Insights into processing and cyclization events associated with biosynthesis of the cyclic peptide kalata B1

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\textbf{Background:} Production of insecticidal and nematocidal cyclic peptides is inefficient in transgenic plants.
\textbf{Results:} Efficient cyclization requires cleavage of the N-terminal propeptide from the mature cyclotide domain and a C-terminus containing a binding motif.
\textbf{Conclusion:} The cyclization motif has Asn at position P1, a small amino acid at position P1’ and a Leu at position P2’.
\textbf{Significance:} Understanding substrate requirements will help produce cyclotides in transgenic plants.

\textbf{SUMMARY}
Plant cyclotides are the largest family of gene-encoded cyclic proteins. They act as host defense molecules to protect plants and are promising candidates as insecticidal and nematocidal agents in agriculture. In order for this promise to be realized a greater understanding of the post-translational processing of these proteins is needed. Cyclotides are cleaved from precursor proteins with subsequent ligation of the N- and C-termini to form a continuous peptide backbone. This cyclization step is inefficient in transgenic plants and our work aims to shed light on the specificity requirements at the excision sites for cyclic peptide production.

Using the prototypic cyclotide kalata B1 (kB1) expressed from the Oak1 gene, MALDI-TOF mass spectrometry was used to examine the cyclization efficiency when mutants of the Oak1 gene were expressed in transgenic \textit{Nicotiana benthamiana}. Cleavage at the N-terminus of the cyclotide domain occurs rapidly with no strict specificity requirements for amino acids at the cleavage site. In contrast, the C-terminal region of the cyclotide domain in the P2, P1, P1’ and P2’
positions is highly conserved and only specific amino acids can occupy these positions. The cyclization reaction requires an Asn at position P1 followed by a small amino acid (Ala, Gly, Ser) at the P1’ position. The P2’ position must be filled by Leu or Ile; in their absence an unusual post-translational modification occurs. Substitution of the P2’ Leu with Ala leads to hydroxylation of the neighbouring proline. Through mutational analysis this novel proline hydroxylation motif was determined to be Gly-Ala-Pro-Ser.

**INTRODUCTION**

Cyclotides are a large family of head-to-tail cyclized proteins from plants (1) that function in host defense against insect predation (2,3), and have exciting potential in agriculture as insecticidal or nematocidal agents and also in drug design applications (4,5). In order for this promise to be realised a greater understanding of the post-translational processing of these proteins is needed. The mechanism of excision and cyclization of cyclotides from their linear precursors involves a fascinating post-translational processing phenomenon that until now has been only partially understood. The joining of the N- and C-termini occurs via a transpeptidation reaction to create a continuous peptide backbone. This circular structure bestows cyclotides with exceptional structural and proteolytic stability (4,6).

Cyclotides, which are the largest group of circular peptides contain between 28 and 37 amino acids, and have a well-defined three-dimensional structure containing exclusively naturally occurring amino acids (7-9). Cyclotides are characterised by the combination of a cyclic peptide backbone with an embedded cystine knot, collectively called the cyclic cystine knot (10). Cyclotides appear to be cyclized by a protease mediated biosynthetic pathway, most likely as the result of an asparaginyl endopeptidase (AEP) that catalyses a cleavage reaction as well as the transpeptidation reaction (3,11,12). Cyclotides are expressed as larger precursor proteins containing an endoplasmic reticulum (ER) targeting signal, an N-terminal pro-peptide (Ntpp), an N-terminal repeat (Ntr) region of 16-20 amino acids, and a cyclotide domain. The N-terminal regions both target the cyclotide precursor to the vacuole where the protein is post-translationally processed and cyclised (13). The Ntr and cyclotide domains can be repeated up to three times in some cyclotide precursors and the repeated domains contain a short hydrophobic C-terminal region (Ctr) of 3 to 7 amino acids (Figure 1A). The prototypic cyclotide kalata B1 (kB1) expressed from the Oak1 gene of *Oldenlandia affinis* contains a single cyclotide domain (Figure 1B).

Genes encoding cyclotide precursors from *Oldenlandia affinis*, the most studied cyclotide-bearing plant (6), encode proteins that have a conserved motif that flanks the upstream and downstream processing sites of each cyclotide domain (14). A recent analysis of a large number of cyclotide precursor sequences showed that similar motifs are present in other plant species (15). The conserved motifs that flank the cyclotide domains of the precursor proteins have been proposed to have a crucial role in the cyclization reaction (Figure 1C) (14,16).

There are two highly conserved residues near the C-terminus of every cyclotide domain. The first is an asparagine or aspartate residue in the P1 position at the C-terminal processing site of the cyclotide domain, which led to the
A proposal that an AEP could be involved in both the cleavage and cyclization of cyclotides (3,11,16,17). Discovery that cyclotides are targeted to the vacuole, which contains AEP’s, where processing and cyclization occurs (13). These enzymes are common in plants and have a role in processing seed storage proteins, where they cleave peptide bonds adjacent to Asn residues. The second highly conserved residue is a leucine located two amino acids down-stream of the Asn in the P2’ position. It has been proposed, but not experimentally verified, that this residue is essential for AEP binding and cyclization (16,18).

Studies of transgenic plants into which modified cyclotide precursor genes are inserted provide a powerful way of probing the role of individual residues in cyclotide processing and cyclization. We recently reported the observation of processing intermediates during the maturation of the O. affinis kalata B1 precursor protein (Oak1) in transgenic Nicotiana benthamiana (16). Substitution of residues surrounding the cleavage sites to produce these intermediates should enable a clearer picture of how cyclotides are cleaved from their precursor proteins and cyclized. The Ntr appears to be cleaved before the cyclotide precursor is cyclized, which suggests the Ctr should be critical in the enzymatic cyclization of the protein. Mutation of this Ctr region should then reveal which residues are essential for cyclization.

Here we focus on the residues at the N- and C-terminus of the cyclotide domain with particular emphasis on the P2, P1, P1’ and P2’ residues at the C-terminus of the cyclotide domain to help delineate the residues that are essential for cyclization. Our examination of the in planta protein products resulting from systematic mutation of key residues within the processing site has enabled a detailed understanding of the recognition site required for binding to the enzyme that catalyses cyclization. During the process of mutating the Ctr of Oak1 a novel proline hydroxylation sequence was discovered that contains the tetrapeptide motif GAPS within the Ctr.

**EXPERIMENTAL PROCEDURES**

**Production of mutant Oak1 constructs**

The Oak1 mutants were generated from the Oak1 gene (Supp Fig 1) using the Phusion site-directed mutagenesis kit (Finnzymes). All constructs were subcloned into pAM9 to incorporate the 35S cauliflower mosaic virus promoter and terminator sequences (19). The genes were then cloned into the binary vector pBIN19. The pBIN19 expression vectors were transformed into Agrobacterium tumefaciens strain 4404. The Agrobacterium was grown on yeast mannitol (YM) plates containing 50 µg/ml kanamycin and 100 µg/ml streptomycin.

**Transient expression**

Agrobacterium cells were taken from YM plates and suspended in a transformation solution containing 10 µM acetylsheringone, 10 mM MgCl₂ at an OD₆ₒ₀ of 1.0 and incubated at room temperature for 2 hours. The cells were then infiltrated into the abaxial side of the leaves of Nicotiana benthamiana plants by forcing the liquid into the tissue using a syringe (16). The full list of constructs expressed in planta is presented in Supp Fig 2.

**Peptide analysis of leaf tissue**

Samples were prepared by grinding N. benthamiana leaves to a powder in liquid N₂ before the addition of 50% (v/v) acetonitrile containing 0.1% (v/v) TFA.
Samples were centrifuged and the supernatant was de-salted and concentrated using C18 ziptips (Millipore). Samples were mixed 1:1 with a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA), spotted onto a sample plate and air dried. All samples were produced with a minimum of three biological replicates. Mass analysis was performed in positive ion reflector mode on either a 4700 Proteomics Analyzer (AB/Sciex, Foster City, USA) or a Bruker ultraflex III MALDI TOF/TOF mass spectrometer (Bruker AXS GmbH, Karlsruhe, Germany). Two hundred spectra at each of 10 randomly selected positions were accumulated per spot between 1000 and 5000 Da using an MS positive ion reflectron mode acquisition method. Calibration was conducted using a mixture of peptide standards (Bruker Daltonics). Data was acquired and processed with either the accompanying 4000 series Explorer software or Bruker flexAnalysis software.

Relative quantification of transiently expressed peptides

Spectra were collected and the integrated peak area corresponding to known Oak1 peptides were taken as 100% of the expressed peptides. This enables the amount of cyclic protein to be assessed within the sample as a percentage of the total amount of cyclotide peptides. All mutants were expressed in triplicate within different leaves to give a minimum of three biological replicates. The standard error of the mean was determined for each peptide mass. This relative quantification method using MALDI-MS was validated against LC-ESI-MS using an independent t-test to assess equality of means (IBM SPSS Statistics v19.0) to determine that the datasets produced by both techniques were not significantly different (Supp fig 3). LC-MS could not be used for this study as it has limitations in sensitivity making it difficult to detect cyclic peptides.

The final relative levels of cyclic peptide produced by each construct were compared to the wt-Oak1 construct and a paired t-test was used to determine if the levels of cyclic peptide were statistically different (represented as p values).

Purification and analysis of kB1-GAOS

The kB1-GAOS peptide was purified from plant extracts using HPLC. Proteins were separated on a C8 column in 0.1% TFA and eluted with 0.089% TFA, 80% acetonitrile. Peaks containing the 3236 Dalton mass were determined using MALDI MS and pooled. Amino acid analysis of the 3236 Dalton HPLC peak was performed by the Australian proteome analysis facility (Sydney Australia) to confirm the presence of hydroxyproline.

Inhibitor infiltrations

The Oak1, and Oak1 L31A constructs were expressed transiently in N. benthamiana with and without the prolylhydroxylase inhibitor 2,4-pyridinedicarboxylic acid monohydrate (PDCA). A 2mM solution of the inhibitor was infiltrated into N. benthamiana leaves 12 h after Agrobacterium infiltration and leaves were harvested at 3.5 dpi.

RESULTS

Expression of the Oak1 precursor in transgenic plants produces both cyclic and linear forms of the kalata B1 protein due to processing inefficiencies (16). In this study we have expressed the Oak1 gene and mutants of this gene in N. benthamiana to determine which amino acids within the processing sites of the Oak1 precursor protein are necessary for
cyclization to occur. When Oak1 is expressed transiently the largest Oak1 precursor protein intermediate observed via mass spectrometry (MS) was that of the cyclotide domain with the Ctr still attached (Figure 2A). In order to determine if substitution of amino acids within the Oak1 precursor had an effect on the amount of cyclic protein produced a novel method of quantitation was utilised. This method involved taking all MALDI-TOF MS peaks corresponding to the Oak1 precursor, i.e. peptides consisting of the kB1 cyclotide domain with varying lengths of Ctr attached, as 100% of the total Oak1 precursor protein expressed. Using this method it was possible to quantitate the amount of cyclic kB1 produced relative to the linear forms in any one sample (Figure 2A right panel). When Oak1 was expressed transiently in N. benthamiana leaves a total of 5.1% (±1.5%) of all peptides expressed were processed to the cyclic form. All of the other expressed peptides were present as linear forms of kalata B1 with varying lengths of Ctr. All peptides produced and their masses are documented in Table 1. The relative amount of each of the peptides produced during expression of the Oak1 mutants is given in Table 2. Analysis of the statistical significance of the levels of cyclic protein as compared to the wt-Oak1 are given Supp Fig 4.

Requirements for cleavage at the N-terminus of the cyclotide domain (P1 and P2)

Post-translational processing of the precursor begins with the excision of the region upstream of the cyclotide domain comprising the Ntpp and Ntr (16). We predict that the Ntr (and regions upstream of it) must be removed from the cyclotide domain before cyclization can occur. The N-terminus of the cyclotide domain is liberated after cleavage next to an upstream residue which for Oak1 is a Lys residue (K-1). Substitution of Lys-1 with an Ala (K-1A) did not produce significant changes in the levels of cyclic kB1, 6.3% (±0.3%) when expressed transiently in N. benthamiana. Substitution of the residue further upstream at position P2 (Leu-2) effected cleavage of the Ntr domain, with normal levels of cyclic protein being produced (5.3% (±1.6)), but a number of peptide products were observed with additional residues at the N-terminus (Table 2). This suggests that the Leu residue is necessary for clean cleavage of the Ntr from the cyclotide domain.
Table 1: Summary of the mutants of Oak1 which were tested for cyclization in N. benthamiana. A tick indicates cyclic peptide was produced. The mass to charge ratio (m/z) is given for each non-cyclic product as well as the corresponding cyclic peptide. –G corresponds to the loss of Gly from the N-terminus of the cyclotide domain and the + indicates amino acids present at the C-terminus of the cyclotide domain.

| Oak1 | Construct | Observed m/z | Assignment of masses |
|------|-----------|--------------|----------------------|
| Oak1-GB | Cyclic kB1 | 2891.1 | 2909.2 | 2966.2 +G | 3079.3 +GL | 3176.4 +GLP |
| L2A | 2852.1 -G | 2891.2 | 2909.2 | 2966.3 +G | 3079.4 +GL | 3176.5 +GLP | 3206 +G +GLPS |
| K2A | 2892.3 -G | 2891.4 | 2909.3 | 2966.3 +G | 3079.5 +GL | 3176.5 +GLP | 3263 +GLPS |
| R2P3A | 2767.2 -G | 2806.3 | 2824.4 |
| R2K | 2863.3 | 2881.3 | 2938.3 +G |
| N2Q | | 2923.3 | 2980.3 +G | 3190.5 +GLP |
| G2A | 2891.5 | 2909.5 | 2966.5 +G | 3079.6 +GL |
| G2F | | 2988.8 -G +F | 3055.8 +F |
| GLP* | 2892.4 -G | 2890.6 | 2898.6 | 2965.6 +G | 3078.6 +GL |
| G* | | 2909.3 | 2966.4 +G |
| GP* | | 2909.5 | 2966.4 +G | 3063.4 +GP |
| GLA* | 2891.2 | 2890.3 | 2965.6 +G | 3078.6 +GL |
| SLP* | | 2891.2 | 2909.3 | 2966.4 +S |
| LGP* | | 2909.0 | 3022.3 +L | 3079.2 +LG |
| GGL* | | 2909.1 | 2966.2 +G | 3032.2 +G | 3136.3 +GGL |
| ALA* | 2891.5 | 2909.5 | 2923.5 -G +A | 2980.6 +A | 3039.6 +AL |
| L3A | | 2909.0 | 2966.5 +G | 3079.6 +GL |
| L3A-L34A | | 2909.2 | 2966.2 | 3150.4 +GA |
| GAPS* | | 3037.5 +GA | 3237.6 +GAOS |
| GAP* | | 3221.4 +GAOS |
| L3I | 2852.1 -G | 2891.1 | 2909.1 | 2966.2 +G | 3079.4 +GI | 3176.4 +GIPS |
| L3A-ΔP32 | 2852.2 -G | 2891.2 | 2909.3 | 2966.4 +G | 3079.5 +GI | 3176.7 +GIPS |
| L3I-A-P32A | | 2909.0 | 2965.9 +G | 3037.0 +GA |
| L3A-P32A | | 2908.9 | 2965.9 +G | 3036.9 +GA |
| L3A-P32N | | 2909.1 | 2966.3 +G | 3037.2 +GA |

* Indicates constructs with a truncated Ctr. These constructs are labelled according to the amino acid sequence of the Ctr.
Cyclotide domain C-terminal P1 and P2 requirements

The last residue of the cyclotide domain before the Ctr is Asn$_{29}$, this residue is in the P1 position and is necessary for cleavage and cyclization to occur. Substitution of this Asn with an Ala resulted in no cyclic protein being made and mutation to Asp produced minimal cyclic peptide (16). Furthermore, substitution of this residue with a glutamine (N$_{29}$Q), also failed to produce any cyclic peptide. The P2 residue immediately preceding Asn$_{29}$ is a highly conserved residue and a search of the cyclotide database (Cybase) revealed that greater than 50% of documented cyclotides contain an Arg at this position (15). When Arg$_{28}$ was replaced with Ala (R$_{28}$A) or Lys (R$_{28}$K), there was a significant decrease in the amount of detectable protein as determined using MALDI MS. Cyclic protein was still produced, but the level of expression for both of these was significantly lower than observed for the wild type Oak1 precursor. Quantification suggested an increased ratio of cyclic-to-linear protein for the R$_{28}$A mutant with 10.3% (±1.2%) of all protein present being cyclic, but the total amount of Oak1 peptides observed was extremely low. The increase in relative amount of cyclic protein was statistically significant compared to wt-Oak1 (p= 0.04).

Ctr Motif Requirements

Minimum Ctr motif for cyclization

The Ctr sequence plays a major role in peptide recognition prior to cyclization. Expressing the wild-type Oak1 precursor in *N. Benthamiana*, produces a series of peptides with different length Ctr regions due to the expressed protein being sequentially trimmed by carboxypeptidases. Not only is cyclic protein produced (5.1%), but also linear kB1 (32.5%), linear kB1 with Gly present at the C-terminus (29.1%), linear kB1 with Gly-Leu (20.4%) and linear kB1 with Gly-Leu-Pro (13.0%) (Figure 2A). In order to determine the minimum Ctr motif required for cyclization to occur efficiently, a series of truncation mutants were produced (Supp Fig 2). Expression of a construct where the Ctr was truncated from the native seven amino acids down to three amino acids (GLP*) did not prevent cyclization with 2.5% (±1.9%) cyclic kB1 observed (Figure 2B). Further truncation of the Ctr to two amino acids (GL*) produced 3.7% (±0.4%) cyclic protein (Figure 2C). The level of cyclic protein in both of these truncation mutants was not significantly different to the wt-Oak1 construct. Shortening the Ctr to a single Gly (G*) resulted in no cyclic peptide formation with >95% of the peptide remaining as the linear +G peptide (Figure 2D). The relative percentages of all of the peptides expressed are presented as histograms in Figure 2, and the standard error of these values are presented in Table 2.
Table 2: Summary of the relative percentages of each Oak1 peptide produced when mutants of Oak1 were expressed transiently in *N. benthamiana*. The standard error of the mean is shown in brackets. -G corresponds to the loss of Gly from the N-terminus of the cyclotide domain and the + indicates amino acids present at the C-terminus of the cyclotide domain. * indicates the level of cyclic protein is significantly different (p<0.05) to the levels of cyclic protein produced by the Oak1 construct.

| Oak1 Mutant | Relative percentages of observed peptides |
|-------------|------------------------------------------|
|             | Linear kB1 –G | Cyclic kB1 | Linear kB1 | Linear kB1 + 1aa | Linear kB1 +2aa’s | Linear kB1 +3aa’s | Linear kB1 +4aa’s |
| Oak1        | 5.1% (±1.5)   | 32.5% (±0.7) | 29.1% (±7.7) +G | 20.4% (±5.8) +GL | 13.0% (±3.3)+GLP |
| L2A         | 0.3% (±0.3)   | 5.3% (±1.6)  | 36.8% (±8.1) | 31.6% (±5.2) +G | 0.3% (±0.3) +K   | 17.2% (±3.9)+GL  |
|             |              |            |             | 0.3% (±0.3) +K  | 0.3% (±0.3) +AK  | 1.2% (±0.5)+GLP  |
| K3A         | 2.1% (±0.7)   | 6.3% (±0.3)  | 30.0% (±1.9) | 38.1% (±1.1) +G | 17.8% (±2.0)+GL  | 5.7% (±0.1)+GLP  |
|             |              |            |             | 3.6% (±1.9) +AK +GL | 3.6% (±1.9) +AK +GL |
| R28A        | 0.8% (±0.7)   | 10.3% (±1.2) | 88.9% (±1.0) | 20.4% (±5.8) +GL | 13.0% (±3.3)+GLP |
|             |              |            |             | 17.8% (±2.0)+GL  | 17.8% (±2.0)+GLP |
| L2A         |              | 7.8% (±0.4)  | 72.9% (±1.7) +G | 5.7% (±1.4) +GL | 17.2% (±3.9)+GLP  |
| N29Q        |              | 3.7% (±0.4)  | 72.9% (±1.7) +G | 5.7% (±1.4) +GL | 17.2% (±3.9)+GLP  |
|             |              |            |             | 5.4% (±1.1) +K   | 5.4% (±1.1) +K   |
| G30F        |              | 5.9% (±5.9)  | 94.1% (±5.9) +F | 94.1% (±5.9) +F | 94.1% (±5.9) +F |
| GLP*        | 2.5% (±1.9)   | 16.3% (±3.0) | 54.3% (±9.2) +G | 19.8% (±1.5)+GL | 6.5% (±3.2)+GLP  |
| GL*         | 1.7% (±0.9)   | 3.7% (±0.4)  | 19.4% (±2.2) | 57.4% (±3.3) +G | 17.8% (±2.0)+GL  |
| G*          |              | 4.1% (±0.3)  | 95.9% (±0.3) +G | 95.9% (±0.3) +G | 95.9% (±0.3) +G |
| GLA*        | 2.6% (±1.1)   | 19.2% (±2.7) | 69.4% (±5.6)+G | 8.7% (±2.4) +GL | 34.3% (±12.1)+SL |
| GL*         |              | 1.8% (±1.5)  | 58.6% (±15.9)+L | 39.5% (±14.7)+LG | 39.5% (±14.7)+LG |
| SLP*        | 2.8% (±2.8)   | 6.3% (±2.3)  | 51.7% (±10.6)+S | 5.0% (±2.9)+SL  | 34.3% (±12.1)+SL |
| LGP*        |              | 2.8% (±2.3)  | 25.3% (±25.3)+G | 18.5% (±18.5)+GG | 56.1% (±6.8)+GGL |
| ALA*        | 0.3% (±0.3)   | 7.0% (±4.5) -G +A | 87.6% (±3.6)+A | 7.4% (±2.4)+AL  | 4.5% (±1.4)+ALP  |
|             |              |            |             | 4.5% (±1.4)+ALP  | 4.5% (±1.4)+ALP  |
| L31A        |              | 2.7% (±1.3)  | 5.5% (±1.3) +G | 15.0% (±0.5) +GA | 15.0% (±0.5) +GA |
| L31A-L32A   |              | 2.7% (±1.3)  | 5.5% (±1.3) +G | 1.5% (±0.5) +GAO | 1.5% (±0.5) +GAO |
| GAPSL*      |              | 3.5% (±3.5) +GA | 85.0% (±1.8) +GAOS –G | 85.0% (±1.8) +GAOS –G |
| GAP*        |              | 3.5% (±3.5) +GA | 85.0% (±1.8) +GAOS –G | 85.0% (±1.8) +GAOS –G |
| GAPS*       |              | 3.5% (±3.5) +GA | 85.0% (±1.8) +GAOS –G | 85.0% (±1.8) +GAOS –G |
| GAP*        |              | 3.5% (±3.5) +GA | 85.0% (±1.8) +GAOS –G | 85.0% (±1.8) +GAOS –G |
| L31I        | 5.6% (±1.9)   | 5.2% (±0.7)  | 29.3% (±4.3) | 35.9% (±3.1) +G | 1.2% (±0.7)+GI  | 10.5% (±5.1) GIP |
|             |              |            |             | 10.5% (±5.1) GIP | 10.5% (±5.1) GIP |
| GIP*        | 3.0% (±1.9)   | 3.9% (±2.2)  | 34.1% (±2.7) | 51.4% (±3.4) +G | 0.4% (±0.4) +GI  | 7.1% (±2.9)+GIP  |
|             |              |            |             | 7.1% (±2.9)+GIP  | 7.1% (±2.9)+GIP  |
**Ctr P1’ requirements**

Having determined that the minimum length of the Ctr required for cyclization is two amino acids, substitutions were made within the Ctr to further clarify the requirements of this motif. The substitutions encompassed the Ctr P1’, P2’ and P3’ positions which are predicted to be required for binding to the enzyme that catalyses cyclization. The Gly at position P1’ (G30) was mutated to Ala, Ser and Phe. Substitution of Gly30 with Ala (G30A), which is a conservative substitution, produced 3.1% (+1.7%) cyclic peptide, whereas mutation to Phe (G30F), a large hydrophobic residue, produced no cyclic protein at all (Table 2). Further to this a mutant with a 3 amino acid Ctr was produced where Gly30 was mutated to Ser (G30S). This construct was labelled kB1-SLP* and produced 2.8% (+2.8%) cyclic protein.

**Ctr P2’ requirements**

To determine if the highly conserved Leu residue at position P2’ of the Ctr (L31) was necessary for cyclization the Leu was substituted with Ala or Ile. Substitution of the Leu to Ala (L31A) resulted a single linear product with an m/z of 3237.4 (Figure 3A). This mass did not correlate with any of the predicted linear cyclotides so it was postulated that a post-translational modification of the peptide had occurred. Sequencing of this peptide by LC-MS revealed that the cyclotide domain was unaltered but the Ctr contained a hydroxyproline (Hyp) at amino acid 32. That is the hydroxylated peptide was composed of the cyclotide domain with a 4 amino acid Ctr region, Gly-Ala-Hyp-Ser. Expression of the L31A mutant was interesting as it still produced cyclic protein 5.2% (+0.7%) and also an additional peptide with the post-translational hydroxylation of Pro32 (kB1-Gly-Ile-Hyp-Ser 3278.4 Da). However the hydroxylated protein was a minor product making up just 10.9% (+3.3%) of all Oak1 peptides identified.

**Ctr P3’ requirements**

To determine if a Pro residue is essential at the P3’ position of the Ctr, this amino acid was mutated to Ala (P32A) in a construct containing a 3 amino acid Ctr (kB1-GLA*). The expression of kB1-GLA* produced 2.6% (+1.1%) cyclic kB1 similar to that found for Oak1.

**Shuffling of the P1’, P2’ and P3’ sites**

Shuffled variants of the Oak1 Ctr containing only three amino acids after the cyclotide domain were produced to further delineate the residues required for cyclization. Expression of mutants where the Leu was moved such as kB1-LGP* and kB1-GGL* or removed such as kB1-GP* resulted in no cyclic protein being produced. Additionally, substitution of both residues either side of the Leu interfered with the cyclization mechanism such that expression of kB1-ALA* only produced 0.6% (+0.3%) cyclic protein (Table 2). This reduction in the amount of cyclic protein produced was statistically significant compared to the relative percentage produced by wt-Oak1 (p = .05). These shuffled variants of the Ctr tripeptide motif revealed that Leu or a similar residue at the P2’ position is essential for cyclization.

**Characterization of the proline hydroxylation motif**

As mentioned previously substitution of Leu with Ala at position 31 resulted in accumulation of a single peptide with an unexpected mass of 3237.4 as a result of hydroxylation of Pro32. MS/MS sequencing of this mass revealed the amino acid...
sequence to be the kalata B1+GAOS peptide (Figure 4). Amino acid analysis further confirmed that a Hyp residue was present in this peptide. The proline at position 32 was found to be essential for the post-translational modification event as deletion of this residue or mutation to an Ala or Asn (double mutants L\(_{31}\)A-ΔP\(_{32}\), L\(_{31}\)A-P\(_{32}\)A and L\(_{31}\)A-P\(_{32}\)N) resulted in proteolytic trimming of the Ctr over time with no cyclic protein formation nor any post-translational hydroxylation events.

**Determination of the minimum motif for hydroxylation**

To determine the minimum motif for hydroxylation of Pro\(_{32}\), a series of Oak1 L\(_{31}\)A truncation mutants were made which corresponded to the sequences kB1-GAPSL*, kB1-GAPS* and kB1-GAP*. The GAPSL* and GAPS* mutants produced only a single mass (3237.4) corresponding to Gly-Ala-Hyp-Ser motif at the Ctr (Figure 3B). Further truncation to GAP* did not produce any masses that corresponded with the presence of Hyp (Figure 3C).

Truncation of the L\(_{31}\)I mutant to a Ctr of only 3 amino acids (kB1-GIP*) also no longer produced Hyp but still produced cyclic peptide (Figure 3E)

**Prevention of carboxypeptidase activity by Hyp**

A time-course was undertaken to examine the products resulting from expression of both L\(_{31}\)A and the double mutant L\(_{31}\)A-ΔP\(_{32}\) in N. benthamiana over a period of eight days. It was apparent that biosynthesis and trimming differed greatly for these two constructs (Figure 5). The L\(_{31}\)A mutant produced a single protein product of m/z 3237 which was the kalata B1-GAOS peptide. This hydroxylated peptide was stable over a longer period of time and was not readily trimmed from the C-terminus. The L\(_{31}\)A-ΔP\(_{32}\) double mutant was trimmed via proteolysis yielding linear kB1 and kB1+G as the major products at day 8. The hydroxylation of the proline prevented the action of carboxypeptidases resulting in a long-lived product.

**Infiltration of a prolylhydroxylase inhibitor**

Transient expression of L\(_{31}\)A in N. benthamiana with and without the prolylhydroxylase inhibitor 2,4-pyridinedicarboxylic acid monohydrate (PDCA) produced a marked difference in the peptides produced. Without PDCA, expression of L\(_{31}\)A resulted in production of the hydroxylated kB1-GAOS peptide (Figure 6A), but in the presence of PDCA production of this peptide was almost completely abolished (Figure 6B). Interestingly, a small peak coinciding with the mass expected for kB1+GAOSLAA (m/z 3435.9) was found in the L\(_{31}\)A PDCA sample, though there was insufficient peptide for sequencing. Expression of the wt-Oak1 precursor with and without PDCA did not affect expression levels or processing producing near identical spectra (Figure 6C and 6D). The sharp reduction in the amount of kalata B1 peptide produced with the PDCA treated L31A construct was unexpected, but may be due to misprocessing in the ER and resultant degradation of the protein product.

**DISCUSSION**

The processing of cyclotides *in planta* is an interesting phenomenon with multiple post-translational processing steps needed to produce the final cyclic peptide. In order for these processing events to occur, specific residues are required for enzyme recognition and binding. The post-translational processing events which produce cyclic protein
appear to start with the cleavage of the Ntpp and Ntr from the precursor sequence. When Oak1 is expressed in *N. benthamiana*, a plant that does not naturally produce cyclotides, it is processed to either cyclic protein, linear cyclotide domain alone, or the cyclotide domain with varying lengths of the Ctr attached.

Cleavage of the Ntr from the cyclotide domain of Oak1 occurs at a lysine residue \((K_{-1})\) at the P1 position immediately upstream of the cyclotide domain. Substitution of this residue significantly increased the relative amount of cyclic to linear protein product substitution of the Leu residue in position P2 further upstream had a greater impact as it resulted in inefficient cleavage of the Ntr producing linear cyclotide with additional residues still present at the N-terminus.

The nature of the residue in the P1 position immediately upstream of the cyclotide domain is probably not important; it can be a Lys, as in the *O. affinis* cyclotides, or an Asn as occurs in other cyclotide subfamilies, as long as the Ntr is surface-exposed and accessible to proteases and cleavage at this junction prior to the cleavage at the Ctr that results in cyclization. This is supported by the recent discovery of cyclotides in the Fabaceae family where the cyclotide domain is directly preceded by the ER signal peptide and the Ntr is absent (20). The cleavage of this signal peptide in the ER would free up the N-terminus of the cyclotide which presumably remains free until the cyclotide precursor reaches the compartment where cyclisation occurs.

Although the residues upstream of the cyclotide domain in positions P2 and P1 are not conserved, the first two residues of the cyclotide domain itself in positions P1’ and P2’ (Gly-Leu) are conserved and these residues appear necessary for cyclization. Substitution of either of these residues resulted in little or no cyclic protein being formed during transient expression of the Oak1 construct (16). These residues are similar across species, with a common theme of a small residue followed by a larger hydrophobic residue (Leu, Ile, or Val).

The residues at the C-terminus of the cyclotide domain in positions P1 and P2 are essential for cyclization. Asn\(_{29}\) in position P1 prior to the Ctr is necessary for cleavage and cyclization to occur with mutation of this residue producing no cyclic protein. The residue at position P2 in the cyclotide domain is not completely conserved, but is generally an Arg. Substitution of this residue did not reduce the relative percentage of cyclic protein produced, but severely reduced the total amount of kalata B1 peptide produced such as to be difficult to detect with MALDI MS. The reduction in peptide levels is likely due to misfolding of the precursor protein resulting in degradation or some other event preventing exit of the protein from the ER.

The fact that Arg\(_{28}\) is not required for cyclization is interesting and allows an earlier proposed self cyclization mechanism to be discounted. We initially speculated that the Arg could act as an electron withdrawing group to activate an intein-type processing event involving the autocatalytic removal of residues on the C-terminal side of Asn\(_{29}\) via the formation of a succinimide/lactone intermediate, with ligation of the free termini to the amide from the N-terminal Gly, facilitated by the activated Asn residue (3,11). For this process to yield mature cyclotide, removal of the N-terminal prodomain must precede the ligation event.

**Ctr motif requirements**

From our hypothesis that the Ctr is essential for cyclization the residues...
required in this tail region were studied to
determine not only the minimum motif
for cyclization, but also the type of amino
acids permitted at each position relative
to the cleavage site. Truncation mutants
of the Ctr revealed that a minimum of two
amino acids are required after Asn\textsubscript{29}
for cyclization to occur. Trimming of the Ctr
beyond the Leu residue in the P2' position
results in complete abolishment of all
cyclic peptide production.

The P1' amino acid within the Ctr
was substituted with various amino acids
and from this it was obvious that small
amino acids are tolerated a large
hydrophobic amino acid was capable of
completely abolishing cyclic peptide
production. This finding is strengthened
by the high conservation of a small
residue in this position in cyclotide
precursors from the majority of species
(15).

The importance of the P2' residue
within the Ctr was exemplified with the
truncation mutants which showed that
Leu is essential for cyclization and this is
not entirely surprising as a Leu in this
position is completely conserved across all
cyclic peptides found in all species except
the Fabaceae which contain an Ile (20).
Cleavage and cyclization occurs two
amino acids from this leucine. It is
proposed that this Leu residue binds
within a hydrophobic pocket of the
asparaginyl endopeptidase acting to hold
the Ctr and position the Asn in an optimal
conformation for cleavage and ligation of
the cyclotide domain. Substitution of
Leu\textsubscript{31} with an isoleucine (L\textsubscript{31}I) which is a
conservative change still produced cyclic
protein in \textit{N. benthamiana} with equal
efficiency to the wild-type Oak1 gene.

The enzyme which carries out the
cyclization reaction, which is most likely
an AEP, requires a small residue in the P1'
position and a larger hydrophobic amino
acid in the P2' position. These are not
completely novel requirements with the
P1' residue often required to be a small
amino acid in the case of naturally
occurring protease inhibitors due to steric
constraints in binding to a protease.
The coagulation proteases such as plasmin
and kallikrein in mammals prefer a
hydrophobic residue such as Leu to bind
in the S2' binding pocket (21).

Whilst changing Leu\textsubscript{31} in the P2'
position to a similar amino acid had little
effect on cyclization, substitution of this
residue with Ala (L\textsubscript{31}A) resulted in
production of a single post-translationally
modified peptide of mass 3236.4 Da.
Sequencing revealed that this peptide was
kalata B1 with a truncated and modified
form of the Ctr containing the amino acid
sequence Gly-Ala-Hyp-Ser. No other
processing intermediates were produced
with this construct, suggesting that the
enzyme which hydroxylates the proline
does so with high efficiency.

The L31I mutant whilst producing
cyclic protein also produced a mass which
matched the post-translational
hydroxylation of Pro\textsubscript{32} with the sequence
kB1 + Gly-Ile-Hyp-Ser (3278.4 Da). The
hydroxylated protein was a minor
product, but it is interesting to note that
such a conservative change could produce
this hydroxylation event.

The P3' position of the Ctr had
little effect on cyclization efficiency.
Shortening the Ctr past this residue did
not prevent cyclization and mutation also
had little effect. This suggests that the
major determinant of specificity are the
P1, P2, P1' and P2' residues. The amino
acids within the Ctr are highly conserved
across species and expression of mutants
where Leu\textsubscript{31} was removed or moved
resulted in no cyclic protein being
produced. It was also interesting to note
that substitution of both residues either
side of the Leu interfered with cyclization.
The mutants expressed in this work reveal a cyclization motif which can be modified within specific limits. The P2 residue could not be modified due to its effect on protein levels, but not due to cyclization constraints. The P1 position requires an Asn though an Asp can function inefficiently at this position. P1’ requires a small amino acid and P2’ requires Leu or Ile for efficient cyclization to occur. These mutants all reinforce the need for an Asn – small aa – Leu, at the C-terminus in order for efficient cyclization to occur (Figure 7).

**The proline hydroxylation motif**

The modification of the P2’ residue had major implications producing post-translationally modified protein products. Mutation to Ala resulted in the production of kalata B1 with a Ctr containing the sequence GAOS. The hydroxylation of this protein appears unusual as it does not coincide with a known hydroxylation motif, though these are poorly characterised in plants (22).

Truncation of the Ctr revealed that the minimum motif for hydroxylation of Pro$_{32}$ was GAPS. Removal of the C-terminal Ser residue prevented hydroxylation of the proline and resulted in proteolytic trimming of the Ctr. Deletion or substitution of the proline residue in the Ctr negates any post-translational modification of the expressed protein and resulted in proteolytic trimming of the Ctr over time. A time course for the processing of the L$_{31}$A and L$_{31}$A-ΔP$_{32}$ mutants showed that the L$_{33}$A mutant produces only the kalata-GAOS product which is quite resistant to breakdown, whilst the L$_{33}$A-ΔP$_{32}$ double mutant was simply trimmed via proteolysis.

Cyclotides are processed inside the ER where protein disulfide isomerase helps with the formation of disulfide bonds within the cysteine knot motif (23). Protein disulfide isomerase are thought to exist in complex with prolylhydroxylases in the ER so the cyclotide precursor would be in close proximity to these proteins when being folded (24). This could account for the high efficiency of the hydroxylation reaction in the L31A mutant.

Whilst the hydroxylation reaction is catalysed by a prolylhydroxylase, it is more difficult to speculate on the enzyme responsible for cleaving the Ctr to produce the single product kB1-GAOS. Some clues are given when the L31A mutant was infiltrated with the prolylhydroxylase inhibitor. This resulted in very little cyclotide protein being produced but the masses which were found coincided with kB1-GAOS as well as kB1-GAOSLAA. This suggests that the prolylhydroxylase is acting to hydroxylate the protein prior to cleavage of the last three amino acids of the Ctr (LAA). The fact that this cleavage reaction is inhibited by a prolylhydroxylase inhibitor also points to the prolylhydroxylase being responsible for the cleavage reaction.

**CONCLUSIONS**

From this work we can conclude that for cyclization to occur the N-terminal propeptide must be cleaved to free up the N-terminus of the cyclotide domain. Also the Ctr is necessary for cyclization with a specific amino acid sequence required for this reaction to take place. This motif requires an Asn at position P1, followed by a small amino acid in the P1’ position and a Leu in the P2’ position.

The finding that a single amino acid substitution in the Oak1 C-terminal cyclization motif can produce a post-translational hydroxylation event suggests that there may be other hydroxylated cyclotides present in nature.
The fact that non-cyclotide producing plants can make the cyclic form of these peptides when expressed recombinantly points to the ability of enzymes, which normally have other functions, to carry out the unique cyclization process in all plants. The AEP’s responsible for this process are predicted to be very similar in both cyclotide and non-cyclotide producing plants. The fact that the cyclization process is very efficient in the cyclotide producing plants may suggest that some optimization of these enzymes or the cyclotide precursor has occurred over time in order to enhance the levels of cyclic versus linear peptide.

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6The abbreviations used are: Hyp, hydroxyproline; Ntpp, N-terminal pro-peptide; Ntr, N-terminal repeat; Ctr, C-terminal repeat; Oak1, O. affinis kalata B1 precursor gene; Oak1, O. affinis kalata B1 precursor protein; kB1, kalata B1; SFTI-1, sunflower trypsin inhibitor 1.
FIGURE LEGENDS

FIGURE 1: A: Cyclotides are expressed as multidomain precursor proteins containing five distinct regions (ER, Ntpp, Ntr, cyclotide and Ctr), which can contain up to three cyclotide domains. B: The Oak1 precursor from Oldenlandia affinis with the amino acid sequence displayed for the residues either side of the cyclotide processing sites. C: Alignment of cyclotide sequences from different plant families showing the conservation of the Asn at the C-terminus, followed by a small amino acid and a Leu. Residues at the N-terminal side (-1) of the cyclotide domain are not strongly conserved. Oak1 is the precursor of kalata B1 from Oldenlandia affinis (3,11), Voc3 is the precursor of cycloviolacin O13 from Viola odorata (25), Gbc1 is from Gloeospermum blakeanum (26) and Viba1 is a precursor from Viola baoshanensis (27).

FIGURE 2: MALDI MS spectra of Oak1 proteins expressed transiently in N. benthamiana. The letters above each peak indicate the amino acids of the Ctr attached to the linear kB1 peptide. A.) Oak1 with full length Ctr (GLPSLAA*) and the proteins produced. B.) Oak1 with the Ctr truncated to GLP. C.) Oak1 with the Ctr truncated to GL* and D.) Oak1 with the Ctr truncated to G*. On the right histograms are given showing the average percentage of all of the peptide species for each of the constructs expressed.

FIGURE 3: MALDI MS spectra of Oak1 proteins expressed transiently in N. benthamiana. A) Oak1 L31A  B) Oak1 L31A with the Ctr truncated to GAPS*  C) Oak1 L31A with the Ctr further truncated to GAP*  D) Oak1 L31I  E) Oak1 L31I with the Ctr truncated to GIP*. Hydroxyproline is represented as an O in single letter amino acid code

FIGURE 4 : MS/MS spectrum of the reduced and alkylated form of the Oak1 L31A product with a precursor ion observed at m/z 1196.03+corresponding to a mass of 3585 Da. Sequence analysis revealed that the peptide sequence was GLPVCGETCGGTNTPGCTCSPVCTRNGAOS where O = Hyp. Singly-charged ions present are labelled.

Figure 5: Time-course for the expression and processing of two Oak1 mutants over 8 days in N. benthamiana. Top: L31A yields kB1 with a truncated and hydroxylated Ctr with m/z 3237, this peptide is resistant to proteolytic breakdown. Bottom: L31A, ΔP32 is trimmed over the 8 days and produces linear kB1 and kB1+G as the predominant products by day 8.

Figure 6: MALDI MS spectra of transiently expressed Oak1 L31A and Oak1 with and without the prolylhydroxylase inhibitor PDCA. A.) Oak1 L31A  B.) Oak1 L31A in the presence of PDCA  C.) Oak1 and  D.) Oak1 in the presence of PDCA.

Figure 7: The specificity requirements of the C-terminal region of the Oak1 cyclotide precursor for production of cyclic kalata B1.
Fig 1
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Fig 4
Fig 7
Insights into processing and cyclization events associated with biosynthesis of the cyclic peptide kalata B1
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