

## Review Article

# Plant derived cyclic peptides

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Cyclic peptides are widespread throughout the plant kingdom, and display diverse sequences, structures and bioactivities. The potential applications attributed to these peptides and their unusual biosynthesis has captivated the attention of researchers for many years. Several gene sequences for plant cyclic peptides have been discovered over the last two decades but it is only recently that we are beginning to understand the intricacies associated with their biosynthesis. Recent studies have focussed on three main classes of plant derived cyclic peptides, namely orbitides, SFTI related peptides and cyclotides. In this mini-review, we discuss the expansion of the known sequence and structural diversity in these families, insights into the enzymes involved in the biosynthesis, the exciting applications which includes a cyclotide currently in clinical trials for the treatment of multiple sclerosis, and new production methods that are being developed to realise the potential of plant cyclic peptides as pharmaceutical or agricultural agents.

## Introduction

Cyclic peptides have received significant attention in the literature having been found in all domains of life [1]. In addition to deciphering how these unusual peptides are produced *in vivo*, developing approaches for producing them *in vitro*, and determination of their structures, bioactivities and potential applications have been key areas of interest. Here, we refer to cyclic peptides as those with a head-to-tail cyclic backbone, rather than cyclized through disulfide bonds or side chain linkages. A disproportionately large number of head-to-tail backbone cyclic peptides have been characterised from plants relative to other organisms. These peptides have been identified in a range of plant families as previously outlined in a comprehensive review published in 2006 by Tan and Zhou [2]. As to why higher numbers of plant derived cyclic peptides have been characterised remains to be seen, but in the meantime, a wealth of information is being gathered on this intriguing and potentially valuable class of molecules.

Recent studies in the field of plant derived cyclic peptides have focussed heavily on three classes, namely orbitides, cyclotides and SFTI-1 related peptides. Orbitides are plant cyclic peptides that do not contain any disulfide bonds or non-natural amino acids. The first orbitide, evolidine, was isolated in the 1950s from the rainforest tree *Melicope xanthoxyloides* and subsequent studies have confirmed its sequence, as summarised by Fisher et al. [3]. These peptides, which contain between 5 and 16 residues, were originally referred to as Caryophyllaceae-type cyclic peptides, but have since been termed orbitides [4–6]. In the 1970s evidence of a new class of plant derived cyclic peptides was emerging based on analysis of an indigenous medicine used in Africa [7]. However, it was more than 20 years later that the sequence and structure of the cyclic peptide kalata B1, originally isolated from *Oldenlandia affinis*, was characterised [8]. Subsequent studies made it clear that kalata B1 was a member of a large family of related peptides and the term cyclotide was coined in 1999 [9]. Similar to orbitides, cyclotides generally do not contain non-native amino acids, but they contain approximately 30 residues including six cysteine residues, which form a cystine knot motif. At the same time as cyclotides were being classified, the first example of another class of plant cyclic peptides was reported

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from sunflower seeds [10]. This peptide was termed sunflower trypsin inhibitor-1 (SFTI-1); it inhibits trypsin in the sub-nanomolar range, is similar to the orbitides in size, but contains a single disulfide bond and subsequent studies have found several related peptides [11,12].

There have been several reviews relating to plant derived cyclic peptides, but studies in the last few years have been particularly exciting and have provided key advances relating to the sequence diversity of plant derived cyclic peptides, their biosynthesis and potential applications. This mini-review provides and overview of these recent findings relating to both fundamental and applied aspects of plant derived cyclic peptides.

## Discovery and characterisation

The early discoveries of plant cyclic peptide sequences were based primarily on tedious chemical/proteomic analyses, but not surprisingly transcriptomic/genomic approaches now facilitate their discovery. A combination of transcriptomic and proteomic approaches has been particularly useful in recent studies on orbitides, cyclotides and SFTI-1-like peptides as outlined below.

### Orbitides

The number of orbitide sequences characterised has significantly increased in recent years. A range of species from the Asteraceae family have been analysed and 46 orbitides identified [6,13]. These peptides are referred to as PLP-1 to PLP-46, with PLP referring to PawL-derived Peptides [12]. It has been estimated that Asteraceae orbitides could number in the thousands based on the abundance within some species and the hypervariability of the sequences [6]. The structures of four of the PLP peptides have been determined using NMR spectroscopy and reveal relatively well-defined structures for such small peptides (6–8 residues in length). PLP-10 contains a single proline residue and the NMR data allowed determination of both the *cis* and *trans* isomers. Although there are examples of orbitides with antimicrobial activity, the bioactivity of the PLP peptides is unclear, as several were tested in antibacterial and antifungal assays but did not display activity.

Orbitides from other plant families have also been found recently, including a cyclic peptide from *Pseudostellaria heterophylla* [14] and a novel orbitide derived from a genetic deletion was detected from flaxseed [15]. Furthermore, a study that revisited the discovery of evoludine from the Rutaceae species *M. xanthoxyloides* also resulted in the discovery of six novel orbitides (xanthoxycyclin A–F) encoded by similar transcripts [3]. The structures of synthetic versions of two of these orbitides were studied using NMR spectroscopy, with the 8-residue peptide xanthoxycyclin D showing a well-defined structure in contrast with the 9-residue peptide xanthoxycyclin F, which appeared to have conformational heterogeneity [3]. The sequences of these peptides differ significantly with the latter containing two proline residues, which might be responsible for the structural distinctions. A major finding from this study was that the new orbitides had diverse C-terminal residues, in contrast with the PLP orbitides, which has significant implications for the biosynthesis of these sub-families of cyclic peptides.

### Cyclotides

Cyclotides represent one of the largest families of cysteine-containing cyclic peptides, and they have been discovered in a range of plant families, including Rubiaceae, Violaceae, Fabaceae, Solanaceae and Cucurbitaceae [16]. Not all species within these families have been found to contain cyclotides, but the Violaceae family appears to be unique in that all species studied so far have contained cyclotides. However, two recent studies on Violaceae species have highlighted some striking differences. Analysis of the medicinal herb *Hybanthus enneaspermus* using RNAseq data revealed the presence of 93 putative cyclotide sequences, and 16 acyclic cyclotides (i.e. cyclotide related peptides that do not contain a cyclic backbone, which have been referred to as acyclotides) [17]. In contrast, analysis of another Violaceae plant species, *Rinorea bengalensis*, showed a large number of acyclotides and only one cyclotide sequence [18]. Despite the lack of the cyclic backbone, several of these peptides have been shown to have cytotoxic activities similar to their cyclic counterparts [18]. New cyclotide sequences have also recently been found in *Palicourea sessilis*, that have potential as immunosuppressants [19].

### SFTI-like peptides

Following on from the original study which identified SFTI-1 [10], it has become clear that SFTI-1 is the prototypic member of a family of related peptides found in the Asteraceae family [20]. These peptides have been termed PDPs (PawS-derived peptides), based on the name of the precursor protein for SFTI-1 [21]. The sequences and structures of this family of peptides has been reviewed in Franke et al. [20]. The discovery of PDPs is primarily based on similarity with the gene sequence encoding SFTI-1, but significant sequence and

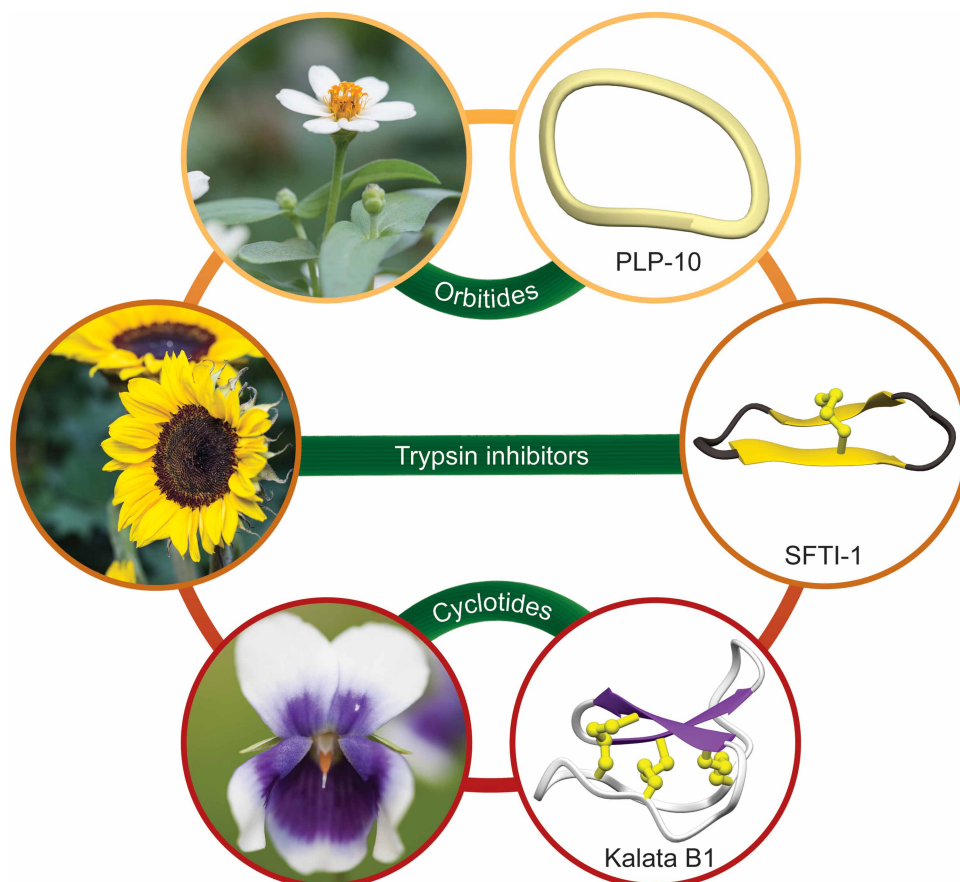
structural diversity has been found. Interestingly, not all PDPs are cyclic. An Asp-Gly cyclisation motif is present in all cyclic PDPs stemming from an N-terminal glycine and C-terminal aspartic acid in the precursor protein. When the C-terminal is an Asn rather than an Asp the resulting mature peptides are acyclic [11,12,22]. The original discovery identified SFTI-1 as a potent trypsin inhibitor, but it has not been as straightforward to elucidate the bioactivity of the related peptides, even for peptides such as PDP-20, which has significant similarity to SFTI-1 but does not inhibit trypsin [11].

To highlight the diversity of the structures and plants from which the cyclic peptides are derived, examples from each of the three main families discussed here are given in Figure 1. The single disulfide bond of SFTI-1 braces the  $\beta$ -strands, whereas the three disulfide bonds in the prototypic cyclotide, kalata B1, form a cystine knot motif whereby two of the bonds with the connecting backbones form a ring through which the third bond threads.

## Biosynthesis

There is still a lot to learn about the biosynthesis of plant derived cyclic peptides, but significant advances have been made within the last decade and remarkable similarities and links have been observed amongst the different classes. The genes for the precursor proteins for several plant cyclic peptides have been characterised and we are beginning to understand how the mature peptides are released.

The first precursor genes of orbitides were cloned from *Vaccaria hispanica* (*Saponaria vaccaria*) [23] for the subfamily referred to as segetalins, and a subsequent study revealed two enzymes involved in their biosynthesis. These enzymes were termed oligopeptidase 1, which catalyses the cleavage of intermediates at the N-terminus,



**Figure 1. Representative structures of plant cyclic peptides from the orbitide, PLP and cyclotide families.**

SFTI-1 (PDB code: 1JBL) from the sunflower *Helianthus annus*, PLP-10 (PDB code: 6AZF) trans-isomer from *Zinnia sp.*, Kalata B1 (PDB code: 1NB1) from *Viola hederacea*.

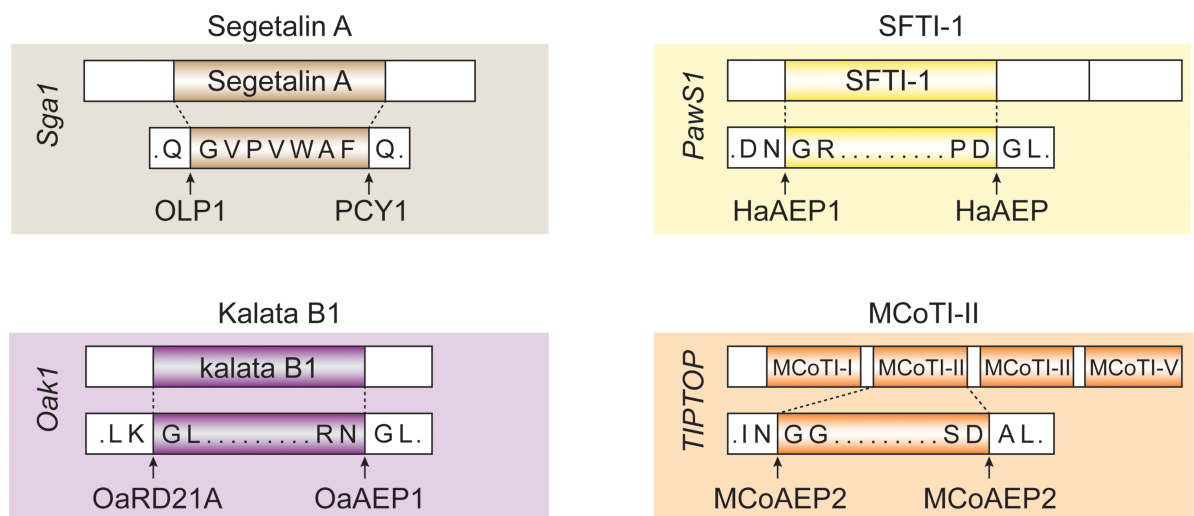
and peptide cyclase 1 (PCY1), a serine protease which is involved in the subsequent step of backbone cyclisation and removal of the C-terminal flanking sequence [24]. However, the mechanisms/processes involved in cyclisation do not appear to be conserved, at least not entirely, as studies on other plant cyclic peptides have identified different classes of enzymes involved in the cyclisation process.

Cyclisation of SFTI-like peptides (PDPs), orbitides from the Asteraceae family (PLPs) and cyclotides all appear to involve asparaginyl endopeptidases (AEP) proteins, despite different precursor protein structures. AEPs recognise a conserved Asp/Asn at the C-terminal processing site, and can facilitate cyclisation via transpeptidation [25–27]. The AEPs characterised from cyclic peptide producing plants include butelase-1, OaAEP1b, VyPALs and HaAEP1 [28–31]. These enzymes are involved in the C-terminal cleavage event, but there is variation in the N-terminal processing site. For kalata-like peptides, a papain-like cysteine protease, termed kalataase, has been shown to be involved in the N-terminal processing [32]. In contrast, the AEP (MCoAEP2) involved in the C-terminal cleavage of the subfamily of cyclotides that inhibit trypsin is involved in both the N-terminal and C-terminal processing [33]. SFTI-1 and related PDP peptides are characteristically buried within a precursor protein that also encodes a napin-type seed storage albumin [20,21]. Despite this unusual ‘hijacking’ of the albumin gene to encode PDPs, a conserved Asn/Asp at the C-terminus of the mature cyclic peptide and prior to the N-terminal residue make it likely that AEPs are also involved in the maturation of PDPs. A summary of the known enzymes involved in processing plant derived cyclic peptides is given in Figure 2.

The three-dimensional structure of the SFTI-1 precursor has been determined using NMR spectroscopy and revealed that the SFTI-1 and the albumin domains are both well folded and separated by a flexible linker [34]. Interestingly, when the recombinant precursor was incubated with a recombinant sunflower AEP (HaAEP), the formation of the cyclic peptide was inefficient [34]. This observation coupled with the range of C-terminal residues found in the orbitides from *M. xanthoxyloides*, which are not consistent with the known plant cyclizing enzymes, suggests that there are cyclizing enzymes yet to be discovered [3].

## Potential applications and production methods

One of the drivers behind many of the studies related to plant cyclic peptides has been exploring the potential of these peptides in agricultural or pharmaceutical applications [35–38]. Orbitides have been shown to have a range of bioactivities including anti-cancer and immunomodulating properties [39], and similarly, cyclotides also have a range of bioactivities including insecticidal and antimicrobial activity [40]. At this stage, perhaps the most exciting is a cyclotide analogue that results in diminished symptoms in an experimental autoimmune



**Figure 2. Overview of the known enzymes involved in plant cyclic peptide processing.**

Schematic presentation of the precursor proteins from the different classes of plant derived cyclic peptides. OLP1 and PCY1 have been identified in orbitide processing, HaAEP for SFTI processing, kalataase and OaAEP for cyclotide processing, MCoAEP2 for trypsin inhibitor cyclotide processing [24,32–34]. The precursor proteins are not drawn to scale; the orbitides and SFTI-1 are much smaller than the cyclotides.

encephalomyelitis multiple sclerosis mouse model following oral administration. There was no report of adverse effects [41] and this analogue is currently in clinical trials [42]. Furthermore, a cyclotide extract from *Clitoria ternatea* is approved as an insecticide [43].

There have also been numerous studies on using the cyclic peptides as scaffolds for the design of pharmaceutical lead molecules as reviewed previously [16,44]. These studies have mainly utilised cyclotides and SFTI-1 as the scaffolds, with small bioactive sequences grafted into one of the inter-cysteine loops of the cyclic peptide. Such studies have generally relied on synthetic methods such as native chemical ligation to produce sufficient quantities for structural and functional studies. However, a recent study involved grafting the cystatin first hairpin sequence into the trypsin inhibitor cyclotide MCoTI-II [45] and utilised recombinant expression of the grafted peptide. Although the grafted peptide was able to bind to papain, a property thought to have significant potential in translational medicine, the grafted peptide did not contain the cyclic backbone, highlighting one of the major limitations of this approach for cyclic peptide production.

For cyclic peptides to be useful as pharmaceutical or agricultural agents it is imperative that they can be produced in sufficient quantities using economical processes. The insecticide from *C. ternatea* is directly obtained from a plant extract and, although this is proving effective in this instance, it has been suggested that limitations of plant extraction, including the developmental stage of a plant, and seasonal fluctuations, could have implications for the viability of this approach [46]. One approach that is being explored for the continuous and uniform production of cyclotides is the use of *in vitro* cultures. A somatic embryo culture of *Viola odorata* has been developed whereby the relative abundance of cyclotides was higher in the somatic embryo extract compared with the natural plant extract [46]. The use of a yeast-based production method has also been used in conjunction with *in vitro* cyclisation using recombinant AEPs [47]. This approach was used to produce a range of cyclic peptides, including an SFTI analogue and cyclotide. The yields were significantly improved over other methods, boding well for future studies in this area.

Given how widespread cyclic peptides are throughout nature, and the complexities associated with the formation of the cyclic backbone, it appears likely that there are significant advantages to compensate for the extra effort involved in producing them. Based on this thought process the efforts going into establishing efficient methods for the production of cyclic peptides are worthwhile. However, the counter argument is that in some cases acyclotides are no less stable than cyclotides because of the stability conferred by the cystine knot motif, and therefore might have less complicated production processes without the cyclic backbone [48]. It appears likely that the formation of three disulfide bonds alone is likely to add complexities to large scale production methods and therefore the differences in the production of cyclotides relative to acyclotides might end up being minimal.

## Conclusions

The peptides mentioned in this mini-review by no means represent the full breadth of cyclic peptides identified in plants, but rather highlight the major classes that have been studied recently. There is exciting potential in the field with a plant cyclic peptide currently in clinical trials and methods being developed for large scale production. The academic curiosity related to the discovery of novel cyclic peptides and their biosynthetic mechanisms it not likely to abate soon, but likely to increase. This curiosity is likely to result in significantly more diverse cyclic peptides being discovered and the potential applications expanding. The cyclotides represent one of the largest families of plant cyclic peptides, not only in terms of number of peptides characterised but also molecular mass. The smallest backbone cyclic peptides have already been discovered in plants, including the recent discovery of the dipeptide cyclo(Arg-Trp) from the Chilean hazelnut cotyledons [49], but it remains to be seen whether the cyclotides represent the largest plant cyclic peptides or whether higher molecular mass peptides/proteins await discovery.

### Perspective

- Backbone cyclisation is a key post-translational modification found predominately in plants.
- Understanding the biosynthesis of plant cyclic peptides is likely to facilitate their production and potential applications.
- Plant cyclic peptides hold promise for the development of new pharmaceutical and agricultural agents.



## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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N.L.D. and D.W. wrote the manuscript.

## Abbreviations

AEP, asparaginyl endopeptidases; PCY1, peptide cyclase 1; PDPs, PawS-derived peptides.

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