3 fungal spores were seeded on 12-well slide chambers and incubated with different concentrations of altides for 24 h at 25°C. Bright field microscopy was performed to observe any morphological changes. From figures 3.10 and 3.11 it is observed that, the hyphae are stunted and more branched than the untreated control with a significant retardation of hyphal growth when *A. alternata*, *A. brassiciola* are treated with aSG1 and aSR1. Together this data indicates that altides are anti-fungal agents that inhibit the growth of chitin-containing phytopathogenic fungi by retarding hyphal elongation at growing ends of the mycelia.

Figure 3.10. Hyphal growth inhibition of fungi treated with aSG1. (A) *Alternaria alternata* (B) *Alternaria brassiciola*. Formation of short stunted hyphae indicated that altides can inhibit hyphal growth at the growing tips of fungal mycelia.

Figure 3.11. Hyphal growth inhibition of fungi treated with aSR1. Significant retardation of hyphal elongation was observed when (A) *Alternaria alternata* and (B) *Alternaria brassiciola* were treated with 87.5 µg/ml of aSR1.

2.7. Heat and proteolytic stability

Thermal and enzymatic degradation cause significant loss of peptides and proteins in herbal medicine. To assess the stability of altides to heat and proteolytic degradation, altides were heated at 100°C for 1 h or incubated with trypsin and pepsin for 6 h. Presence and extent of degradation was assessed from the UPLC chromatographs and MALDI-TOF MS. Greater than 90% of altides remained stable to thermal degradation after 1 h while >75% of altides were stable against proteolytic degradation by trypsin and pepsin after incubation for 6 h (Fig. 3.12). In the heat and enzyme stability tests, linear peptides DALK and RV-14 (RLYRRGRLYRRNHV), respectively, synthesized in-house served as controls. From the figure we can see that DALK degraded completely within 1 h of heat treatment and RV-14 within 6 h of incubation with trypsin and pepsin. Together, this data provides satisfactory evidence of the stability of altides against thermal and enzymatic degradation.

Figure 3.12. Stability assays of altides. Thermal stability of (A) aSR1 and (B) aSR2 at 100°C. DALK, a linear peptide synthesized in our laboratory was used as an internal standard. (C) Trypsin stability assay of aSR1. (D) Pepsin stability assay of aSR1. Altide, aSR1 remained stable to thermal and enzymatic degradation.

2.8. Bioactivity assays

Cytotoxic, hemolytic and anti-bacterial effects of altides, aSG1 and aSR1 were studied. In the cytotoxicity assay, Vero cells were treated with upto 100 μ M of altides aSG1 and aSR1 and their viability was assessed using the PrestoBlue reagent. After treatment, it was found that altides did not demonstrate any cytotoxic effect on Vero cells at concentrations upto 100 μ M. The hemolytic effect of altides was studied using human type O erythrocytes with 0.1% Triton X-100 as the positive control. Altides, aSG1 and aSR1 did not display any hemolytic effects upto 100 μ M.

The anti-bacterial activity of altides, aSG1 and aSR1 was tested against gram-positive *Staphylococcus aureus* and gram negative *Escherichia coli* using the radial diffusion assay. Cyclotide, cT8, a potent anti-microbial CRP isolated from *Clitoria ternatea* in our laboratory was used as a positive control [40]. Altides, aSG1 and aSR1 showed negligible anti-bacterial effect at concentrations upto 100 μ M.

3. Discussion

Hevein-like peptides are integral in plant defense against fungi and insect pathogens. They can be divided into three sub-classes, namely, 6C-, 8C- and 10C-hevein-like peptides based on their cysteine content [49]. Thus far, four 6C-hevein-like peptides have been reported, namely, two Ac-AMPs from *A. caudatus* [45], Ar-AMP from *A. retroflexus* [50] and IWF-4 from *B. vulgaris* [46]. In this study we have isolated and characterized six novel 6C-hevein-like peptides from two varieties of *A. sessilis*, expanding the existing library of 6C-hevein-like peptides. Altides are 29-30 amino acid residues in length and adopt the cystine-knot disulfide arrangement previously found in CRPs like cyclotides [40, 157] and cystine knot-type of α -amylase inhibitors (CKAIs) [158]. This knotted topology renders altides highly stable against thermal and enzymatic degradation. Altides bind to chitin oligosaccharides with dissociation constants in the micromolar range with aromatic residues occupying the chitin-binding domain mediating responsible the binding interaction. Altides could inhibit the growth of commonly occurring phyto-pathogenic fungi with IC_{50} in the range of 1.12 μ g/ml to 69.02 μ g/ml and were non-cytotoxic and non-hemolytic to Vero cells and type O erythrocytes respectively.

3.1. Sequence conservation of altides

Sequence comparison of altides within the green variety revealed >89% homologies with aSG1 and aSG3 differing by only one amino acid residue (Gly-31 of aSG3 is absent in aSG1). Altides of the red variety were 85% identical to each other where aSR2 and aSR3 differ by only one amino acid residue (Gly-30 of aSR2 is absent in aSR3). Sequence comparison of altides between red and green varieties showed 75%-90% homology.

Altides shared a sequence homology of 70%-90% with other 6C-, 8C- and 10C-hevein-like peptides from different plant families. Sequence alignment of altides with previously reported 6C-hevein-like peptides shows that the N-terminal is highly variable when compared to the C-terminal. The C-terminal Gly is conserved in all altides and IWF-4 but is replaced by Arg in Ac-AMP2 and Ar-AMP. A common observation in hevein-like peptides is the difference of one amino acid residue at the C-terminal. For example, aSG2 and aSG3 differ by only one glycine residue at the C-terminus. A similar observation was made among aSR2 and aSR3. This phenomenon could be due to different post-translational processing or proteolytic degradation by carboxypeptidase during the purification process [47]. The molecular diversity of altides is evident from our results as none of the loops are absolutely conserved and vary by 2-3 residues even in altides from the same plant species. Except for the six cysteine and seven glycine residues, variations are also observed in the chitin-binding domain where aSR1 comprises a Phe residue instead of Tyr. This variation may have arisen due to a point mutation in the gene encoding aSR1 and is unlikely to affect

the binding interaction with chitin as the aromatic ring of Phe can still facilitate CH- π stacking interactions [79].

Among all altides, aSR1 showed highest homology (79.6%) to Ac-AMP2 isolated from *A. caudatus*. This data suggests that there could be a close evolutionary relationship between altide aSR1 from *A. sessilis* var. red and *A. caudatus*. Although altides, Ac-AMPs, Ar-AMPs and IWF-4 are isolated from plants belonging to the Amaranthaceae family, they show some sequence diversity but retain the same function. This suggests that 6C-hevein-like peptides evolved from a common ancestral gene that is probably retained throughout the Amaranthaceae family due to its integral role in plant defense. Screening more plants from Amaranthaceae and closely-related plant families may help gain further insight into the exact evolutionary relationship among 6C-hevein-like peptides.

3.2. Biosynthesis of altides

The precursor organization of altides comprises three domains - an ER signal domain, a mature CRP domain and a C-terminal tail. This precursor organization is similar to thionins [159] but differs from cyclotides [94, 157, 160], CKAls [158] and ω -conotoxins [161] that comprise an N-terminal pro-domain before the mature domain and lack a C-terminal tail (Fig. 3.13). The presence of a signal peptide indicates that the bioprocessing of altides follows the secretory pathway as suggested for many CRP [91, 162, 163].

This feature distinguishes them from small peptides (5-12 amino acid residues in length) that are synthesized by multi-enzyme complexes [90]. An interesting feature of the precursor is the presence of a C-terminal tail of 25-33 amino acid residues that bears no sequence homology to any known conserved domains in the NCBI database. The role of the C-terminal tail is yet to be understood.

Sequence comparison of altide precursors with Ac-AMP2, IWF-4 and Ar-AMP showed that they were highly homologous with Ac-AMP2 (81%) and Ar-AMP (66%). The precursors of altides were five residues longer than IWF-4 and their signal peptides shared no sequence homology. Due to high sequence identity between the clones of altides, Ac-AMP2 and Ar-AMP it can be presumed that they evolved by gene duplication from the same ancestral gene by divergent evolution. In divergent evolution, a single ancestral gene undergoes variations to help the plant improve its survival and adapt to environmental changes [164].

Knowledge of the gene organization and precursor structure of altides can be used to develop transgenic crops with enhanced resistance to fungal pathogens. The transgenic approach could prevent the use of toxic chemical pesticides that are harmful for human consumption and adversely affect the environment. Thus far, 6C-hevein-like peptides have not been used to develop transgenic crops. However, closely related α -amylase inhibitors α -AI1 and α -AI2 have been cloned in plants which are deemed fit for human consumption [165]. Therefore, insight into the gene organization and bioprocessing of 6C-hevein-like peptide opens up new avenues for transgenic crop research and can be implemented for the development of pest-resistant crops.

6C-Hevein-like peptides

Cystine-knot α amylase inhibitor (CKAI)

Cyclotide (Kalata B1)

Figure 3.13. Comparison of the precursor organization of 6C-hevein-like peptides with other knottin-type CRPs. Precursors of hevein-like peptides differ from cyclotides and cystine-knot α -amylase inhibitors (CKAIs) as they do not comprise an N-terminal pro-domain (NTPP) or an N-terminal repeat (NTR) before the C-tail. CKAIs lack the C-tail while cyclotides comprise a short C-tail of 7 amino acid residues.

3.3. Binding interaction of altides with chitin oligosaccharides

Analysis of the binding interaction and the residues involved in binding of altides, aSG1 and aSR1 was performed by ^1H -NMR titrations with chitobiose and chitotriose respectively. From our experiments we found that aromatic residues, Tyr-19, Tyr-21, and Tyr-28 from aSG1 and Phe-18, Tyr-20, and Tyr-27 from aSR1 respectively are involved in the binding interaction (Fig 3.14). These aromatic amino acid residues occupy loops 3 and 4 of altides that comprise the conserved chitin-binding domain S-X-(F/W/Y)-X-(F/W/Y)-C-G-X₄-Y. Similar binding motifs were observed when Ac-AMP2, hevein, pseudohevein and WGA [76-78, 166] were titrated with chitin oligosaccharides. This data indicates that the chitin-binding domain is conserved throughout the hevein-like peptide family and the peptide-sugar complexes are stabilized by CH- π stacking interactions and van der Waal's contacts between the aromatic residues and the sugar moiety. The dissociation constant K_D of the binding interaction between aSG1 and chitobiose was two orders of magnitude higher than that of aSR1 and chitotriose. This significant difference can be due to the multivalent nature of the binding interaction [58], where increased number of binding sites in chitotriose lead to increased binding affinity. Similar observations were made when hevein was titrated with chitin oligosaccharides of increasing chain length (GlcNAc)_n (n=1-3) where, a difference of one-order of magnitude was observed per GlcNAc residue.

Figure 3.14. Chitin-binding motif of aSG1. ^1H -NMR titrations with chitobiose showed that Gln-18, Tyr-19, Tyr-21, and Tyr-28 were involved in the binding interaction.

The dissociation constant of altides is similar to hevein even though they comprise a C-terminal truncation indicating that the C-terminal does not play a major role in binding. This observation has previously been made by Aboitiz *et al.* who synthesized a truncated hevein analog (HEV32) and reported that there was no significant difference in the binding constant upon titration with chitotriose [79]. Together, this data provides insight into the nature of the binding interaction of altides with chitin oligosaccharides and shows that the chitin-binding domain is conserved throughout the hevein-like peptide family. Knowledge of the binding interaction can be used for efficient design of putative anti-fungal agents that have increased affinity for chitin. The dissociation constant K_D of the binding interaction between aSG1 and chitobiose was two orders of magnitude higher than that of aSR1 and chitotriose. This significant difference can be due to the multivalent nature of the binding interaction [58], where increased number of binding sites in chitotriose lead to increased binding affinity. Similar observations were made when hevein was titrated with chitin oligosaccharides of increasing chain length (GlcNAc)_n (n=1-3) where, a difference of one order of magnitude was observed per GlcNAc residue. Together, this data provides insight into the nature of the binding interaction of altides with chitin oligosaccharides and shows that the chitin-binding domain is conserved throughout the hevein-like peptide family. Knowledge of the binding interaction can be used for efficient design of putative anti-fungal agents that have increased affinity for chitin.

3.4. Anti-fungal activity of altides

Six phytopathogenic fungal strains (*A. alternata*, *A. brassiciola*, *C. lunata*, *F. oxysporum*, *R. solani* and *V. dahliae*) were susceptible to treatment with altides aSR1 and aSG1 as indicated by the formation of arc-shaped inhibition zones at growing hyphal tips. The IC₅₀ of altides against these fungi ranged from (1.12 – 69.02 µg/ml) depending on the peptide and strain tested and is comparable with other 6C-hevein-like peptides like, Ac-AMP2, IWF-4 and Ar-AMP [45, 46, 50]. The potency of all 6C-hevein-like peptides is higher than hevein whose IC₅₀ is in the range of 90-1250 µg/ml.

Morphological changes like short and stunted hyphae with increased branching were observed when *A. alternata* and *A. brassiciola* were treated with aSG1 and aSR1. Similar morphology was observed when IWF-4 was incubated with *Cercospora beticola* [46]. These observations indicate that altides and IWF-4 inhibit fungal growth through the same mechanism. It was speculated that, as a consequence of their chitin-binding property, altides can disrupt the “steady-state” model of hyphal growth [100]. According to the “steady-state” model, during hyphal growth, the tip of the hypha is plastic, comprises nascent chitin chains and gradually gets rigidified at the base by cross-linking of newly-synthesized chitin chains with β-glucans. In this manner, a ‘steady-state’ of plasticity and rigidification is maintained for efficient hyphal growth. Due to the plasticity of the hyphal apex, the nascent chitin chains are highly susceptible to chitinases and anti-fungal agents. Thus, altides may inhibit the growth of fungi by binding to

these nascent chitin chains and preventing the rigidification process resulting in the formation of short and stunted hyphae in *A. alternata* and *A. brassiciola*.

Taken together, our data highlights the sequence diversity, biosynthesis pathway, chitin-binding interaction and anti-fungal property of a novel suite of 6C-hevein-like peptides from two varieties of *A. sessilis*. Our data has helped expand the existing library of 6C-hevein-like peptides from four to ten. The altide aSR1 is the smallest 6C-hevein-like peptide isolated thus far with a binding affinity to chitin oligosaccharides comparable to hevein. Although the anti-fungal activity of altides is established, further insights are needed into their putative mechanism of action. This data can be used to develop robust anti-fungal agents with increased potency against fungal pathogens.

Chapter 4

Morintides: Novel 8C-Hevein-like Peptides from *Moringa oleifera*

1. Introduction

Moringa oleifera is a drought-resistant tree that can grow to a height of 10 m and its trunk can reach a diameter of 45 cm [167]. Due to its high medicinal and nutritional value, *M. oleifera* is now incorporated in marketed health formulations which are remedies for a variety of health disorders (Fig. 4.1) [168]. *M. oleifera* seeds are the best known natural coagulants which are commonly used to purify turbid water. It is speculated that the coagulation properties are due to dimeric proteins in the seeds which are highly stable [169]. The seeds, stem, roots and leaves of *M. oleifera* are reported to exhibit anti-fungal effects [111, 118]. However, there is no report on the peptide and protein components that contribute to the putative therapeutic value of *M. oleifera* leaves. Since hevein-like peptides are primarily involved in plant defense against fungi, we screened the leaves of *M. oleifera* for putative hevein-like peptides.

Figure 4.1. Nutritional benefits of *Moringa oleifera*. Moringa leaves are sold as health formulations to combat malnutrition around the world. Figure adapted from <http://www.tentree.com/blog/benefits-of-the-moringa-oleifera-tree/>

In this study, we report three anti-fungal 8C-hevein-like peptides from *M. oleifera*, designated morintides and abbreviated as mO1, mO2 and mO3. Sequencing using LC-ESI-MS/MS showed that morintides mO1 and mO2 are 44 amino acid residues in length while mO3 is 35 amino acid residues long. Transcriptome data analysis revealed that full-length precursors of morintides contain three domains, a signal peptide, a mature CRP domain and a short C-terminal tail. Morintides are the first 8C-hevein-like peptides that have a significantly short C-terminal tail unlike other 8C- and 10C-hevein-like peptides that contain a long C-terminal tail encoding for another bioactive protein like Barwin or chitinase [88, 170, 171]. As a consequence of its chitin-binding property mO1 inhibited the growth of two phytopathogenic fungal strains with IC_{50} in the micromolar range by inhibiting the growth of fungal hyphae. Together our data provide an insight into the sequence, biosynthesis and anti-fungal activity of morintides.

2. Results

2.1. Screening and isolation of peptides from *M.oleifera*

Screening of *M. oleifera* leaves revealed a cluster of peptides around 4 kDa. A scale-up extraction of 3 kg of plant material was performed by blending in equal volume of water. Putative CRPs were isolated by multiple rounds of SCX- and RP-HPLC and isolated peptides were profiled using MALDI-TOF MS in the mass range of 2-5 kDa. The MS profile of *M. oleifera* indicated that two peptides with m/z of 4536.71 Da and 4463.76 Da were present in the leaf extract and were designated morintide mO1 and mO2 respectively. The purified putative CRPs were subjected to reduction with DTT for 2 h followed by alkylation with IAM for 1 h at 37°C. After reduction and alkylation of the disulfide bonds, a mass shift of +474 Da was observed indicating the presence of eight cysteine residues and confirming that the isolated peptides were CRPs (Fig. 4.2).

Sequencing of morintides was performed by nanospray MS/MS. The mass difference between b and y ions was used to deduce the primary sequence of mO1 and mO2. Since tandem MS cannot differentiate isobaric residues like Leu/Ile, they were assigned based on the transcriptome data. From proteomic and transcriptomic analysis, morintides were 44 amino acid residues in length.

Figure 4.2. Reduction and alkylation of morintides. Mass spectra of reduced-alkylated morintides revealed that a peak with m/z 5010 was of highest abundance. The increase in mass by approximately 474 Da indicates that morintides comprise 8 cysteine residues.

The primary sequence comprised high content of cysteine (eight residues), glycine (seven residues), asparagine (five residues) and glutamine (five residues) making up nearly 50% of the peptide sequence. The glutamine residue at the N-terminal was spontaneously converted to pyroglutamic acid making the peptide resistant to degradation by exopeptidases. Sequence comparison of morintides revealed that their primary sequences are highly homologous and differ in one amino acid position: Gln-15 in mO1 was replaced by Gly-15 in mO2. Sequence alignment revealed a cysteine-spacing pattern of X₂**C**-X₈**C**-X₄**CC**-X₅**C**-X₆**C**-X₅**C**-X₃**C**-X₃ with an absolutely conserved chitin binding domain SQYGFCX₄Y in loops 3 and 4, where X represents any amino acid. BLAST analysis revealed that morintides belonged to the hevein-like peptide family, shared greater than 54% homology with 8C-hevein-like peptides and highest homology (64.3%) with hevein and Pn-AMPs (Table 4.1).

Table 4.1. Consensus sequences of morintides.

Peptide/ Loop	Peptide Sequence					
	1	2	3	4	5	6
	<div style="text-align: center;"> Chitin-binding domain </div>					
<i>mO1</i>	--QNC	GRQAGNRAC	CANQL	CCSQYGFC	GSTSEY	CSRANGCQSNCRGG
<i>mO2</i>	--QNC	GRQAGNRAC	CANGL	CCSQYGFC	GSTSEY	CSRANGCQSNCRGG
<i>Hevein</i>	--EQC	GRQAGGKLC	PNNL	CCSQWGW	CGSTDEY	CSPDHNCQSNCKD--
<i>Fa-AMP1</i>	--AQC	GAQGGGATC	PGGL	CCSQWGW	CGSTPKY	CG--AGCQSNCK--
<i>Fa-AMP2</i>	--AQC	GAQGGGATC	PGGL	CCSQWGW	CGSTPKY	CG--AGCQSNCR--
<i>Pn-AMP1</i>	--QQC	GRQASGRLC	GNRL	CCSQWGY	CGSTASY	CG--AGCQSQC
<i>Pn-AMP2</i>	--QQC	GRQASGRLC	GNRL	CCSQWGY	CGSTASY	CG--AGCQSQC

Sequence alignment with previously reported 8C-hevein-like peptides including hevein (29), Fa-AMPs (62) and Pn-AMP (38). The chitin-binding domain is located in loops 3 and 4 and is highlighted by a red arrow. Asterisks indicate the specific residues involved in binding to chitin and the disulfide pattern is indicated at the bottom of the table.

2.2. Modeled structure of morintide mO1

Structure modeling for mO1 was performed using the online server SWISS-MODEL. The best three results were all based on the structures of hevein (PDB ID: 4wp4, 1q9b and 1hev) as templates. The model using the structure 1HEV had the best QMEAN score. The modeled structure has two β strands consisting of residues Cys17-Ser19 (β 1) and Phe23-Gly25 (β 2), forming antiparallel β -sheets (Figure 4.3 A) while residues Ser28-Cys31 form a one-turn α -helix. From the model generated it was clear that mO1 is a typical 8C-hevein-like peptide with four disulfide bonds. Due to the high structural and sequence homology between hevein and mO1 it was presumed that the disulfide bond pattern would be the same in both peptides. The disulfide bonds Cys I-Cys IV and Cys II-Cys V link the N terminal loop and the strands β 1 and β 2 respectively. The disulfide bond Cys III-Cys VI makes the α -helix lie close to the strands β 1 and β 2. The disulfide bond Cys VII-Cys VIII makes the C-terminus bend, lying beside the strand β 1. The two β strands consist all of hydrophobic residues, located in the center of the structure. The four positive charged residues (Arg-10, Arg-5, Arg-33 and Arg-42) are distributed in the N-terminus and C-terminus. There is only one negative charged residue (Glu-29) located in the C-terminal region. The structure of mO1 is very similar to hevein (Figure 4.3 B). For both mO1 and hevein, the two strands β 1 and β 2 consist mostly of hydrophobic residues. Hevein contains more charged residues than mO1, and has five negative charged residue (Glu-1, Asp-28, Glu-29, Asp-34 and Asp-43) and four positive charged residues (Arg-5, Lys-10, His-35 and Lys-42).

Figure 4.3. Modeled structure of morintide mO1. The structure of hevein modeled using hevein (1HEV) had the best QMEAN score. The modeled structure has two anti-parallel β -sheets and a one turn α -helix. The eight cysteine residues are presumed to have the same disulfide pairing as hevein, Cys I-Cys IV, Cys II-Cys V, Cys III-Cys VI, and Cys VII-Cys VIII.

2.3. Chitin binding activity

To assess the chitin binding activity of morintides, mO1 was incubated with chitin beads for 4 h at 4°C. RP-UPLC analysis showed that mO1 bound to chitin beads within 1 h (Fig. 4.4 A). On using elution buffers with up to 1 M NaCl, mO1 could not be eluted from the beads indicating a strong binding interaction. The use of 0.5 M acetic acid with heating at 100°C effectively eluted mO1 in 1 h (Fig. 4.4 B). This data indicated that mO1 bound strongly to chitin beads as it could be eluted using a strong elution buffer and heating at high temperature.

Figure 4.4. Chitin-binding activity of morintide mO1. (A) Incubation with chitin beads for 1 h at 4°C resulted in binding of mO1 within 1 h. (B) Elution of bound mO1 was performed at 100°C for 30 min in 0.5 M acetic acid.

2.4. Anti-fungal activity

The disc diffusion assay was performed to test the vulnerability of seven strains of phytopathogenic fungi to mO1. Fungi were inoculated and allowed to grow at 25°C for 24-72 h or until a radial colony was formed. Morintides of designated concentration were loaded on discs placed at the growing mycelial tips. Out of the seven strains *A. alternata* and *A. brassiciola* showed the formation of crescent-shaped inhibition zones indicating that they are susceptible to treatment with mO1 (Fig. 4.5 A).

To obtain the half-maximal inhibitory concentration (IC_{50}) of mO1, micro-broth dilution assay was performed against the susceptible fungi. After treatment with different morintide concentrations for 24 h, the cells were fixed with methanol and stained with 0.1% methylene blue. Absorbance was measured at 650 nm and a dose-response curve was generated from which the IC_{50} calculated was in the range of 25.5 µg/ml to 60.43 µg/ml after incubation for 24 h at 25°C depending on the fungal strain tested (Fig. 4.5 B).

To observe any morphological changes in fungi sensitive to mO1, the fungal spores were treated with mO1 and grown on slide chambers for 24 h at 25°C. Bright field microscopy showed that treatment of fungal spores with mO1 resulted in morphological changes like short and thick hyphae with swollen tips when compared to the untreated control. The mO1 treated fungal mycelia were closely bunched together and did not branch out as extensively the untreated fungi (Fig 4.6). Together these data suggest that mO1 inhibits fungal growth by retarding the growth of budding hyphae in a dose-dependent manner.

Figure 4.5. Anti-fungal activity of morintide mO1. (A) Formation of arc-shaped inhibition zones at the growing tips of fungal mycelia indicates the susceptibility of *A. alternata* and *A. brassicicola* to mO1. (B) dose-response curves generated from the microbroth dilution assay were used to calculate the IC_{50} values.

Figure 4.6. Hyphal growth inhibition assay of morintide mO1. Treatment of *A. alternata* and *A. brassiciola* spores with mO1 resulted in the formation of thicker and less branched hyphae than the control well indicating that mO1 could inhibit hyphal elongation consequently retarding the growth of fungi.

2.5. Heat and proteolytic stability

Traditional medicine requires heating therapeutic decoctions to 100°C for prolonged time periods and peptides are usually considered unstable to thermal degradation. In addition, bioavailability of therapeutic agents is significantly reduced due to susceptibility to proteolytic degradation. To study if mO1 could be the primary bioactive principle in decoctions of *M. oleifera*, stability of mO1 against thermal and enzymatic degradation was assessed. Morintide, mO1 was incubated at 100°C for 1 h or with pepsin and carboxypeptidase A for 6 h. By computing the area under the curve from RP-UPLC chromatograms it was observed that more than 95% of the peptide remained intact after heating for 1 h and on treatment with proteolytic enzymes (Fig. 4.7). More than 50% of linear control peptide RV-14 was degraded within 1 h of heating at 100°C while WV-14 was completely digested in 4 h by carboxypeptidase A and only 50% of WV-14 remained intact after 6 h of pepsin treatment. Thus, this data establishes the high stability of mO1 against thermal and enzymatic degradation and suggests that mO1 could be the active principle contributing to the medicinal value of *M. oleifera*.

Figure 4.7. Thermal and enzymatic stability assay. Morintide mO1 was stable after (A) heating at 100°C for 1 h while control peptide (B) RV-14 was degraded within 1 h. Enzymatic stability of mO1 against (A) carboxypeptidase and (B) pepsin was assayed upto 6 h. Control peptide WV-14 was degraded in the presence of (C) carboxypeptidase and (D) pepsin in 6 h.

2.6. Biosynthesis of morintides

A total of 78,561 transcripts with contig length between 201-22191 bp were obtained from transcriptome of RNA extracted from fresh *M. oleifera* leaves. These transcripts were analyzed using in-house software, Protein Analyzer 1.0 to look for conserved cysteine motifs. Three precursor sequences, designated *mo1*, *mo2* and *mo3* were obtained. Sequence comparison of precursors of *mo1* and *mo2* revealed that the signal peptide is absolutely conserved, the mature domain comprises a variation of one amino acid residue and the C-terminal tail varies at three positions, namely, Asp-67, Glu-71 and Gly-76 of *mo1* are replaced by Ala-67, Gly-71 and Ser-76 in *mo2*. The precursor sequence, *mo3* was detected only at the transcriptome level and contained a C-terminal truncation. The mature domain was different from mO1 by six amino acid residues: Gln-2, Asn-8, Ala-10, Ala-12, Ser-25, and Glu-28 in mO1 were replaced by Gln-2, Gly-8, Thr-10, Pro-12, Thr-25 and Ala-28 in mO3, respectively. These precursor sequences were aligned with previously reported 8C-hevein-like peptides including hevein from *H. brasiliensis* [88] and Pn-AMP1 from *Pharbitis nil* [47] (Fig. 4.8). From sequence alignment it was observed that morintides share similar precursor organization as other 8C-hevein-like peptides and comprise three domains, a 20-amino acid long ER signal peptide followed by a 44-amino acid long mature CRP domain and a short 15 amino acid-long C-terminal tail. The presence of an ER signal peptide indicates that morintides are ribosomally synthesized peptides [90] and their biosynthesis is through the secretory pathway [91]. A striking feature that distinguishes the precursors of mO1 from hevein is the significantly short C-tail

(15 residues). Hevein comprises a long C-terminal tail, 142 amino acid residues in length which encodes for a different bioactive protein. These bioactive proteins are usually lectins with a Barwin domain capable of binding to tetrameric N-acetylglucosamine [88]. This phenomenon leading to the truncation of the C-terminal tail may have occurred by divergent evolution since *M. oleifera* and *H. brasiliensis* belong to orders Brassicales and Malphigiales respectively which are two distinct clades within the plant kingdom.

Figure 4.8. Sequence alignment of putative morintide precursors and 8C-hevein-like peptides. Precursor organization is conserved and comprises a signal peptide domain, a mature CRP domain and a C-terminal tail. The C-terminal tail of hevein is significantly longer than morintides, mO1 and mO2 as it encodes for a bioactive protein comprising the Barwin domain.

3. Discussion

In this study, three novel 8C-hevein-like peptides, collectively named morintides were isolated, sequenced and characterized by proteomic, transcriptomic and biochemical methods. Morintides, mO1 and mO2 are 44 amino acid residues in length, were detected at proteomic and transcriptomic level and differ by one amino acid residue: Gln-15 in mO1 is replaced by Gly-15 in mO2 while mO3 was 35 amino acid residues in length and detected only at the transcriptome level. Morintides are homologous to hevein and other 8C-hevein-like peptides like Pn-AMPs [47] and Fa-AMPs [51]. Structural analysis of mO1 revealed that three disulfide bonds were arranged to form the cystine knot motif and the fourth disulfide bond was located at the C-terminal. Morintide mO1 inhibited the growth of *A. alternata* and *A. brassiciola* in the disc diffusion assay with an IC_{50} of 25.5 μ g/ml to 60.43 μ g/ml, respectively. Transcriptomic analysis revealed that morintide precursors consist of three domains: a signal peptide domain, a mature CRP domain and a short C-terminal tail suggesting that their bioprocessed through the secretory pathway.

3.1. Sequence Comparison of Morintides

Sequence comparison of morintides with reported 8C-hevein-like peptides revealed that, in addition to the eight conserved cysteine residues, three glutamine (Gln-6, Gln-20, Gln-36), two serine (Ser-19, Ser-37) and Leu-16 were absolutely conserved in all 8C-hevein-like peptides.

Figure 4.9. Sequence logo of aligned mature domains of 8C-hevein-like peptides. Based on the cysteine pattern the sequence can be divided into six loops. None of the loops are absolutely conserved indicating that there is high molecular diversity among 8C-hevein-like peptides. Loops 3 and 4 comprise the conserved chitin-binding domain which is highlighted in green.

Based on the cysteine-pattern the peptide sequences can be divided into six loops. The length of the loops is conserved with variation observed only in loop 5 of Fa-AMPs and Pn-AMPs that comprise three residues as opposed to five residues seen in hevein and mO1. The N-terminal of all 8C-hevein-like peptides except Fa-AMPs comprises a glutamate residue which is spontaneously converted to pyroglutamic acid conferring enhanced proteolytic stability against exopeptidases. The C-terminus usually comprises a positively charged residue followed by glycine, serine or aspartate residues in mO1, Pn-AMP1 and hevein respectively.

A sequence logo of the aligned morintides and 8C-hevein-like peptides was used to illustrate the similarity and frequency of occurrence of amino acid residues at each position (Fig. 4.9). The height of the stack indicates the overall sequence conservation while the frequency of an amino acid at a particular position is indicated by the height of the symbols. From the sequence logo it is clear that the extent of molecular diversity is high among 8C-hevein-like peptides as none of the loops show absolute sequence conservation. Loops 3 and 4 comprise the chitin-binding domain where a serine and glutamine are absolutely conserved. The aromatic amino acid residues in loop 3 are highly variable while a tyrosine residue in loop 4 is absolutely conserved. It is speculated that such variations in the primary sequences of closely-related peptides arise by divergent evolution and lead to functional diversity in the peptide family.

Similar modulation of function by natural diversity was observed in 10C-hevein-like peptides from *Triticum kiharae* where mutation of Ser to Gly in the chitin-binding domain reduced binding affinity to chitin but resulted in a gain of function. These natural variants could bind to fungal metalloproteases and inhibit the degradation of plant chitinases resulting in resistance to fungal infections in *Triticum kiharae* [101].

3.2. Biosynthesis of morintides

Transcriptome data analysis revealed that morintides are expressed as a three-domain precursor comprised of a signal peptide, a mature domain and a C-terminal tail and are processed via the secretory pathway [91]. In the secretory pathway, ribosomally synthesized peptides are directed to the endoplasmic reticulum where the signal peptide is cleaved by signal peptidase (SPase1) followed by cleavage of the mature domain from the C-terminal tail. Sequence alignment of morintide precursors with other 6C-, 8C- and 10C-hevein-like peptides showed that this precursor organization is conserved throughout the hevein-like peptide family with variations primarily in C-terminal domain length (Fig. 4.10). Sequence alignment revealed that the 15-amino acid long C-terminal domain of morintide is rich in glycine residues and differs from hevein and 10C-hevein-like peptides which have a significantly longer C-terminal tail (142-200 amino acid residues) coding for proteins with a Barwin domain or a glycine or serine-rich hinge region [170] followed by the catalytic domain of Class I chitinases [171]. The glycine-rich nature of the C-tail of morintides suggests that

it could be a remnant of the hinge-region in chitinases and morintides could be truncated chitinases.

The hevein-like peptide family is an interesting family of CRPs as it could be divided into three sub-classes making it an ideal family to study genetic diversity. Genetic divergence within the plant phyla result from mutations in the mature domain which also lead to functional diversification. For example, the signal peptide and C-terminal tail of morintide mO1 and mO2 precursors are highly homologous, but a point mutation in the mature domain results in replacement of Gln-15 in mO1 to Gly-15 in mO2. Similar genetic diversity has also been observed in other CRPs like clotides cT4-cT12 of the cyclotide family isolated from *Clitoria ternatea* [93], allotides of cystine knot α -amylase family isolated from *Allamanda cathartica* [158] and α - and β -hordothionin of the thionin family isolated from *Hordeum vulgare* [172].

Figure 4.10. Representation of precursor arrangement in the hevein-like peptide family. The precursor organization is conserved among 6C-hevein-like peptides, 8C- and 10C-hevein-like peptides including hevein and Ee-CBP have long C-terminal tails coding for proteins with a Barwin domain or a chitinase domain. Morintides and WAMP-1a are exceptions of the 8C- and 10C-sub-classes and comprise short C-terminal tails with no homology to any known proteins in the NCBI conserved domains database.

A phylogenetic tree was constructed using the precursor sequences of morintides and nine other hevein-like peptides to study their evolutionary relationship (Fig. 4.11). Data analysis showed that morintides can be grouped as a separate cluster within the 8C-hevein-like peptide family, attributed by the significantly short C-terminal tail of morintides (15 amino acid residues) when compared to hevein (142 amino acid residues). The precursor structure of hevein is a molecular skeleton of hololectins like class-I/IV chitinases and comprises a chitin-binding domain followed by a long C-terminal chitinase domain [70]. A similar phenomenon was observed among 10C- hevein-like peptides where the C-terminal tail of WAMP-1 and WAMP-2 from *T. Kiharae* seeds lacks the chitinase domain and [89] is significantly shorter than Ee-CBP from *E. europaeus* [70]. Andreev *et al.* speculated that *wamp* genes were “remnants” of chitinase genes and were either formed by a frame shift deletion of a portion of the DNA sequence coding for the chitinase domain or by alternative splicing of chitinase pre-mRNA followed by reverse transcription and genomic integration. These “truncated” genes were then selected by evolution as they coded for anti-fungal peptides which conferred the plant with resistance to fungal infections [89]. The distinct nature of *wamp* genes also suggested that excision of the chitinase domain from class-I chitinases led to the evolution of class-II chitinases that lack this domain. It can be speculated that a similar phenomenon occurs in the evolution of 8C-hevein-like peptides which will be discussed in detail in chapter 5.

Figure 4.11. Phylogenetic tree of reported hevein-like peptides. 6C-, 8C- and 10C-hevein-like peptides are grouped in separate clusters. Morintides form a different cluster within the 8C-hevein-like peptide cluster due to their significantly short C-terminal tails.

3.3. Anti-fungal activity of morintides

Out of seven fungal strains tested, two strains, namely, *A. alternata*, *A. brassiciola* were susceptible to treatment with mO1 as indicated by arc-shaped inhibition zones around growing mycelia. The IC₅₀ calculated from dose-response curves was in the range of 25.5 µg/ml to 60.43 µg/ml and is similar to other 8C-hevein-like peptides, Pn-AMPs and Fa-AMPs but more potent than hevein [47, 51]. Morphological changes like swollen hyphal tips, stunted growth and less-branched hyphae were observed on treatment of fungal spores with different concentrations of mO1. Similar changes in morphology have been observed with other 8C-hevein-like peptides including hevein [38] and Pn-AMPs [47]. However, the mechanism of action against fungi is still unclear. It was speculated that hevein inhibits growth of fungal pathogens by interrupting the steady-state model of hyphal elongation similar to 6C-hevein-like peptides. However, Pn-AMPs could also inhibit non-chitin containing fungi suggesting that there could be a different mechanism involved. Fluorescence studies on fungi treated with labelled Pn-AMPs indicated that the small size and highly basic PI of Pn-AMPs allows them to easily penetrate through the fungal cell wall. Once they reach the plasma membrane alterations in membrane polarity could cause leakage of cytoplasmic material subsequently inhibiting fungal growth [47, 52, 99]. The mechanism of action of morintides remains to be elucidated; however, the anti-fungal activity of morintides can be attributed to their chitin-binding property as they have neutral PI values.

3.4. Comparison of 6C- and 8C-hevein-like peptides

An intriguing feature of the hevein-like peptide family is the molecular diversity leading to the existence of three sub-classes with distinct cysteine content. Since we have studied two of these sub-classes we could gain further insight into their evolution and functional diversity. The 6C-hevein-like peptides are considered “truncated” versions of 8C-hevein-like peptides that comprise a C-terminal truncation of about 10 amino acid residues including two cysteines [45, 46, 50]. The 6C-hevein-like peptides are 29-30 amino acid residues in length and based on the cysteine-spacing pattern can be divided into four loops. The loop lengths are conserved with four to six amino acid residues per loop. In the 40-45 amino acid long 8C-hevein-like peptides, the sequence can be divided into six loops where loop 2 is significantly longer than loop 2 of 6C-hevein-like peptides and comprises eight amino acid residues. Loops 3 and 4 of 6C- and 8C-hevein-like peptides comprise the chitin-binding domain.

A sequence logo of the chitin-binding domains of 6C- and 8C-hevein-like peptides sheds light on the sequence conservation of this domain within each sub-class (Fig. 4.12). The overall sequence of the chitin-binding domain is conserved with variations only in the aromatic amino acid residues following the conserved Ser residue.

6C-hevein-like peptides

8C-hevein-like peptides

Figure 4.12. Comparison of chitin-binding domain of 6C- and 8C-hevein-like peptides. The Ser residue at position I, Gly at position IV and Tyr at position V are absolutely conserved. In 6C-hevein-like peptides Phe is preferred at position II while Tyr at position III is highly conserved. The aromatic amino acid residues at position II and III are variable in 8C-hevein-like peptides.

In 6C-hevein-like peptides a higher occurrence of Tyr and Phe is seen at position II of the chitin-binding domain while positions III and IV comprise an absolutely conserved Tyr residue. However, in 8C-hevein-like peptides, Trp and Tyr are preferred in positions II and III with an absolute conservation of Tyr residue at position IV. Despite these variations in amino acid sequences, altides and hevein bind with similar affinity to chitin oligosaccharides indicating that variations in the aromatic amino acid residues do not significantly affect binding affinity. The 8C-hevein-like peptides contain two additional loops due to the two additional cysteine residues. The C-terminal of altides and morintides comprise a Gly residue however, other 8C-hevein-like peptides comprise positively charged residues like Lys and Arg [32, 47, 51].

Sequence variations among peptides of the same family are known to increase functional diversity. Since one of the primary functions of hevein-like peptides in plants is defense against fungal infections, we studied the anti-fungal activity of altides and morintides against seven phytopathogenic fungi. Out of the seven strains tested, both altides and morintides could inhibit *A. alternata* and *A. brassiciola* but inhibitory activity against the remaining five strains was dependent on the peptides and strain being tested (Table. 4.2). For example, altide aSG1 could inhibit *V. dahliae* while mO1 showed no inhibitory activity against this strain. On comparing the potency of altides and morintides against *A. alternata* and *A. brassiciola* it was evident that altides were more potent inhibitors of these fungi. Thus, it can be speculated that variations in peptide sequence may have a role in broadening the spectrum of anti-fungal activity of hevein-like peptides.

Table 4.2. Comparison of anti-fungal activity of aSG1, aSR1 and mO1. *A. alternata* and *A. brassiciola* were sensitive to all three peptides whereas fungal growth inhibition of these peptides against the remaining strains was more strain-specific. “+” indicates inhibitory activity and “-“ indicates no fungal growth inhibition.

Fungal strain	Anti-fungal activity		
	aSG1	aSR1	mO1
<i>Alternaria alternata</i>	+	+	+
<i>Alternaria brassicola</i>	+	+	+
<i>Aspergillus niger</i>	-	-	-
<i>Curvularia lunata</i>	-	+	-
<i>Fusarium oxysporum</i>	+	-	-
<i>Rhizoctonia solani</i>	-	+	-
<i>Verticillium dahliae</i>	+	-	-

Insights into the precursor organization of altides and morintides can help shed light on their evolutionary relationship. Comparison of the precursor sequences of altides and morintides shows that the precursor organization is conserved and comprises a signal peptide, a mature CRP domain and a C-terminal tail. The signal peptide of altides is four residues longer than morintides while the C-terminal tail is longer by 10 residues. It has previously been reported that the C-terminal tail may comprise sorting signals that can decide the fate of the peptide after secretion from the Golgi apparatus [173-176]. Thus we performed a BLAST analysis of the C-terminal tails to identify any sorting signals within the peptide sequence. However, no sequence homology with any conserved domains in the database was obtained thereby making it difficult to decipher their functional significance. While hevein is obtained from a chimeric precursor that encodes for another bioactive protein with a Barwin domain the short C-tail of morintides is similar to the 6C-hevein-like peptides [37]. Thus, it can be assumed that morintides and altides share a close evolutionary relationship although they are isolated from two different plant species. It is possible that morintides and altides arose by divergent evolution from hevein as their short length and integral role in defense against fungal infections were more energetically favorable for plants. This evolutionary relationship is discussed in more detail in Chapter 5.

CHAPTER 5

EST-Based *in silico* Identification of Hevein-like Peptides in Plants

1. Introduction

Bioactive peptides, commonly found in all organisms from bacteria to plants and animals, are highly diverse in their structure and display considerable functional diversity [177]. These bioactive peptides are interesting candidates to study the interplay between natural diversity and evolution in creating an arsenal of peptides with diverse biological functions [178]. The conventional approach of identifying putative bioactive peptides is bio-guided fractionation, where the crude plant extract is fractionated by HPLC and each fraction is screened for bio-activity [179]. Although this method has been used for decades in the discovery of novel peptide biologics, it is time-consuming and laborious. Thus, there is a need to develop a robust and high-throughput method by which novel peptides can be identified in plants at a rapid rate. Recently, a lot of progress has been made in the field of genomics, proteomics and transcriptomics providing a vast expanse of data and new avenues to discover bioactive peptides. So far, there are more than 73 million expressed sequences tags (ESTs) in the NCBI database (dbEST) which is expanding substantially each year [180]. A combination of bioinformatics and computational algorithms provides a platform to virtually screen a variety of plant genomes at an exponential rate as compared to the conventional approach [181]. This *in silico*-based approach has been

previously used for the identification of bacteriocins [182] and lantibiotics [183]. However, thus far, this method has not been widely used for screening CRPs.

The hevein-like peptide family is an interesting family of CRPs due to its natural sequence diversity allowing it to be divided into three sub-classes, namely, 6C-, 8C- and 10C-hevein-like peptides. The molecular diversity of hevein-like peptides and their role in plant defense against fungal pathogens makes them interesting candidates to study their evolution and distribution in plants. Thus far, ten 6C-hevein-like peptides, five 8C-hevein-like peptides and five 10C-hevein-like peptides have been isolated and characterized from Amaranthaceae (10 peptides), Euphorbiaceae (1 peptide), Polygonaceae (2 peptides), Convolvulaceae (2 peptides), Poaceae (2 peptides), Celastraceae (1 peptide) and Eucommiaceae (2 peptides) (Fig. 5.1). Although hevein-like peptides are widely distributed in plants, knowledge of their origin, evolution and distribution is still limited. Thus, the aim of this chapter is to gain insights into the expression, distribution and evolution of hevein-like peptides across different plant families using a high-throughput bioinformatics-guided approach.

Taking advantage of the vast expanse of the EST-based database a BLAST search was performed using the reported hevein-like peptides as queries to identify their potential homologs. The resulting hits were filtered based on characteristics of hevein-like peptides and a total of 385 novel hevein-like peptides were identified from 124 different plant families.

Figure 5.1. Distribution of reported hevein-like peptides in plants. A total of 20 hevein-like peptides have been reported to date and they are distributed in 7 different plant families. The Amaranthaceae family has the highest number of hevein-like peptides and accounts for 50% of the reported peptides.

A phylogenetic tree was constructed to understand the evolutionary pattern of hevein-like peptides and their biosynthesis pathway was explored. Sequence alignment revealed that the precursor organization is conserved throughout the hevein-like peptide family. The three-domain precursor is comprised of a signal peptide, a mature CRP domain and a C-terminal tail. In 8C- and 10C-hevein-like peptides, chimeric precursors were observed, where the C-terminal tails coded for other bioactive proteins like Barwin and rare lipoprotein-like double-psi beta barrel (RlpA-DPBB-1) domain. Together our data sheds light on the sequence diversity, distribution, evolution and biosynthesis of the hevein-like peptide family.

2. Results

2.1. EST-based discovery of hevein-like peptides in plants

Two major databases, namely NCBI (www.ncbi.nlm.nih.gov) and OneKp (www.onekp.com) were used to search for novel hevein-like peptide sequences in plants. The reported peptide sequences of 6C-, 8C- and 10C-hevein-like peptides were used as queries to search against the EST nucleotide database comprising nucleotide sequences translated in six open reading frames (ORFs). Initially, a total of 626 peptide sequences were obtained from both databases of which 24 were 6C-hevein-like peptides, 565 were 8C-hevein-like peptides and 37 were 10C-hevein-like peptides which were pooled into different datasets. There was a considerable overlap in the sequences obtained from both databases, thus, replicate sequences were filtered manually. After the first round of filtering a total of 161 sequences with identical precursor sequences from the same plant or from different species of the same genus were deleted. Of these deleted sequences, four were 6C-hevein-like peptides, 150 were 8C-hevein-like peptides and seven were 10C-hevein-like peptides. After filtering the data, a total of 465 putative hevein-like peptide sequences remained (Fig. 5.2 A). However, the likelihood of class I chitinase sequences appearing in the hevein-like peptide datasets is high since the chitin-binding domain of class I chitinases is essentially a hevein-like peptide linked to a catalytic domain by a hinge region [60, 96] (Fig. 5.2 B).

Figure 5.2. EST-based screening of hevein-like peptides. (A) Flow chart of data mining of putative hevein-like peptides from NCBI and OneKp databases. (B) Comparison of organization of hevein-like peptides and class I chitinases. The chitin-binding domain of class I chitinases is essentially a hevein-like peptide.

Therefore, the 6C-, 8C- and 10C-hevein-like peptide datasets were pooled and filtered a second time using the Blast2GO PRO software to eliminate chitinase sequences [151]. After scanning all the datasets, a total of 81 chitinase sequences were deleted. Thus, after two rounds of filtering, we obtained 385 putative hevein-like peptides, that is, 20, 6C-hevein-like peptides, 349, 8C-hevein-like peptides and 16, 10C-hevein-like peptides. This high incidence of hevein-like peptides in plants suggests that they are a widely distributed family of CRPs and makes them interesting candidates to study their origin, distribution and evolutionary relationship.

2.2. Distribution and Occurrence of hevein-like peptides in the plant kingdom

From EST-based data mining of hevein-like peptides, a total of 124 plant families belonging to 52 orders expressing hevein-like peptides were found from NCBI and OneKp databases (Fig. 5.3 A). The 20 novel 6C-hevein-like peptides were distributed in 18 plant species from 4 plant families only comprising dicot angiosperms. These families are derived from orders Asterales, Caryophyllales and Boraginales which are closely-related and grouped under the common clade Asterids. The Amaranthaceae family had the highest number of 6C-hevein-like peptides suggesting that they are an important source of these peptides. The novel 8C-hevein-like peptides were found in 271 plant species from 117 different plant families originating from non-vascular bryophytes (mosses) to more-evolved vascular plants including gymnosperms and angiosperms.

A **B**

Figure 5.3. Distribution of putative hevein-like peptides in plants. (A) Novel putative hevein-like peptides were distributed in 124 plant families belonging to 52 orders. The order Cupressales comprising gymnosperms had the highest number of hevein-like peptides closely followed by Fabales from angiosperms. (B) 8C- and 10C-hevein-like peptides were distributed among angiosperms, gymnosperms and Bryophyta indicating that they are the most diverse and widespread sub-classes of hevein-like peptides.

A total of 3.6% 8C-hevein-like peptides were found in non-vascular plants, originating from the lineages of the first terrestrial plants on Earth, namely, Bryophyta and Marchantiophyta while 17% were distributed in the order Cupressales from gymnosperms and 10% in Fabales from angiosperms (Fig. 5.3 B). The 16 novel 10C-hevein-like peptides were distributed in 14 species from six different plant families. One sequence each was found in the Plagiochilaceae and Gnetaceae families of Marchantiophyta and gymnosperms, respectively. Majority of the 10C-hevein-like peptides were detected in monocot and dicot angiosperms with 88% in the Poaceae family and 6% in the Rutaceae family belonging to orders Poales and Sapindales, respectively (Fig. 5.3 B). Thus, it can be speculated that there is a close evolutionary relationship between 10C-hevein-like peptides from monocot and dicot angiosperms. Interestingly, 15 hevein-like peptides were found multiple times in more than one plant species. While some sequences were found in plants of the same genus and different species, others were found in plants belonging to the same family (Appendix A).

To further understand the distribution and evolution of hevein-like peptides we constructed cladograms of the putative hevein-like peptide producing plants. The expression of 6C-hevein-like peptides was restricted to eudicots belonging to Asterids and the closely-related, sister clade Caryophyllales [184] (Fig. 5.4 A).

Figure 5.4. Summary cladogram of the distribution of hevein-like peptides. (A) 6C-hevein-like peptides were exclusively found in eudicots belonging to orders Asterids and Caryophyllales. (B) 10C-hevein-like peptides were distributed in Bryophyta and Tracheophyta including Gymnosperms and monocot and eudicot angiosperms.

Within Asterids, 6C-hevein-like peptides occur in Asterales and Boraginales suggesting that closely-related orders, Gentianales and Solanales may also contain these peptides and examining plant families within these orders maybe a means of discovering new 6C-hevein-like peptides. In contrast to 6C-hevein-like peptides, 10C-hevein-like peptides occurred in Bryophyta and Tracheophyta (Fig. 5.4 B). However, the incidence of 10C-hevein-like peptides in bryophytes and gymnosperms was limited to Marchantiidae and Gnetidae, respectively.

Majority of 10C-hevein-like peptides were prevalent in monocots from the Poaceae family and eudicots from the Rutaceae family. The 8C-hevein-like peptides were widely dispersed in plants and occurred in Bryophyta and Tracheophyta including gymnosperms and angiosperms (Fig. 5.5). Among Bryophytes, 8C-hevein-like peptides were found in the basal grade Marchantiidae and the main clades like Jungermaniidae, Bryidae and Lycopodiidae suggesting that these peptides originated early in the evolution of Bryophytes. In gymnosperms, majority of 8C-hevein-like peptides were observed in the most abundant order Cupressales from the sub-class Pinidae and also in the less common Gnetales and Ginkgoales indicating that these peptides are ubiquitous among gymnosperms. These peptides were also observed in ferns from the sub-class Polypodiidae.

Figure 5.5. Summary cladogram of the distribution of 8C-hevein-like peptides in plants. The 8C-hevein-like peptides are the most diverse and widespread sub-class of the hevein-like peptide family and are distributed in Bryophyta, gymnosperms and angiosperms.

The 8C-hevein-like peptides occurred in orders Poales, Arecales and Asparagles belonging to the Commelinids clade of monocot angiosperms. These peptides were also distributed in 85 plant families from higher order Rosids and Asterids comprising eudicot angiosperms. This widespread distribution of 8C-hevein-like peptides suggests that they are highly diverse and were probably the first member of the hevein-like peptide family to be expressed in plants. Taken together, the widespread expression of hevein-like peptides across the plant kingdom suggests that they are probably the largest family of CRPs and are indispensable to plants probably due to their defense roles against fungi and insect pests.

2.3. Sequence alignment and comparison of hevein-like peptides

To perform sequence comparison of the putative hevein-like peptides from different plant families, multiple sequence alignment using ClustalW was performed using previously reported hevein-like peptide sequences and the putative peptides obtained from databases. Similar to previously reported 6C- and 8C-hevein-like peptides, the putative peptides displayed conserved cysteine-spacing patterns. However, among the putative 10C-hevein-like peptides, a novel cysteine motif that differed from previously reported Type I, Type II and Type III was observed and designated Type IV (Fig. 5.6).

6C- Hevein-like Peptides

8C- Hevein-like Peptides

10C- Hevein-like Peptides

Type I
(Ee-CBP-like)

Type II
(WAMP-like)

Type III
(EAFP-like)

Type IV*
(pA-1-like)

*-disulfide pairing not determined

Figure 5.6. Cysteine-spacing and disulfide connectivity of hevein-like peptides. The putative 6C- and 8C-hevein-like peptides had similar cysteine-spacing patterns as their previously reported counter-parts. A novel cysteine-spacing motif was observed in a 10C-hevein-like peptide from *Plagiochila asplenioides* and was designated as Type IV. The disulfide connectivity of the new motif is yet to be determined.

2.3.4. 6C-Hevein-like peptides

From sequence alignment of five previously reported 6C-hevein-like peptide sequences and 20 putative peptides obtained from EST databases a sequence logo was obtained (Fig. 5.7). It was observed that the peptides are 29-30 amino acid residues in length and have a high content of glycine (7 residues), proline (2 to 3 residues) and aromatic amino acid residues (3 residues) which make up approximately 60% of the sequence (Appendix B). Apart from the cysteine residues, glycine at positions 2, 7, 12, 19, 22, 24 and 29, Ser-16, Pro-10, Tyr-20 and Tyr-27 are highly conserved (numbered according to Ac-AMP2). The 6C-hevein-like peptides can be divided into four loops which are about four to six residues in length. Loop 1 is the most variable while loop 2 comprises absolutely conserved residues Pro-10 and Gly-12 (Fig. 5.7). Loops 3 and 4 comprise the conserved chitin-binding domain SX(F/W)X(Y/W)CGX₄Y except one peptide from *Kochia scoparia* where the second aromatic residue in the chitin-binding domain is replaced by histidine. The disulfide bonds in previously reported 6C-hevein-like peptides form the stable cystine-knot motif where three disulfide bonds are formed between Cys I-Cys IV, Cys II-Cys V and Cys III-Cys VI. Due to the highly conserved cysteine pattern among all 6C-hevein-like peptides, we assume that the putative peptides also comprise the cystine-knot motif similar to altides and Ac-AMPs where the disulfide connectivity has been confirmed by NMR studies [45, 87].

6C-Hevein-like Peptides

8C-Hevein-like Peptides (Gymnosperms)

8C-Hevein-like Peptides (Angiosperms)

Figure 5.7. Sequence logo of putative 6C- and 8C-hevein-like peptides. The cysteine spacing is absolutely conserved among 6C- and 8C-hevein-like peptides. The 6C-hevein-like peptides can be divided into four loops and comprise the chitin-binding domain in loops 3 and 4 (highlighted in box). The N-terminal of 8C-hevein-like peptides from gymnosperms and angiosperms are starkly different but in both cases the composition of the chitin-binding domain is conserved.

2.3.5. 8C-Hevein-like peptides

Sequence alignment of 349 putative 8C-hevein-like peptides from EST databases with previously reported sequences revealed that the cysteine spacing is highly conserved and the peptide backbone between cysteine residues can be divided into six loops (Fig. 5.7). Interestingly, the N-terminal of sequences expressed in gymnosperms comprises the motif (D/EP) and differs from that of angiosperms and bryophytes which comprise the motif (A/E/QQN) (Appendix C). Although there is high sequence variability at the N- and C-termini, the length and sequences of loop 3 and loop 4, which comprises the chitin-binding domain, are conserved.

Along with the conserved cysteine residues, Ser-19 (numbered according to hevein) is absolutely conserved while Gly-25 is replaced by Val-25 or Asp25 in three sequences, namely, *Austrotaxus spicata*, *Dryas octopetala* and *Marchantia paleacea* (Appendix C). Each of these three sequences originates from a gymnosperm, an angiosperm and a liverwort respectively suggesting that there could be a close relationship between peptides from all three clades. Interestingly, Tyr-30 known to be integral for chitin-binding activity, is replaced by His-30 in all 8C-hevein-like peptides expressed in gymnosperms and in 19 sequences from eudicots belonging to Rosids, Asterids, Gunneridae, Ranunculales and Asparagales. Structural analysis of hevein revealed that the first three disulfide bonds form the cystine knot motif while the fourth disulfide bond is formed between the last two cysteine residues [66]. Due to conserved cysteine-spacing

pattern between the putative peptides and hevein it can be assumed that the disulfide pairing is identical.

2.3.6. 10C-Hevein-like peptides

The three previously reported sequences were aligned with 16 putative 10C-hevein-like peptides (Appendix D) which revealed that there are four distinct cysteine spacing patterns designated Type I to Type IV.

Type I, similar to Ee-CBPs from *E. europaeus* [52], is observed in three peptides from the order Sapindales comprising eudicot angiosperms. Sequence comparison with Ee-CBP showed that loop 1 of the putative hevein-like peptides is shorter than Ee-CBP and comprises four amino acid residues. Loop 2 shows high sequence conservation in peptides from the Rutaceae family but bears no homology with Ee-CBP. In loop 5 of the peptide from *Poncirus trifoliata*, Tyr-30 (numbered according to Ee-CBP) is replaced by His-30. Loop 6 and loop 8 are the most variable and loop 7 is partially conserved (Fig. 5.8). Based on the conserved cysteine spacing, it can be assumed that the disulfide connectivity is similar to Ee-CBP and follows the pattern Cys I-Cys IV, Cys II-Cys V, Cys III-Cys VI, Cys VII-Cys X and Cys VIII-Cys IX [99].

Nine monocot angiosperms from the order Poales displayed a cysteine-spacing similar to Type II previously observed in WAMPs from *T. kiharae* [53]. Sequence comparison with WAMP-1b from *T. kiharae* revealed that loop 1 was the most variable while loops 2 and 3 were highly conserved. Loop 2 comprised three residues while loop 3 comprised an absolutely conserved Leu residue.

Type I
(Ee-CBP-like)

Type II
(WAMP-like)

Type III
(EAFP-like)

Type IV
(pA-1-like)

Figure 5.8. Sequence logo of putative 10C-hevein-like peptides. Four distinct cysteine-spacing patterns were observed among 10C-hevein-like peptides and designated Type I to Type IV. While Type I to Type III have been previously reported, a novel pattern, Type IV was observed in a Marchantiophyte called *Plagiochila asplenioides* with a modification in the chitin-binding domain. The chitin-binding domain is indicated in the box.

Loops 4 and 5 comprise the conserved chitin-binding domain. Interestingly, the conserved Ser-20 residue in the chitin-binding domain in loop 4 of peptides from *Triticum aestivum* and *Elymus spicatus* is replaced by Gly-20 similar to WAMPs reported from *Triticum kiharae* [53] (Appendix C). Loop 6 comprises the conserved motif GXGX, except in *Elymus spicatus*, where Gly-33 is replaced by Asp-33 (Fig. 5.8). Loop 7 comprises a conserved Glu-37 residue which is replaced by His-37 in one peptide from *Oryza sativa* Japonica. It can be assumed that the putative hevein-like peptides from Poales share the same disulfide connectivity as WAMPs from *Triticum kiharae*, Cys I-Cys V, Cys II-Cys VI, Cys III-Cys X, Cys IV-Cys VII, Cys VIII-Cys IX, since they belong to the same plant family [73].

The Type III cysteine-spacing pattern, similar to EAFPs from *E. ulmoides* Oliv [48], was observed in one gymnosperm from the order Gnetidae. However, on sequence comparison with EAFP-1, no absolute sequence conservation was observed except for the cysteine residues (Fig. 5.8). This could be because the putative peptide is observed in a gymnosperm as opposed to EAFPs isolated from eudicot angiosperms. Despite the high sequence variation observed in the all loops of both peptides, loops 4 and 5 are more similar as they comprise the chitin-binding domain. Due to conserved cysteine-spacing it can be presumed that the disulfide pairing is similar to EAFPs.

A novel cysteine-spacing pattern, that we designated Type IV was observed in *Plagiochila asplenioides*, a Bryophyte from the order Marchantiidae. Intriguingly, the additional cysteine residue in this peptide replaces the Ser residue of the chitin-binding domain. This is the first instance of a 10C-hevein-like peptide from a Bryophyte with a substitution of the conserved Ser residue by a Cys residue in the chitin-binding domain. Further studies need to be done to isolate and elucidate the disulfide pattern, structure and chitin-binding affinity of the putative peptide.

From this data it is clear that 8C- and 10C-hevein-like peptides are the most diverse sub-classes of the hevein-like peptide family. This can be attributed to their widespread distribution across the plant kingdom from mosses and gymnosperms to highly evolved eudicots. In contrast, the 6C-hevein-like peptides seem to be more recent members of the hevein-like peptide family and comprise loops that are less variable than 8C- and 10C-hevein-like peptides. An interesting feature of the 10C-hevein-like peptides is the disulfide promiscuity resulting from four distinct cysteine-spacing patterns. Despite the variations in the length of the primary sequences of 6C-, 8C- and 10C- hevein-like peptides, the length of the loops comprising the chitin-binding domain are conserved across the hevein-like peptide family. Thus, this peptide family shows universality in diversity in their primary and secondary structure as shown by the great degree of diversity as well as conservation of certain key residues involved in binding chitin.

2.4. Precursor organization of hevein-like peptides

One of the advantages of using EST-based databases is that the full-length precursor sequence of the target peptides can be studied. Since knowledge of the sequence diversity was obtained from the primary and secondary structure of the putative peptides, we analyzed the precursor sequences to gain insights into their putative processing sites and pathway followed in their *in planta* synthesis. Gene cloning studies of 6C-, 8C- and 10C-hevein-like peptides have shown that the precursor organization is conserved throughout the family and comprises a signal peptide domain, a mature CRP domain and a C-terminal tail with variations only in domain length [86, 89, 98]. This precursor organization suggests that hevein-like peptides are ribosomally synthesized and are first translated as a three-domain precursor. The presence of a signal peptide suggests that the biosynthesis follows the secretory pathway. The biosynthesis of hevein-like peptides involves excision of the signal peptide from the mature domain followed by excision of the C-terminal tail and oxidative folding of the mature peptide to form the disulfide bonds.

Sequence alignment of the precursors of putative hevein-like peptides from the EST database revealed that they comprise a similar precursor structure. The residue at the N-terminal processing site is Ala or Gly in majority of the putative peptides sequences. However, 8C-hevein-like peptides from *Austrocedrus chilensis*, *Brassica napus*, *Callitris gracilis*, *Papuacedrus papuana* and *Populus trichocarpa* comprise a Ser residue while *Cycas micholitzii* comprises a Lys residue at the N-terminal cleavage site. The C-terminal of hevein-like peptide

precursors comprises multiple glycine repeats and resembles the hinge region of chitinases. Thus it is difficult to predict the exact processing site at the C-terminal tail.

An interesting feature of hevein-like peptide precursors is the diversity of their C-terminal tail. In 6C-hevein-like peptides the length of the precursor varies from 56 to 90 amino acid residues. BLAST analysis of the C-tail in the NCBI conserved domain database showed no homology to any known domains. The precursors of 8C- and 10C-hevein-like peptides are significantly longer than 6C-hevein-like peptides. This can be attributed to the chimeric nature of the precursors where the C-tails encode for other bioactive protein domains. In 8C-hevein-like peptides, the mature domain is followed by either a short (11-35 amino acid residues) or a long C-tail (35-250 amino acid residues) where the short C-tails shared no homology with any known protein sequences in the database. In 10C-hevein-like peptides, C-tails of peptides from Poaceae family comprise the lysozyme-like protein domain while those from Rutaceae family bear no sequence homology to any known proteins. The C-terminal tails bearing homology to the RlpA-DPBB-1 domain were only observed in five peptides expressed in Bryophyta. The Extensin domain was observed in one peptide from *Glycine max*. A phylogenetic tree was constructed using the precursor sequences of the putative hevein-like peptides to gain insight into their probable evolutionary pattern (Fig. 5.9).

Figure 5.9. Phylogenetic Distribution of putative hevein-like peptides. (A) Phylogenetic tree of the putative hevein-like peptides. Color strips 1, 2 and 3 represent the number of cysteine residues, the C-terminal domains and the clade in which the peptides are expressed, respectively.

From the phylogenetic tree it is clear that the hevein-like peptides are grouped into distinct clusters based on their C-tail composition and the clade to which the plants belong. From the consolidated data of 6C-, 8C- and 10C-hevein-like peptide precursors, 48.8% of the C-tails comprised the Barwin domain, 5.5% comprised the lysozyme-like domain, 1.3% comprised the RlpA-DPBB1 domain and 0.8% comprised the Extensin domain (Fig. 5.10). Thus, although the precursor organization is conserved across the hevein-like peptide family, variations in processing sites and C-terminal tail composition indicate that there may be species or family-specific mechanisms or processing enzymes that need to be further elucidated.

Figure 5.10. Distribution of C-tail domains in hevein-like peptides. Majority of the putative hevein-like peptide precursors comprise a C-terminal tail with a Barwin domain. The 10C-hevein-like peptide precursors from the Poaceae family comprise C-tails with a lysozyme-like domain while five peptides from the clade Bryophyta comprise the RlpA-DPBB-1 domain. The Extensin domain was observed in one peptide from *Glycine max*.

3. Discussion

3.1. Distribution and evolution of hevein-like peptides

Insights into the distribution and evolution of hevein-like peptides have been gained using EST-based nucleotide databases. A total of 385 putative hevein-like peptides were identified in 124 different plant families originating from non-flowering and flowering plants. Knowledge of the distribution of hevein-like peptides in the plant kingdom helps shed light on the origin and evolution of this peptide family. The occurrence of 8C-hevein-like peptides in Bryophytes suggests that they may have appeared about 450 million years ago when the first terrestrial plants (mosses and liverworts) diverged from their algal ancestors [185] (Fig. 5.11). Interestingly, 10C-hevein-like peptides were also found in the clade Marchantiidae of Bryophyta which suggests that 8C- and 10C-hevein-like peptides are the most ancient members of the hevein-like peptide family. The 6C-hevein-like peptides are relatively recent members of this peptide family as they are expressed exclusively in Asterids which diverged from core eudicot Rosids about 128 million years ago [186].

Figure 5.11. Summary cladogram showing the major evolutionary groups of hevein-like peptides. The timeline of evolution of hevein-like peptides is indicated as million years ago (Mya). The expression of 8C- and 10C-hevein-like peptides in Bryophyta suggests that they originated ~450 million years ago in the first terrestrial plants on Earth. The 6C-hevein-like peptides are more recent members of the hevein-like peptide family as they are only expressed in Asterids which diverged from class Rosids ~125 million years ago. It is speculated that the 6C- and 10C-hevein-like peptide sub-classes arose by divergent evolution from their 8C-hevein-like peptide ancestors in closely related plant species.

The distribution of 6C-hevein-like peptides is similar to cyclotides which have only been found in eudicot angiosperms [187]. However, the evolutionary pathway of 6C- and 10C-hevein-like peptides is still unclear.

The similarity in length of the 8C- and 10C-hevein-like peptides with variations only in the number and positions of cysteine residues in the mature domain allow us to speculate that they arose from point mutations in 8C-hevein-like peptides (Fig. 5.12). The four distinct cysteine-spacing patterns of 10C-hevein-like peptides are observed in orders Marchantiidae, Gnetidae, Poales and Sapindales. Since Marchantiidae is the most ancient among the three clades, it can be assumed that the origin of 10C-hevein-like peptides was in Bryophytes. Based on the summary cladogram in figure 5.10 it can be presumed that the distinct cysteine-spacing patterns of 10C-hevein-like peptides evolved from 8C-hevein-like peptides of Marchantiidae, Poales and Sapindales, respectively by divergent evolution. Thus, it is possible that after the divergence of 10C-hevein-like peptides from their 8C-ancestors, adaptive evolution and positive Darwinism played a role in retaining their expression due to the increased resistance to fungal pathogens in plants. The most recent members of the hevein-like peptide family are the 6C-hevein-like peptides which are exclusively expressed in eudicots. Majority of the 6C-hevein-like peptides are expressed in the Amaranthaceae family belonging to the order Caryophyllales.

Figure 5.12. Putative evolutionary pathways of hevein-like peptides. The 10C-hevein-like peptide sub-class may have divergently evolved from 8C-hevein-like peptides in orders Sapindales, Poales, Gnetidae and Marchantiidae by point mutations resulting in two additional cysteine residues. The “truncation” of 8C-hevein-like peptides giving rise to 6C-hevein-like peptides may have occurred in order Caryophyllales.

Thus, it can be assumed that the truncation of the C-terminal comprising the last two cysteine residues of 8C-hevein-like peptides may have occurred in Caryophyllales and resulted in the divergent evolution of 6C-hevein-like peptides. However, the mechanism behind the truncation still remains to be elucidated. The 6C-hevein-like peptides could then be selected by positive Darwinism due to their small size and increased potency against fungal pathogens. A similar evolutionary pattern has been observed in chitinases and snake venom toxins that confer functional diversity and ability to target different prey [178, 188].

An alternate mechanism for the evolution of 10C-hevein-like peptides (WAMPs) from *Triticum kiharae* (wheat) has been suggested by Andreev *et al.* who proposed that WAMPs could be truncated Class I chitinases [89]. It is well known that Class I chitinases evolved by the incorporation of a chitin-binding domain in the gene coding for the catalytic domain by a transposition event [189]. On sequence comparison, they observed a striking similarity between the chitin-binding domains of Class I chitinases from cereals and WAMP-1b. Thus, they speculated that frame-shift deletions or alternative RNA splicing of chitinase genes may result in the expression of “truncated chitinases” comprising only the chitin-binding domain. This interesting observation suggests that, during the evolution of class I chitinases, a reverse phenomenon also took place where deletions in the chitinase gene resulted in the expression of a single chitin-binding domain lacking the catalytic domain. However, this theory is specific to WAMPs and cannot explain the distinct cysteine-spacing patterns of 10C-hevein-

like peptides from other plant families. Thus, more studies are required to gain a deeper insight into the origin of 10C-hevein-like peptides.

The evolution pattern of hevein-like peptides differs from cyclotides which arose from their linear counterparts by convergent evolution where, the mutations to insert essential Asp/Asn residues at the point of cyclization occurred independently in Asterids and Rosids about 125 million years ago [190]. Together, this data suggests that hevein-like peptides are an ancient, highly diverse class of CRPs and that the sub-classes of hevein-like peptides arose due to divergent evolution between closely related plant species.

3.2. Universality in diversity of hevein-like peptides

The highly diverse nature and widespread distribution of hevein-like peptides in plants makes them interesting candidates to study molecular diversity and the effect of sequence variations on functional diversification. Our results highlight the high molecular diversity of hevein-like peptides as shown by the extensive variation in their loop lengths and residue composition. The 6C-hevein-like peptides show relatively high sequence conservation when compared to 8C- and 10C-hevein-like peptides. This could be attributed to their distribution in closely related plant families resulting in less sequence variation. In 8C-hevein-like peptides, apart from the eight cysteine residues, none of the other residues show absolute conservation. In 10C-hevein-like peptides only two glycine residues in loops 3 and 4 are absolutely conserved suggesting that they may play an important role in stabilizing the conformation of the peptide while binding to chitin.

It is commonly believed that the variable residues; not involved in biological functions are prone to mutations as opposed to more conserved residues and they are located in the interior pocket in the peptide [191]. Thus, the surface residues are more prone to insertions, deletions and substitutions. However, in the case of hevein-like peptides, mutations in important residues in the chitin-binding domain are observed. Interestingly, in the loops comprising the chitin-binding domain, the tyrosine residue known to be crucial for binding to chitin is replaced by histidine in 8C-hevein-like peptides from 2% of gymnosperms and 8% of angiosperms. A similar phenomenon is observed in a 10C-hevein-like peptide from *Poncirus trifoliata* belonging to the Rutaceae family and a 6C-hevein-like peptide from *Kochia scorpioides*. Thus, it can be speculated that there may have been a gene transfer event from gymnosperms to angiosperms during the evolution of 8C- and 10C-hevein-like peptides. However, further analysis of the gene organization needs to be done to validate this hypothesis. We speculate that the chitin-binding property of these peptides may not be drastically affected since the imidazole ring of the histidine residue can retain the CH- π stacking interaction with the GlcNAc moiety. Intriguingly, the conserved serine residue of the chitin-binding domain is replaced by a glycine residue in 10C-hevein-like peptides from wheat species. A similar observation was made in WAMPs from *Triticum kiharae*, where this mutation led to decreased affinity for chitin oligosaccharides [53]. However, this loss of binding affinity is compensated by Ser-36 in loop 6 which not only confers resistance against fungal chitinases but also inhibits them [101]. The replacement by histidine and glycine residues in the

chitin-binding domain could also alter the net charge of the peptides, increase their cationicity and amphiphilicity conferring them anti-bacterial properties in addition to fungal defense [73]. Thus, it is possible that hevein-like peptides underwent multiple mutations during evolution to confer the plant an arsenal of defense peptides to target diverse microbial pathogens. Together, these observations suggest that the promiscuity of hevein-like peptides is integral for plant defense against multiple pathogens [192].

All hevein-like peptides are stabilized by a cystine-knot motif formed by the first three disulfide bonds [67]. This cystine-knotted scaffold has been observed in many CRPs and insect toxins [193, 194]. In hevein-like peptides this core cystine-knot motif is conserved and additional disulfide bonds are present in 8C- and 10C-hevein-like peptides. A similar phenomenon is observed in snake venom toxins, where, additional disulfide bonds are known to confer specificity to different receptors and also alter the receptor-binding efficiency [178]. However, thus far, the effect of additional disulfide bonds on the binding efficiency of hevein-like peptides has not been elucidated. Taken together, these observations show that despite the sequence diversity of hevein-like peptides, key features of the primary and secondary structure are conserved indicating the universality in diversity of this peptide family.

3.3. Precursor organization and biosynthesis of hevein-like peptides

Knowledge of the precursor organization of hevein-like peptides provides insights into their bio-processing pathway. The precursor organization comprising a signal peptide, a mature CRP domain and a C-terminal tail suggests that they are ribosomally synthesized peptides. Similar precursor structure is observed in thionins [159] and varies from precursors of cystine-knot α -amylase inhibitors (CKAIs) which comprise an N-terminal pro-domain before the mature domain [94]. It can be presumed that the folding of hevein-like peptides to form the cystine knot occurs in the endoplasmic reticulum by virtue of their signal peptide. Subsequently they may be transported to the Golgi apparatus or vacuoles where cleavage from the C-terminal tail occurs before secretion of the peptide [195, 196]. The signal-peptide guided biosynthesis of hevein-like peptides could facilitate natural evolution due to the relatively small number of processing enzymes and the high multiplicity of pathways towards similar types of precursors. Thus, the convergent evolution of processing pathways across different plant families confers an evolutionary advantage since access to chemical diversity is increased at a low genetic cost [197].

The natural diversity of hevein-like peptides is evident from the chimeric nature of the 8C- and 10C-hevein-like peptide precursors. Their C-terminal tails encode bioactive proteins with a Barwin domain or RlpA-DPBB-1) domain. The Barwin domain is a protein domain found in a lectin from Barley and is known to bind to tetrameric N-acetylglucosamine while the RlpA-DPBB-1 domain is a transglycosylase which targets glycan chains [88, 198]. A similar precursor

organization was observed in cyclotides from *Clitoria ternatea* where the C-tail comprised the legume albumin PA1a [93, 199]. However, the 6C-hevein-like peptides and some 8C- and 10C-hevein-like peptides comprise short C-terminal tails that have no homology to any known protein domains.

The diversity in precursor organization could lead to functional diversity of hevein-like peptides. The hevein-like peptides with chimeric precursors could function like “guided missiles”, where the chitin-binding domain of hevein-like peptides facilitates binding to the fungal cell wall and the Barwin and RlpA-DPBB-1 domains either increase binding efficiency or enzymatically digest the chitin chains, respectively. This mechanism involving enzymatic digestion of the chitin cell wall is similar to chitinases and cellulases [60, 200, 201]. Thus, it can be speculated that Class I chitinases evolved from 8C- or 10C-hevein-like peptide precursors comprising catalytic domains.

Evaluation of the important residues involved in the bio-processing of hevein-like peptide precursors was performed by aligning the putative peptide precursors. It was observed that excision between the signal peptide and the mature domain occurs at either a glycine or alanine residue. A variety of endoproteases in the vacuole or Golgi apparatus may be responsible for cleavage of the C-tail from the mature domain. One such family of endoproteases is the asparaginyl endoproteinases (AEPs) responsible for the cleavage and cyclization of cyclotides [202]. In hevein-like peptides, the C-terminal cleavage site is occupied by a glycine residue suggesting that a glycine endoproteinase might be responsible for cleavage of the mature domain from the C-tail.

3.4. Applications of hevein-like peptides

The discovery that hevein-like peptides are more numerous than originally anticipated will be of value in developing strategies to utilize this diverse class of CRPs for agrochemical and pharmaceutical applications.

3.4.7. Development of transgenic crops

Plant fungal pathogens adversely affect a myriad of agricultural crops leading to major losses in the agricultural industry and posing a threat to food safety [203]. Thus, effective and sustained control of agricultural crops against fungal pathogens is becoming increasingly important. Production of transgenic plants with increased resistance to fungal infections could alleviate the problem. However, expression of non-proteinaceous inhibitors in plants is cumbersome as a consequence of their complex bio-processing pathways [165]. Knowledge of the gene structure and *in vitro* anti-fungal activity of hevein-like peptides can be used for the development of transgenic plants with increased resistance to fungal pathogens. Previously, transgenic expression of 8C-hevein-like peptide, *pnAMP-h2* cDNA in tobacco plants has resulted in increased resistance to oomycete, *Phytophthora infestans* known to cause black shank disease [98]. Similarly, Gao *et al.* have successfully expressed a defensin gene in potato plants which demonstrated resistance against *V. dahliae* in greenhouse and field tests comparable to commonly used fumigants [204]. Thus, hevein-like peptides serve as less-toxic alternatives to commercially used fungicidal agents and their ease

of expression in plants makes them promising candidates for the development of transgenic crops.

3.4.8. Scaffold for development of peptidyl bioactives

Cysteine-rich peptides possessing a cystine-knotted structure serve as scaffolds to graft multiple linear bioactive peptides or proteins from plants, animals or microorganisms. Thus, cystine-knotted hevein-like peptides possess several features that make them interesting candidates for engineering stable bioactive peptides with increased bioavailability. First, hevein-like peptides are easily available by chemical synthesis or molecular biology methods. Second, cystine-knotted peptides are tolerant to sequence variations in multiple loops thus; the structure of hevein-like peptides makes them potential scaffolds for grafting linear bioactive peptides [205-207]. This technique has previously been used to graft bradykinin antagonists and melanocortin onto cyclotide kalata B1 [208, 209], human kallikrein-related peptidase 4 inhibitor onto sunflower trypsin inhibitor-1 [210] and angiogenic peptides onto *Momordica cochinchinensis* trypsin inhibitor-II [211]. Third, the cystine-knotted scaffold confers high resistance to degradation by proteolytic enzymes thereby increasing the bioavailability of grafted linear peptides [40, 158]. Due to the lectin-like property of hevein-like peptides, they can be used for the development of targeted therapeutics with specificity for glyco-peptides on the plasma membrane. These peptide conjugates would have the added advantage of increased bioavailability along

with high specificity resulting in lesser off-target side-effects which are common among small molecule drugs.

Thus, for the first time, the distribution and evolution of hevein-like peptides in plants have been studied using a bioinformatics-guided approach. The unprecedented distribution of hevein-like peptides in bryophytes, gymnosperms and angiosperms suggests that they are integral for plant defense against fungi. Insights into the sequence diversity of 6C-, 8C- and 10C- hevein-like peptides show that the key residues involved in binding to chitin are relatively more conserved as compared to the remaining residues in the sequence. On analyzing the precursor organization, it was found that 8C- and 10C-hevein-like peptides show great diversity in the C-terminal tail which encodes for other bioactive proteins like Barwin and RlpA-DPBB-1. Thus, combined knowledge of distribution, sequence diversity and biosynthesis allowed us to propose a probable evolutionary pathway of hevein-like peptides. We presume that 6C- and 10C-hevein-like peptides arose by divergent evolution from their 8C-hevein-like peptide ancestors in closely related plant families.

Figure 5.13. Updated timeline of hevein-like peptides. About 56 years after the discovery of hevein, our study on the distribution of hevein-like peptides in plants has expanded our knowledge on this highly diverse family of CRPs. We have discovered 385 novel hevein-like peptides across the plant kingdom and also suggested a possible evolutionary pathway for the hevein-like peptide family, the largest family of constitutively expressed CRPs.

Summary, Conclusion and Future Outlook

Hevein-like peptides are a family of cysteine-rich peptides that are rich in glycine and aromatic residues. They play an integral role in plant defense against fungal pathogens by binding to chitin, a polysaccharide of repeating N-acetylglucosamine units (GlcNAc) and the primary constituent of fungal cell walls. An interesting feature of the hevein-like peptide family is that they can be divided into three sub-classes, namely 6C-, 8C- and 10C-hevein-like peptides based on the number of cysteine residues they contain. The molecular diversity and defense role of hevein-like peptides in plants prompted us to isolate and characterize novel hevein-like peptides from medicinal herbs like *Alternanthera sessilis* and *Moringa oleifera*. We also used bioinformatics and proteomic methods to gain insights into the distribution, diversity and evolution of the hevein-like peptide family.

Chapter 3 discusses the isolation and characterization of six novel 6C-hevein-like peptides, collectively named altides, from red and green varieties of *A. sessilis*. This finding is significant as it expands the existing library of 6C-hevein-like peptides from four to ten. Our data signifies the high molecular diversity of altides as none of the loops are absolutely conserved even among altides from the same plant species. Structural analysis of aSG1 using 2D-NMR showed that the three disulfide bonds form a cystine knot conferring altides stability against thermal and enzymatic degradation.

From ^1H -NMR titration experiments we found that the binding affinity of altides, aSG1 and aSR1 to $(\text{GlcNAc})_2$ and $(\text{GlcNAc})_3$ was in the micromolar range and decreased by two-orders of magnitude with an increase in one GlcNAc unit. The binding motif comprised a conserved Ser, Gly and three aromatic residues occupying the chitin-binding domain. Since the binding interaction with chitin oligosaccharides was established, we studied the anti-fungal effects of altides against seven commonly occurring phytopathogenic fungi. Altides, aSG1 and aSR1 showed broad-spectrum anti-fungal effects against four of the seven tested strains with IC_{50} in the micromolar range. Microscopic analysis of fungi treated with altides showed that the hyphae were stunted and branching was reduced suggesting that altides inhibit fungi by retarding hyphal growth. Knowledge of the binding interaction with chitin oligosaccharides and anti-fungal property of altides can be used to develop robust anti-fungal agents with increased binding affinity to chitin.

Gene cloning studies of aSG1 revealed that altides are synthesized as a precursor protein and are processed to attain the mature peptide. The altide precursors comprise a signal peptide domain, a mature domain and a C-terminal tail suggesting that their biosynthesis is through a secretory pathway. Knowledge of the gene organization and precursor structure may be useful for the development of transgenic crops with increased resistance to fungal pathogens.

Chapter 4 describes the isolation and characterization of three novel 8C-hevein-like peptides, called morintides, from *M. oleifera* using proteomic and transcriptomic methods. Morintides mO1 and mO2 are 44 amino acid residues in

length and differed in only one amino acid residue while mO3 is 35 amino acid residues in length. Sequence comparison with previously reported 8C-hevein-like peptides showed the cysteine-spacing pattern is conserved and the sequences can be divided into six loops. The N-terminal of all 8C-hevein-like peptides comprises a Gln residue that is spontaneously converted to pyroglutamate, enhancing resistance against exopeptidases. Apart from the cysteine residues, three Gln, one Leu and two Ser residues are absolutely conserved suggesting that they may play a significant role in structure stabilization. Computer modeling of the structure of morintide mO1 revealed that the first three disulfide bonds form a cystine knot while the fourth disulfide bond is at the C-terminal.

Studies on the chitin-binding property of morintide, mO1, revealed that mO1 binds completely to chitin beads, and its elution required heating at 100°C under acidic conditions, suggesting a strong binding interaction between mO1 and chitin. As a consequence of its chitin-binding property, mO1 could inhibit the growth of two phytopathogenic fungi, *A. alternata* and *A. brassiciola*. Morphological changes like short and thick hyphae with reduced branching were observed on treatment of *A. alternata* and *A. brassiciola* with mO1. Transcriptomic analysis revealed that morintides have a similar precursor organization as other hevein-like peptides and comprise a signal peptide domain, mature domain and a C-terminal tail. However, the C-tail of morintides is only 15 amino acid residues in length in contrast to the 142 amino-acid-long C-tail of hevein which encodes a protein with a Barwin domain. This diversity of precursor

organization may hold clues to the evolutionary pattern of 8C-hevein-like peptides.

In chapter 5, the distribution, evolution and diversity of the hevein-like peptide family was studied using an EST-based in silico approach. A total of 385 novel hevein-like peptides were discovered in 124 plant families belonging to 52 orders. This widespread distribution of hevein-like peptides suggests that they are probably the largest family of constitutively expressed CRPs. Sequence alignment and comparison of 6C-, 8C- and 10C-hevein-like peptides shed light on the molecular diversity of this peptide family. While 6C- and 8C-hevein-like peptides had conserved cysteine-spacing patterns, 10C-hevein-like peptides had four distinct cysteine-spacing motifs which we designated Type I to Type IV resulting in distinct disulfide pairing. While cystine patterns Type I, Type II and Type III have been previously reported, Type IV is a novel motif found during our EST-based search in a Marchantiophyte, *Plagiochila asplenioides*. Further studies will be done to isolate this peptide and elucidate its disulfide pattern and structure. This promiscuity of the cysteine residues and disulfide bonds in 10C-hevein-like peptides further highlights the molecular diversity of this vast peptide family.

Knowledge of the precursor structure of hevein-like peptides was used to construct a phylogenetic tree and gain insight into their putative evolutionary pathway. A striking feature of hevein-like peptide precursors is the diversity of their C-terminal tail. While 6C-hevein-like peptides comprise short C-tails that bear no sequence homology to any known proteins, certain 8C- and 10C-hevein-

like peptides comprise chimeric precursors where the C-tails encode proteins with Barwin, RlpA-DPBB-1, lysozyme-like or Extensin domains. These chimeric precursors may function like “guided missiles” where the hevein-like peptide domains bind to GlcNAc units and the catalytic domains digest the chitin chains.

Phylogenetic analysis suggests that 8C-hevein-like peptides are the most ancient members of the hevein-like peptide family as they are expressed in Bryophytes, the first terrestrial plants on Earth. In addition, our analysis showed that 6C- and 10C-hevein-like peptides are evolved from their 8C-hevein-like peptide ancestors by divergent evolution. We propose that 10C-hevein-like peptides are originated from point mutations in 8C-hevein-like peptides resulting in the insertion of two additional cysteine residues, whereas, the 6C-hevein-like peptides are originated from the C-terminal truncations of 8C-hevein-like peptides. This hypothesis provides an evolutionary pathway of hevein-like peptides for the first time

In conclusion, this thesis presents the discovery, isolation and characterization of novel hevein-like peptides from *A. sessilis* and *M. oleifera* with insights into their binding interaction with chitin oligosaccharides and anti-fungal properties against phyto-pathogenic fungi. In addition, EST-based data mining has expanded the existing library of hevein-like peptides from 20 to 385. This unprecedented number of hevein-like peptides distributed across the plant kingdom further validates the integral role of this peptide family in plant defense. Our data also sheds light on the distribution and evolution of hevein-like peptides and further advances our understanding of this diverse family of CRPs. Taken together, this thesis highlights the molecular diversity of one of the largest families of

constitutively expressed CRPs and opens up new avenues for the development of non-toxic, peptide-based anti-fungal agents that can be of commercial and agricultural value.

Publications and Presentations

1. **Kini, S. G.**, Nguyen, P. Q., Weissbach, S., Mallagaray, A., Shin, J., Yoon, H. S., and Tam, J. P. (2015) Studies on the Chitin Binding Property of Novel Cysteine-Rich Peptides from *Alternanthera sessilis*, *Biochemistry* 54, 6639-6649.
2. **Kini, S. G.**, Wong, K. H., Tan, W. L., and Tam, J.P., 8C_S-Hevein-like-Peptides: A Novel Sub-Class of Hevein-like Peptides Isolated from *Moringa oleifera* (in manuscript)
3. **Kini, S. G.**, Wong, K. H., Tan, W. L., and Tam, J.P., EST-based in silico identification of novel hevein-like peptides in plants (in manuscript)
4. **Kini, S. G.**, Nguyen, P. Q., Weissbach, S., Mallagaray, A., Shin, J., Yoon, H. S., and Tam, J. P Studies on the Chitin Binding Property of Novel Cysteine-Rich Peptides from *A. sessilis*, Peptide Therapeutics Symposium, San Diego, California, USA, Oct 2015
5. **Kini, S. G.** and Tam, J. P., Hevein-like Peptides from *Alternanthera sessilis*: Bringing Down the “Walls” of Fungal Pathogens, International Peptide Symposium, Singapore, Dec 2015

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Appendix

Appendix A. Replicate sequences of putative 6C- and 8C-hevein-like peptides in plants

6C-Hevein-like Peptides

Plant	Family	Peptide Sequence
<i>Amaranthus retroflexus</i>	Amaranthaceae	EGECNNGQCPAGLCCSQYGYCGSGPDYCG
<i>Chamaseyca mesebyranthemum</i>	Euphorbiaceae	
<i>Flaveria bidentis</i>	Asteraceae	
<i>Portulaca mauui</i>	Portulacaceae	
<i>Alternanthera sessilis</i>	Amaranthaceae	SGECNMYGRCPAGYCCSVYGYCGLGPKYCG
<i>Atriplex hortensis</i>	Amaranthaceae	
<i>Atriplex prostrata</i>	Amaranthaceae	
<i>Chamaseyca mesebyranthemum</i>	Euphorbiaceae	
<i>Heliotropium calcicola</i>	Heliotropiaceae	

8C-Hevein-like Peptides

Plant	Family	Peptide Sequence
<i>Lycopersicon cheesmanii</i>	Solanaceae	QQCGRQRGGALCGGNLCCSQFGWCGSTPEYCSQGCQSQCRG
<i>Solanum lycopersicum</i>	Solanaceae	
<i>Capsicum annuum</i>	Solanaceae	QQCGRQRGGALCGGNLCCSQFGWCGSTPEYCSQGCQSQCSG
<i>Nicotiana tabacum</i>	Solanaceae	
<i>Actinidia chinensis</i>	Actinidiaceae	ENCGRQAGGALCPGGQCCSKWGWCGTTPDHC GTDCQSQCGG
<i>Actinidia deliciosa</i>	Actinidiaceae	
<i>Actinidia eriantha</i>	Actinidiaceae	
<i>Medicago truncatula</i>	Fabaceae	
<i>Arachis hypogaea</i>	Fabaceae	EQCGYQVGGAHCANSLCCSKYGWCGTTPDYCSPDAGCQSNCWG
<i>Arachis ipensis</i>	Fabaceae	
<i>Glycine max</i>	Fabaceae	EQCGRQAGGQTCNNLCCSQYGWCGNTTEYCSPSKNCQSNCWG
<i>Widdringtonia cedarbergensis</i>	Cupressaceae	
<i>Populus nigra</i>	Salicaceae	EQCGKQAGGQTCNNLCCSQYGWCGD TDDYCSPSKNCQSNCKG
<i>Populus tremula</i>	Salicaceae	
<i>Populus trichocarpa</i>	Salicaceae	
<i>Lathyrus sativus</i>	Fabaceae	EQCGRQAGGATCPNNLCCSQYGYCGD TDDYCSPSKNCQSNCHG
<i>Pisum sativum</i>	Fabaceae	
<i>Galega orientalis</i>	Fabaceae	EQCGRQAGGKTCNNLCCSQYGYCGNTDDYCSPSKNCQSN CQG
<i>Glycyrrhiza glabra</i>	Fabaceae	
<i>Bursera simaruba</i>	Burseraceae	EQCGRQAGGKLCNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSG
<i>Vitex agnus castus</i>	Lamiaceae	
<i>Eutrema salsugineum</i>	Brassicaceae	QQCGSQAGGQTCGNICCSQYGYCGTTADYCSPDNNCQSN CWG
<i>Thellungiella halophila</i>	Brassicaceae	
<i>Limnanthes douglassii</i>	Limnathaceae	QQCGSQAGGRTCPNNICCSQYGYCGTTEDYCSPSKNCQSN CWG
<i>Trochodendron araliodes</i>	Trochodendronaceae	
<i>Lycium sp</i>	Solanaceae	QQCGRQAGGKTCQGNVCCSQYGYCGTDDYCSPSKNCQSN CQG
<i>Tellima breviflora</i>	Saxifragaceae	
<i>Juglans nigra</i>	Juglandaceae	QQCGKQAGGKTCNNLCCSQYGYCGSTDDYCSPSKGCQSN CQS
<i>Phellodendron amurense</i>	Rutaceae	

Appendix B. Full-length precursors of putative 6C-hevein-like peptides

6C-Hevein-like Peptides

Plant name	Signal peptide	Mature domain	C-Terminal tail
<i>Aerva lanata</i>	MMSMKSCLMIMVIVALVMVEPSMG	TGECKHGKCPNGLCCSQYGYCGSGPAYCGGA	NEQQAALLQRTGVTNTGECKHGKCPNGLCCSQYG
<i>Aerva persica</i>	MMNMKKFLILMVVIALVMAEPSMG	AGECRNGRCPSGLCCSRYGYCGSGPAYCG	KSNMLHFFSVTAVSLRTLPELILEHNHIIVACLLTSKVNMYLST
<i>Alternanthera caracasana</i>	MMNMKKFLILMVVIALVMAEPSMG	AGECRNGRCPSGLCCSRYGYCGSGPAYCG	SAQEQAALLQRNGGVTTDTTGAHP
<i>Alternanthera sessilis</i>	MMKSFVIMMVMSLMVGTSMG	SGECNMYGRCPAYGCCSVYGYCGLGPKYCG	NAEQQVAEQQVAHPASVNTTANLPQTRKVRAPLGVNTP
<i>Alternanthera sessilis</i>	MVNMKSFVIMVIMALVFVEPSMG	TGECVNGLCPRGLCCSQYGYCGKGYCGS	ADEQRAALLERTAAKHDAHVDGTTAP
<i>Alternanthera sessilis</i>	MMNMKKFLIVMVVALVMVEPSMG	APGQCKHGRCPSGLCCSQYGYCGTGPAYCG	GAAEQRAALLQRTGSVTADTTDTTKAP
<i>Amaranthus retroflexus</i>	MNMKSLVLMVILALMMVQPSMG	DGECVNGSCPNGLCSSQFYGYCGSGPAYCG	GADEQRAALLQRIGVKTRKIPTTKITGAGAP
<i>Amaranthus retroflexus</i>	MANNMKKFMMVMVVALVMVGPSMG	EGECNNGQCPAGLCCSQYGYCGSGPDYCG	AAAQQAALLKRTGATSATTTTTQAP
<i>Atriplex hortensis</i>	MMKSFVIMMVMSLMVGTSMG	SGECNMYGRCPAYGCCSVYGYCGLGPKYCG	DAEQQGAQPASVNTTANLPQTRKVRAPLGVETP
<i>Atriplex prostrata</i>	MMKSFVIMMVMSLMVGTSMG	SGECNMYGRCPAYGCCSVYGYCGLGPKYCG	NAEQQVAEQQVAHPASVNTTANLPQTRKVRAPLGVNTP
<i>Atriplex rosea</i>	MMKMKSFVIMVIMTLMVGTSM	QSGQCTDGQCPNGLCCSQYGYCGSGPAYCG	GSVEQAQADPSTDNPQVVVGAKAP
<i>Beta maritima</i>	MKSFVIVMLVMSMMVATSM	SGECNMYGRCPPGYCCSKFYCGVGRAYCG	DAEQKVEDHPSNDADVPKFVGAGAP
<i>Chamaseyca mesebyranthemum</i>	MANNMKKFMMVMVVALVMVGPSMG	EGECNNGQCPAGLCCSQYGYCGSGPDYCG	AAAQQAALLKRTGATSATTTTTQAP
<i>Chamaseyca mesebyranthemum</i>	MMKSFVIMMVMSLMVGTSMG	SGECNMYGRCPAYGCCSVYGYCGLGPKYCG	NNAEQQVAEQQVAHPASVNTTANLPQTRKVRAPLGVNTP
<i>Flaveria bidentis</i>	MANNMKKFMMVMVVALVMVGPSMG	EGECNNGQCPAGLCCSQYGYCGSGPDYCG	AAAQQAALLKRTGATSATTTTTQAP
<i>Heliotropium calcicola</i>	MMKSFVIMMVMSLMVGTSMG	SGECNMYGRCPAYGCCSVYGYCGLGPKYCG	NAEQQVAEQQVAHPASVNTTANLPQTRKVRAPLGVNTP
<i>Heliotropium tenellum</i>	MMNMKKFLILMVVIALVMAEPSMG	AGECRNGRCPSGLCCSRYGYCGSGPAYCG	SAEQQXSVTAVSLRTLPELILEHNHIIVACLLTSKVNMYLST
<i>Heliotropium texanum</i>	MVVIALVMAEPSMG	AGECRNGRCPSGLCCSRYGYCGSGPAYCG	SAQEQAALLQRTG
<i>Kochia scopari</i>	MMKSFVIMMVMSLIVGTSM	EGECKKGRCPRGLCCSKHGYCGRGPEYCG	RDAEQAVDTTDLLQARKVRAPVGVKTP
<i>Portulaca mauii</i>	MANNMKKFMMVMVVALVMVGPSMG	EGECNNGQCPAGLCCSQYGYCGSGPDYCG	AAAQQAALLKRTGATSATTTTTQAP

Appendix C. Full-length precursors of putative 8C-hevein-like peptides

8C-Hevein-like Peptides	Plant name	Signal peptide	Mature domain	C-Terminal tail
<i>Actinidia deliciosa</i>		MKRAGMFGVWVLAILYLATDGAA	QFCGRQAGGRKCPKKICCSKWGYCGTTPDFCLPSNGCQSNCKK	RLVDGGESASNVMATYHYYNPEQVGWDLRAVSSYCSCTWDNDMPLAVRKKYGWA
<i>A. deliciosa</i> (cont.)				AFCGPAGPRGQASCGKCLRVTNTATGAETTVSIVDRCSNGGLDLDITGVFQGLD
<i>A. deliciosa</i> (cont.)				TDGQGNARGHLIVDYQFVNCG
<i>Actinidia eriantha</i>		MVGMFVWAVAILYLAAGGAA	QQCGRQAGGRLCDNNLCCSQMWGYCGNTDEYCLPSNNCQSNCKG	SGGDSASNVRRATYHFYNPEQVGWDLRAVSAYCSTWDADKPLAWRKKYGTAF
<i>Actinidia eriantha</i>				GPVGPGRQASCGKCL
<i>Actinidia setosa</i>		MFGVWVLAILYLATDGAA	QYQCGRQAGGRKCPNKVCCSKWGYCGTTEPFCFLPSKGCQSNCKK	RLVGGGESASNVMATYHYYNPEQVGWDLTAVSSYCSCTWDANMPLALRKK
<i>Actinidia chinensis</i>		MKMRVCVLVSVLAILVIRGSA	ENCGRQAGGALCPGGQCCSKWGCCTPDHCGTDCQSQCGG	TPSPGPSGSDITNLIPRSTFDRMLLHRNDGACPGKNFYTYDGFIAAARSFG
<i>Agathis robusta</i>		MEYKNLVCVVLVAVVIGESSVSVKG	DPTCSPAGQVYCNSSGRCCSKFNWCGSTAAYCQRPNCIAQCWP	VTALTLESLLSLSNYTSEATMXC
<i>Ajuga reptans</i>		MAFFLIQTKPLLTLTLTALFLAGGTQVSG	QNCGCASNECCSQMWGYCGTTNDYCGRGCQSGPCFAP	PSTNGASVPDIVTDAFFNGIADQAA
<i>Alangium chinense</i>		MSKKQQSKMGRSINLCAALIACLTVAATA	QQCGRQAGGRLCPGNICCSQMWGYCGTTDEYCLPSQNCQSNCKP	GGGESASNVRRATYHFYNPEQVGWDLNAVSAYCSTWDANKPLAWRRYGTAF
<i>A. chinense</i> (cont.)				GPAGPRGQAACGRCLRVTNTGTGTQTTVRIVDQCSNGGLDLDVGVFRQLDITDQ
<i>A. chinense</i> (cont.)				GYARGHLIVNYQFVDCDGLNELNALLPILDFQ
<i>Allium commutatum</i>		MKTVLLVTTILALAFALFTNSYA	QQCGGQAGGALCSNGLCCSQYGYCGSTSDYCGPGCQSQCG	GGGAGGGAGGGGGV
<i>Alnus glutinosa</i>		MERLSVCLVLFCLLATATA	QQCGSQAGGKTCADNLCCSQYGYCGTTDDYCLPSNNCQSNCKSSG	DSSTGQASNVRRATYHFYNPEQNGWDLNAVSAYCSTWDANKPLAWRSKYGWA
<i>A. glutinosa</i> (cont.)				FCGPVGPGRQDSCGKCLKVTNSATGAQTTVRIVDQCSNGGLDDEGEFRKID
<i>A. glutinosa</i> (cont.)				TDGRGYAQGHLTVSYEFV
<i>Amentotaxus argotaenia</i>		MALYKLSVTVLVMLMFTAMLRVMA	DPTCSPAGNFFNCCNRCCSRFNWCGTGSPYCGRGNICIAQCP	PAVTALTYSYFSKNLTSANP
<i>Amentotaxus argotaenia</i>		MKKSLLLIQFVLAVISTSSNVIVRG	DPTCSPAGRFFNCCNRCCSKFNWCGSTSDYCARPNCVAQCWP	VTTLTYSKSLLTITNNTP
<i>Amentotaxus argotaenia</i>		MVRTGTHHSTMAETRLKLAFYVLVLMVAVKGLG	DPTCSPAGNFWCDTGRCCSIYNWCGSTAECYCAQGNCLAQCWSS	VAALNHYYQGLSLLNQMLTASLNNP
<i>Amentotaxus argotaenia</i>		MAKVKMLVFSILAIIVLDLGLGLPAFAA	QCGRQDRGRRCPRLCCSQFGYCGSTPAYCCAGCQSQ	CKCPQGAFAATAEEQQSLNIIIP
<i>Ammopiptanthus mongolicus</i>		MKKVGVAGLVVFLCLIVTAIA	EQCGSQAGGKKCPNNLCCSQYGYCGNSEDYCSPSKNCQSNCWG	GGGGESTSNVRATYHYYPQEHGWDLNAVSAYCSTWDAGKPSYWSKYGTAF
<i>A. mongolicus</i> (cont.)				CGPVPHGQASCGKCLRVTNTGTGAQEIIVRIVDQCSNGGLDLDVGVFNRLD
<i>A. mongolicus</i> (cont.)				GRGYQQGHLIVNYQFVDCGNELEELTNPSIPSIILDALQ
<i>Ammopiptanthus mongolicus</i>		MKKVGVVGLVVFLCLIVTAIA	EQCGSQAGGKKCPNNLCCSQYGYCGDSEDYCSPSKNCQSNCWG	GGGGESASNVRRATYHYYPQEHGWDLNAVSAYCSTWDAGKPSYWSKYGTAF
<i>A. mongolicus</i> (cont.)				GPVGPBGQASCGKCLRVTNTGTGAQEIIVRIVDQCSNGGLDLDVGVFNRLD
<i>A. mongolicus</i> (cont.)				GRGYQQGHLIVNYQFADCGNELEKLTNPSPSIILDALQ
<i>Anemone hupehensis</i>		MGNRNLSSVLLLLCLIGVATA	ENCGRQVGATCPGNHCCSQWGWCGTDEHCLPSKNCQSNCRG	GGSSGGGGGGSATNVRRATYHYVNPAGIGWDLYRASAYCSTWDGNKPLEWRKYGW
<i>A. hupehensis</i> (cont.)				TAFCPVGPGRQASCGRCLRVTNTXTLQ
<i>Anomodon attenuatus</i>		MARHGEMLIVLVSSLLALTALAA	QQCGSQAGNRRCPNNLCCSQYGYCGTDPYCGNGCQSQCHG	GGGGGGSTGKASYSPYIPISACYGNDESQFPNRYMTAVSDGNPNLWQNRQCGG
<i>A. attenuatus</i> (cont.)				KHYRIRCQNGCRNGDTITIKVVEFCPNGCHGRVFDLSEEAFAIAADKNVGVIT
<i>A. attenuatus</i> (cont.)				VQYEPVNSADAIPWAQEKLIAEVGRIGK
<i>Aphanopetalum resinosum</i>		MVNFSCEFLLMLCIACVARA	EECGRQAGGRTCSGNICCSQYGYCGTSDDYCSPSKNCQSNCAG	GGSGGGGGGGGQSTSNVRATYHYYNPEQHWDLTAVSAYCSTWDAGKPLAWRKR
<i>A. resinosum</i> (cont.)				HGWTAFCPGAPGRGQAACGKCLRVTNSWTGAKTTVRIVDQCSNGGLDLDVGVFQ
<i>A. resinosum</i> (cont.)				KLDTNGRGYQQGHLIVSYEFVNCGDGIYNPL
<i>Apios americana</i>		MKGFEVCLAVLLCLMVTAIA	EQCGRQAGGQTCPDNLCCSQYGYCGNTEDYCSPSKGCQSNCG	GGSGGGGGGGGESASNVRRATYHYYPEKHWDLNAVSAYCSTWDAGKPSYWSRK
<i>A. americana</i> (cont.)				YGMGXCGPVGPGRQDSCGKCLQVTNTGTGAQTTVRIVDQCSNGGLDLDVGVFNR
<i>A. americana</i> (cont.)				LDTDGRGYQQGHLIVNYQFVDCGNQLDLTKPLFSIILDA
<i>Apios americana</i>		MKGFEVCLAVLLCLMVTAIA	EQCGRQAGGQTCPDNLCCSQYGYCGNTEDYCSPSKGCQSNCG	GGSGGGGGGGGESASNVRRATYHYYPEKHWDLNAVSAYCSTWDAGKPSYWSRK
<i>A. americana</i> (cont.)				YGMGXCGPVGPGRQDSCGKCLQVTNTGTGAQTTVRIVDQCSNGGLDLDVGVFNR
<i>A. americana</i> (cont.)				LDTDGRGYQQGHLIVNYQFVNCG
<i>Arabidopsis thaliana</i>		MKIRLSITILLSYTVATVAG	QQCGRQGGGRTCPGNICCSQYGYCGTTADYCSPTNNCQSNCWG	SGPXGPGESASNVRRATYHFYNPAQNNDLRAVSAYCSTWDADKPYAWRSKYGT
<i>A. thaliana</i> (cont.)				AFCGPAGPRGQASCGKCLRVTNRTRTNAAVTVRIVDQCSNGGLDLSMFNQIX
<i>Arabis alpina</i>		MKRLDILTIILLAYTVALVAG	QQCGSQAGGKTCSGNICCSQYGYCGTTADYCSPANCKQSNCWGSG	PPPSGGESASNVRRATYHYYNPEQNNWDLRAVSAYCSTWDADKPYAWRSKYGWA
<i>A. alpina</i> (cont.)				FCGPAGPRGQASCGKCLRVTNRTRTNAAVTVRIVDQCSNGGLDLDVAMFNRI
<i>A. alpina</i> (cont.)				TDGNGYQQGHLIVDYQFVDCGNELETSKNIILVSA
<i>Arachis duranensis</i>		MARVGTCIVLSLCLIMVTMAMG	EQCGYQVGGAYCANSLLCCSKYGCWCGTTYDYCSPDAGCQSNCWG	GSTPTPTPTPTPTGTPVRATYHYLPEQNGWDLMAVSAYCSTYDANKPLSWRSK
<i>A. duranensis</i> (cont.)				YGTAFCPGVPSPGFPACGRCILVTNSATKASEIIVRIVDQCSNGGLDLDVGVFN
<i>A. duranensis</i> (cont.)				RIDTDGRGYQQGHLVDVTSF
<i>Arachis duranensis</i>		MARVGTCIVLSLCLIMVTMAMG	EQCGYQVGGAYCANSLLCCSKYGCWCGTTYDYCSPDAGCHSNCWG	GSTPTPTPTPTPTGTPVRATYPL
<i>Arachis hypogaea</i>		MKMGNKLPILLVAMALVMIASVSA	QNCGCASGLCCSKYGYCGTDAYCGDGCQEGPCYSS	PSTPSTPSSSGGVNVADVVTQDFNSSGGVNVADVVTQDFNSSIINQASANCE
<i>A. hypogaea</i> (cont.)				GKNFYTRDAFLSALNSYSTFGQLSSTDSSKREIAAFAHFTHETGYFCYINEQN
<i>A. hypogaea</i> (cont.)				GASHNYCDSSYTYQCPNPNKYVGRPLQLTWNYNYGAGNANNFDGLNAPETV
<i>Arachis hypogaea</i>		MGRVGTCIVVSLCLMVTMATG	EQCGYQVGGAHANSLLCCSKYGCWCGTTYDYCSPDAGCQSNCWG	GPTPTPTPTPTPTGTPVRATYHYLPEQNGWDLMAVSAYCSTYDANKPLSWRSKY
<i>A. hypogaea</i> (cont.)				GTAFCPGVPSPGFPACGRCILVTNSATKASEIIVRIVDQCSNGGLDLDVGVFN
<i>A. hypogaea</i> (cont.)				IDTDGRG
<i>Arachis hypogaea</i>		MLNAKLPYLLSLTLLLLLLGAKA	EQCGRQAGGALCPNNYCCSQFGWCGTDDYCLASKGCQSQCRG	PPTPSPRPPSPAPPGGDVANVISSIFDQMLKYRNDPRCHANGFCTYNAFITA
<i>A. hypogaea</i> (cont.)				ARSFNGFGTTGGDATRKKEEAFLGQTSHETGGWAS

<i>Arachis ipaensis</i>	MSYTKIILNMLSLILTLFLLHGGAA	EQCGSQGGGALCPNNLCCSRWGC GTDDYCLVGNCGCSQCKD	SPTPTPTPTPTPTSPSPSGGDISKYINSSMFDPMIKYRNDPRCHANGFYTNDAFIN
<i>Arachis ipaensis</i>	MGRVGTCLIVVSLCLMVTMATG	EQCGYQVGGAHCA NSLCCSKYGC GTTYDYCSPDAGCQSNCWG	GTPTPTPTPTPTPTGTPVRATYHYHYPEQNGWDLMAVSAYCSTYDANKPLSWRSK
<i>A. ipaensis (cont.)</i>			YGWTAFCGPNSPGFFAACGRCLIVTNSATKASEIVRIVDQCNNGGLLDLVGVFAR
<i>A. ipaensis (cont.)</i>			RGHLIVDYQFVNCGGDTTSENEPLFSIMDVAVD
<i>Argemone mexicana</i>	MATKNLSIFALVLFLLTTIVSIASA	QQCGRQAGGQTCPNGLCCSQYGYCGDTDDYCLPSNNCKSKCKG	SSGGGGGGGGSGESASNV RATYHYHYPNAQNGWDLTRVSAYCSTWDANKPLAWR
<i>A. mexicana (cont.)</i>			KYQGWTAFCGPGVPRGQASCGRCLRVNTRGTGAQITVRIVDQCSNGGLDLVDYD
<i>A. mexicana (cont.)</i>			VFKPIDTDGQGYLRGHMFVDYQFVNCGD
<i>Asparagus densiflorus</i>	MKEFLILTLFLTISSSILSSA	EQCGSQAGGAVCPNGLCCSKFYCGTTSAYCCDGCQSQGTP	TFNPPSGGGGVSSSIISSSLFNQMLLH
<i>Astilbe chinensis</i>	MERMNLCMIIMLLPYLVSSVAA	QQCGRQAGGRTCANNLCCSQFYCGSTA EYCS TNCQSNCSNG	GGGGGGQSASNV RATYHFYNP EQNGWNLNAVSAYCSTWDANKPLEWRKRYGWTA
<i>A. chinensis (cont.)</i>			FCGPGVGRGRDSCGKCLRVNTNTGTGTATVRIVDQCSNGGLDLDA GVPFKKLDTN
<i>A. chinensis (cont.)</i>			GRGNAQGHLIVNYQFVNCGD
<i>Astragalus membranaceus</i>	MGNFGIGLVVVVSILGCLIVTAIG	EQCGRQGGGQTCPNNCCSQYGWCGDTDEYCSPSKNCQSNCKG	DTGGGESASNV RATYHYHYP EQNGWDLNAVSAYCSTWDAGKPYSWRSKYGWTA
<i>A. membranaceus (cont.)</i>			CGPGVPRGQASCGRCLRVNTNTGTGAQQTVRIVDQCSNGGLDLVGVPFNRLD TDG
<i>A. membranaceus (cont.)</i>			RGYQQGHLIVNYQFVDCGNELEXPPSCLRMWTTKLLIYPLCCSN
<i>Astragalus propinquus</i>	MGNFGIGLVVVVSILGCLIVTAIG	EQCGRQGGGQKCPNNLCCSQYGWCGDTDEYCSPSKNCQSNCKG	DTGGGESASNV RATYHYHYP EQNGWDLNAVSAYCSTWDAGKPYSWRSKYGWTA
<i>A. propinquus (cont.)</i>			FCGPGVPRGQASCGRCLRVNTNTGTGAQQTVRIVDQCSNGGLDLVGVPFNRLD
<i>A. propinquus (cont.)</i>			TDGRGYQQGHLIVNYQFVDCGNELDITKPLFSILHHA
<i>Athrotaxis cupressoides</i>	MALNKVSIIVLVAIVFLMGNVGVMA	DPTCSPSGNFWC NNGRCCSIYNWCGSGAAYCAKGNCLAQ CWP	KAAALNQSSLNLSQQNLTS P SP
<i>Athrotaxis cupressoides</i>	MALNKVSIIVLVAIVFLMGNVGVMA	DPTCSPSGNFWC NNGRCCSIYNWCGSGAAYCAKGNCLAQ CWP	GVTAVTTYSLTQRNLTS TKTNP
<i>Athrotaxis cupressoides</i>	MTNRKYGG EKIKIALYMLLVLMASVMVGK	DPTCSPAGNFWCNSGRCCSIYNWCGSTSDYCASGNCLAQ CWP	KAAALNQSSLNLSQQNLTS P SP
<i>Atropa belladonna</i>	MEMEKLSTILLALVLF SIAAGGNA	QQCGRQGGGALCSGNLCCSQFGWCGSTPEYCSPSQGCQSRCSG	AAGGESAQNV RATYHIYNPQNVGDLNAVSAYCSTWDANKPFAWR SKYGTW
<i>A. belladonna (cont.)</i>			AF CGP
<i>Austrocedrus chilensis</i>	MGEYKSTVSVL MVIVLLGCNIGLPMVMG	DPTCSPSGNFFC NNGRCCSVYNWCGTGNAYCGKGNCLAQ C SP	GVTAVTTYTLNGKNLTSNNRN
<i>Austrocedrus chilensis</i>	MADRRLRLGLHILLVMVIVEKVNG	DPTCSPAGNFWC NTGRCCSIYNWCGSTSDYCASGNCLAQ CWP	NAVALNQSTLQVAKNLTSNSSP
<i>Austrocedrus chilensis</i>	MAYKSSSLALYMLGLMLIRVVKS	DPTCSPAGNFWC NTGRCCSIYNWCGSTSDYCASGNCLAQ CWS	NTVALNKSC LNVSLHNRSSPSP
<i>Austrotaxus spicata</i>	MAKVIE TMLLLCSIFAFIVIGLPSSVSAA	QCGKDADGALCSSLCCSEWGCNTPDHCGAGCQSQCG	EEPTPTPTPTPTPAGEGVASII
<i>Austrotaxus spicata</i>	MLITIFVATEMEMVMA	DPTCSPRGNFFCNSGRCCSKFNWCGSGAQYCAKGNCLAQ CWP	VSALTKYSYQILLAGRNLTSATVNNP
<i>Austrotaxus spicata</i>	MAENRLRLAFYMLVLMAMVVEG	DPTCSPAGNFWC NTGRCCSIYNWCGSTAAYCAQGNCLAQ CWP	TVAALNNSSLTL LQQRNLTSIHQNNP
<i>Basella alba</i>	MRFWAVFLLSWFSATLGGLAQ	EQCGSQAGGALCPGGLCCSQYGYCGTTSAYCGEGCQSQCT	SNVPPPPPTPTPTPTPTPT
<i>Bauhinia tomentosa</i>	MGKLVVLSLVVALVLCIAEA	EQCGKQAGGQICPNNLCCSQYGWCGSTDDYCN TGKGCQSN CAG	GGGGSGESASNV RATYHYHYP EQHGWDLNAVSAYCSTWDAGKPYSWRSKYGTW
<i>B. tomentosa (cont.)</i>			AF CGPAGPTGAAACGKCLRVNTNAWTAQITVRIVDQCSNGGLDLVGVPFNRL
<i>B. tomentosa (cont.)</i>			DTNGRGYQQGHLIVNYQFIDCGNELIHP LLSILGH
<i>Bazzania trilobata</i>	METKASVAILALFLILSPTFIAG	QQCGSQAGGRTCPNGNCCSQWGCWGTDDY CAGGCQSQCG	SNRCGRDFWGRVCSNRCCSAWGC GTDSLACGSGCQSQCHGLGLPTPV SQ
<i>B. trilobata (cont.)</i>			QLAMIMLNLSTLPAHLM EVAKLI
<i>Boehmeria nivea</i>	MERLGLGLGLLALTMLASHFGGARA	EQCGRQAGFAICPNGLCCSQYGWCGDTDYCSPDKNCQSNCWG	GTPTPTPPSGSETIVRATYHYHYPRENNWDL LAVSAFCSTWDASKPYSWRS
<i>B. nivea (cont.)</i>			KYGWTAFCGPGSGPTGRDSCGRCLRL
<i>Boswellia sacra</i>	MSLYKCSWLLALCTLASTLGALA	EQCGSQAGGALCPGGLCCSKFGWCGSTSDYCSSGCQSQC	NGASPS
<i>Boykinia jamesii</i>	MEKRLNLCMIIMLLCLVASAIA	QQCGKQAGGRTCANNLCCSQYGYCGSTA EYCF TNCQSNCSNG	GGGGESASNV RATYHYHYP EQNGWDLNAVSAYCSTWDANKPLEWRKRYGWTA
<i>B. jamesii (cont.)</i>			FCGPGVPRGQASCGRCLRVNTNTGTGTATVRIVDQCSNGGLDLDA GVPQQLD
<i>B. jamesii (cont.)</i>			TNGRGNAQGHLIVNYQFINCGD
<i>Brassica napus</i>	MKIRLSIAIILLSYTAAMVAG	QQCGRQAGGQTCAGNICCSQYGYCGTTADYCSPDNNCQSNCWG	SGPPSGSGESATGV RATYHYHYPNAQNNWDLRAVSAYCSTWDADKPYSWRSKY
<i>B. napus (cont.)</i>			GWTAFCGPAGPRGQASCGRCLRVNTRTNAAVTVRIVDQCSNGGLDLDVAMF
<i>B. napus (cont.)</i>			NQIDTDGDCYRKGH LIVDYQFVDCGNELIDQPADFKNILVSATDRV
<i>Brassica napus</i>	MALTKLSLVFLCFLGLYSETVKS	QNGCAPNLCCSQFYCGSTDA YCGTGCRSGPCRSPG	GTPSPPGGSGVSGIVTQAFLLTVLSIKPVVVA PGRISTPVLISLTPQILS
<i>B. napus (cont.)</i>			QTLPIRLLDVKLQPCLLISPTRLDISAT
<i>Brassica napus</i>	MATHNVLLKNVLMIFLFTLTIMTETAFS	QNCGKTGCKGNMCCSRWGYCGTTNAYCGTGCGSQSGPKCS	KPKPTPTPSGSGLNAGPRGTIASVVTPAFFNGIMSKVGRGCPAKGFYTRQA
<i>B. napus (cont.)</i>			FIVAAQSFAAYKGTVAKREIAAMLAQFSHESGSFCYKEE IAR
<i>Brassica oleracea</i>	MKIRLSIAIILLSYTAAMVAG	QQCGRQAGGQTCAGNICCSQYGYCGTTADYCSPDNNCQSNCWG	GGPPSGSGESATGV RATYHYHYPNAQNNWDLRAVSAYCSTWDADKPYSWRSKY
<i>B. oleracea (cont.)</i>			GWTAFCGPAGPRGQASCGRCLRVNTRTNAAVTVRIVDQCSNGGLDLDVAM
<i>B. oleracea (cont.)</i>			FNQIDTDGDCYRKGH LIVDYQFVDCGNELIDQPADFKNILVSATDRV
<i>Brugmansia sanguinea</i>	MEKLSSTILLALVLFITIGAGANA	QQCGSQRGGALCSGNLCCSQFGWCGSTPEYCSPSQGCQSQCSG	SGPTPGGGGSAQNV RATYHIYNPQNVGDLNAVSAYCSTWDANKPYAWRSK
<i>B. sanguinea (cont.)</i>			YGWTAFCGPAGPRGRDSCGKCLRVNTRTRAQTIVRIVDQCSNGGLDLIN V
<i>B. sanguinea (cont.)</i>			FRQIDTDGVGNQGGHLIVNYQFVDCGNVNVPLLSVVDKE
<i>Bryum argenteum</i>	MAQVGQTQGMWIMRVASLLVQMQLVAVVVA	QQCGSQAGGQNC PGG LCCSQYGYCGTTAEYCGGGCQSQCG	SGGFSADGEKAASPTTSGKASYTTAPYVPSACNGNDNQFPANMLFAAGGD
<i>B. argenteum (cont.)</i>			SSSANIWNSGKNCKNFFNIQCTGKGCRRNNV I VKVVDRCPNGCTGGRAFD
<i>B. argenteum (cont.)</i>			LSSQAFAAIADPAVGVIDIKYSPA
<i>Bursera simaruba</i>	MGRVMNTFIVVLLCLTGVAIA	EQCGRQAGGKLCPNNLCCSQWGCWGSTDEYCSPDHNCQSNCKDSG	EGVGGGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWRXASMA
<i>B. simaruba (cont.)</i>			GLHSAPVSEHTANPPVESA
<i>Caioophora chuquitensis</i>	MGAKMGRGLGVVMLVTIFIAVMIGCAAA	QQCGRQGGGRTCQNNLCCSQYGYCGSTDDYCLPSNGCQSNCRPSG	PGESANNV RATYHFYDPERVGWDLRAVSAYCSTWDADKPLAWRKYGWTAFC
<i>C. chuquitensis (cont.)</i>			GPAGPRGRDSCGRCLRVNTNTGTGAQITVRIVDQCSNGGLDLDFNGVFKRIDT

<i>C. chuquitensis (cont.)</i>			DGQGYAKGHLIVNYQFVNCNDGVNSLLSSIFDL
<i>Cajanus cajan</i>	MGKLGGLVLLCLIATAVA	EQCGSQAGGKTCPNNLCCSQYGWCGNAEDYCSPSKNCQSNCG	GGGESASNV RATYHYYPQELHGWDLNAVSA YCSTWDAGEPYSWRSKYGWTA F
<i>C. cajan (cont.)</i>			CGPVGPRGQASC GKCLRV TNTNGSGPDHCEDR
<i>Callitris gracilis-March</i>	MAFTSSSVAMVMILGLLVMVKS	DPTCSPAGGFWCNI GRCCSIYNWCGSTWEYCAPGNCLAQ CWP	YSRPLNHSSFFHP PHNRSTTSP
<i>Callitris macleayana-March</i>	MEMKKVMVMVMVMVGVGVR SVEG	EPTCSPAGRIYCS PGRCCSKFNWCGSSRAYCGPNCIAQCHSP	RTPLEYATSTLLSATNFTNP
<i>Callitris macleayana-March</i>	MQILAERADMAPNKLRI GLYVLLVLVIVQING	DPTCSPAGNFLCNSGRCCSIYNWCGDTS DYCAAGNCLAQC	WPNLALNQSALNLAQRNITSSSP
<i>Callitris macleayana-March</i>	MVMNKLCMGIIILLALVGLKVKG	DPTCSPAGSFCLNSGRCCSKYNWCGTSDY CASGSCLAQCWP	NAVALNQSALHVAKQNLTS SSP
<i>Calocedrus decurrens</i>	MAYKISSSLPHMLLVLLVMNVKG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDY CASGNCLAQ CWP	NALALANHSSCLNVI SHRNRTSAPAP
<i>Calocedrus decurrens</i>	MEAKAVAFILAVTLAAPLLTLA	ENCGSQGGGKACSGGECCKWGC GTTDDHCLPDRGCQSNCHG	GGGGGGGKAYNVRSTYHEYYPERNWNLYAVSA YCSTWDGEKPLWWRQKYGW
<i>C. decurrens (cont.)</i>			TAFCGPVGPGRGQASC GKCLRV TNRETGAQT VRIVDQCSNGGLDLDINVFRQ
<i>C. decurrens (cont.)</i>			LD TNGQGNARGHLMVDYEFV
<i>Camelina sativa</i>	MNIRLCITIIILLSYTAATVAG	QQCGRQAGGRTCPGN ICCSQYGYCGTTP EYCS PANNCQSNCGG	SGPSGSGESAKNVRATYHIYNPAQNNWDLRAVSA YCSTWDADKPYAWRSKYGW
<i>C. sativa (cont.)</i>			TAFCGPAGPRGQASC GKCLRVNRTRTNAAVTVRIVDQCSNGGLDLDVAMFNQI
<i>C. sativa (cont.)</i>			DTDRNGYQQGHLIVDYQFVNCGD
<i>Camellia sinensis</i>	MAKIGLSMALIMVFPFLALATA	QQCGSQAGGQTCPNNLCCSQYGYCGT TDDYCS PKGCSNCQTG	GGGASGESATNVRATYHYYPQNGWDLNAVSA YCSTWDANKPLAWRSKYGW
<i>C. sinensis (cont.)</i>			AFCGPVGAHQASC GKCLRV TINTGTGAQQT VRIVDQCSNGGLDLDG VFNKLD
<i>C. sinensis (cont.)</i>			TDGKGYAQGHLTVNYQFVDCGD
<i>Cannabis sativa</i>	MGKLSILVLALVLVVVAIGGARA	EQCGRQAGGVKCPNGLCCSQHGWCGNTFDYCS PQAQCQSNCG	GPTPPSGETVVRATYHFYFPAENNWDLMAVSA F CSTWDANKPFSWRSKYGW
<i>C. sativa (cont.)</i>			AFCGPGSGPRGRDSCGKCLRLRNVTGATITARIVDQCSNGGLDLDFNTIFKP
<i>C. sativa (cont.)</i>			LDTDGRGYQQGSMQVGYTFVDCGNLSEDEHQHPLSSIIEEKTQGV
<i>Cannabis sativa</i>	MGKLSILVLALVLVVVAIGGARA	EQCGRQAGGVKCPNGLCCSQFGWCGD TFDYCS PQAQCQSNCG	GPAPPSGETVVRATYHFYFPAENNWDLMAVSA F CSTWDANKPFSWRSKYGW
<i>Capsella rubella</i>	MKIRLSIAIILLSYTAAMVAG	QQCGRQAGGRTCPGN ICCSQYGYCGTTP EYCS PANNCQSNCG	GSAPSGSGESAKNVRATYHIYNPAQNNWDLRAVSA YCSTWDADKSYAWRSKY
<i>C. rubella (cont.)</i>			GWTAFCGPAGPRGQASC GKCLRVNRTRTNAAVTVRIVDQCSNGGLDLDVAMF
<i>C. rubella (cont.)</i>			NQIDTDRNGYQQGHLIVDYQFVNCGD
<i>Capsicum annuum</i>	MEKRTSTTILALVLFIVSGVANA	QQCGRQGGALCGNLC CSQYFGWCGSTPEYCS PQSQCSQCSG	SGPTPEGSAQNV RATYHLYNPQNVG WDLNAVSA YCSTWDANKPLAWRSKYGW
<i>C. annuum (cont.)</i>			AFCGPVGPGRGQASC GKCLRV TNRRTRAQT VRIVDQCSNGGLDLDINVFRQI
<i>C. annuum (cont.)</i>			DTDGNGNQGHLMVDYQFVNCGDNVNVPLLSVVD TQ
<i>Capsicum annuum</i>	MKFQVVILVLPALLLTRTSA	QNCGRQAGGRVCANRLCCSQFGFCGRTREYCGPGCQSNCR	YATDTTGEGENVSND EHKNNGGTSAATTRCCWGFFS
<i>Carica papaya</i>	MGSRAFAFTIVLVGLLGAATA	QNCGRQAGGRTCANNLCCSQFGFCGTTDDHCS PAKNCQSNCRG	SSDTGGGTATNVRATYHFYFNPQNGWDLRAVSA F CSTWDADKPLAWRRHGWT
<i>C. papaya (cont.)</i>			AFCGPVGPGRGQASC GKCLRV TNETGAQT VRIVDQCANGLDLDA GVFRQLD
<i>C. papaya (cont.)</i>			TNGQGNARGHLIVNYSFVN LW
<i>Cassytha filiformis</i>	MVEG	DPTCSPAGRCYNDGRCCSKFNWCGTGAAYCSKGN CIAQCPPG	VSPLSLSQSLLSIANHTAQ P
<i>Castanea mollissima</i>	MERLGMCVVLLCLVANAI A	QQCGWQVGKTCSDNLCCSQYGYCGT TDDYCS PSKNCQSNCG	GGGGGGGGGGESASNV RATYHYYNPEQHG WDLNAVSA YCSTWDASKSYAWR
<i>C. mollissima (cont.)</i>			SKYGWTAFCGPAGPRGQASC GKCLRV TNATWGAQT VRIVDQCSNGGLDLD
<i>C. mollissima (cont.)</i>			VGVSNDWQMEEDMLKATLW
<i>Casuarina glauca</i>	MERVSFCVLLSLCLSASVAA	QQCGRQAGGRTCANNLCCSQYGYCGSTDDYCS PSKNCQSNCKSG	GGGGESASNV RATYHFYFNPQNGWDLRAVSA YCSTWDADKPLSWRRKYGWTA F
<i>C. glauca (cont.)</i>			CGPAGPRGQAACGKCLRV TNSGTGAQT T VRIVDQCSNGGLDLDA GVFNKLD
<i>C. glauca (cont.)</i>			TDGRGYAQGHLIVSYQFVDCGNGGPVESA
<i>Celtis occidentalis</i>	MSRLCIWALVLVAVGRAAA	EQCGRQAGGAICPNGLCCSQFGWCGNTAEHCS PQAQCQSNCG	GPPSGETVVRATYHFYFNPQNNWDLRAVSA F CSTWDADKSFWSRSKYGWTA
<i>C. occidentalis (cont.)</i>			FCGPGSGPRGRDSCGRCLRLRNIRTGATIT A
<i>Celtis occidentalis</i>	MMGRLCILALVLLAIGGAAA	EQCGRQAGGAICPNGLCCSQYGWCGNTAEYCS PQEGCQSNCG	GPTPGPPSGETVVRATYHYYPENNWDLRAVSA YCATWDADKPYAWRSK
<i>C. occidentalis (cont.)</i>			YGWTAFCGPTGPTGRDSCGRCLRLNRTRTGATITARIVDQCSNGGLDLDF
<i>C. occidentalis (cont.)</i>			NTIFRPLDTCGVYQQGSMQVGYTFVDCGDGISDHDHDP LSSIILEKSYGV
<i>Cercidiphyllum japonicum</i>	MGRRVNLCMVFLLCVVVAIATA	QQCGRQAGGQTCANNLCCSEFGYCGSTDDYCS PSKNCQSNCKSG	SSGGGGGGGGESASNV RATYHFYFNPQNGWDLNAVSA YCSTWDANKPLAWR
<i>C. japonicum (cont.)</i>			RKYGWTAFCGPVGPGRGQASC GKCLRV TNSGTGTQATVRIVDQCSNGGLDL
<i>C. japonicum (cont.)</i>			DEGVFRQLD TDGKNAQGH LTVNYQFVDCGD
<i>Cercis canadensis</i>	MGKLGVSLLVLA LVCVIERDIAEA	EQCGRQGGGRTCPNNLCCSQYGWCGNDDYCS PSKGCQSNCG	GGGGGESASNV RATYHYYPQEQHG WDLNAVSA YCSTWDAAPKPSWRSKYGW
<i>C. canadensis</i>			TAFCGPAGPRGQAACGKCLRV TNAGTGAQT T VRIVDQCSNGGLDLDVGVF
<i>C. canadensis</i>			NRLD TDGRGYQQGHLIVNYQFVDCGNDIINPLLSILNP
<i>Cicer arietinum</i>	MGKLELGSIVAILTLGCLICTAIG	EQCGRQGGGKTCPNNLCCSQYGYCGSDDYCS PSKNCQSNCHG	SGESGESASNV RATYHYYPQEQHG WDLNAVSA YCSTWDASKPYSWRSKYG
<i>C. arietinum (cont.)</i>			WTAFCGPVGPGRGQASC GKCLRV TINTGTGAQET VRIVDQCSNGGLDLDVG
<i>C. arietinum (cont.)</i>			VFNRI DTDGRGYQQGHLIVNYQFVDCGNQLDIT HPLL SIFNA
<i>Cicer arietinum</i>	MGKLELGSIVAILTLGCLICTAIGE	QCGRQGGGKTCPNNLCCSQYGYCGSDDYCS PSKNCQSNCHG	SGESGESASNV RATYHYYPQEQHG WDLNAVSA YCSTWDASKAYSWRSKY
<i>C. arietinum (cont.)</i>			GWTAFCGPVGPGRGQASC GKCLRV TINTGTGAQET VRIVDQCSNGGLDLD
<i>C. arietinum (cont.)</i>			VGVFNRI DTDGRGYQ
<i>Cinnamomum camphora</i>	MAYSSKVLIALAVICCSWFMVEA	QQCGRQAGGRVCDGGLCCSQWGYCGSTDEYCS PQGCQSNCG	GSSPSPPGEGTLVRSTYHFYFNPQHG WDLNAVSA F CSTWDADKPLSWRSK
<i>C. camphora (cont.)</i>			YPWTAFCGPGGPTGRDACGKCLRV TNQGTGAQIVVRIVDQCSNGGLDL
<i>C. camphora (cont.)</i>			DVAAFNQIDTDGRGYAQG
<i>Citrus aurantium</i>	MVLLICSMIAIAA	QQCGRQAGGRTCANNLCCSQYGYCGSTDEYCS PSKNCQSNCRPG	SSSSGDGQASNV RATYHFYFNPQNGWDLNAVSA YCSTWDANKPLSWRR
<i>C. aurantium (cont.)</i>			KYGWTAFCGPFGRGQASC GKCLRV TNRA TRAQT VRIVDQCSNGGLD

<i>C. aurantium (cont.)</i>			LDAGVFRQLDTRRGNAQGHLMVDYQFVNCGD
<i>Clematis chinensis</i>	MKHLHLGVVLLYLASLLLGASAQA	QCGRDAGGALCANNLCCSQFGCGDTPPYCGDGCQSQCRPG	STPTPTSTPTPTPTGGDLGSLIPESLFNEMLKHRSDPACQGGFYTYSAF
<i>C. chinensis (cont.)</i>			INAATSFAGFATTGNTDVRREIAAFAQTSHETTGGWATAPDGPYAW
<i>C. chinensis (cont.)</i>			GYCFIRERGDNPQDFCRPDPQWPCAPNR
<i>Cleome viscosa</i>	MRRVGVLISVVLICIAATAIA	DKPQCGRNADGKKCPNNLCCSAWFGCGSTEDYCSPEKHCQDNCWY	SAKDVFAGYAAYNAEQNWDVLAVNAFCAESDADKPYSWRSKYGWTA
<i>C. viscosa (cont.)</i>			FCGPVGPGRKDACGKCLNVKNSKSAQITVRIVDECNGGGLNLDVDFV
<i>C. viscosa (cont.)</i>			FKKLDTDGDGIRKGHLTVDYEFVNCGDDDAELINSDARKALFVIDRV
<i>Conopholis americana</i>	MGRLSFCLVLLLSLISLLLLAATTSAG	EQCGRQASGKKCDNNLCCSQYGYCGSTGEFCLLSKNCQSNQCS	TTSGGGGGGCGNTGGGGESASNVVRATYHLYNPEQNGWNLNAVSGYCSTWD
<i>C. americana (cont.)</i>			ANKPLAWRSKYGWTAFCGPVGAHQASCGKCLKVTNTGTGAQTTVTRIV
<i>C. americana (cont.)</i>			DQCSNG
<i>Coriaria nepalensis</i>	MGKLISHCFVLILSLLASAATA	QQCGRQAGGKTCANNLCCSQYFGCTTDDYCSPSKQCQSNCKSG	GVGGGSGRSRK
<i>Crassula perforata</i>	MWRVGLRLFVYVFLCCATSAHVSA	QICGKQAGGKTCPGNICCSQWGYCGTSDDYCSPSNNCQSNCKG	GGGDKASNVVRATYHYNPEQHGWDNLNAXXALTVLHGMLTSLWHGARNTAGL
<i>C. perforata (cont.)</i>			RSVDLLVPAVKLHAASA
<i>Crossopetalum rhacoma</i>	MMALAMAMTMMVEA	DPSCSPAGRQYCNDRCCSKFNWCGTGAAYCGKNCIGQCP	PRVSPLPLSQITLSITNHTTKTQAHHNP
<i>Cucumis melo</i>	MKTYSLIILSFAFLGAASA	EQCGRQANGALCPNNLCCSQFGFCGDTDDYCKNGCQSQCRG	SSTPTPSGGSGVSGSIISESLYNQLMKYSRDPRCPSNGFYTYNAFITAAR
<i>C. melo (cont.)</i>			SFTPTFGTTGDATTRKREIAAFFGQTSHETTGGSWSTAPDGPYAWGYCFI
<i>C. melo (cont.)</i>			RERNQQTCTYCTPSQQWPCAPGQQYYVGRGPIQLTHNYNYPAGKAIGATL
<i>Cucumis melo</i>	MKSQSLCLILAFAPLLGSTSA	EQCGRQANGALCPNNLCCSQYGWCGTDAYCKDGCQSQCRG	STSPTPPSTPSGSGVSGSII TESLYKQMLKYSTD SRCP SNGFYTYNAFIT
<i>C. melo (cont.)</i>			AAQSFPGFGTTGDDATRKRELAAFFGQTSHETTGGSWASAPDGPYAWGYC
<i>C. melo (cont.)</i>			FIREINQDVYCTPSSQWPCVSGQKYVGRGPIQLTHNYNYPAGNALSLN
<i>Cunninghamia lanceolata</i>	MPESRLRSAYVYLLVLMVAVMKVKVG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCASGNCLAQCWP	NAVALNQYTFNLTQQNLTSAP
<i>Cupressus dupreziana</i>	MGQYKSTVATLLVIVFLGCNIGLPMVMG	DPTCSPKGNFFCNSGRCCSIYNWCGTGAAYCAKGNCLAQCSP	GVTAVTYYTLTGKNLTSTHNN
<i>Cupressus dupreziana</i>	MAERRIRWALCMLFVLMVAVVING	DPTCSPAGNFWCNSGRCCSQYNWCGTSDYCASGNCLAQCWP	NAMALNQSSLINVSMMKNITSASP
<i>Cupressus dupreziana</i>	MAYKISTLTLMHLLLLMINVKG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCASGNCLAQCWP	NAISFVNHSSCLNVII SHNRTSPSP
<i>Cycas micholitzii</i>	MEIKNVMLTMLALLCVAFGMKGVRC	EQCGWQVGGAACDGLCCSQYFGFCGSTTEYCGQCQSKCSG	GGGAGESADNIRATYHEYYPERNHNDLNAVSAYCWTDWASKPLWWRQ
<i>C. micholitzii (cont.)</i>			KYGTAFCGPVGPRGQASCGKCLRVNTNRDTSQATVRIVDQCSNGGL
<i>C. micholitzii (cont.)</i>			DLDINVFQKLDTNRGVAQGHLMVNYEFVDCGDGDGVDAQLLR
<i>Datura metel</i>	MDKLSSTMLLALVLFITIGAGANA	QQCGRQGGGALCSGNLCCSQFGWCGSTPEYCLPSQCQCSQCSG	SGPTPGGGGASAQNVVRATYHIYNPQNVGWDLNAVSAYCWTDWANKP
<i>D. metel (cont.)</i>			YAWRSKYGWTAFCGPAGPGRGRDSCGKCLRVNTNRTRKAQTVIRIVDQCS
<i>D. metel (cont.)</i>			NGGLDLIDINVFQKLDITDGVGNQQGHLLVNYQFVNCGDNVNVPLLSVVDKE
<i>Deutzia scabra</i>	MTRSPLYTMFFLFLFLAAITIGTA	QQCGRQASGQTCANNLCCSQYGYCGTDDYCLPSNNCQSNCKSG	GGGESASNVVRATYHYYYPEQNGWDLNKVSAYCSTWDASKPLAWRRKYGW
<i>D. scabra (cont.)</i>			TAFCGPVGPRGQNSCGKCLRVNTNTGTGAQETVRIVDQCSNGGLDLDWG
<i>D. scabra (cont.)</i>			VFQRLDTNNGYAQQGHLTVNYQFVDCGD
<i>Dicranum scoparium</i>	MDQQGQRILVASVFVILQLMALTA	EQCGRQAGGALCPGGLCCSQFGFCGTTPEYCENGCSQSNCG	SGSPSPGPGSGTGRASFYTAPYVPSACFGFDQGGQFPANMFFAAGDSAG
<i>D. scoparium (cont.)</i>			ANINWNGHNCGRNYRIRCGQNGCRGSGAITIKIVDRCPNGCSGGRTF
<i>D. scoparium (cont.)</i>			DLSSQAFAAIADPNVGVITVSYSLASPHDDVAVPWEKEQLIAEVGHGGQ
<i>Dillenia indica</i>	MGLKVYLCMLLVSSIIGALA	QQCGSQAGGKTCPNNLCCSQYGYCGSTDPYCLPSNNCQSNCKG	GGGSSSGGGGNXGGESASNVVRATYHLYNPQQHNWDLNAVSAYCWTDAN
<i>D. indica (cont.)</i>			KPLSWRSKYGWTAFCGPVPHGQAACGKCLRVNTNTGTSGQAIVRIVDQC
<i>D. indica (cont.)</i>			SNGGLDLIDINVFQKLDTNNGYAQQGHLVINYQFVDCGD
<i>Dioon edule</i>	MEASNKVLTLALLCVVVVFRREVGR	QQCGRQVSGRECDGNLCCSQFGYCGSTPEYCDPSQCQSKCTGAG	GEKAENIRATYHEYYPERNHNDLLAVSAYCWTDWANKPLSWRQRYGWTA
<i>D. edule (cont.)</i>			FCGPVGPGRQAAACGKCLRVNTNRDTSQATVRIVDQCSNGGLDLIDINV
<i>D. edule (cont.)</i>			FQKLDTNRGVAQGHLMVNYEFVNCGDGDGVADARVELLR
<i>Diselma archeri</i>	MAYKSSSLAVLMILGLMLIRAKS	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCAPWNCLAQCWP	YAVALNQSSLNLSQHNRSSSTSP
<i>Diselma archeri</i>	MTDSRLRLGLHILLVLMVIVQTVNG	DPTCSPAGNFWCDSGRCCSIYNWCGTSDYCASGNCLAQCWP	DAVALNQSTLNSVSNQNNLTLS
<i>Dombeya burgesiae</i>	MSNLRLYLVLFSLCIASVAAA	QQCGRQAGGRTCANNLCCSQHGVCYTTEYCSPSQNCQSNCRG	TPTTGGGESASNVVRATYHFYNPAQNGWDLNKVSAYCSTWDANKPLEWRRRYGW
<i>D. burgesiae (cont.)</i>			TAFCGPVGPRGQASCGRCLRVNTNRATRAQTVTRIVDQCSNGGLDLVAMFQQ
<i>D. burgesiae (cont.)</i>			IDTDGRGHAQQGHLMVDYQFVNC
<i>Dombeya burgesiae</i>	MDKVKSILFVLLVSSVGTTLG	EQCGRQAGGALCPDNLCCSQYGWCGSTDPYCLPENNCQSNCRS	PRPGPGPGPGPEATVRSTYHFYNPEQHGWDLMAVSAYCSTYDAGKPLS
<i>D. burgesiae (cont.)</i>			WRSKYGWTAFCGPVGPFPAAACGRCLRVNTNRTRGAQQTVRIVDQCSNGG
<i>D. burgesiae (cont.)</i>			LDLDVGVFNRLD
<i>Draba hispida</i>	MKRLNFIFILFVYTAATVIG	QQCGSQAGGQTCPGNICCSQYGYCGTTADYCSPANNCQSNCWG	SGPSGPGESASNVVRATYHYNPEQNNWDLRAVSAYCWTDADKPYAWRS
<i>D. hispida (cont.)</i>	MKRLDIITFILLAYTAAMVAG	QQCGRQAGGKTCPGNICCSQYGYCGTGADYCSPAKNCQSNQCQGTG	KYGTAFCGPVGPRGQASCGKCLRVNTRNTN
<i>Draba hispida</i>			PSGPGESATNVVRATYHYNPEQNNWDLRAVSAYCWTDADKSYAWRSKYG
<i>D. hispida (cont.)</i>			WTAFCGPAGPRGQASCGKCLRVNTRNTASVTVRIVDQCSNGGLDLVAM
<i>D. hispida (cont.)</i>			FNRIDTDGNGYQQGHLLVDYQFVDCGNELIDQSDSKNILVSALDRV
<i>Draba ossetica</i>	MKRLDIFTFILLAYTAAMVAG	QQCGRQAGGQTCPGNICCSQYGYCGTTEDYCSPANNCQSNQCQGTG	PGDSASNVVRATYHYNPEQNNWDLRAVSAYCWTDADKPYAWRSKYGWTA
<i>D. ossetica (cont.)</i>			FCGPAGPRGQASCGKCLRVNTRNTAAVTVTRIVDQCSNGGLDLVAMFN
<i>D. ossetica (cont.)</i>			RIDTDGFGYQQGHLLVDYQFVDCNDSKNILVSAIDRV
<i>Drimys winteri</i>	MVFLCLAAAA	EAQQCGRQAGGRTCAGNLCCSQYGYCGTADDYCLPSNNCQSNCKSSG	VGGGGGESASNVVRATYHFYNPEQNGWNLSVSAYCWTDWANKPLAWR
<i>D. winteri (cont.)</i>			SKYGTAFCGPVPHGQASCGRCLRVNTNATSAQATVRIVDQCSNGG

<i>D. winteri</i> (cont.)			LDLDDGVFKRLD TDGRGNAQGH LIVNYQFINCGD
<i>Elaeocarpus sylvestris</i>	MGKSNLCMVLF LCVVASAIA	QQCGKQGGGKK CANKLCCSQYGYCGTTPPYCSKSQGCQSNCKG	SVGDVAGESASNVKATYHHYNPQQNDWD LKAVSASCSTSHANKPVEWR
<i>E. sylvestris</i> (cont.)			RKYGWTAFSGGQDACGKCLRVNTNTATRAQATVRVVDQSSNGGLDMD
<i>E. sylvestris</i> (cont.)			AAVFEQLDTDGK GNAQGHLMVNYQFVDCAD
<i>Ephedra sinica</i>	MKILSDICFNRKMGKVAMMLMLVAAGVVVG	EQCGRQAGGKVCDDGMCCSQFGWC GTTNEYCGAGCQSNCG	GGGGGESASGVRSTYHEYYPERHNWDLNAVSA YCSTWDASKPLSWRKK
<i>E. sinica</i> (cont.)			YGWTAFCGPVGRGQASCGKCLKVNTSGTGASATVRIVDQCSNGGLD
<i>E. sinica</i> (cont.)			LDVNVFNRIDTDGRGHAQGH LIVSYQFVNCGNLAEAFSSALA
<i>Eschscholzia californica</i>	MATKNLFTIFLLFLTATASVKA	QQCGRQGGGSTCRDNLCCSQYGYCGNTDAYCLPSNNCQSNCRG	GGGGGGGGGGGESADNVRATYHYFYPEQNGWDLNRVSAYCSTWDAN
<i>E. californica</i> (cont.)			KPLAWRQKYGWTAFCGPVGRGQASCGKCLRVNTNRGTGAQTI VRI V
<i>E. californica</i> (cont.)			DQCSNGGLDLDDEGVFKQLDTNGRGYADGHMFVNYQFVNCGD
<i>Euptelea pleiosperma</i>	MVLLCLTAGATA	QNCGRQAGGRTCDGNICCSQWGF CGTTNDHCLPSNNCQSNCKSG	GGSGGGGESASNV RATYHIYNPEQNGWDLNRVSAYCSTWDANKPLE
<i>E. pleiosperma</i> (cont.)			WRRKYGWTAFCGPVGRGQASCGRCLRVNTNTGTGSQVTVRIVDQCS
<i>E. pleiosperma</i> (cont.)			NGGLDLDDEGVFKQLDTNGRGNAQGHMIVNYQFVDCGD
<i>Eutrema halophilum</i>	MKKSRLSIAIILLSYTVATVAG	QQCGSQAGGQTCPGNICCSQYGYCGTTADYCS PDNNCQSNCWG	SGPSGPGESASNV RATYHYFYNPEQNNWDLRAVSAYCSTWDADKPY
<i>E. halophilum</i> (cont.)			AWRSKYGWTAFCGPAGPRGQASCGKCLRVNRTNRNAVTVTRIVD
<i>E. halophilum</i> (cont.)			QCSNGGLDLDVAMFNR LDTDGVGYQQGH LIVDYQFVDCGNDLIH
<i>Eutrema salsugineum</i>	MKKSRLSIAIILLSYTVATVAG	QQCGSQAGGQTCPGNICCSQYGYCGTTADYCS PDNNCQSNCWG	SGPSGPGESASNV RATYHYFYNPEQNNWDLRAVSAYCSTWDADK
<i>E. salsugineum</i> (cont.)			PYAWRSKYGWTAFCGPAGPRGQASCGKCLRVNRTNRNAVTVTR
<i>E. salsugineum</i> (cont.)			IVDQCSNGGLDLDVAMFNR LDTDGVGYQQGH LIVDYQFVDCGNDLIHQPADS
<i>Fagopyrum tataricum</i>	MKSVKAITNLLLSLVSFLIFSSVSEAA	QCGAQGGGATCPGGLCCSQWGCSTGPKYCGAGCQSN C	RPNVAGAQNDVDPDAVKPTTNAHP
<i>Falcatifolium taxoides</i>	MTMMVEA	DPSCSPAGRQYCNDRCCSKFNWCGTGAAYCGKGCIGQCP	PRVSPLPLSQITLSITNHTTKTQAHPNP
<i>Falcatifolium taxoides</i>	MAMEHKATMFVLLMGVVATFPLLGA	ENCGRQAGGQICNGGECCSQYGC GTTPDHCGTGCCQSNCG	GGGGEKATNVRSTYHEYNPQNINWDYKASVYCSTWDGGKPLSWRKKYGT
<i>F. taxoides</i> (cont.)			AFCCGPVGRGQASCGKCLRVNTNRETSQTTVRIVDQCSNGGLDLDVNVFNQLD
<i>F. taxoides</i> (cont.)			TNGQG HARGHLMVDYEFVNCGDGEWVGAQSS
<i>Ficus pumila</i>	MGRLCFCVLVALLFLVVGSSA	EQCGKQASGKTCPNLCCSQYGCWGSTDEYCSRSKGCQSNCG	GGGDGASANNIRATYHYYNPEQNGWDLNAVSA YCSTWDAGKPYSWRSKYGT
<i>F. pumila</i> (cont.)			AFCCGPAGPTGQASCGKCLRVNTNTYTQAQLTVRIVDQCSNGGLDLDYNTAFRK
<i>F. pumila</i> (cont.)			LDTDGRGYQQGH LIVNYQFVDCGNGFDGNPLLSIVGDQ
<i>Ficus religiosa</i>	MGRLCFCVLVALLFLVVGSSA	EQCGRQAGGKTCPNSLCCSQYGCWGSTDEYCSPSKGCQSNCG	GSSGASATNIRATYHYYNPEQNGWDLNAVSA YCSTWDAGKPYSWRSKYG
<i>F. religiosa</i> (cont.)			WTAFCGPAGPTGQAACGKCLRVNTNTQTQLTVRIVDQCSNGGLDLDYN
<i>F. religiosa</i> (cont.)			TAFRKLDTNGL
<i>Flaveria bidentis</i>	MKTFLLSSFLLLLASVSSA	QNCGRQAGNAPCSNGNCCSQYGYCGNTPAHCLPENNCQSQCRP	SGGGGGGGGXAVAAAGVVVLDPSLLRPLCRCSSTETTRDVTQMDFTLT
<i>F. bidentis</i> (cont.)			MLSLMLQQRIMDSAPLEVSMIKRESSRLSSLKPPMKQQVDQLHQMVS
<i>F. bidentis</i> (cont.)			LHGDIALLENKTRRIGTVTRLLGRVPKATLVEDLSNSLITITMGNLESQ LIRT
<i>Fokienia hodginsii</i>	MAESRLRLALYTL LVLMAVTVNG	DPTCSPAGNFWCDSGRCCSIYNWCGSIDYCASGNCLAQCW P	DAAALNQSSSLTVFRNLTSVSP
<i>Fokienia hodginsii</i>	MAESRLRWTLYTL LVLMAVMVKVNG	DPTCSPAGNFWCNSGRCCSQYNWCGSTSDYCASGNCLAQCW P	NALALNQYSSNVSHQNTSLSP
<i>Fokienia hodginsii</i>	METKAVAFILAVTLAAPLLCAA	QNCGSQAGGKVCSGGECCSKYGC GTTDDHCLPDRGCQSN CAG	GGGGGGSGEKASNV RSTYHEYYPERNNWSLYAVSA YCSTWDGEKPLWWRQKYG
<i>F. hodginsii</i> (cont.)			WTAFCGPVGRGQASCGKCLRVNTNRDTGAQATVRIVDQCSNGGLDLDINVFRQL
<i>F. hodginsii</i> (cont.)			DTNGQNAKHGLMVDYEFVDCDGLNLEEAQVM
<i>Fontinalis antipyretica</i>	MVRVATLVALLCSLLHLASA	QQCGRAVNNTPCANLLCCSQAGYCGTTDAYCVTGCCQSGPCR	TTFSPPPPPTP
<i>Galax urceolata</i>	MGMVILTVALI IYLS TTAVA	QQCGQQAGGTL CANNLCCSQYGYCGTTDDYCLPSNNCQSNCRPG	GGGGGGESASNV RATYHYYNPAQVNWDLNAVSA YCSTWDAGMPLAWRQKYGT
<i>G. urceolata</i> (cont.)			AFCCGPVGP TGQASCGKCLQVTNVRTGAQATVRIVDQCSNGGLDLDVSVFQQQL
<i>G. urceolata</i> (cont.)			DTDNGY AQGH LTVNYQFVDCDGLKRFQQLPISNN
<i>Galax urceolata</i>	MKTAVAILIAFVLAKVLRGSA	EQCGSQAGGALCPGGLCCSKFGWCGSTSDYCGNCQSQCG	GSTPTPGPSGGGVSLISADLFNQMLLHRSDASC PAHN
<i>Galega orientalis</i>	MGNFGVCFVLVLTGLCLIATTIA	EQCGRQAGGKTCPNLCCSQYGYCGNTDDYCSPSKNCQSN CQG	GGGGGGGGGGGESASNV RSTYHYYPETVGGTKR
<i>Geranium carolinianum</i>	MECINVVLVLCVAVALAGGAVG	QQCGRQGGGRSCGNMCCSQYGYCGTSDDYCSPSKGCQSN CAG	GGGGGGGQASNV RATYHYYNPEQHGWDLNAVSA YCSTWDAGKPLSWRKKYGT
<i>G. carolinianum</i> (cont.)			AFCCGPVGRGQASCGKCLRVNTNTWTGAQATVRIVDQCSNGGLDLDVGVFQRLD
<i>G. carolinianum</i> (cont.)			TNGRGYAQGHMIVNYQFVNCGNLLELDLVGVF
<i>Geranium maculatum</i>	MKFFWSVVAVALVSLIGGEMAAA	QNCGRQAGGRRCANRLCCSQFGFCGSTHEYCGFGCQSQCP R	AAASVDPDAATAGDQATAAGNP
<i>Geum quellyon</i>	MGVVGNLVL LVLISGCV A	QQCGKQGGKTCAGNLCCSQYGYCGTTDDYCSQSKGCQSN CAG	GGGGGGGGGGGESANNVRSTYHLYNPQQHGWDLGAVSA YCATWDASKPYS
<i>G. quellyon</i> (cont.)			WRSKYGWTAFCGPVGRGQAACGKCLRVNTNTATRAQTTVRIVDQCSNGGL
<i>G. quellyon</i> (cont.)			DLDVGVFRQLD TDGSGYARGHLIVNYQFINCG
<i>Gleditsia sinensis</i>	MKGKVVVVAVLVCLIGRAIG	EQCGRQAGGQCPDNLCCSQFGYCGSTDDYCSPSKNCQSNCKG	DDSGGASASNV RATYHYYPQLHGWDLNAVSA YCSTWDASKPYSWRSKYGTA
<i>G. sinensis</i> (cont.)			FCGPAGPHCQAACGKCLRVNTNTGTGAQITVRIVDQCSNGGLDLDVGVPNRLD
<i>G. sinensis</i> (cont.)			TDGRGYQQGH LIVNYQFIDCGNELDFNPVLSILNH
<i>Gleditsia triacanthos</i>	MKGKGVVLVAVLVCLIGRAIG	QQCGRQGGGKQCPDNLCCSQFGYCGTTDDYCSPSKNCQSNCKG	DDGGGASASNV RATYHYYPQLHGWDLNAVSA YCSTWDASKPYSWRSKYG
<i>G. triacanthos</i> (cont.)			WTAFCGPAGPHGQASCGKCLRVNTNTGTGAQITVRIVDQCSNGGLDLDV
<i>G. triacanthos</i> (cont.)			GVFNRLD TDGRGYQQGH LIVNYQFIDC
<i>Glycine max</i>	MGKAWVGLVVLCLIVTAIA	EQCGRQAGGQTCPNLCCSQYGCWGNTEYCSPSKNCQSNCWG	GGGGESASNV RATYHYYPEQHGWDLNAVSA YCSTWDASKPYSWRSK
<i>G. max</i> (cont.)			YGWTAFCGPVGRGRDSCGKCLRVNTNTGTGANTIVRIVDQCSNGGL
<i>G. max</i> (cont.)			DLDVGVFNRLD TDGRGYQQGH LIVNYQFVDCGNELDLTKPLLSILDAP

<i>Glycine max</i>	MGKAWGLVVLVLLCLIVTAIA	EQCGRQAGGQTCNNLCCSQYGCWGNTEEYCSPSKNCQSNCWG	GGGGGGGGGGESASNVRAITYHYEPEQHGWDLNAVSAYCSTWDASK
<i>G. max (cont.)</i>			PYSWRSKYGTAFCGPVGPRGRDSCGKCLRVTINTGTGANTIVRIVD
<i>G. max (cont.)</i>			QCSNGGLDLDVGVFNRIIDTDRGRYQQGHLIVNYQFVDCGNELDLTKPLLSILDAP
<i>Glycyrrhiza glabra</i>	MEKIGVGLAVLLLAELIVTAMG	EQCGRQAGGKTCNNLCCSQYGYCGNTDDYCSPSKNCQSNCCG	GGGGGGESASNVRAITYHYEPEQHGWDLNAVSATCSTWDASKPYSWRSKYGMT
<i>G. glabra (cont.)</i>			AFCGPVGPRGQASCGKCLRVTINTGTGAQATVRIVDQCSNGGLDLDVGVFNRI
<i>G. glabra (cont.)</i>			IDTDRGRYQQGHLIVNYQFVNCGNELVDLMTNPLLSILDH
<i>Glycyrrhiza uralensis</i>	MKMRFRFETGVVVLVCLMMVVGSSRG	EQCGRQAGGAVCPGGLCCSKFVGWCGSSPDYCGDGCQSQCG	GGGGGGGDLGSIISRDTFNQMLKHRNDGGCPAKGFYTYDAFIAAAKAFPNFA
<i>G. uralensis (cont.)</i>			KTGDTPTTRKREVAAPFGQTSHETT
<i>Gompholobium polymorphum</i>	MGKVGVGVVVLVCLIVRAIA	EQCGQAGGQTCNNLCCSQYGYCGNTDDYCSPSKNCQSNCWG	GGGGGGGGESASNVXAPHIITINRSYMDGT
<i>Gossypium hirsutum</i>	MDKVKFVMLFVFLVSLLGATMG	EQCGRQAGGALCPNNLCCSQYGCWGNTEEYCSPEKNCQSNCRSG	PPPTTGEATVRSITYHFNPEQNGWDLMAVSAYCSTWGANKPYSWRSKYGWTA
<i>G. hirsutum (cont.)</i>			FCGPVGPSPFAACGRCLRVRNRSRTGAQEIIVRIVDRCSNGGLDLDVGVFNRI
<i>G. hirsutum (cont.)</i>			TDGVGHAQGHILTIRYDFVDCGNGFNPLLASVIDS
<i>Gossypium raimondii</i>	MGNLSLSLVSLLLCLLATAAS	QQCGRQAGGRTCANNLCCSQFYCGTDDYCSPSRGCCSNCRG	GGGSGGGESASNVRAITYHYNPAQNGWNLNAVSAYCSTWDANKPLAWRQKY
<i>G. raimondii (cont.)</i>			GWTAFCGPVGPGRQASCGRCLRVTNRATRAQVTVRIVDQCSNGGLDLVAM
<i>G. raimondii (cont.)</i>			FQQIDTDGRGHAQGHLMVDYQFVAC
<i>Gyrocarpus samericanus</i>	MTRSHSVLWLVLLVVTLLLVTKA	QQCGRQAGGRICDNNLCCSQFGWCGSTDEYCLPTRGCQSNCRG	GGGGGGGGGGGGGGQATVRSITYHLYNPEQHGWSLTAVSAYCSTWDADKP
<i>G. samericanus (cont.)</i>			LWWRQKYGWTAFCGPVGPRGRDSCGKCLRVTNQATGAQRVVRIVDQCANGG
<i>G. samericanus (cont.)</i>			LDLDINVRQLDTDGRGMAQGHILTIRYQFVNCNDGLTELDAAS
<i>Gyrostemon ramulosus</i>	MKTIILSLFLCLSLLAATTA	QQCGRQAGGRTCANNLCCSQYGYCGNTDDYCSPAKNCQSNCW	DGGESASNVRAITYHFNPEQNNWDLRAVSAYCSTWDADKPYSWRSKYGW
<i>G. ramulosus (cont.)</i>			TAFCGPVGPRGQASCGKCLRVTINTGTGAQATVRIVDQCSNGGLDLDV
<i>G. ramulosus (cont.)</i>			VFNKIDTDGRGYQQGHLIVNYQFVNCGDGLSADDDHQLLLSIINA
<i>Halocarpus bidwillii</i>	MKMEAVRMALVMVLAMGVNMMMVVEG	EPTCSPAGRIYCSGPRCCSRFNWCGDTPAYCGRGNCIANCHX	QIHLSLTLNTLSTATP
<i>Halocarpus bidwillii</i>	MKMEKMRMLMMLAMAMMMMVVVEG	DPSCNPAAKIYCNPGRCCSKFNWCGTLTSYCGKDYCIAQCPPG	VSPLSHSQIHLSIANLTTTKPQAHVHNT
<i>Hamamelis virginiana</i>	MGRVMNTFIVVLLCLTGVAIA	EQCGRQAGGKLCNNLCCSQWGCWGSTDEYCSPDHNCQSNCKDS	GEGVGGGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSWR
<i>H. virginiana (cont.)</i>			SXMAEQVWLDCLIRRRSTRPILLWKVLECDKYRDNWS
<i>Hedera helix</i>	MKNAGLFLVLLCLVAGGTA	QQCGSQGGGKTCANNLCCSQYGYCGTDEYCSPSKNCQSNCKS	NGGDSSSGESASNVRAITYHMYEPEKNGWNLNAVSAPCSTWDADKPLEWR
<i>H. helix (cont.)</i>			SKYGTAFCGPVGPRGQASCGKCLRVTINTGTGAQATVRIVDQCSNGGL
<i>H. helix (cont.)</i>			DLDVGVFRKLDTDGKNAQGHILVNYQFVNC
<i>Heuchera sanguinea</i>	MEILNLCLTILMLTCLVASATA	QQCGRQAGGRTCANNLCCSQYGYCGTTAEFTNNCQSNCSAG	GGGESATNVRAITYHYZNPANQNGWNLNAVSAYCSTWDANKPLAWRKKY
<i>H. sanguinea (cont.)</i>			WTAFCGPAGPRGQASCGKCLRVTNRTRTGAQATVRIVDQCSNGGLDL
<i>H. sanguinea (cont.)</i>			DGVFKPLDTDGNGYQGHILTIVNYQFVNCGD
<i>Hevea brasiliensis</i>	MGRVLNIFIVVLLCLTGVAIA	EQCGRQAGGKLCNNLCCSQWGCWGSTDEYCSPDHNCQSNCKNSG	EGVGGGSASNVLATYHLYNSQDHGWDLNAASAYCSTWDANKPYSW
<i>Hevea brasiliensis</i>	MNIFIVVLLCLTGVAIA	EQCGRQAGGKLCNNLCCSQYGCWSSDDYCSPSKNCQSNCKG	GGGGGGG
<i>Hevea brasiliensis</i>	MTNIDMRASFTVSCLLLASLLPGTSA	EQCGRQAGGALCPGGLCCSQFGWCGNTPDYCGATCQSQCGDG	DLTSIISRATFNQMLKHRNDGGCPAKGFYTYDAFISAAKAFP
<i>H. brasiliensis (cont.)</i>			TFGTTGGLLRHVKGIAAL
<i>Hibiscus cannabinus</i>	MPKLSLCLVSLLLCLLANAAA	QQCGRQAGGQTCANNLCCSQYGYCGTDDYCSPSKSCQSNCRPG	GGGESASNVRAITYHFNPAQNGWDLNAVSAYCSTWDANKPLAWRQKYGTAF
<i>H. cannabinus (cont.)</i>			GFVGPGRQASCGRCLRVTNRGTGAQVTVRIVDQCSNGGLDLVAMFQQIDTDR
<i>H. cannabinus (cont.)</i>			RGYAQGHLMVDYQFVTC
<i>Hibiscus cannabinus</i>	MDKVKFVMLFVFLVSLVGATMG	EQCGRQAGGALCPNNLCCSQYGCWCGSTDEYCLPENKQSNCRG	PQPQPQPQPGGGGATVRSITYHYNPEQNNWDLNAVSAYCSTWDANKPYAW
<i>H. cannabinus (cont.)</i>			RSKYGTAFCGPVGPFPAACGRCLNVRNRTGAQTTRIVDQCSNGGLDL
<i>H. cannabinus (cont.)</i>			DWGVFRQLDTDGEGYRQGHMTVRYDFVDCGNGFNPLLASVVDN
<i>Humulus lupulus</i>	MKWCFSVFIIFSFLIGGALG	EQCGRQAGGASCNGLCCSQYGCWCGTNDYCSQAQGCQSNCKG	SSTFTNPTGTGETVVRATYHYNPPQNNWDLRAVSAYCSTWDADKPYSWR
<i>H. lupulus (cont.)</i>			KYGTAFCGPS
<i>Humulus lupulus</i>	MKLNTCLWTILWFGILVGLVISDDDDKP	ECGRDAFGEFCPSQCCSQWGYCGNTSVYCGRCQSNCWNS	PPPTTPTPPPPSPCPPSPPPKPCPPSPPPKPC
<i>Hypocoum procumbens</i>	MRTINLCVIFLLSLALAVNA	QQCGRQAGGATCQNNLCCSQYGYCGSTDAYCLPSNNCQSNCRG	SSPGTGGGESASNVRAITYHFNPEQNGWDLGRASTYCATWDANKPLEWRR
<i>H. procumbens (cont.)</i>			YGTAFCGPVGPRGQASCGKCLRVTNRTRTGTATVRIVDQCSNGGLDLDDG
<i>H. procumbens (cont.)</i>			VFKQLDSGGRGDFGHLTVNYQFVNCGD
<i>Impatiens balsamifera</i>	MRGENVAMMMIVFSLISTAALVG	QQCGRQAGGRLCDNNLCCSQWGYCGSTDEYCLTSGNCQSNCRG	GGGSEGSASNVRAITYHFNPPQVNNLYAVSAYCSTWDGDKPLAWRKK
<i>I. balsamifera (cont.)</i>			YGTAFCGPSGPTGQASCGKCLLVNTRTGAKATVRIVDQCSNGGLDL
<i>I. balsamifera (cont.)</i>			DEGVFRQIDTDGSGYAQGHILVSYQFVDCGNSLPFYSS
<i>Ipomoea nil</i>	MKYCTMFIVLLGLGSLLLTPTTIMA	QQCGRQASGRLCGNGLCCSQWGYCGSTAAYCGAGCQSQCKSTA	ASSTTTTANQSTAKSDPAGGAN
<i>Isoetes tegetiformans</i>	MVLAMATMMIMVEG	DPTCSPAGRYCNDGRCCSKFNWCGTGAAYCSKGNCAQCSPG	VSPLSLSQSLLSIANHTAQ
<i>Itea virginica</i>	MGRDSLCLILLCLVAYATNA	QQCGKQAGGKTACAGDCCSQYGYCGTDDYCSSKNCQSNCKG	GDGQGSASNVRAITYHYNPEQHGWDLNAVSAYCSTWDANKPLAWRQKYGW
<i>I. virginica (cont.)</i>			TAFCGPAGPRGQASCGKCLRVTINTYTGTTTTRIVDQCSNGGLDLVGVF
<i>I. virginica (cont.)</i>			QKLDNTRGQYAGHLIVNYEFVNCGDGINPLFSLIDA
<i>Juglans nigra</i>	MERLCVWLVLVSLCLFAAATTA	QQCGKQAGGKTCNNLCCSQYGYCGSTDDYCSPSKGCQSNCCS	SSSGGESASNVRAITYHFNPEQNGWNLNAVSAYCSTWDANKPLAWRQKYGWTA
<i>J. nigra (cont.)</i>			FCGPVGPGRQAACGKCLRVTNSGTGAQATVRIVDQCSNGGLDLVYAGVFQ
<i>J. nigra (cont.)</i>			KLDTDGKGYAQGHILVSYQFVNCGD
<i>Juniperus scopulorum</i>	MAERRLRWALCMFFVLMAVVING	DPTCSPAGNFLCNSGRCCSKYNWCGSTSDYCASGNCLAQCW	NALALNQSTLINVSMERNITSAP
<i>Larrea tridentata</i>	MLKMENASILLVFLFLTATA	QQCGSQAGGRICDGNLCCSQYGCWGSTAEYCSPDQRCQSNCWG	PNPPQPPSPGGEGASNIRATYHMYNAEQNGWNLVAVSAYCSTWDGDKPL

<i>L. tridentata</i> (cont.)		
<i>L. tridentata</i> (cont.)		
<i>Lathyrus sativus</i>	MGKFGTCLVVLTLVLCIATTIA	EQCGRQAGGATCPNNLCCSQYGYCGTDDYCSPSKNCQSNCHG
<i>L. sativus</i> (cont.)		
<i>L. sativus</i> (cont.)		
<i>Laurelia sempervirens</i>	MGTWAFIFLFLASLLCASA	QQCGSQRGGAVCTGRLCCSQFGYCGNTDPYCGAGCQSKCSSG
<i>Lepidosperma gibsonii</i>	MRKISTYIELFSLAIVCLGALCLTSQ	AQTCGSQAGGALCADNMCCSQYGYCGTSDYCGTGCSQCG
<i>Limnanthes douglassii</i>	MKIPSSFTLLLFLCLIGTSLA	QQCGSQAGGRTCPNNICCSQYGYCGTTEDYCSPSKNCQSNCWG
<i>L. douglassii</i> (cont.)		
<i>L. douglassii</i> (cont.)		
<i>Limonium spectabile</i>	MSPLALVSFLCLALAIGTVTA	QQCGSQAGGRTCANNLCCSQYGYCGSTDAYCATGCSNCKTG
<i>L. spectabile</i> (cont.)		
<i>L. spectabile</i> (cont.)		
<i>Linum hirsutum</i>	MERLRVANISFTLVLMTLATIAAA	QNCGRQAGGATCANNLCCSQFGFCGTTDDHCNPSKNCQSNCRPSG
<i>L. hirsutum</i> (cont.)		
<i>L. hirsutum</i> (cont.)		
<i>Linum leonii</i>	MRASVLSIVLLCFFTAIARA	QNCGRQAGGQTCAANNLCCSQYGYCGTTDDHCSPSKNCQSNCRPG
<i>L. leonii</i> (cont.)		
<i>L. leonii</i> (cont.)		
<i>Linum lewisii</i>	MRASVLSIVLLCFFTAIARA	QNCGRQAGGQTCAANNLCCSQYGYCGTTDDHCSPSKNCQSNCRP
<i>L. lewisii</i> (cont.)		
<i>L. lewisii</i> (cont.)		
<i>Linum macraei</i>	MERLNIVAMVRYILLLCFFTAIASG	QDCGRQAGGRTCAANNLCCSQWGFCTTDDHCNPSKNCQSNCRP
<i>L. macraei</i> (cont.)		
<i>L. macraei</i> (cont.)		
<i>Linum strictum</i>	MERSSTNIVAMVRYILLLCFFTALASA	QNCGRQAGGRTCAGNLCCSQYGFCTTDDHCNPSKNCQSNCRPG
<i>Linum usitatissimum</i>	MKASMLVTTLCLFLAAIASA	QNCGRQAGGQTCAANNLCCSQYGYCGTTDDHCSPSKACQSNCKP
<i>L. usitatissimum</i> (cont.)		
<i>L. usitatissimum</i> (cont.)		
<i>Liriodendron tulipifera</i>	MASHLGLWLLFLMVCAASIAA	EQCGRQAGGATCPGNLCCSQYGWCGSTDEYCTNNCQSNCKPSG
<i>L. tulipifera</i> (cont.)		
<i>L. tulipifera</i> (cont.)		
<i>Loropetalum chinense</i>	MEKLSLSMVFIMFLAGMDGAIA	QQCGKQAGGKTCPGNICCSQYGYCGTDDYCSPSKNCQSNQCQ
<i>L. chinense</i> (cont.)		
<i>L. chinense</i> (cont.)		
<i>Lotus japonicus</i>	MARLVEAVLAVLLPCLIVTAMG	EQCGSQAGGQLCPNNQCCSQYGWCGNTEDYCSSKGCQSRCWG
<i>L. japonicus</i> (cont.)		
<i>L. japonicus</i> (cont.)		
<i>Lotus japonicus</i>	MKMRLGIAILVSFILVGWCRG	EQCGSQAGGALCPGGICCSKYGWCGSTSEYCGEGCQSQCGG
<i>L. japonicus</i> (cont.)		
<i>Lotus japonicus</i>	MKMRLGIAILVSFILVGWCRG	EQCGSQAGGAVCPGGICCSKYGWCGSTSEYCGEGCQSQCGG
<i>L. japonicus</i> (cont.)		
<i>Lotus japonicus</i>	MKFQVVILVLPALLLTRTSA	QNCGRQAGRVCANRLCCSQFGFCGTTREYCGAGCQSNCR
<i>Lycium barbarum</i>	MEKLITLLILFLSIALGANA	QQCGRQKGGALCSGNLCCSQYGWCGSTPEYCSPSQGCQSRCSG
<i>L. barbarum</i> (cont.)		
<i>L. barbarum</i> (cont.)		
<i>Lycium sp</i>	MGKISISMLLIICVAYGASA	QQCGRQAGGKTCQGNVCCSQYGYCGTDDYCSPSKNCQSNQCQ
<i>Lycium sp</i> (cont.)		
<i>Lycium sp</i> (cont.)		
<i>Lycopersicon cheesmanii</i>	MEKMNLSSSTPILLALVLCISLTSVNTA	QQCGRQRGGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCRG
<i>L. cheesmanii</i> (cont.)		
<i>L. cheesmanii</i> (cont.)		
<i>Maesa lanceolata</i>	MAKTSSLPFIALLCLVASAAA	QQCGSQAGGKTCANNLCCSQYGYCGTDDYCLPSKNCQSNCKSG
<i>M. lanceolata</i> (cont.)		
<i>M. lanceolata</i> (cont.)		
<i>Magnolia grandiflora</i>	MSSQLGLCLLFLMSCAASIT	AEQCGRQAGGATCPGNLCCSQYGWCGTTDEYCTNNCQSNCRPSG
<i>M. grandiflora</i> (cont.)		
<i>M. grandiflora</i> (cont.)		
<i>Malus domestica</i>	MKLQTLIILSLSLLLGISA	EQCGSQAGGAVCPNGLCCSQFGWCGTTSYCAAGCQSQCSS
<i>Manihot esculenta</i> subsp. <i>Peri</i>	METVKSNICMFLLLCLTGGAI	EQCGSQAGGQLCPNNLCCSRYGWCGTTEYCSDGCCQSNCKG

SWRSKYGWTAFCGPVGPRGQASCGKCLRVTNRAQTIVRIVDQCS
 NGGLDLDGVSVFQRLDTDGRGYQQGHLMDVYQFVNCBGDVSSM
 SGGESASNV RATYHYYPQEQHGWDLNAVSAYCSTWDASKPYSWRS
 KYGWTAFCGPVGPRGQASCGKCLRVRNSGTGAETVRIVDQCSN
 GGLDLDVGVFNRIIDTDRGRYQQGHLIVSYQFVDCGNELDMINPLFSIMDAKQ
 GSTPTPTPSGGGGAVGSLSLDQFENMLK
 GDGGGG
 GGGGGGGGGGGGETATNVRATYHMYEPEKHNDWYMAVSAYCSTWDANKP
 YSWRSKYGWTAFCGPVGPRGQASCGKCLRVTNTYTGAQQTIVRIVDQCSN
 GGLDLDVGVFNRLDITNGRGYAQGHILVINYQFVDCGDNVNSVMSE
 GGGGSPSGGGGASASNV RATYHLYNPQNGWNLYAVSAYCSTWDG
 GKSLAWRSKYGWTAFCGPVGPRGRDSCGKCLRVTNTGTGAQTIVRIV
 DQCSNGGLDLDVNVFRQLDITNGRGNAMGHMIVNYQFVDCRD
 GGGGGGGGGGESASNV RSTYHLYNPEQNGWNLNAVSAYCSTWDANKPLAWRQ
 KYGWTAFCGPVGPRGQASCGRCLRVRNRGTGAETVRIVDQCSNGGLDLD
 VGVFRRIIDTDRGIAQGHILMDVYQFVNCGD
 GGGSGGGGGGESASNV RATYHLYNPEQNGWNLNAVSAYCSTWDAGKPLA
 WRQKYGWTAFCGPVGPRGQASCGRCLRVTNRGTGASETIVRIVDQCSNGG
 LLDLDVGVFNRIIDTDRRGNAQGHILMDVYQFVNCGD
 GGGSGGGGGGESASNV RATYHLYNPEQNGWNLNAVSAYCSTWDAGK
 PLAWRKYGWTAFCGPVGPRGQASCGRCLRVTNRGTGASETIVRIVDQCS
 NGGLDLDVGVFNRIIDTDRRGNAQGHILMDVYQFVNCGD
 AGGGGSGSPSGGGGESASNI RATYHLYNPEQNGWNLNAVSAYCSTWDANKPLA
 WRQKYGWTAFCGPVGPRGQASCGRCLRVTNRTGAQTIVRIVDQCSNGGL
 LLDLDVGVFRRLDITDRGNAQGHILMDVYQFVNCGD
 GGGGGGGGGGVVAVRLMSGRRIICTTLSTRVTGT
 ASGGGGTGGGGGDSATNVRATYHMYEPEKIGNWNLMTASAYCSTWDASK
 SLAWRKYGWTAFCGPVGPTGQASCGRCLRVTNKGTGAQTIVRIVDQCS
 NGGLDLDVGVFRRIIDTDRGNAQGHILMDVYQFVNCGD
 GPPSGGEGATVRATYHLYNPEQNGWDLNAVSAYCSTWDASKSYAWRSRY
 RWTAFCGPVGSPGQASCGKCLSVTNRTGAQTIVRIVDQCSNGGLD
 LDEGVFRQLDITDGSYQAQGHILMDVMDVYQFVNCGD
 GGGGGGGGGGSASNV RATYHYYPQEQHGWDLNAVSAYCSTWDANKPL
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 GGLDLDAGMFRQLDITDRGNAQGHILVINYEFVNCGDGINNPLSIVDV
 PTPPTPGGESASNVYASYHYYPQEQVWDYSGTGATWDAGKSLAWRSKYGWTAF
 CGPVGPRGQASCGRCLRVTNSRTGAQQTIVRIVDQCANGGLDLDVGVFSKLDITD
 VGVYQQGHMTVSYQFVDCGNELELSKPLLSIIDAA
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 YPSFGTTGDTSTRKREIAAFF
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 GPTPTPTPGGGAQVRATYHYINPNQVNGWDLNAVSAYCSTWDANKPYSWR
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 DINVRQIDITDGVGNHQGHILVIVN
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 RSRYRWTAFCGPVGPRGRASCGRCLRVTNRTGAQTIVRIVDQCSN
 GGLDLDVGVFRQLDITDRGYAQGHILMDVYVFNVCNCDLGVES
 TPKPTPTPTPSGGGVDVSSLVSSSVFDQMLKYRNDGRC
 GGGGGGGGQIARNVRATYHLYNPQNHGWDLNAVSAFCSTWDANKPYSW

M. esculenta (cont.)
M. esculenta (cont.)
Manoao colensoi MEKAKIVAVVMVLAMVLMVEG
Marchantia paleacea MLFYRSPMALVLEPLVVPFLQVSSA
M. paleacea (cont.)
Marchantia polymorpha MYTKVIRVATILLLLFHLSSA
M. polymorpha (cont.)
M. polymorpha (cont.)
Medicago truncatula MGKLVVLILVCLIAATIA
M. truncatula (cont.)
M. truncatula (cont.)
Meliosma cuneifolia MGSFTLYVCLISVVCLLAGASA
Metasequoia glyptostroboides MAQSRLRLALYILLVMAVTVKG
Metasequoia glyptostroboides MGMETKAVVFALAVAVPFLCAA
M. glyptostroboides(cont.)
M. glyptostroboides(cont.)
Metasequoia glyptostroboides MLVCLLIVALIVVGPAPFA
Microbiota decussata MAEIRLRMGVMDLLVLIVAVMVG
Microstrobos fitzgeraldii MATMMVEG
Microstrobos fitzgeraldii MEKARAVALVMVLAMVLSVTMMVEG
Microstrobos fitzgeraldii MEKVRLMAVLMVLLMAMTMMVEG
Microstrobos fitzgeraldii MIVVAMAVMVQG
Mitella pentandra MEILNLCLTIMLLTCLVGSATA
M. pentandra (cont.)
M. pentandra (cont.)
Monotropa uniflora MRIVAVVSLSLLSIIGVFA
Monotropa uniflora MGSPMFDICVALMIICLTITTAIGGAA
M. uniflora (cont.)
M. uniflora (cont.)
Morinda citrifolia MAMALELGLVLVFCGCCIASIAA
M. citrifolia (cont.)
M. citrifolia (cont.)
Moringa oleifera MAKLSFSLFLLLCLVATATA
Morus notabilis MARLFYCMVLVFLWLVLVGALA
M. notabilis (cont.)
M. notabilis (cont.)
Morus nigra MGRISFSLIVLGLAWLAVGSRG
M. nigra (cont.)
M. nigra (cont.)
Mydocarpus sp MKTDGLCLVLFLSLVAGAAA
Mydocarpus sp (cont.)
Mydocarpus sp (cont.)
Myriophyllum aquaticum MKYMSSILVALALILGVASGAG
Nageia nagi MEKIRVMALVMVLAMAMTIMVEG
Neocallitropsis pancheri MANRRLNGFPHILLVLIVIVQEVNG
Neocallitropsis pancheri MASKSSSLAVLVMILGLMLIRVKS
Nicotiana tabacum MGKLSLTLFALVLYVIAAGANA
N. tabacum (cont.)
N. tabacum (cont.)
Nicotiana tabacum MRLCKFTALSSLLFLSLLLSASA
N. tabacum (cont.)
N. tabacum (cont.)
Nyssa ogeche MKFSAVITVCLLLVLTLYQGSAA
Ochna mossambicensis MEKSSILYNILIFVLVITITKTAMA
O. mossambicensis (cont.)
O. mossambicensis (cont.)
Oncotheca balansae MAENRLRLAFSMLVLMAMVMVG
Oncotheca balansae METKTKAVVFLLVALLSLCAA
O. balansae (cont.)
O. balansae (cont.)

EPTCSPAGRIYCSRGRCCKSFNWC GTGPAYCGKSNCIAQCS
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DPSCSPTVTGRISCNRGRCCKSFNWC GTGVSYCGKDYCVAQC
EPTCSPAGRIYCSRGRCCKSKYNWC GTGPAYCSKSNCIAQCS
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DPTCSPAGRFWCNSGRCCSIYNWC GTWEYCAPGNCLAQCWP
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SGGGSGGGGESATNVRATYHLYNPEQHNWDLNAVSAYCSTWDANKPIA
WRRKYGWTAFCGPAGPRGQACGKCLRVNTNRTGAQTTVRIVDQCSN
GGDLDDIAAFRQIDTDGRGNAQGHILVNYQFVNCGD
SVDADVHGYLSLLQEKNLTSAHQNNP
GGGEKGYNVRSTYHEYPERHNWDLAYSAYCSTWDGGRPLWRRKYGW
TAFCGPVGPGRQASCGKCLRVNRTGSAQTVRIVDQCSNGGLDLDI
NVFKQLDNTGQGVARGHLMVDYEFVDCGDGAGASQFL

<i>Ophioglossum vulgatum</i>	MGFLNRRRLNLHVLVLLLLQQWSRVGRA	QQCGSQAGGAVCSNNLCCSKYGYCGSTDYFCGSGCQSGPCSSG	GGGTPAPSGGGGVSSSIITSSIFDTFLNHRITDGCSPANGFYTYNAFITAATAAYN
<i>O. vulgatum (cont.)</i>			GFGTGTGATTQKRELAFFAHAAHETSEGCKYKEINKQTYCQAT
<i>O. vulgatum (cont.)</i>			AQWSCASGKQYYGRGLQITWN
<i>Papaver setigerum</i>	MATKNLSVTALLFLTAIFSAVSA	QQCGSQAGGRTCPGNLCCSQYGYCGDTNEYCLPSNNCQSNCRG	PSVPGTGESASNVRTYHIYSPAQNGWDLRVSAYCSTWDGNPLAWRQKYG
<i>P. setigerum (cont.)</i>			WTAFCGPVGRGQASCGRCLRVTNRTGGAQTTRIVDQCSNGGLDLEEGV
<i>P. setigerum (cont.)</i>			FRQIDTNGIGINDGHMFVDYQFVNCGD
<i>Papuacedrus papuana</i>	MAEKRLRLALYMLGLMLIRVRT	DPTCSPAGNFWCKSGRCCSIYNWCGSTDYCAPGNCLAQCW	YIAGLNQSCNLVSPHNSSASP
<i>Papuacedrus papuana</i>	MAENKLRMGLYILLVLLIVKIYG	DPTCSPAGNFWCNSGRCCSIYNWCGSTDYCASGNCLAQCW	DAVALNQSSLQVAKQNTSNSSP
<i>Papuacedrus papuana</i>	METKAATVTFILAATLAAPLLCGG	QNCGSQAGGKVCSSGSECCSKWGCCTTDDHCLPDRGCQSNCRG	GGGGGGGGSGEKAFNVNSTYHEYYPERHNWDLVAVSAYCSTWDGGK
<i>P. papuana (cont.)</i>			PLWWKQYGWTAFCGPVGARGQPSCKGCLRVTNRDTSQATVRIV
<i>P. papuana (cont.)</i>			DQCSNGGLDLINVRQLDTNGQGNARGHLMVDYQFVDCGDGSEFEGVHEFM
<i>Parasitaxus usta</i>	MERAKIVAFVMVLAVAMTMMIMVEG	DPTCSPAGRVYCNPRCCSKFNWCGTTRANCGKNCIAQCHP	AVSPLSLYQAFLSITNHTAQ
<i>Parasitaxus usta</i>	MKMERAKIVAFVMVLAMAMTMMIMVEG	DPTCSPAGRQYCNDRCCSKFNWCGTGAAYCSKNCIAQCP	PGVSPLSLSQSSLSIANHTAQ
<i>Parasitaxus usta</i>	MANAGVVLVWAVASSLAALA	EDCGKAAGKLCFPGGLCCSQGWGCCTADHCKGNCQSCSG	GGPTPTPTPXRSGLLHHHSHKRLQYVVEAQERKGLPRQE
<i>P. usta (cont.)</i>			FLLLQRLHYCCQFLPLRNLHRRSHHQEKRRARFLRPNIA
<i>P. usta (cont.)</i>			RNRDWMGIRPRWVRVGVLLPQRKWRRLQLQPLGALCREAVL
<i>Passiflora caerulea</i>	MGRGMKSWLVLLCLFLGGAMA	EQCGQAGGKTCPGNICCSQYGYCGTDEYCSPDNNCQSNCKG	GGGGGGGGGQSAKNVRATFNDYNPQQHGWDLNAVSAYCATWDAGKPYSWRSKYG
<i>P. caerulea (cont.)</i>			WTAFCGPVGPQGRDSCGKCLRVTNRTGAQTTRIVDQCSNGGLDLVNVFNQ
<i>P. caerulea (cont.)</i>			LDTDGNNGFQGHLLIVDYQFVDCGDSVNNPLYSIIEDRLM
<i>Peganum harmala</i>	MVKLSLYIVLSLSILAATTTA	QQCGRQAGGRLCPNNLCCSQFYGYCGSSDDYCSPSKNCQSNCKPSG	GGGGGGGGGASASNVRTYHLYNPQIGWDLMAASAYCATWDANKPLAWRRK
<i>P. harmala (cont.)</i>			YGWTAFCGPAGPRGRESCKGCLRVTNRTGAQTTRIARIVDQCSNGGLDLIAVFR
<i>P. harmala (cont.)</i>			QIDTDGRGHAQGHLMVDYQFVNCGNEISEIFLGEASS
<i>Pereskia aculeata</i>	MKTPTIPMKFSLLSLLILLMGAHEGEA	QQCGAQAGGALCRDGLCCSIWGYCGSTDYCGPDNCQSQCPG	SSPPPPPSPTPPPPPPPLPCPGGDLSDIFP
<i>Peumus boldus</i>	MASQKSLVWAMAMICCSCLATKTEA	VQCGRQAGGQLCNGNLCCSQFGWCGSTDEYCLPSSGQSNCKTSG	GTTVRATYHLYNP
<i>Phaseolus vulgaris</i>	MKGARVVILQLFLCLIVTATA	QQCGRQAGGSRCSGNLCCSQFGWCGNTAEYCSPSQNCQSNCWG	SGGSASNVRTYHYYPQEQHGWDLNAVSAYCSTWDAGKSYAWRSKYGWTAFCGP
<i>P. vulgaris (cont.)</i>			VGPTGRDSCGRCLRVNTGTGAQTTRIVDQCSNGGLDLVGVFNRDLD
<i>P. vulgaris (cont.)</i>			NGQGYRGHLIVNYEFVDCGNELNLALHAPN
<i>Phellodendron amurense</i>	MERLCVWLVLSLCLFAAATTA	QQCGKQAGGKTCNNLCCSQYGYCGSTDYCSPSKGCQSNQCS	SSSGGESASNVRTYHFNPEQNGWNLNAVSAYCSTWDANKPLAWRSKY
<i>P. amurense (cont.)</i>			GWTAFCGPVGRGQAACGKCLRVNTSGTGAQTTRIVDQCSNG
<i>P. amurense (cont.)</i>			GLDLDYAGVFKQLDYGKGYAQGHLLIVSYQFVNCGD
<i>Phellodendron amurense</i>	MIFSLCLLATAAA	QHCGRQAGGRTCPNNLCCSQFYGYCGTDDYCSPSKNCQSNQCSAG	GGGGGGGQ
<i>Philonotis fontana</i>	MARQGGSMVMLLASAFVVLSLMSLTAA	EQCGRQAGGKTCPDNLCCSQWGYCGNTDEFCKNDQSQCG	GGGGGGGGGQSGTQASFYGPEYVPSACFGDPNQFPANR
<i>P. fontana (cont.)</i>			HFAAGDGSNPNIWXSLRLPTPTWV
<i>Philonotis fontana</i>	MARQGGSMMLLASGIIMVLSLMSQLTAA	EQCGRQAGNCKPKDQCCSYWGWCGDTEFCEHGQSQSCSG	GGGAPGGSTGQASFYGPPSPNACYGFDTLGFLPANLYIAAAGDGNPNLWDN
<i>P. fontana (cont.)</i>			GRNCGRFFRIQCQKGRADRGTITVKIVDR
<i>Phyllocladus hypophyllus</i>	MEKMRMMALVVVLAMAMTMVVEG	EPTCSPAGRIYCSQGRCCSKFNWCGTGPAYCSKSNCAQCS	AGISPLPQSQILLITNLTHVANTP
<i>Phyllocladus hypophyllus</i>	MAKKATILVVMGVAVALLCVA	QNCGRQAGGVCSGGECCSQYGYCGTTPDHCGTGCQSNCG	GGGGGEGKYNVNSTYHEYYPERINWDYITASVYCTWDGKPLSWRNKYGWT
<i>P. hypophyllus (cont.)</i>			AFCGPVGRGQASCGKCLRVTNRTGAQETTRIVDQCSNGGLDLVNVFNR
<i>P. hypophyllus (cont.)</i>			LDTNGQGYARGHLMVDYEFVNCGDGQTVAVE
<i>Physcomicromitrium sp</i>	MIINMNVFLIATIVGRDVA	QCTAPPRSDFQCGPANGNASCPGKCCSWWGWGATSFYCNQRSLSCTS	LTSSPTPAKTPTLAPTVPNTTPTPVLTPAKTPSRIPPISVRGASVFNW
<i>Physcomicromitrium sp (cont.)</i>			CGTSKSIALTFDESAFMEPEITNLNLDKRLQMKATFF
<i>Pilgerodendron uviferum</i>	MAAIRLRLGLHMLLILMAVMVKVG	DPTCSPAGNFWDCSGRCCSIYNWCGSTDYCASGNCLAQCW	NTPLELNQSTLTVAQNLTSPSP
<i>Pilgerodendron uviferum</i>	MQQYKSTMTVLVVIVLLGCSIGIPVMVG	DPTCSPRGNFFCNSGRCCSIYNWCGTGPAYCGKNCCLAQCS	GVTAVTYSLNGKNLTSNNRN
<i>Pinus taeda</i>	METSVCNMKSMKFSAMAIALLTMATMNLVYFVSA	EQCGQAGGALCPGGLCCSKWGCNTDDHCGDQCSQCGG	STPTPSPPTGGQGVASIIITESIFNELLKHRNDAGCKASGFYTYSA
<i>P. taeda (cont.)</i>			FIAADNAPFSFGTAGDVATRKRELA
<i>Pinus parviflora</i>	MALYKLSVTVLVLMFTAMLRVMA	DPTCSPAGNFFCNNGRCCSRFNWCGTGPAYCYGRGNCAQCP	PAVTALTYSFSKAKNLTANP
<i>Pisum sativum</i>	MRNFGVGVVVLTLAYLIATTIA	EQCGRQAGGATCPNNLCCSQYGYCGTDDYCSPSKNCQSNCHG	SGGGSGESASNVRTYHYRPEQHGWDLNAVSAYCSTWDASKPYSWRSKYGWTA
<i>P. sativum (cont.)</i>			FCEPVGPRGQASCGKCLRVNRSGTGAQETTRIVDQCSNGGLDLVGVFN
<i>P. sativum (cont.)</i>			RIDTDGRGYQGHLLIVSYQFVDCGNEVDMINPLPSWMLT
<i>Platycladus orientalis</i>	MAYKIVSLALYMLLMLMNVKG	DPTCSPAGNFWCNSGRCCSIYNWCGSTDYCASGNCLAQCW	NALSLLNHSSCLNVPINHNRSSVSP
<i>Platycladus orientalis</i>	MKEVAVVMVMIIVMAVSVEG	DPTCSPAGRFFCNSGRCCSKYNWCGSTDYCAKNNCIAQCW	GFTATSSPLTIANTVPLHNNP
<i>Platycladus orientalis</i>	MRIKIRMADSRSMGLYLILVFMVTVNIHG	DPTCSPAGNFWCNSGRCCSVYNWCGSTDYCASGNCLAQCW	NAVALNQSTLISVSQKNITVSVP
<i>Plumbago zeylanica</i>	MEKNIVRRQPLFLIIYTLASLAVAAVA	QQCGRQAGGRTCPNGLCCSQYGYCGTDDYCLSKNCQSNCKG	SGGSPSTGCGGGSASNVRTYHLYNPQQNGWSLYAVSAFCSTWDGDKP
<i>P. zeylanica (cont.)</i>			LSWRSKYGWTAFCGPVGRGRDSCGKCLRVTNRTNAQA
<i>Podocarpus coriaceus</i>	MEKIRVMALVMVLAIAMTMMVEG	DPSCSPAGRQYCNDRCCSKFNWCGTGPAYCSKNCIGQCPP	GVSPPLAQIIPSIANLTQPEAHHPN
<i>Polypodium plectolens</i>	MEGQAKLNI AFLP LLLSSTALLSCWA	QQCGSQAGGALCANNLCCSQYGYCGSTSAYCAVPGCQSCG	GSSSTGGSSGVSSIITSDIFNQFFSQNSASG
<i>Polyscias fruticosa</i>	MGSQTHNVTKARLLVFLCLVAGGTA	QQCGRQGGGKTCANNLCCSQYGYCGTDDYCSPSKNCQSNCSHG	GGVVGGESASNVRTYHIYEPEKKGWDLNAVSAFCSTWDADKPLEWRSKYG
<i>P. fruticosa (cont.)</i>			WTAFCGPVGHGQASCGKCLRVNTGTGAETTRIVDQCSNGGLDLV
<i>P. fruticosa (cont.)</i>			GVFRQLDITDGKGNAQGHLLIVNYQFVNC
<i>Populus deltoids</i>	MRTVYDTFLIVLLCLLAGATG	EQCGKQAGGQTCNNLCCSQYGYCGTDDYCSPSKNCQSNCKG	SGGESASNVRTYHLYNPQDHGWDLNAVSAYCSTWDASKPYSWRSKYGWTA

<i>P. deltoids (cont.)</i>		
<i>P. deltoids (cont.)</i>		
<i>Populus euphratica</i>	MQPPGMERLSLLIVFLCLAATGIA	QNCGRQAGGRTCANNLCCSEWGFCTSDHCHSPSKCQSNCRASG
<i>P. euphratica (cont.)</i>		
<i>P. euphratica (cont.)</i>		
<i>Populus euphratica</i>	MRRVYDTFLIVLLCLIGGATG	EQCGKQAGGQTCPNNLCCSQFGWCGTDDYCSPSKNCQSNCKG
<i>P. euphratica (cont.)</i>		
<i>P. euphratica (cont.)</i>		
<i>Populus euphratica</i>	MKIWTFTVFSLLLSLLLGCSA	EQCGSQAGGALCPGGLCCSQFGWCGKNNDYCGTGCSQCSQG
<i>P. euphratica (cont.)</i>		
<i>Populus nigra</i>	MRRVYDTFLIVLLCLIAAGATG	EQCGKQAGGQTCPNNLCCSQFGWCGTDDYCSPSKNCQSNCKG
<i>P. nigra (cont.)</i>		
<i>P. nigra (cont.)</i>		
<i>Populus tremula</i>	MRRVYDTFLIVLLCLIGGAAG	EQCGKQAGGQTCPNNLCCSQYGWCGTDDYCSPSKNCQSNCKG
<i>P. tremula (cont.)</i>		
<i>P. tremula (cont.)</i>		
<i>Populus trichocarpa</i>	MKRLSLFMVFLCLAGTAIA	QNCGRQAGGQTCANNLCCSQWGYCGTSDHCHNPSKNCQSNCRSS
<i>P. trichocarpa (cont.)</i>		
<i>Populus trichocarpa</i>	MKRLSLFMVFLCLAATAIA	QNCGRQAGGQTCANNLCCSQWGYCGTSDHCHNPSKNCQSNCRSSG
<i>P. trichocarpa (cont.)</i>		
<i>P. trichocarpa (cont.)</i>		
<i>Populus trichocarpa</i>	MESQI	INCGRQAGGRTCANNLCCSEWGFCTSDHCHSPSKNCQSNCRP
<i>Prumnopitys andina</i>	MEKISLMVFVMVLAMMMVEG	DPSCNPAGKIYCNPGRCCSKFNWCGTLESYCGKNYCIAQCP
<i>Pseudotaxiphyllum elegans</i>	MARHGKMLMLIALAVSLLALTADG	EQCGSQADGATCPNNLCCSKWGYCGTSDAYCNPNEGCQSNCWG
<i>P. elegans (cont.)</i>		
<i>P. elegans (cont.)</i>		
<i>Pseudotaxus chienii</i>	MKKRLTLFMALLIIIISSGSVSG	DPTCSPAGRFNCTGRCCSKFNWCGSTAEBYCASPCKIAQCWP
<i>Pseudotaxus chienii</i>	MAENRLRAFYMVLVMAVMVEG	DPTCSPAGNFWCNTGRCCSIYNWCGSTAAYCAQGNCLAQCP
<i>Pyrenacantha malvifolia</i>	MDRIAILLAWSLLVLIGTAMG	QQCGRQAGGRLCANNMCCSQWGYCGTDDYCLPSNGCQSCQCRG
<i>P. malvifolia (cont.)</i>		
<i>P. malvifolia (cont.)</i>		
<i>Quercus petraea</i>	MERLGLCSVVLLCLVANAIA	QQCGWQVGKTCNNLCCSQYGYCGTDDYCSPSKNCQSNCCG
<i>Q. petraea (cont.)</i>		
<i>Q. petraea (cont.)</i>		
<i>Quillaja saponaria</i>	MKFMYTFFVFTLILVLKISA	EQCGKQAGGQVCPNGLCCSQFGFCGTTNEYCETGRGCQSCKPSPG
<i>Q. saponaria (cont.)</i>		
<i>Q. saponaria (cont.)</i>		
<i>Raphanus raphanistrum subsp.</i>	MKIKLSITLILLSYTAAMVAG	QQCGRQASGRTCAGNICCSQYGYCGTTADYCSPDNNCQSNCWG
<i>R. raphanistrum (cont.)</i>		
<i>R. raphanistrum (cont.)</i>		
<i>Reseda odorata</i>	MRDLSLILLIAILCLCVAAEKARG	QQCGRQAGGRTCANNLCCSQYGYCGTDDYCSPSKGCQSNCRG
<i>R. odorata (cont.)</i>		
<i>Retrophyllum minus</i>	MERAKIVAFVMVLAMATMMIMVEG	DPTCSPAGRVYCNPGRCCSKFNWCGTTRANCGKNCIAQCHP
<i>Rhamnus japonica</i>	MASLRLGHCLSLVLLIFLVAACATA	QQCGQVGKGTCSGNLCCSQYGYCGTDDYCSPSKNCQSNCWG
<i>R. japonica (cont.)</i>		
<i>R. japonica (cont.)</i>		
<i>Rhodiola rosea</i>	MEIVKWVVFISLGLLATATA	QNCGRQAGSATCANNQCCSQWGYCGTDDHCLPSNNCQSNCRTEG
<i>R. rosea (cont.)</i>		
<i>R. rosea (cont.)</i>		
<i>Rhodiola rosea</i>	MRSMGNVGLFSVLFFILSAYVTS	AQICGKQAGGKTCAGNICCSQWGYCGTDDYCLPSNNCQSNCKG
<i>R. rosea (cont.)</i>		
<i>R. rosea (cont.)</i>		
<i>Ricinus communis</i>	MGSVIDVLMVVLLCSLGVAFG	EQCGRQAGGKLCNNLCCSQYGWCGSTDDYCSPSKNCQSNCKG
<i>R. communis (cont.)</i>		
<i>R. communis (cont.)</i>		
<i>Robinia pseudoacacia</i>	MGKVKVGLVIFACLIVSAIG	EQCGRQAGGQTCNNLCCSQYGYCGTDDYCSPSKNCQSNCRG
<i>R. pseudoacacia (cont.)</i>		
<i>R. pseudoacacia (cont.)</i>		
<i>Roridula gorgonias</i>	MTYPLVLLSLAAILVTCCWA	EQCGSQAGGALCPGGLCCSKFGWCGTGDYCGDGCQSCQG

FCGPAGPRGQASCGKCLRVTNTRTGAQTTARIVDQCSNGGLDLVDNVF
RTIDTDGDGYXKGHLIVNYQFVDCXDSINSPKPLLSIIDDQ
SGSGGGSSGGGESASNVBRATYHFYNPEQNGWDLNAVSAYCSTWDANKPLAW
RRQYGTWAFCGPVGPRGQASCGKCLRVTNTRTGAQTTARIVDQCSNGGL
DLDAVFRQIDTDGRGNAQGHILVNYQFVNC
GGGESASNVBRATYHLYNPQEHGWDLNAVSAYCSTWDASKPYSWRSKYGW
TAFCGPAGPRGQASCGKCLRVTNTRTGAQTTVRIVDQCSNGGLDLVDN
VFRTIDTDGDGYAKGHLMVNYQFVDCGDSINSPKPLLSIIDDQ
GAGGGDLGSIISAKFDEMLKHRNDGGCPGKGFYTYNAPISAANAFPGFTTGDA
DTRKREIAAFLGQTSHETTGGWQTAPDGPYAWGCVFKEQNPGSYCSPSSTYP
GGGESASNVBRATYHLYNPQDHGWDLNAVSAYCSTWDASKPYSWRSKYGWTAFC
GPAGPRGQASCGKCLRVTNTRTGAQTTVRIVDQCSNGGLDLVDNVFRTIDTDGD
GYAKGHLIVNYQFVNCGDSINSPKPLLSIIDDQ
GGGGGESASNVBRATYHLYNPQDHGWDLNAVSAYCSTWDASKPYSWRSKYGWTA
FCGPAGPSGGQASCGKCLRVTNTRTGAQTTARIVDQCSNGGLDLVDNVFRTID
TDGDGYAKGHLIVNYQFVDCGDSINSPKPLLSIIDDQ
DTGGGGESASNVBRATYHLYNPQDHGWDLNAVSAYCSTWDANKPLAWRRKYGW
TAFCGPVGPRGQASCGKCLRVTNTRTGAQTTVRIVDQCSNGGLDLDAVFRQI
DTDGRGNAQGHILVNYQFVNCGD
SGGGTGGGGESASNVBRATYHLYNPQDHGWDLNAVSAYCSTWDANKPLAWRR
KYGWTAFCGPVGPGRGQASCGKCLRVTNTRTGAQTTVRIVDQCSNGGLDL
AGVFRQIDTDGRGNAQGHILVNYQFVNC
SGGGGGGGGGGESASNVBRATYHFYNPEQNGWDLNAVBRAYCSTWDANKPLA
PGVSPSPSQILLSIANLTAQPAHVDLNP
EVHALDRGLGDPRCGQAGGGDIGNFGRCCSIFDFCGSTDAYCNGDCAS
QCPRNGGLRASTGKASYTYRVPSACYGNDESQLPRNRHMAAVSDGHP
NLWKNCGCGKHVRVRCCEGNGCRNADAITIKVVDRC
GVTATATATATPTATAXPTATATATPSPLLTITNYTTTPNNRHP
TVAALNNSSLTLQQRNLTSIHQNNP
GGGGTEVRATYHFYNPDQVHWDLNAVSAPCSTWDASKPLWRRSKY
GWTAFCGPVGPQQAACGKCLRVRNKRKTGASTTVRIVDRCSNG
GLDLDNVNFRALTDGSGYAQGHILVITYEFVDCGDSNGGDDALLITSVTDNRNI
GGGGGGGGGGGESASNVBRATYHLYNPQEHGWDLNAVSAYCST
WDAGKSYAWRSKYGWTAFCGPAGPRGQASCGKCLRVTNTRTGAQTTV
RIVDQCSNGGLDLVDGVFQRLDTDGRGYAQGHLMVNYQSVDRGDGFNPLLSII
TGGGPTPKPTXAVVVAMLPLLLALIFSTNCLNIETMANARAKDFILTML
SSLRQDLLVGLVPQVILARGRLTLWLKHPMKPQEDGVVPMQAHMHG
VIALSEKKTGLHIVIPAKGHVLLANYIMAEDLFN
GGPSGSGESASNVBRATYHLYNPQNNWDLNAVSAYCSTWDANKP
YSWRSKYGWTAFCGPVGPGRGQASCGKCLRVRNTRTNAAVTVR
IVDQCSNGGLDLVAMF
GGDSGGQASNVBRATYHLYNPERNNWDLRAVSAYCSTWDADK
PYSWRSKYGWTAFCGPVGPGRGXG
AVSPLSLYQAFLSITNHTAQ
GGDSGSGESASNVBRATYHFYNPEQNGWDLNAVSAYCATWDA
SKPYSWRSKYGWTAFCGPVGPGRGQASCGKCLRVTNTRTG
AQRTVRIVDQCSNGGLDLDAVFTQDLDGSGYAQGHILVINYDFVNCNDLVD
TGGGGQASNVBRATYHLYNPQNIQWDLNTASVPCSTWDADKPLAWRS
RHGWTAFCGPVGTGGRDSGKCLRVTNTRTGAQAVVRIVDQCSNG
GLDLDNVNFRQIDTDG
GGGGEKASNVBRATYHLYNPQEHGWDLNAVSAYCSTWDANKPLAWRK
YGTWTAFCGPVGPGRGQASCGKCLRVTNTRTGAQTTVRIVDQCSNG
GLDLDVGVFNKLDTNQGYAKGHLMVNYEFVNCNGLDYQLLSSTTISDQ
GGGGESASNVBRATYHFYNPQHGWDLNAVSAYCSTWDANKPYSWRSKYG
WTAFCGPAGPRGQTCGKCLRVTNTRTGAATTVRIVDQCSNGGLDL
DVNVFRQLDTDGIGYQGHILVNYQFVNCGDSFNPLFSIMDDQ
GGGESASNVBRATYHYQPELHGWDLNAVSAYCSTWDAGKPYSWRSKYGWTA
FCGPVGPHGQASCGKCLRVTNTRTGAQETVRIVDQCSNGGLDLVDGVFNRLD
TDGLGYQRGHLIVNYQFVECGNELELENNPLLSILDAH
GGATPTPG

<i>Ruellia brittoniana</i>	MALSFSHIKPSMFFALAAAILLAAIAPALG	QNCGCDARLCCSRWGYCGSTIEYCGQCGSQGPCFTSPPSNG	NSVSNIVTEAFFNGI
<i>Salix dasyclados</i>	MRIYDAFVIVLLCLRIGAIG	EQCGSQAGGQTCPNNLCCSQFGWCGDTDDYCSPSKNCQSNCKG	GGGGESASNVRATYHLYNPQDNGWDLNAVSAYCSTWDASKPYSWRSKYG
<i>S. dasyclados (cont.)</i>			WTAFCGPAGPRGQASCCKCLTVTNTRTGARTTVRIVDQCANGGLDLD
<i>S. dasyclados (cont.)</i>			VNVFRTIDTDGDGYAKGHLIVNYQFVDCGDSVKNPBFLPSIIEDQ
<i>Salix fargesii</i>	MRIYDTFVIVLLCLRIGAIG	EQCGSQAGGQTCPNNLCCSQYGWCGDTDDYCSPSKNCQSNCKG	GGGGESASNVRATYHLYNPQDNGWDLNAVSAYCSTWDASKPYSWRSKY
<i>S. fargesii (cont.)</i>			GWTAFCGPAGPRGQASCCKCLTVTNTRTGARTTVRIVDQCANGGLDLD
<i>S. fargesii (cont.)</i>			DVNVFRTIDTDGDGYAKGHLIVNYQFVDCGDSIKIPEPLFSIIEDQ
<i>Saxegothaea conspicua</i>	MEVGQKATIFVFMIAVALPLFGSG	ENCGRQAGGQVCNGBECCSQWGWCGTTPDHCGTCQCSNCG	GGEKGTNVRSTYHMYNPQNINWDYAAASVYCSTWDGGNPLSWRKKYGWT
<i>S. conspicua (cont.)</i>			AFCGPVGPGRQASCCKCLRVTNRNTRTAQTTVRIVDQCSNGGLDLDVNV
<i>S. conspicua (cont.)</i>			FNQLDITNGQGYAQGHMVDYEFVNCGDGETLAVESSEPLLLRSVAHEEENLMP
<i>Schizolaena sp.</i>	MGRSLQLAVALLLCLLGAV	AQQCQGWQVGGQTCPDNLCCSQYGYCGTDDYCSPSKNCQSNCKG	GGGGGGGGGSESASNVRATYHLYNPEQHWDLNAVSAYCSTWDASKPYSWR
<i>Schizolaena sp. (cont.)</i>			SKYGTWAFCGPVGPHGQAACGKCLRVTNSTGAQETTVRIVDQCSNGGLD
<i>Schizolaena sp. (cont.)</i>			LDVGVPQRDLTDGRGYAQGHLIVNYQFVDCGDGITSSTGPSFLNKLEET
<i>Schwetschkeopsis fabronia</i>	MKSVLFVVIVVLSLVASIEA	ACSKTSPCANGLCCSQYGYCGTTAEYCGAGCQSQCG	GTPATPKPTTSGGGGTGKGYITYHLYNQDGLSTTVACSDGANGLMTRWGYS
<i>S. fabronia (cont.)</i>			TLSPMYPNVAAFSGATWNSPNCCKCAKLTKGAKTAYTVTI DQC GGAPAGYT
<i>S. fabronia (cont.)</i>			AHFDVAPGAFTNLFGSTSQGVGSVDWSFVANSFCQGNRG
<i>Sciadopitys verticillata</i>	MAECKLRWAFILVLLGLMLVLA	DPTCSPAGNFWCNSGRCCSKYCNWCGDTEYCGEGNCIAQCWA	SVSAVTEYSVNLQKQNLTTAQKNP
<i>Sciadopitys verticillata</i>	MTTMKKLVVFLVMGVAAAPLLCAA	ENCQQVGGVCGPGECCSQYGWCGTTHAHCAGCQSNCRTP	SPSPSGGGDKASNVRSYTHEYYPERHNWDLVAVSAYCSTWDGKKPQWWRQ
<i>S. verticillata (cont.)</i>			KYGTWAFCGPVGPGRQASCCKCLLVKNRETGSQATVRIVDQCSNGGLDLD
<i>S. verticillata (cont.)</i>			INNVFKQLDITNGQVARGHLMVDYEFVNCGDGVEDSPFLRSVT DAN
<i>Selaginella apoda</i>	MAASTSRVVLALALMLVVALIAVQA	DPTCSPAGRQYCNPRCCSKFNWCGTGRAYCGRGNCIAGPCWR	SAAGVNNDDQFQVSAANFSSYPAVVHGLATEEELP
<i>Sequoiadendron giganteum-Gla.</i>	MAESRLRLALHMLIVLMTVTVKG	DPTCSPAGNFWCNSGRCCSIYNWCGSTSDYCASGNCLAQCWP	NAALNQSSSLNVSERNLTSGSP
<i>Serenoa repens</i>	MDKIRVMVLMALAMAMTMVVEA	DPSCSPAGRQYCNDRCCSKFNWCGTGAAYCGKNCIGQCPC	RVSPPLPSQITLSITNHTTKTQAHHPNP
<i>Sideroxylon reclinatum</i>	MGLRVGSVWMTMMVMCATIVGTAVG	QQCGRQAGGALCENNLCCSQMGYCGTTDEYCLPSNNCQSNCRG	GGTPPSGEGTTVRSTYHLYNPQNVGWDLNAVSAYCSTWDAGKPLWWRQYK
<i>S. reclinatum (cont.)</i>			WTAFCGPVGTGQAACGKCLLVNTGTGAQATVRIVDQCSNGGLDLDVNV
<i>S. reclinatum (cont.)</i>			FNQIDTDGKGYYQQGHLTVKYQFVDCGDFVFSII
<i>Simmondsia chinensis</i>	MKWAATMVLLLALPYLLVLSSA	QRCGRQGGGQRCRGLCCSNYGYCGTGSAYCAPGSCSQSCHH	RFVDMGQDFITEATEGYA
<i>Solanum lycopersicum</i>	MKPHVILMILALLLTATSA	QQCGRQARGRACANRLCCSQYGFCCGSTRAYCGFCQCSNCR	YDATGKGQKLNDEHKNNNDGHN
<i>Solanum lycopersicum</i>	MNKLSSSTPILLALVLCISLTSVTNA	QQCGRQGGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCRG	GPTPTPTPGGGAQVRATYHIYNPQNVGWDLNAVSAYCSTWDANKPYSWRSKY
<i>S. lycopersicum (cont.)</i>			GWTAFCGPVGPGRGRDSCGKCLRVTNTRTGATTVRIIXQC SNGGLDLDINV
<i>S. lycopersicum (cont.)</i>			FRQIDTDGVG
<i>Solanum torvum</i>	MEKLNSTILLGVVLFISIGAIANA	QQCGRQRGALCDGGLCCSQFGWCGSTPEYCSPSQGCQSQCSG	SGPTPEGSAQNV RATYHLYNPQNVGWDLVAVSAYCSTWDGNKP
<i>S. torvum (cont.)</i>			YSWRSKYGTWAFCGPVGPGRKPPAASA
<i>Solanum tuberosum</i>	MKLPIFTTLLSLLFSLLLLIASA	EQCGSQAGGAICASGLCCSKFGWCGNTDTYCGPNCQSQPCPS	VPSPKLPITDGPGLGDQLGSIISNSMFDQMLKHRNDNVCKGNFNYSYNA
<i>S. tuberosum (cont.)</i>			FITAARSYPGF GTTGDI TTRKREIAAFFAQTSHT
<i>Solanum tuberosum</i>	MVKLSCAPILLVLILCISLTSVANA	QQCGSQRGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCSG	SGPDPGQGGSAQNV RATYHIYNPQNVGWDLNAVSAYCSTWDANKPYAWR
<i>S. tuberosum (cont.)</i>			SKYGTWAFCGPVGPGRGRDSCGKCLRVTNTRTGATTVRIVDQCSNGGL
<i>S. tuberosum (cont.)</i>			DLDINVFRQIDTDGVGNQQGHLIVNYQFVNCGDNVNVP LLSV
<i>Solanum tuberosum</i>	MVKLSCGPILLALVLCISLTSVANA	QQCGRQRGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCTG	SGPDPGQGGSAQNV RATYHIYNPQNVGWDLNAVSAYCSTWDANKPYAWRS
<i>S. tuberosum (cont.)</i>			KYGTWAFCGPVGPGRGRDSCGKCLRVTNTRTGATTVRIVDQCSNGGLDLD
<i>S. tuberosum (cont.)</i>			DINVFRQIDTDGVGNQQGHLIVNYQFVNCGDNVNVP LFSVI
<i>Solanum dulcamara</i>	SMEKLSSTILLAVVLFISIGAVANA	QQCGRQRGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCSG	SGPIPTPGGSAQNV RATYHFYNPQNVGWDLNAVSAYCSTWDANKPLAWRR
<i>S. dulcamara (cont.)</i>			KYGTWAFCGPVGPGRGQASCCKCLRVTNTRTGATTVRIVDQCSNGGLDLD
<i>S. dulcamara (cont.)</i>			DINVFRQIDTDGLGNQQGHLIVNYEFVDCGDNVNVPLIMSVVDKE
<i>Solanum lasiophyllum</i>	MEKMNLSSSTPILLALVLCISLTSVTNA	QQCGRQRGALCGNLCSSQFGWCGSTPEYCSPSQGCQSQCRG	GPTPTPTPTPGGGAQVRATYHIYNPQNVGWDLNAVSAYCSTWDANKPYSWR
<i>S. lasiophyllum (cont.)</i>			SKYGTWAFCGPVGPGRGRDSCGKCLRVTNTRTGATTVRIVDQCSNGGLDLD
<i>S. lasiophyllum (cont.)</i>			INNVFRQIDTDGVGNQQGHLIVNYQFVNCGDNVNVP LSVVDKE
<i>Solanum sisymbriifolium</i>	MEKLNSTTSILLALILFILA AVAKA	QQCGRQGGALCAGNLCCSQFGWCGSTPEYCSPSQGCQSRCSG	SGSTPTTPGGSQNV RATYHLYNPQNVGWDLNAVSAYCSTWDANKPLAWRR
<i>S. sisymbriifolium (cont.)</i>			KYGTWAFCGPVGPGRGRDSCGKCLRVTNTRTGATTVRIVDQCSNGGLDLDI
<i>S. sisymbriifolium (cont.)</i>			NVFRQIDTDGVGNQQGHLIVNYQFVDCGDN
<i>Solanum sisymbriifolium</i>	MSKLSSTILLGMVLFISIGAIANA	QQCGRQMGALCGNLCSSQYGWCGSTPEYCSPSQGCQSQCSGSG	PIPTPGGSAQNV RATYHLYNPQNVGWDLNAVSAYCSTWDANKPYAWRS
<i>S. sisymbriifolium (cont.)</i>			KYGTWAFCGPVGPGRGQASCCKCLRVTNTRTGATTVRIVDQCSNGGLD
<i>S. sisymbriifolium (cont.)</i>			LDINVFRQIDTDGVGYQQGHLFVDYEFVNCGDNVNVP LKSVVDKE
<i>Sphagnum lescurii</i>	MGQYKSTVAALLVIIMFLGCNIGLPMVMG	DPTCSPKGNFFCNSGRCCSVYNWCGSGAS YCAKGNCLAQCWP	GVTAVTITYTLTGKNLTSTHNN
<i>Stangeria eriopus</i>	MEYKNLVCVVLVLA VVIGESSVSVKG	DPTCSPAGQVYCN SGRCCSKFNWCGSTAAYCQRPN CIAQCWP	GVTALTLSESLLSL SNYTSEAA TMAAENNNP
<i>Staphylea trifolia</i>	MNRVRICMVLLGLVAGAIA	QQCGSQAGGRCDNNLCSSQYGYCGTDEYCLPSNNCQSNCKG	SSPTGGESASNVRATYHYYYPEQNGWDLNAVSAYCSTWDANKPLEWRRKY
<i>S. trifolia (cont.)</i>			GWTAFCGPVGPGRGQASCCKCLRVTNTRTGATTVRIVDQCSNGGLDLD
<i>S. trifolia (cont.)</i>			GVFQQLDITNGNGIAQGHLIVNYQFVNCGD
<i>Strychnos spinosa</i>	MRNLILILLSLTTFSTILA	EQCGSQAGNALCPNGLCCSQYGWCGSTPEYCTTGQCSQCG	GSSTPIPSNPPAAPGGGGGGDIGSIIXPS
<i>Sundacarpus amarus</i>	MEKISMMALVMVLAXEG	DPSCNPMKAVYCNPRCCSKFNWCGTLESYCGKNYCIAQCP	TGISPLSPSQILLSTANLTAQTQVHVDPNP

<i>Synsepalum dulcificum</i>	MGMRVASVNTVMVMCATIIGTAVA	QQCGSQAGGALCANNLCCSQWGYCGTDEYCLPSNGCQSNCRG	GGTPPGPPTGEGATVRSTYHLYNPQNVGWDLNAVSAYCSTWDASKPLSWRQ
<i>S. dulcificum</i> (cont.)			KYGWTAFCGPVPGTGAACGKCLLVNTGTGAQATVRIVDQCSNGGLDLVD
<i>S. dulcificum</i> (cont.)			NVFNQIDTDRRGYQQGYLTVNYQFVDCGQVPSII
<i>Taiwania cryptomerioides</i>	MVESRLRLPLHILLVMTVEVRVRG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCASGNCLAQCWP	NAVALNQYTFNTSQLNLTSASP
<i>Talinum sp</i>	MKLYVVCALITTLPCFLGTITSA	EQCGSQAGSALCPNKLCCSKFNGWCGSTDYQYCGPGCQSQCTPSG	PSPTPTPTPSGGSDVGSIIISPTLFDQMLKHRNENS
<i>Tamarix hispida</i>	MTIMSSKHAYTLTLLLSLAISGATAV	QCQTQAGGQLCPNNLCCSQYWGCGSTDDYCSQANNQCSNCNG	GGGSGPPSTGGSGESATNVNRATYHLYNPQQNGWNLYAVSAYCSTWDGDK
<i>T. hispida</i> (cont.)			PLSWRSQYGWTAFCGPGVGRGQAACGKCLRV
<i>Tapiscia sinensis</i>	MEKSGLLTALLLCLLAVATA	QQCGRQAGGRTCANNLCCSQYGYCGTTAEYCSQNCQCSNCSFG	GGGCGGGGGGESASNVNRATYHLYNPEQHGWDLNAVSAYCSTWDANKPLA
<i>T. sinensis</i> (cont.)			WRQKYGWTAFCGPGVGRGQASCGRCLRVNTRGTSAQETVRIVDQCSNGG
<i>T. sinensis</i> (cont.)			LDLDVGAFLNQLDTRRGYAQGHLMVDYQFVNCGD
<i>Tarenaya hassleriana</i>	MNKQGLSIAFVLLLYTAAVAG	EQCGRQAGGRTCPDNLCCSQYGYCGTDEYCSPEKNCQSNCWG	GGGGGGGGGGESASINIRATYHYYNPEQHNWDLRAVSAYCSTWDAKKPYTW
<i>T. hassleriana</i> (cont.)			RSKYGWTALCGPVGPRGQASCGKCLRITNTRTRAQVTARIVDQCGNGGLDM
<i>T. hassleriana</i> (cont.)			DVGVFNRLDTDGVGYYQQGHLIVDYQFVDCGNELLESNQNTLPSFLDGV
<i>Taxodium distichum</i>	MAESRFRALYIILLVMAVVNVKVG	DPTCSPAGNFWCNSGRCCSIYNWCGNTSDYCASGNCLAQCWP	NAVALDQSNLSVSRQNLTSAFP
<i>Taxodium distichum</i>	MALNKVSIITVLVGLMLLMGNVGVT	DPTCSPAGNFWCNSGRCCSIYNWCGTGPAICGKGNCLAQCSP	GVAAVTTYSLAQKNLTSACLINP
<i>Taxodium distichum</i>	MLLVLMAMVMNVKVG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCAFNGCLAQCWP	NVLALNQSSLSAVQHNLTSASP
<i>Taxodium distichum</i>	MGLNKVSIIVLVVVLLMGNVGVM	DPTCSPKGNFWCNSGRCCSIYNWCGTGPAICSKGNCLAQCSP	GVTAVTTYSLTQKNLTSVNP
<i>Taxodium distichum</i>	MRMSRLTYAICMLLVMAVMLKVVKVG	DPTCSPAGNFWCNSGRCCSIYNWCGTSDYCASGNCLAQCWP	YAVALNESSLNSQQNLTSALHGNP
<i>Taxus baccata</i>	MAENKLRLAFYMLMLTVMVEG	DPTCSPAGNFWCNTGRCCSIYNWCGSTAAYCAQGNCLAQCWP	TVAADVNSSFSLLQHNLTSHTNP
<i>Taxus baccata</i>	MVIAVILIIISVSG	DPTCSPAGRFFCNTGRCCSKFNWCGSTAAYCASRNCIAQCWP	GVATATPSLLTITNYTTPKNHHP
<i>Tellima breviflora</i>	MEILNLCLTLMILLCLVASATA	QQCGRQAGGRTCANNLCCSQYGYCGTTAEFCTTNCQSNCKPG	GGGGESAXNVNRATYHYYNPAQNGWNLNAVSAYCSTWDANKPLAWRQK
<i>T. breviflora</i> (cont.)			YGWTAFCGPAGPRGQASCGRCLRVNTRTGAQVTARIVDQCSNGGL
<i>T. breviflora</i> (cont.)			DLDYSGVFKPLDTDGNGYAQGHILVINYQFVNCGD
<i>Tellima breviflora</i>	MGKISISMLLIICVAYGASA	QQCGRQAGGKTQGNVCCSQYGYCGTTDDYCSPSKNCQSNCCG	GGGGGGGGGGGGAASNVNRATYHYYNPEQHGWDLNAVSAYCSTWDAKKSL
<i>T. breviflora</i> (cont.)			AWRKKYGWTAFCGPAGPRGQASCGKCLRVTNATTGAKTTVRIVDQCSNGGL
<i>T. breviflora</i> (cont.)			DLDVGVFNKLDTDGRGYAQGHLTVNIEFVDCGGINNPLSSIIEA
<i>Thalictrum thalictroides</i>	MANKIICITFLLCLIGVATA	QNCGRQAGGATCPGNICCSQWGWCGTTDDHCLPSNNCQSNCRG	GGPGPGPG
<i>Thellungiella halophila</i>	MKKSRLSIAIILLSYTVATVAG	QQCGSQAGGQTCPGNICCSQYGYCGTTADYCSPDNNCQSNCWG	SGPSPGSESASNVNRATYHYFNPEQNNWDLRAVSAYCSTWDADKPYAWRSKY
<i>T. halophila</i> (cont.)			GWTAFCGPAGPRGQASCGKCLRVTNRTRNAVVTVRIVDQCSNGGLDLDVAM
<i>T. halophila</i> (cont.)			FNRLDTDGVGYYQQGHLIVDYQFVDCGNDLIHQPADSKNILLVSAIDRV
<i>Theobroma cacao</i>	MKNITQKMGNLSLCLVFLVSLLASTATA	QQCGRQAAGRTCANNLCCSQFYGYCGTTNEYCSPSKSCQSNCWPSG	GGGGGESASNVNRATYHYFNPPQNGWDLNAVSAYCSTWDANKPLAWRQYGW
<i>T. cacao</i> (cont.)			TAFCGPVGPRGQASCGRCLRVNTRGTAEAIVRIVDQCSNGGLDLDAAVFQ
<i>T. cacao</i> (cont.)			QIDTDGRGYAQGHLMVDYQFVNCGD
<i>Theobroma cacao</i>	MDKVNTVSRLLVFLVSLVGAAVA	EQCGWQAGGTICPDNLCCSQYGWCGNTDAYCLPENNCQSNCKSSG	PGGETATVSTYHYFNPEQHGWDLMAVSAYCSTWDASKPFWSRSKYGWT
<i>T. cacao</i> (cont.)			AFCGPVGPTTFAACGRCLRVNTRTNAQEIIVRIDRCSNGGLDLVDV
<i>Theobroma cacao</i>	MKNITQKMGNLSLCLVFLVSLLASTATA	QQCGRQAAGRTCANNLCCSQFYGYCGTTDEYCSPSKSCQSNCWPSG	GGGGGESASNVNRATYHYFNPPQNGWDLNAVSAYCSTWDANKPLAWRQYKG
<i>T. cacao</i> (cont.)			WTAFCGPVGPRGQASCGRCLRVNTRGTAEAIVRIVDQCSNGGLDLDAAVF
<i>Thuidium delicatulum</i>	MVRVAPLVALLCSLHFASA	QQCGRQVNNLTADPLNCCSQYGYCGTTDAYCVTGQSGPCR	TFSPPPPPAPPSPSPPPPPRPPSPSPSSGAGKIINRKLFEALYP
<i>Tinospora cordifolia</i>	MEKTNIFVLLLCALGIVASA	QQCGRQAGGRTCPGNLCCSQWGYCGTNEYCSNNCQSNCRPG	AGPGGGGESANNVRATYHIYNPASNWDYLRASVYCATWDANKPLEWRRR
<i>T. cordifolia</i> (cont.)			YGWTAFCGPVGPRGQASCGRCLRVNTRTGAQATVRIVDQCSNGGLDLVD
<i>T. cordifolia</i> (cont.)			GVFRQLDTDGNGNAKGHLIVNYQFVNC
<i>Torreya nucifera</i>	MKTAKVVFVLVAVAVGGPXCTA	QNCGSQVDRVCSGGECCSKWFGCGTTDDHCLPERGCQSNCRG	GGSGEKAYNVIRSTYHEYPERHNWDLAVSAYCSTWDGGRPLWWRQYKWT
<i>T. nucifera</i> (cont.)			AFCGPVGPRGQASCGKCLRVTNTRGTSQATVRIVDQCSNGGLDLINVFN
<i>T. nucifera</i> (cont.)			KLDTNQGVARGHLMVDYEFVCGDGAHAQFL
<i>Torreya nucifera</i>	MAEKRLRLAFSVLVFMAVKGLA	DPTCSPAGNFWCNSGRCCSIYNWCGSTAAYCAQGNCLAQCWP	SASALDPYQYGLNLLNQNLTTSPNLNP
<i>Torreya nucifera</i>	MALYKLSVTVLVMLMLTVMGMVMA	DPTCSPSGNFFCNDGRCCSRFNWCGTGPPYCGKGNCLIAQCP	PSVTALTYSLSGKNLTSANP
<i>Torreya taxifolia</i>	MAEKRLRLAFSVLVFMAVKGLA	DPTCSPAGNFWCNSGRCCSIYNWCGSTAAYCAQGNCLAQCWP	SASALDRYQYGLNLLNQNLTTSPNLNP
<i>Torreya taxifolia</i>	MKMALYKLSVTVLIMLPMFTGMAMA	DPTCSPAGNFFCNSGRCCSKFNWCGSGAQYCGKGNCLIAQCP	PSVTALTRYLSGKNLTSANP
<i>Trianthemum portulacastrum</i>	MKLCLVTSVLVIVILPCLLERTSA	EQCGRQAGGARCTGLCCSQYGWCGTTNAYCGTGCGQSGCPG	GGGGGGSTPTPSLP
<i>Triticum aestivum</i>	MAKLAMPLAATFLTGLAVLLLSAAGPAVA	QNCNCPAGMCCSQWGYCGTGPDYCGAGCQSGPCTVV	SSGAAAAETSGGKPVGSETR
<i>Triticum turgidum</i>	MAKLAMPLATATFLAFGLAVLLLSAAGPAVA	QNCNCPAGMCCSQWGYCGTGPDYCGAGCQSGPCTVA	SSGAAAAESGGKPVGERAHP
<i>Triticum turgidum subsp. Durum</i>	MAKLAMPLATATFLAFGLAVLLLSAAGPAVA	QNCNCPAGMCCSQWGYCGTGPDYCGAGCQSGPCTVA	AAAAEASGGKPVGS
<i>Trochodendron aralioides</i>	MKIPSSFTLLLFLCLIGTSLA	QQCGSQAGGRTCPNNICCSQYGYCGTDEYCSPSKNCQSNCWG	GGGCGGGGGGGGGETATNVNRATYHYMEPEKHNDWYMAVSAYCSTWDANKPY
<i>T. aralioides</i> (cont.)			SWRSKYGWTAFCGPVGPRGQASCGKCLRVTNNTYGAQQTVRIVDQCSNGGL
<i>T. aralioides</i> (cont.)			LDLDVGFNRDLDTNTRGYAQGHILVINYQFVDCGDNVNSVMSE
<i>Trochodendron aralioides</i>	MSSPNLWMVLLCLAAGATA	QQCGRQAGGRTCDGNLCCSQYGYCGTTDDYCSPSKNCQSNCCG	GGGGGGGGGESANNVRATYHYYNPEQNGWDLNAVSAYCSTWDAGKSLAWRSK
<i>T. aralioides</i> (cont.)			YRWTAFCGPVGPRGQASCGKCLRVTNTRTGAQRTVRIVDQCSNGGLDLDA
<i>T. aralioides</i> (cont.)			VAFQQLDTDGNGYAQGHILVINYQFVDCGNLITPLPILLSILD
<i>Tropaeolum majus</i>	MKLLSFIAILSMVLVITTAA	PQCGQRIRIGGRCPKGQCCSRFGYCGSGPAHCAPANCYSQCKL	TEPTVTPNVAGGGGGGGGEXKNTLYVLCVYV
<i>Tropaeolum majus</i>	MVNLFLIAIFLSLTVSGIA	QQCGRQAGGKTCDNLCCSQYGYCGTTDDYCSPAKGCQSNCKG	GGGESASNVNRATYHLYNPEQHGWDLFAVSAYCSTWDGTGTPKPSWRSKYGW

<i>T. majus (cont.)</i>			
<i>T. majus (cont.)</i>			
<i>Tropaeolum peregrinum</i>	MVKAFVIASFVIFLSLAIGGIA	QQCGRQAGGQTCRDNLCCSQYGYCGTTDDYCSPAKNCQSNCKG	TAFCGPVGPRGQASCGKCLKVTNTRTGANEIVRIVDQCGNGGLDLDVGVF
<i>T. peregrinum (cont.)</i>			NRLD TDNGYAQGH LTVNYQFVNCGDAFNSVLR SITY
<i>T. peregrinum (cont.)</i>			SGESASNV RATYHNYNPEQHGWDLMAVSAYCSTWDATKPYSWRSKYGWT
<i>Urginea maritima</i>	MKA WLLACFL LASLLGSSA	QHCGRQAGGKLC PDNLCCSKWGYCGPTADHCGDECQSGPCYDKQ	AF CGPVGPRGQASCGKCLKVTNTRTGANEIVRIVDQCSNGGLDLDVGVF
<i>Vaccaria hispanica</i>	MVTMKS FALLTLLITLPYLMGVTTA	FQCGRQAGGARCSNGLCCSQFYGYCGSTPPYCGAGQCQSQCALD	NRLD TDGIGYAQGH LTVNYLFVNCGDAFNSLLRSVTY
<i>Vigna unguiculata</i>	MGKAGVAIVLLLC LIVTATA	QQCGRQAGGRTCSGNLCCSQYGYCGNT E EYCSPSQNCQSNCWG	PPPPSSPPPP
<i>V. unguiculata (cont.)</i>			VPT EALAQAWAQALQAQAAAGAP
<i>V. unguiculata (cont.)</i>			GGSSGGGSASNV RATYHYYPQLHGWDLNAVSAYCSTWDAGKSYAWRSKY
<i>Viola canadensis</i>	MGRLSILVLVFFLGFVGSVLA	QDCGRQAGGRTCANNLCCSQWGF CGTTDDHCSPSKNCQSNCRG	GWTAFCGPVGPTGRDSCGKCLRV TINTGTGSQTTVRIVDQCSNGGLDLDVG
<i>Vitex agnus castus</i>	MGRVMNTFIVVLLCLTGVAIA	EQCGRQAGGKLC PNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDSG	VFNRLD TDGQYQRGHLIVNYEFVDCGDNNLNLVLDTPK
<i>Vitis vinifera</i>	MEGKI IYMAVLLSLVACAAA	QQCGRQAGGRTCANNLCCSQYGYCGTTAEYCSPSQSCQSNCSG	GTPTPTPKKC
<i>Widdringtonia cedarbergensis</i>	MGKAWVGLVVLLCLIVTAIA	EQCGRQAGGQTC PNNLCCSQYGYCGNT E EYCSPSKNCQSNCWG	EGVGGGSASNV LATYHLYNSQ
<i>W. cedarbergensis (cont.)</i>			GGSSGGGTGASASNV RATYHYIIRSNDGT
<i>W. cedarbergensis (cont.)</i>			GGGGGGGGGSESASNV RATYHYIYEPQHGWDLNAVSAYCSTWDASKPYSWR
<i>Widdringtonia cedarbergensis</i>	MKDSKLRRLHISII LMVVGQTVNG	DPTCSPAGNFWDCSGRCCSIYNWCGDTS DYCASENCLAQCWP	SKYGTWTAFCGPVGPRGRDSCGKCLRV TINTGTGANTIVRIVDQCSNGGLDLD
<i>Xanthocercis zambesiaca</i>	MAKLWGVFVVVLC SIATRIA	EQCGWQAGGQLCPNGLCCSQYGYCGSTADYCSPDKNCQSNCWG	VGVFNRI DTDGRGYQ
<i>X. zambesiaca (cont.)</i>			NAAALNQSTMNVAQNNLTSLS P
<i>X. zambesiaca (cont.)</i>			GGGGGSASNV RATYHYYPQLHGWDLNAVSAYCSTWDAGKPYSWRSKYGW
<i>Ziziphus jujuba</i>	MGKLSHCVFVLVLLCLVAGAAA	QQCGRDVGKKTCDGNLCCSQYGYCGST E EYCSPSKNCQSNCWG	TAF CGPVGPRGQEACGKCLRV TINTGTQAQEI VRIVDQCSNGGLDLDVGVF
<i>Z. jujuba (cont.)</i>			NRLD TDGRGYQGH LTVNYEFVECGNELEELTTPLLSNI LE
<i>Z. jujuba (cont.)</i>			GGGGGGGDSGEGSASNV RATYHYYNPEQHGWDLNAVSAYCSTWDANKPY
			SWRSKYGTWTAFCGPSGPRGQASCGKCLRV TINTRTGAQKIVRIVDQC
			SXQWRIRFGCWGIQPIGH

Appendix D. Full-length precursors of putative 10C-hevein-like peptides

10C-Hevein-like Peptides

Plant name	Signal peptide	Mature domain	C-terminal tail
<i>Citrus aurantium</i>	MRLIGSLILFSLVLSFVLGGSA	GNCGSGVVCPGGECCSRFGWCGLTDDYCEGCGSNQNVVCGE	CDPDDGTAGDGGELGKIIISRMKFEDLLEYRNDKRCPCRCFYTYDAFIEAA
<i>C. aurantium (cont.)</i>			KAPPAFGNSGNETMRKREIAAFFAQTGHETTGGWDPAPGGEYAWGYCFNR
<i>C. aurantium (cont.)</i>			EVGAASSDYCDPNYPCRGKYVGRGPIQLSWNYNY
<i>Citrus sinensis</i>	MRLTWSLLIFSLVLSFVLGGSA	QNCGSFVVCPPGGECCSRFGWCGLTDDYCEGCGSNQNVVCGE	CDPDDGTAGDGGELGKIIISRMKFEDLLEYRNDKRCPCRCFYTYDAFIEAA
<i>C. sinensis (cont.)</i>			KAPPAFGNSGNETMRKREIAAFFAQTGHETTGGWDPAPGGEYAWGYCFNR
<i>C. sinensis (cont.)</i>			EVGAASSDYCDPNYPCRGKYVGRGPIQLTWNVCNLYRCGEALGVD
<i>Cuscuta pentagonia</i>	MKLTSSSSSSVLVITTMALAMLIATDDAF	QCGGQAGGALCANRWCCSRWGYCGLSCEYCGNSCQSYCHPDCRGV	AIKFDNETDVVNTKPM DAGGLN
<i>Elymus spicatus</i>	MKPHMSTRGTRVAAILLAVVLAAMLATAVNG	AQKCGDQARGAKCPNCLCCGKYGFCGSTPDYCDVGCQSQCRCGRDG	VVGQALPTESDPTRAAASASATGLNLTASTRGP
<i>Gnetum montanum</i>	MISAKMKQRPAMMRIVVAALVMSMLVTEG	SILVTEVSSGGECCNCVDKLCGEGFCCSKWGFCTGGEAWCGTGNCQCQCPS	VPPPPSPRPPSPRPPSPRPPSPRPPSPRPPSPRPPSPRPPSLTSALL
<i>Lolium perenne</i>	MMRGLSVVAILAAAFVSAHA	QQCGSQAGGATCANCLCCSQYGYCGSTSA YCGAGCQSQCNCGCG	TPPTTPSGGVSSIIISQSLFDQMLLH
<i>Oryza sativa Japonica Group</i>	MRALAVVVVATAFAVAVVRG	EQCGSQAGGALCPNCLCCSQYGWCGSTSA YCGSGCHSQCSGSGCG	GGPTTPSGGGSGVPSIAVALALPTDASXTERRGVP
<i>Oryza sativa Japonica Group</i>	MRALAVVAVATAFLAAAVHA	EQCGSQAGGAVCPNCLCCSQFGWCGSTSDYCGAGCQSQCSAAGCG	GGGPTTPSGGGSGVASIVSR
<i>Panicum virgatum</i>	MMRGGGAVLTAVALVLLAVAMAMSTTVRA	QQCGSEAGGKLCNCLCCSQWGYCGSTSDYCGDGCQSQCHCGCDVAS	IIISRSVFDLLPYRDDALCPARGFYTYDAFIAANAFPGFGTTGDAETR
<i>P. virgatum (cont.)</i>			KRELVAFLAQTSHLTGGGTHSPGGPYAWGYCFKEQVEDLRADYCRPSN
<i>P. virgatum (cont.)</i>			RWPCVAGKQYYGRAPSSSPGTTTTPGREGIGG
<i>Plagiochila asplenioides</i>	MGTIHKSSASVIMRLILIGALVGVALA	VRECGPSNPKGGECCSQFGSCGCSNAYCGKGCIDNCNG	CGGRWATSAGSNQATYYTTYIPSSCYGFDTSKFPAGNLIAAASSDVFRNR
<i>P. asplenioides (cont.)</i>			AACGTYIEITCKGSATGGGNPCRNPAVKVMVVDLCPGCNQNSFDLSKEA
<i>P. asplenioides (cont.)</i>			FSKIADLDAGRIISIAKRVSKYHRTVEETITEVV
<i>Poncirus trifoliata</i>	MRLIGSLLVFSLVLSFVLGGSA	QNCGSVVCPGGECCSQYGWCGITTDHCCGGCQSDCNQVVCGE	CDPDDGTAGDGGELGKIIISREMFALLEYKTDHRCPCRCF
<i>Solanum dulcamara</i>	MKFSLALVFMILALLVTTTYA	EQCGRQSGRRKCPNRLCCSKFGWCGTTCEYCGSGCQSNCRGGCATT	MFSNETVNNSGKHLEDGTLN
<i>Triticum aestivum</i>	MRAFALFAVLAMAAAMAVAE	QCGSQAGGATCPNCLCCSRFGWCGSTSDYCGDGCQSQCSGCGSTP	VTPTTPSGGGVSSIIISRALFDRMLLHRNDGACQAKGFYTYDAFVAAAGAF
<i>T. aestivum (cont.)</i>			RGFGTTGSTDTRKREVAFLAQTSHETTTGGWATAPDGAFWGYCFKQERG
<i>T. aestivum (cont.)</i>			ATSNYCTPSAQWPCAPGKSYVGRGPIQLSHNNY
<i>Triticum aestivum</i>	MKPDMSATALRAPRVAAILLAVVLA AVLATAVNG	DQKCGDQARGAKCPNCLCCGKYGFCGSDAYCGEGSCQSQRCGRCD	DVVGQALPAEPGSTRATAASSASATGLNLATTGGP
<i>Triticum aestivum</i>	MSAPRAPPSLGLATAAMAILAVLAAALATTARA	QTCGSQAGGARCPNCLCCSRFGFCGSGSEWCGAGCQSQCSGCPAP	GGQGVASIVPRDLFERLLLHRNDAACLARGFYTYDAFLAAAAAFPAFAGT
<i>T. aestivum (cont.)</i>			SEGLSVETRKRREVAFLGQTSHETTTGGVAVRARRPLLVGATASSRERDPPS
<i>T. aestivum (cont.)</i>			DYCPQRP
<i>Triticum turgidum</i>	MRGVVVVAMLAFAVSAHA	EQCGSQAGGATCPNCLCCSKFGFCGTTSDYCGTGCSQCNGCSGG	TPVPVPTPSGGVSSIIISQSLFDQMLLHRNDAACLAKGFYNYGAFVAAANS
<i>T. turgidum (cont.)</i>			FSGFATTGSTDVKKREVAFLAQTSHETTTGGWPTAPDGPYSWGYCFNQER
<i>T. turgidum (cont.)</i>			GATSDYCTPSSQWPCAP

Plant name**Accession number****6C-Hevein-like Peptides**

<i>Aerva lanata</i>	PDQH-2053815
<i>Aerva persic</i>	GBCQ-2018132
<i>Alternanthera caracasana</i>	WGUG-2017653
<i>Alternanthera sessilis</i>	BWRK-2077393
<i>Alternanthera sessilis</i>	LUNL-2061656
<i>Alternanthera sessilis</i>	OYST-2062883
<i>Amaranthus retroflexus</i>	WMLW-2009398
<i>Amaranthus retroflexus</i>	WMLW-2061382
<i>Atriplex hortensis</i>	AKTA-2039893
<i>Atriplex prostrata</i>	MUCT-2036997
<i>Atriplex rosea</i>	PDEX-2042561
<i>Beta maritima</i>	FVXD-2056435
<i>Chamaseyce mesebyranthemum</i>	RHAU-2012379
<i>Chamaseyce mesebyranthemum</i>	RHAU-2051397
<i>Flaveria bidentis</i>	QBGG-2040842
<i>Heliotropium calcicola</i>	RGMN-2104521
<i>Heliotropium tenellum</i>	DIHD-2019944
<i>Heliotropium texanum</i>	MDJK-2144026
<i>Kochia scopari</i>	WGET-2036072
<i>Portulaca mauii</i>	BLWH-2018971

8C-Hevein-like Peptides

<i>Actinidia deliciosa</i>	FG478012.1
<i>Actinidia eriantha</i>	FG507620.1
<i>Actinidia setosa</i>	FG481050.1
<i>Actinidia chinensis</i>	FG459770.1
<i>Agathis robusta</i>	MIXZ-2008294
<i>Ajuga reptans</i>	UCNM-2047888
<i>Alangium chinense</i>	FWBF-2012524
<i>Allium commutatum</i>	KBXS-2000239
<i>Alnus glutinosa</i>	FQ341594.1
<i>Amentotaxus argotaenia</i>	IAJW-2003903
<i>Amentotaxus argotaenia</i>	IAJW-2018454
<i>Amentotaxus argotaenia</i>	IAJW-2018453
<i>Amentotaxus argotaenia</i>	IAJW-2126957
<i>Ammopiptanthus mongolicus</i>	JZ389716.1
<i>Ammopiptanthus mongolicus</i>	JZ471525.1
<i>Anemone hupenhensis</i>	ZUHO-2009007
<i>Anomodon attenuatus</i>	QMWB-2011191
<i>Aphanopetalum resinosum</i>	TOKV-2053682
<i>Apios americana</i>	NXOH-2005138
<i>Apios americana</i>	NXOH-2005139
<i>Arabidopsis thaliana</i>	R90112.1
<i>Arabis alpina</i>	TZWR-2041647

<i>Arachis duranensis</i>	GW946290.1
<i>Arachis duranensis</i>	GW956564.1
<i>Arachis hypogaea</i>	FS977976.1
<i>Arachis hypogaea</i>	JK205505.1
<i>Arachis hypogaea</i>	JK209988.1
<i>Arachis ipaensis</i>	GW960675.1
<i>Arachis ipaensis</i>	GW991589.1
<i>Argemone mexicana</i>	BFMT-2017722
<i>Asparagus densiflorus</i>	FGRF-2048625
<i>Astilbe chinensis</i>	CKKR-2060572
<i>Astragalus membranaceus</i>	HJMP-2014725
<i>Astragalus propinquus</i>	MYMP-2058807
<i>Athrotaxis cupressoides</i>	XIRK-2000808
<i>Athrotaxis cupressoides</i>	XIRK-2000807
<i>Athrotaxis cupressoides</i>	XIRK-2000806
<i>Atropa belladonna</i>	BOLZ-2159794
<i>Austrocedrus chilensis</i>	YYPE-2002937
<i>Austrocedrus chilensis</i>	YYPE-2002936
<i>Austrocedrus chilensis</i>	YYPE-2029866
<i>Austrotaxus spicata</i>	BTTS-2032679
<i>Austrotaxus spicata</i>	BTTS-2072841
<i>Austrotaxus spicata</i>	BTTS-2072606
<i>Basella alba</i>	CTYH-2105366
<i>Bauhinia tomentosa</i>	JETM-2091845
<i>Bazzania trilobata</i>	WZYK-2087147
<i>Boehmeria nivea</i>	ACFP-2028246
<i>Boswellia sacra</i>	FCCA-2043010
<i>Boykinia jamesii</i>	OOVX-2042183
<i>Brassica napus</i>	FG577475.1
<i>Brassica napus</i>	DW153474.1
<i>Brassica napus</i>	EE445484.1
<i>Brassica oleracea</i>	EX114407.1
<i>Brugmansia sanguinea</i>	AIOU-2068307
<i>Bryum argenteum</i>	JMXW-2009405
<i>Bursera simaruba</i>	JSZD-2010254
<i>Caiophora chuquitensis</i>	VTLJ-2010251
<i>Cajanus cajan</i>	GW350060.1
<i>Callitris gracilis-March</i>	IFLI-2000417
<i>Callitris macleayana-March</i>	RMMV-2006113
<i>Callitris macleayana-March</i>	RMMV-2002319
<i>Callitris macleayana-March</i>	RMMV-2004820
<i>Calocedrus decurrens</i>	FRPM-2002883
<i>Calocedrus decurrens</i>	FRPM-2008761
<i>Camelina sativa</i>	XM_010419957.1
<i>Camellia sinensis</i>	FS957358.1
<i>Cannabis sativa</i>	BJSW-2013786
<i>Cannabis sativa</i>	PDIE-2001895
<i>Capsella rubella</i>	XM_006298557.1

<i>Capsicum annuum</i>	BM066673.1
<i>Capsicum annuum</i>	EL814425.1
<i>Carica papaya</i>	EX282008.1
<i>Cassytha filiformis</i>	VYLQ-2036312
<i>Castanea mollissima</i>	GO920272.1
<i>Casuarina glauca</i>	LNER-2050327
<i>Celtis occidentalis</i>	KYAD-2004733
<i>Celtis occidentalis</i>	KYAD-2050740
<i>Cercidiphyllum japonicum</i>	NUZN-2000485
<i>Cercis canadensis</i>	RKFX-2042782
<i>Cicer arietinum</i>	XM_004494647.2
<i>Cicer arietinum</i>	GR404613.1
<i>Cinnamomum_camphora</i>	BCGB-2043744
<i>Citrus aurantium</i>	FC874612.1
<i>Clematis chinensis</i>	JG580697.1
<i>Cleome viscosa</i>	UPZX-2042161
<i>Conopholis americana</i>	FAMO-2092069
<i>Coriaria nepalensis</i>	NNGU-202373
<i>Crassula perforata</i>	KWGC-2013004
<i>Crossopetalum rhacoma</i>	IHCQ-2093561
<i>Cucumis melo</i>	CN845588.1
<i>Cucumis melo</i>	JG498505.1
<i>Cunninghamia lanceolata</i>	OUOI-2010547
<i>Cupressus dupreziana</i>	QNGJ-2000610
<i>Cupressus dupreziana</i>	QNGJ-2067951
<i>Cupressus dupreziana</i>	QNGJ-2000609
<i>Cycas micholitzii</i>	XZUY-2048192
<i>Datura metel</i>	JNVS-2004019
<i>Deutzia scabra</i>	OTAN-2004858
<i>Dicranum scoparium</i>	NGTD-2096910
<i>Dillenia indica</i>	EHNF-2006841
<i>Dioon edule</i>	WLIC-2119047
<i>Diselma archeri</i>	GKCZ-2070649
<i>Diselma archeri</i>	GKCZ-2068869
<i>Dombeya burgessiae</i>	WYIG-2035881
<i>Dombeya burgessiae</i>	WYIG-2180644
<i>Draba hispida</i>	GTSV-2005036
<i>Draba hispida</i>	GTSV-2008655
<i>Draba ossetica</i>	LJQF-2059299
<i>Drimys winteri</i>	WKSU-2129629
<i>Elaeocarpus sylvestris</i>	THHD-2009061
<i>Ephedra sinica</i>	VDAO-2033117
<i>Eschscholzia californica</i>	RKGT-2004462
<i>Euptelea pleiosperma</i>	QTJY-2059221
<i>Eutrema halophilum</i>	BY825221.1
<i>Eutrema salsugineum</i>	XM_006408097.1
<i>Fagopyrum tataricum</i>	JK729372.1
<i>Falcatifolium taxoides</i>	QHBI-2007953

<i>Falcatifolium taxoides</i>	ROWR-2009883
<i>Ficus pumila</i>	HQ224868.1
<i>Ficus religiosa</i>	EDHN-2049024
<i>Flaveria bidentis</i>	ZNZC-2010710
<i>Fokienia hodginsii</i>	UEVI-2033737
<i>Fokienia hodginsii</i>	UEVI-2008402
<i>Fokienia hodginsii</i>	UEVI-2056865
<i>Fontinalis antipyretica</i>	DHWX-2066666
<i>Galax urceolata</i>	ADHK-2058358
<i>Galax urceolata</i>	UFHF-2060702
<i>Galega orientalis</i>	AY952142.1
<i>Geranium carolinianum</i>	VKGP-2118929
<i>Geranium maculatum</i>	YGCX-2138168
<i>Geum quellyon</i>	DHAW-2015118
<i>Gleditsia sinensis</i>	VHZV-2087875
<i>Gleditsia triacanthos</i>	GEHT-2054485
<i>Glycine max</i>	FG990284.1
<i>Glycine max</i>	XM_003554661.3
<i>Glycyrrhiza glabra</i>	PEZP-2068102
<i>Glycyrrhiza uralensis</i>	FS265023.1
<i>Gompholobium polymorphum</i>	VLNB-2018423
<i>Gossypium hirsutum</i>	DW516477.1
<i>Gossypium raimondii</i>	XM_012637384.1
<i>Gyrocarpu samericanus</i>	BSVG-2014470
<i>Gyrostemon ramulosus</i>	UAXP-2077977
<i>Halocarpus bidwillii</i>	OWFC-2045614
<i>Halocarpus bidwillii</i>	OWFC-2009897
<i>Hamamelis virginiana</i>	DMKC-2000892
<i>Hedera helix</i>	EITK-2139802
<i>Heuchera sanguinea</i>	ERIA-2073359
<i>Hevea brasiliensis</i>	HS993022.1
<i>Hevea brasiliensis</i>	HS994451.1
<i>Hevea brasiliensis</i>	JZ538450.1
<i>Hibiscus cannabinus</i>	OLXF-2017798
<i>Hibiscus cannabinus</i>	OLXF-2090625
<i>Humulus lupulus</i>	AQGE-2045110
<i>Humulus lupulus</i>	ES655104.1
<i>Hypecoum procumbens</i>	NMGG-2058911
<i>Impatiens balsamifera</i>	JEXA-2062741
<i>Ipomoea nil</i>	NHAG-2048417
<i>Isoetes tegetiformans</i>	PKOX-2006241
<i>Itea virginica</i>	UWFU-2035071
<i>Juglans nigra</i>	DXQW-2058926
<i>Juniperus scopulorum</i>	XMGP-2055217
<i>Larrea tridentata</i>	UDUT-2047060
<i>Lathyrus sativus</i>	KNMB-2055925
<i>Laurelia sempervirens</i>	WAIL-2059125
<i>Lepidosperma gibsonii</i>	WBIB-2057434

<i>Limnanthes douglassii</i>	CRNC-2000081
<i>Limonium spectabile</i>	WOBD-2081469
<i>Linum hirsutum</i>	HNCF-2000289
<i>Linum leonii</i>	AEPI-2048092
<i>Linum lewisii</i>	BHYC-2052042
<i>Linum macraei</i>	KCPT-2102059
<i>Linum strictum</i>	TXMP-2007103
<i>Linum usitatissimum</i>	OGSY-2094087
<i>Liriodendron tulipifera</i>	CK764856.1
<i>Loropetalum chinense</i>	TQOO-2139966
<i>Lotus japonicus</i>	FS322371.1
<i>Lotus japonicus</i>	AV421457.1
<i>Lotus japonicus</i>	BI419767.1
<i>Lotus japonicus</i>	BM060586.1
<i>Lycium barbarum</i>	LWCK-2001931
<i>Lycium sp</i>	OSMU-2011251
<i>Lycopersicon cheesmanii</i>	UGJI-2125875
<i>Maesa lanceolata</i>	DTOA-2089413
<i>Magnolia grandiflora</i>	WBOD-2101820
<i>Malus domestica</i>	EB105992.1
<i>Manihot esculenta</i> subsp. <i>Peruviana</i>	JG999405.1
<i>Manoao colensoi</i>	CDFR-2008765
<i>Marchantia paleacea</i>	IHWO-2059313
<i>Marchantia polymorpha</i>	JPYU-2036258
<i>Medicago truncatula</i>	XM_013595074.1
<i>Meliosma cuneifolia</i>	AALA-2011529
<i>Metasequoia glyptostroboides</i>	NRXL-2000516
<i>Metasequoia glyptostroboides</i>	NRXL-2058286
<i>Metasequoia glyptostroboides</i>	NRXL-2001780
<i>Microbiota decussata</i>	XQSG-2037376
<i>Microstrobos fitzgeraldii</i>	BBDD-2007819
<i>Microstrobos fitzgeraldii</i>	BBDD-2070246
<i>Microstrobos fitzgeraldii</i>	BBDD-2004959
<i>Microstrobos fitzgeraldii</i>	BBDD-2071547
<i>Mitella pentandra</i>	DAYQ-2052183
<i>Monotropa uniflora</i>	LRTN-2089067
<i>Monotropa uniflora</i>	LRTN-2016473
<i>Morinda citrifolia</i>	ULGV-2007220
<i>Moringa oleifera</i>	CZPV-2001465
<i>Morus notabilis</i>	XM_010103989.1
<i>Morus nigra</i>	XVJB-2002447
<i>Mydocarpus sp</i>	AJFN-2095016
<i>Myriophyllum aquaticum</i>	IUSR-2074233
<i>Nageia nagi</i>	UUJS-2000623
<i>Neocallitropsis pancheri</i>	JDQB-2004699
<i>Neocallitropsis pancheri</i>	JDQB-2064704
<i>Nicotiana tabacum</i>	FS393955.1
<i>Nicotiana tabacum</i>	FS392106.1

<i>Nyssa ogeche</i>	VUSY-2130965
<i>Ochna mossambicensis</i>	TVCU-2008600
<i>Oncotheca balansae</i>	PVGM-2013088
<i>Oncotheca balansae</i>	PVGM-2142572
<i>Ophioglossum vulgatum</i>	QHVS-2011139
<i>Papaver setigerum</i>	FNXH-2023053
<i>Papuacedrus papuana</i>	OVIJ-2005726
<i>Papuacedrus papuana</i>	OVIJ-2001691
<i>Papuacedrus papuana</i>	OVIJ-2082654
<i>Parasitaxus usta</i>	JZVE-2002559
<i>Parasitaxus usta</i>	JZVE-2002557
<i>Parasitaxus usta</i>	JZVE-2003275
<i>Passiflora caerulea</i>	SIZE-2011788
<i>Peganum harmala</i>	OBTI-2041811
<i>Pereskia aculeata</i>	JLOV-2001160
<i>Peumus boldus</i>	KRJP-2083636
<i>Phaseolus vulgaris</i>	FE686122.1
<i>Phellodendron amurense</i>	PGKL-2070733
<i>Phellodendron amurense</i>	PGKL-2050474
<i>Philonotis fontana</i>	ORKS-2002446
<i>Philonotis fontana</i>	ORKS-2002448
<i>Phyllocladus hypophyllus</i>	JRNA-2063583
<i>Phyllocladus hypophyllus</i>	JRNA-2063388
<i>Physcomitrium sp</i>	YEPO-2070125
<i>Pilgerodendron uviferum</i>	ETCJ-2001405
<i>Pilgerodendron uviferum</i>	ETCJ-2008954
<i>Pinus taeda</i>	CF396457.1
<i>Pinus parviflora</i>	IIOL-2071721
<i>Pisum sativum</i>	GH719715.1
<i>Platycladus orientalis</i>	BUWV-2040465
<i>Platycladus orientalis</i>	BUWV-2044272
<i>Platycladus orientalis</i>	BUWV-2045303
<i>Plumbago zeylanica</i>	CB818080.1
<i>Podocarpus coriaceus</i>	SCEB-2044852
<i>Polypodium plectolens</i>	ZQYU-2018462
<i>Polyscias fruticosa</i>	EDBB-2084228
<i>Populus deltoids</i>	CK318298.1
<i>Populus euphratica</i>	XM_011040263.1
<i>Populus euphratica</i>	AJ778967.1
<i>Populus euphratica</i>	AJ776776.1
<i>Populus nigra</i>	DB889512.1
<i>Populus tremula</i>	DN496890.1
<i>Populus trichocarpa</i>	DN485080.1
<i>Populus trichocarpa</i>	XM_006375777.1
<i>Populus trichocarpa</i>	DT474845.1
<i>Populus trichocarpa</i>	BU874345.1
<i>Prumnopitys andina</i>	EGLZ-2020031
<i>Pseudotaxiphyllum elegans</i>	QKQO-2004260

<i>Pseudotaxus chienii</i>	YLPM-2033428
<i>Pseudotaxus chienii</i>	YLPM-2037863
<i>Pyrenacantha malvifolia</i>	QZZU-2062507
<i>Quercus petraea</i>	CU656360.1
<i>Quillaja saponaria</i>	OQHZ-2016253
<i>Raphanus raphanistrum</i> subsp. <i>Maritimus</i>	EY897555.1
<i>Reseda odorata</i>	SWPE-2000451
<i>Retrophyllum minus</i>	VGSX-2061426
<i>Rhamnus japonica</i>	EILE-2037280
<i>Rhodiola rosea</i>	ZJUL-2011613
<i>Rhodiola rosea</i>	JUL-2011100
<i>Ricinus communis</i>	PAZJ-2060672
<i>Robinia pseudoacacia</i>	JK974151.1
<i>Roridula gorgonias</i>	HUSX-2031570
<i>Ruellia brittoniana</i>	AYIY-2073961
<i>Salix dasyclados</i>	IEPQ-2024372
<i>Salix fargesii</i>	RZTJ-2087618
<i>Saxegothea conspicua</i>	QCGM-2071011
<i>Schizolaena</i> sp.	WMUK-2091805
<i>Schwetschkeopsis fabronia</i>	IGUH-2160246
<i>Sciadopitys verticillata</i>	YFZK-2040645
<i>Sciadopitys verticillata</i>	YFZK-2040888
<i>Selaginella apoda</i>	LGDQ-2003229
<i>Sequoiadendron giganteum-Glaucum</i>	QFAE-2000266
<i>Serenoa repens</i>	HXJE-2113249
<i>Sideroxylon reclinatum</i>	OXYP-2002914
<i>Simmondsia chinensis</i>	CVDF-2094468
<i>Solanum lycopersicum</i>	BW688395.1
<i>Solanum lycopersicum</i>	FS204664.1
<i>Solanum torvum</i>	FS109782.1
<i>Solanum tuberosum</i>	AJ878108.1
<i>Solanum tuberosum</i>	AM907316.1
<i>Solanum tuberosum</i>	CN216370.1
<i>Solanum dulcamara</i>	GHLP-2043688
<i>Solanum lasiophyllum</i>	DLAI-2102397
<i>Solanum sisymbriifolium</i>	NMDZ-2017071
<i>Solanum sisymbriifolium</i>	NMDZ-2017724
<i>Sphagnum lescurii</i>	GOWD-2075409
<i>Stangeria eriopus</i>	KAWQ-2005435
<i>Staphylea trifolia</i>	PTLU-2099986
<i>Strychnos spinosa</i>	GGJD-2019841
<i>Sundacarpus amarus</i>	KLGF-2006233
<i>Synsepalum dulcificum</i>	WRPP-2126204
<i>Taiwania cryptomerioides</i>	QSNJ-2000100
<i>Talinum</i> sp	LKKX-2068262
<i>Tamarix hispida</i>	EH051455.1
<i>Tapiscia sinensis</i>	WWKL-2010000
<i>Tarenaya hassleriana</i>	XM_010526614.1

<i>Taxodium distichum</i>	FHST-2007221
<i>Taxodium distichum</i>	FHST-2004844
<i>Taxodium distichum</i>	FHST-2004843
<i>Taxodium distichum</i>	FHST-2003453
<i>Taxodium distichum</i>	FHST-2061111
<i>Taxus baccata</i>	WWSS-2000781
<i>Taxus baccata</i>	WWSS-2000780
<i>Tellima breviflora</i>	CTSS-2001567
<i>Tellima breviflora</i>	CTSS-2043698
<i>Thalictrum thalictroides</i>	GBVZ-2064238
<i>Thellungiella halophila</i>	AK352622.1
<i>Theobroma cacao</i>	XM_007030502.1
<i>Theobroma cacao</i>	CU471310.1
<i>Theobroma cacao</i>	CU495512.1
<i>Thuidium delicatulum</i>	EEMJ-2032575
<i>Tinospora cordifolia</i>	JG647038.1
<i>Torreya nucifera</i>	HQOM-2008514
<i>Torreya nucifera</i>	HQOM-2011119
<i>Torreya nucifera</i>	HQOM-2011118
<i>Torreya taxifolia</i>	EFMS-2012045
<i>Torreya taxifolia</i>	EFMS-2012045
<i>Trianthema portulacastrum</i>	TNVE-2099453
<i>Triticum aestivum</i>	BG604435.1
<i>Triticum turgidum</i>	BF293407.1
<i>Triticum turgidum subsp. Durum</i>	AJ716574.1
<i>Trochodendron aralioides</i>	SWOH-2054532
<i>Trochodendron aralioides</i>	SWOH-2054532
<i>Tropaeolum majus</i>	GH162958.1
<i>Tropaeolum majus</i>	GH164293.1
<i>Tropaeolum peregrinum</i>	MYZV-2053677
<i>Urginea maritima</i>	KOFB-2059461
<i>Vaccaria hispanica</i>	JZ158858.1
<i>Vigna unguiculata</i>	FG848837.1
<i>Viola canadensis</i>	NJLF-2011087
<i>Vitex agnus castus</i>	DMLT-2086965
<i>Vitis vinifera</i>	FQ438078.1
<i>Widdringtonia cedarbergensis</i>	AUDE-2007932
<i>Widdringtonia cedarbergensis</i>	AUDE-2002285
<i>Xanthocercis zambesiaca</i>	ZSSR-2105305
<i>Ziziphus jujuba</i>	ZHEE-2005858

10C-Hevein-like Peptides

<i>Citrus aurantium</i>	DN622484.1
<i>Citrus reticulata</i>	EY794233.1
<i>Citrus sinensis</i>	CF835995.1
<i>Cuscuta pentagonia</i>	AHRN-2012568
<i>Elymus spicatus</i>	FF360240.1
<i>Gnetum montanum</i>	GTHK-2007511

<i>Lolium perenne</i>	GR513336.1
<i>Oryza sativa Japonica Group</i>	CI631870.1
<i>Oryza sativa Japonica Group</i>	CI756576.1
<i>Panicum virgatum</i>	FL945970.1
<i>Plagiochila asplenoides</i>	NWQC-21549421
<i>Poncirus trifoliata</i>	EY833419.1
<i>Solanum dulcamara</i>	GHLP-2050216
<i>Triticum aestivum</i>	BQ805737.1
<i>Triticum aestivum</i>	GH724149.1
<i>Triticum aestivum</i>	JZ886728.1
<i>Triticum turgidum subsp. Durum</i>	AJ612726.1