D1A root-structure-regulator peptide in *Medicago truncatula*: Circular dichroism structure-activity studies

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Abstract

Symbioses between nitrogen-fixing rhizobial bacteria and legumes are of great agricultural importance. We have recently-discovered a 15-amino acid peptide-hormone that contributes to root-nodule formation, essential for rhizobium-legume symbiosis. Manual application of these peptide-hormones increases nodule-formation, as does elevating activity of the corresponding gene in *Medicago truncatula.* The structure/bio-activity relationship of these hormones has however, until now, remained enigmatic. We report structural characteristics essential for the activity of our hormone, dubbed "D1A". D1A variants, with alterations in key amino-acids, were analysed via ultra-violet circular dichroism (CD), which generates structure-specific spectra. Spectra were compared to existing literature. Reconciliation of structural data with previously-obtained hormonal-activity data suggests a 'hairpin' shape to be biologically-relevant, potentially affecting receptor-binding. We speculate D1A possesses a flexible central sequence of amino acids, facilitating this hairpin structure. Structural stabilisation requires interactions between the distal, aromatic amino acids, with natural hormone-structure also requiring proline-hydroxylation. Mono-hydroxylation resulted in hairpin de-stabilisation, whilst non-hydroxylation resulted in elevated hairpin character.

Keywords

Peptide-hormone, circular dichroism, rhizobia-legume symbiosis, root-structure regulation

Introduction

Obtaining or "fixing" nitrogen in mineral form is essential for plants, with availability of nitrogen often limiting plant-growth (Nosengo, 2003). Whilst most plants obtain nitrogen from soil deposits, leguminous plants can form symbioses with nitrogen-fixing bacteria known as rhizobia (Parniske & Downie, 2003). Rhizobia, residing in leguminous root-structures known as nodules, fix atmospheric nitrogen, exchanging this for plant-synthesised metabolites. The possibility of increasing nodulation (i.e. nitrogen-fixation) is thus of great agricultural interest, especially given the many environmental costs associated with the production and (over)-use of artificial nitrogen fertilisers. Herein we report on the structure of a hormone, composed of a chain of amino-acids linked together (otherwise known as a peptide), that limits lateral-root initiation and increases nodule formation in *Medicago truncatula*.

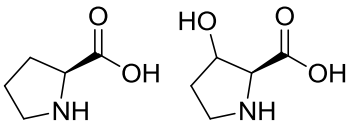
The peptide at the heart of this study, dubbed "D1A", is a 15-amino-acid (or 15-residue) peptide, studied in the model legume *M. truncatula.* Over-expression of the D1A-gene or direct application of peptide to *M. truncatula* roots profoundly affected overall root architecture. Increased nodulation, inhibited lateral root formation and formation of galls (or "root-outgrowths") was observed. A *D1A*-like gene was previously identified by bioinformatic computer-screening in *Arabidopsis* by Ohyama *et al.* (Ohyama, Ogawa, & Matsubayashi, 2008). D1A computational-modelling, using the PEPFOLD program (Imin et al., 2012; Maupetit, Derreumaux, & Tuffery, 2009), suggests D1A assumes a stabilising "hairpin" (known to structural biologists as a "β-hairpin") structure in which the chain of amino acids folds in on itself (Figure 1). The significance of this hairpin-structure, as well as the amino-acid composition of the hormone, is however, still unclear.

To gain insight into D1A's structure/biological-activity relationship, a series of modified D1A-peptides were generated, investigating three main structural characteristics (Figure 1, Table 1):

i) **Hairpin structure**. By changing glycine to alanine, we hoped to alter the central 'turn' region, disrupting the hairpin structure (D1G-to-A)

ii) **Effect of proline-hydroxylation**. In nature proline amino acids within proteins and/or peptides are often hydroxylated, adding an oxygen and hydrogen atom to the proline ring-structure (Fig. 1). In D1A, prolines at positions 4 and 11 on the peptide are hydroxylated. Synthetic peptides D1B and D1C were mono-hydroxylated (Proline-11) and un-hydroxylated respectively to investigate the effect of proline-hydroxylation.

Figure 1: Structures of proline and hydroxyproline

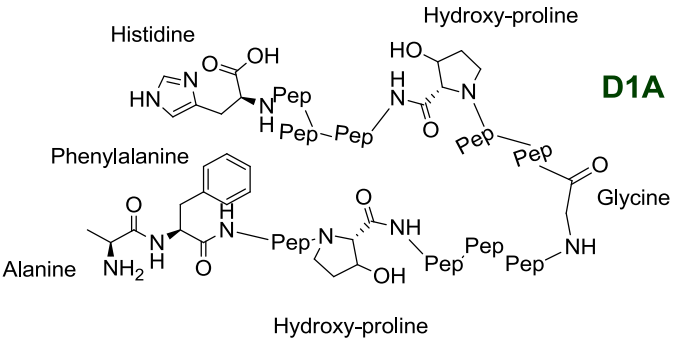


proline hydroxyproline

iii) **Effect of the N and C-terminal residues**. Computer-modelling suggests a hydrophobic interaction involving the histidine and phenylalanine side chains, known as π-stacking, contributes to maintenance of hairpin structure. Peptides D1 MINUS N and D1 MINUS C were synthesised lacking D1A terminals respectively.

Figure 1: **A:** The "hairpin" structure of D1A. Amino acids within the hormone relevant to our study have been labelled. Other amino acids have been labelled "Pep". **B:** Structures of the synthesised D1A-derivatives. For clarity, the "hairpin" hormone-shape has been omitted.

A



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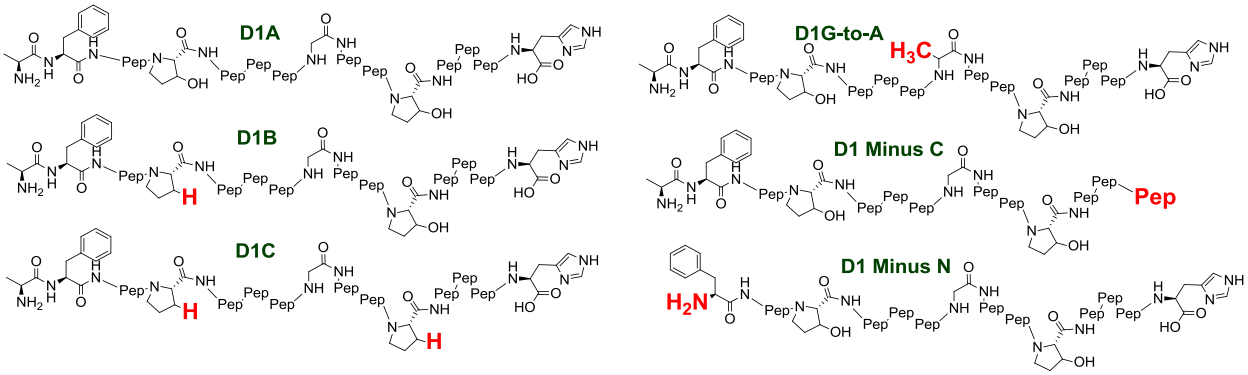
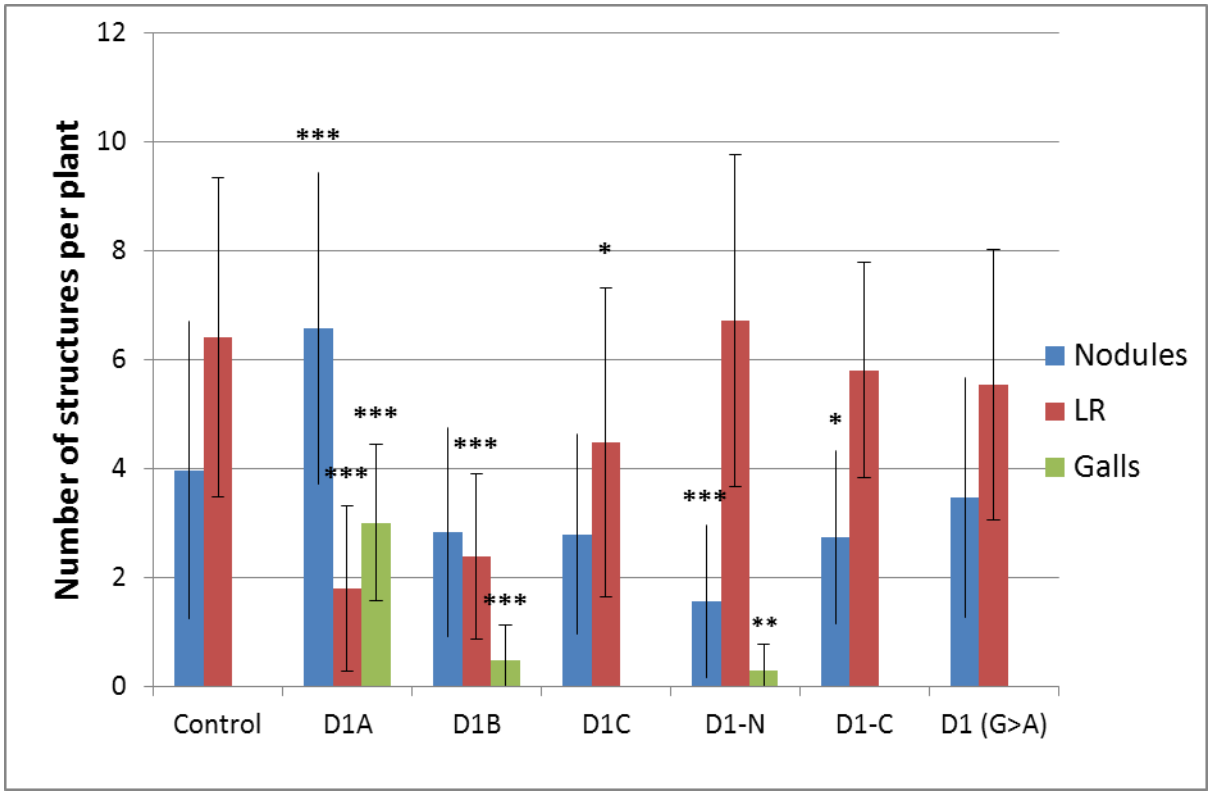


Table 1: The sequences of D1A and the synthetic derivatives investigated. Deviations from D1A sequence have been highlighted in red. X = other amino acid

Peptide	Peptide Sequence														
Amino acid number:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
D1A	**Ala**	**Phe**	**X**	**HyPro**	**X**	**X**	**X**	**Gly**	**X**	**X**	**HyPro**	**X**	**X**	**X**	**His**
D1B	**Ala**	**Phe**	**X**	**Pro**	**X**	**X**	**X**	**Gly**	**X**	**X**	**HyPro**	**X**	**X**	**X**	**His**
D1C	**Ala**	**Phe**	**X**	**Pro**	**X**	**X**	**X**	**Gly**	**X**	**X**	**Pro**	**X**	**X**	**X**	**His**
D1GtoA	**Ala**	**Phe**	**X**	**HyPro**	**X**	**X**	**X**	**Ala**	**X**	**X**	**HyPro**	**X**	**X**	**X**	**His**
D1 MINUS C	**Ala**	**Phe**	**X**	**HyPro**	**X**	**X**	**X**	**Gly**	**X**	**X**	**HyPro**	**X**	**X**	**X**	**-**
D1 MINUS N	**-**	**Phe**	**X**	**HyPro**	**X**	**X**	**X**	**Gly**	**X**	**X**	**HyPro**	**X**	**X**	**X**	**His**

These synthetic peptides, as well as D1A, were externally-applied to *M. truncatula* roots and their biological activity measured using the following criteria: nodule, gall and lateral root number per plant (Figure 2, unpublished data).

*Figure 2: Comparison of M. truncatula nodule number, root length (LR) and gall number upon exogenous application of D1A and derivatives. * = P < 0.05, ** = P < 0.01, *** = P < 0.001 (Student's t-test), error bars represent standard deviation. LR = lateral roots.*



In our present study, we sought to rationalise the biological data above by analysing the peptides' structures using circular dichroism (CD) spectroscopy, paying particular attention to deviations in spectral readings relative to D1A. CD uses helically-polarised light (either a left or right-handed helix) (Greenfield, 2007; Juban, Javadpour, & Barkley, 1997; Kelly & Price, 2000; Pelton & McLean, 2000). Based upon the degree to which the peptide sample differentially absorbs left versus right-handed helically-polarised light (measured in molar ellipticity, deg.cm^2.dmol^{-1}), a spectrum characteristic of a particular folding-structure is obtained.

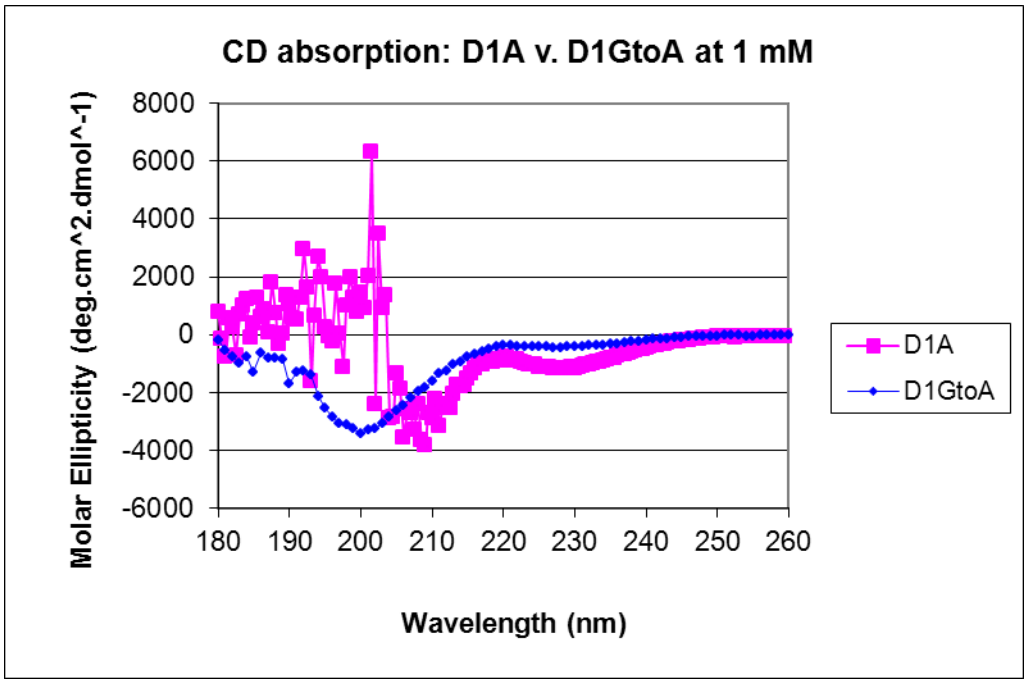
Thus, CD spectra are useful in structural-determination, especially given ease of data-collection (Greenfield, 2007; Juban, et al., 1997; Kelly & Price, 2000; Pelton & McLean, 2000). In addition to qualitative comparisons of obtained spectra with literature, we attempted the use of linear-regression software to quantify spectral changes. Such techniques (Greenfield & Fasman, 1969; Perczel, Park, & Fasman, 1992; Reed & Reed, 1993) use a protein/peptide spectral reference set to estimate an unknown peptide's structure.

Results

Several D1A residues contribute to the β-hairpin

The CD spectrum of D1GtoA was markedly different to D1A, with a shift in minimum-molar-ellipticity from 210 nm to 200 nm, characteristic of an unstructured peptide. This observation was consistent with predictions previously made using the PEPFOLD program (unpublished data).

Figure 3: D1A and D1GtoA spectra, in H_2O at 25°C, 1 mM peptide concentration. The difference between peptides is markedly visible.



Turning to the contribution of other residues in D1A towards hairpin-structure, we found histidine (residue-15) removal (D1 MINUS C) also resulted in a minimum-shift towards 200 nm (Figure 4). In contrast, alanine (residue-1) removal (D1 MINUS N) resulted in a slight increase in β-hairpin character, with spectral minimum-shift to 212 nm.

Hydroxylation was also found to contribute to hairpin-structure, with the mono-hydroxylated D1B apparently forming an intermediate between hairpin and random-structure; the minimum occurring at 205 nm. Curiously, the non-hydroxyprolinated D1C had increased β-hairpin-character, with minimum wavelength increase (to 214 nm) and accentuation of secondary minimum ~227 nm both indicative of a tightly-bound β-hairpin (Figure 5) (Kozin et al., 2001).

Figure 4: D1A, D1 MINUS N and D1 MINUS C CD spectra. D1A and D1 MINUS N spectra are essentially superimposable; D1 MINUS C displays a random structure

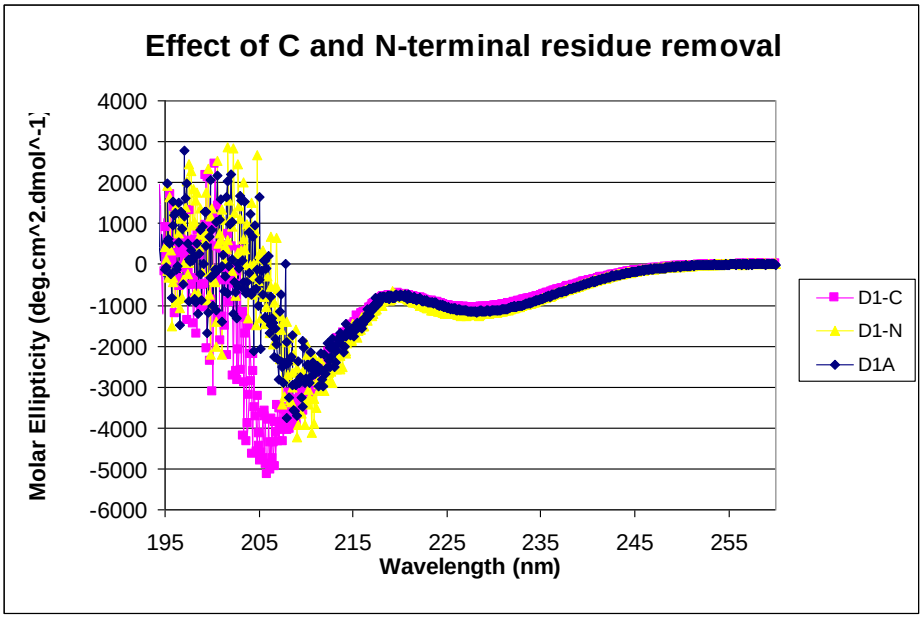
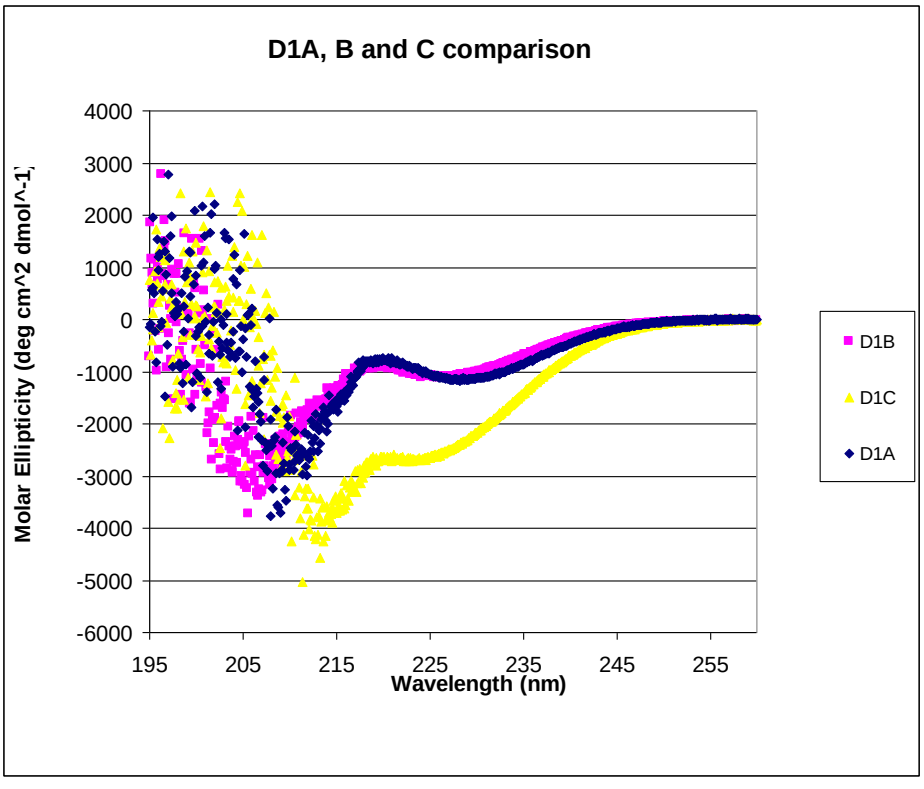


Figure 5: D1A, D1B and D1C CD spectra. D1B's minimum is intermediate, at 205 nm whilst D1C's is 214 nm; typical of hairpin-structure.



Structural analyses corroborated with linear-regression techniques

To confirm our qualitative structure-estimations we employed two linear-regression methods, LINCOMB and MLR. Estimations concurred with our qualitative comparisons, suggesting a β-hairpin structure for D1A, indicated by the weighting of β-turn/sheet (protein-structures similar to β-hairpins) spectra by LINCOMB (64.3%) and MLR (58.2%). No appreciable amount of α-helical structure (another structure common in proteins/peptides) was predicted for our peptides.

Relative to D1A, D1GtoA was reduced in β-hairpin-character with a concurrent - increase in random-structure. A similar result was obtained for D1 MINUS C. The intermediate nature of D1B's structure was tentatively supported by the estimation of β-turn/sheet character by LINCOMB which, at 58%, is between that of D1A (64.3%) and D1GtoA (57.6%). Interestingly, β-turn/sheet content for D1C was estimated to be in excess of that observed for D1A using both LINCOMB (98.4%) and MLR (74.6%). This phenomenon was also observed for D1 MINUS N using LINCOMB, in which β-turn/sheet content was overestimated at 108.9%, but not MLR in which β-turn/sheet content estimate was comparable to D1A (58.1%) (Table 2).

Table 2: Structure-estimations of D1A and synthetic derivatives using linear-regression computer-algorithms LINCOMB and MLR

Spectral Analysis						
Peptide	LINCOMB			MLR		
	α-helix	β-turn/sheet	Random	α-helix	β-turn/sheet	Random
D1A	-4.7	64.3	40.4	4.3	58.2	37.5
D1B	-1.2	58	43.2	1.5	56.8	41.7
D1C	1.6	98.4	0	4.6	74.6	20.8
D1GtoA	-1	57.6	43.4	2.6	56.7	40.7
D1 MINUS C	-1.2	57.8	43.4	2.3	56.7	41
D1 MINUS N	-8.9	108.9	0	4.7	58.1	37.2

Discussion

Various aspects of D1A important for structural-stability/biological-activity

Small peptide-hormones have been shown to regulate several plant physiological pathways, with evidence growing that they work with other plant hormones to control plant growth and development (Matsubayashi & Sakagami, 2006). Well-defined structure seems required for all peptide-hormones to protect them from enzymatic degradation, with known peptide-hormones divided into two classes: cysteine-rich and cysteine-poor. The former peptide class exhibits well-defined tertiary structure resulting from disulphide bonds between

cysteine amino acids. The latter, of which D1A is an example, forms stable folding-structures to prevent degradation.

Structural-modelling predictions vindicated

CD estimations of D1A and D1GtoA are consistent with PEPFOLD-program-estimated structures (Maupetit, et al., 2009). D1A spectra display a prominent 210 nm minimum, consistent with β-hairpin structures reported previously and confirmed by nuclear magnetic resonance experiments (Kozin, et al., 2001; Tedford, Fletcher, & King, 2001); we are endeavouring to replicate NMR results with D1A.

D1GtoA, in contrast, displays a 200 nm minimum suggesting a random-structured peptide (Bewley, John, Rodolfo, & Clore, 2002; Freitas et al., 2007). This is consistent with our expectation that substitution of glycine by alanine in the "turn-region" would destroy hairpin-structure, resulting from alanine's increased bulk (Yang & Ad, 2012).

C-terminal His residue may participate in π-stacking interactions important for β-hairpin structure

The C-terminal His-residue is also important for maintaining β-hairpin structure. Removal of His-15 caused spectral-minimum-shift towards 200 nm, indicating increased random coil character. This concurs with bioassays for D1 MINUS C, which found exogenous application of D1 MINUS C did not lead to D1A-application phenotypes (Figure 2).

Many β-hairpin structures require additional stabilisation, whether by disulphide linking or hydrogen-bonding (Tedford, et al., 2001). We speculate an interaction exists between His-15 and Phe-2 aiding stabilisation. This concurs with the PEPFOLD-predicted D1 MINUS C structure, indicating close proximity between these residues (unpublished data). Such unsaturated/aromatic moiety interactions are known as π-stacking and have been observed

contributing to biomolecular structure (Huang et al., 2012). Confirmation of π-stacking interaction between His-15 and Phe-2 could be obtained by replacing either residue by alanine; both synthetic derivatives would lose β-hairpin structure if the residues are required for π-stacking. NMR data would be definitive, with characteristic π-stacking NMR signals well-documented (Huang, et al., 2012).

The N-terminal residue, although not contributing to β-hairpin-stabilisation, is important for maintenance of native D1A structure. The spectral minimum for D1 MINUS N experienced a slight shift to higher wavelength compared to D1A, suggesting an, albeit marginal increase in β-hairpin structure. N-terminal residue removal may allow better access for His-15/Phe-2 π-stacking interactions.

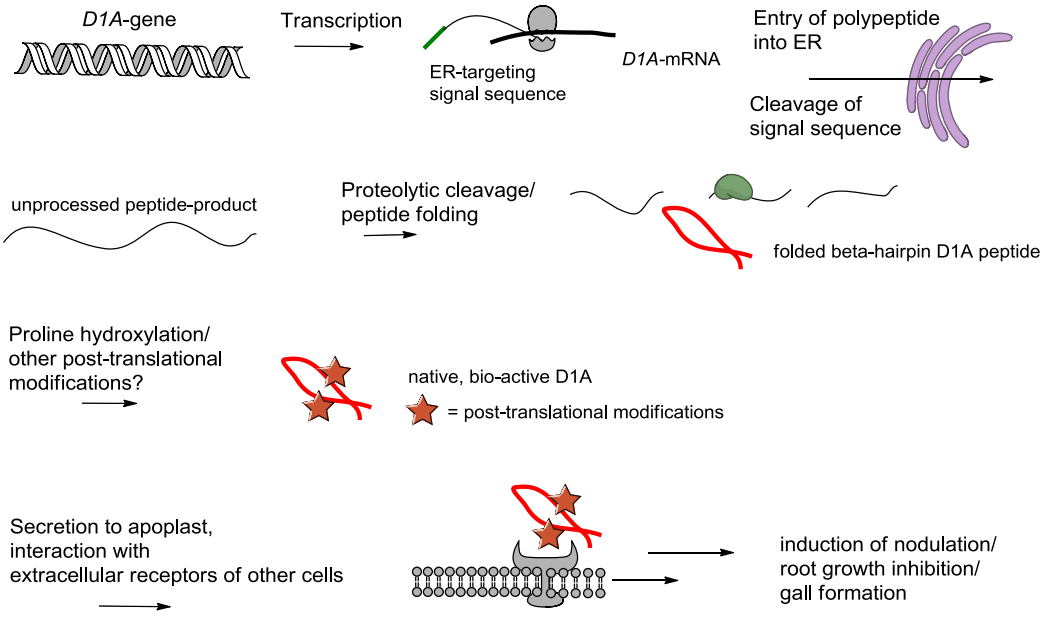
Hydroxyprolination important for structure and bioactivity

The influence of hydroxyprolination, a post-translational modification, upon structure and bio-activity is also apparent. Following biosynthesis of the peptide-chain but prior to secretion, peptides may undergo post-translational modifications essential for biological activity (Matsubayashi, Ogawa, Morita, & Sakagami, 2002; Matsubayashi & Sakagami, 2006). Proline-hydroxylation at positions 4 and 11 are the only known post-translational modification of D1A (Ohyama, et al., 2008), and were found essential for biological activity (unpublished data). Our understanding of D1A bio-synthesis and activity is summarised below (Figure 6).

Herein, we have demonstrated that proline-hydroxylation also affects D1A's structure. D1B, with mono-proline-hydroxylation at position 11, possesses slightly-increased random-structure. Bio-activity is decreased, with nodulation comparable to no-peptide controls. Intriguingly, β-hairpin interaction in non-hydroxylated D1C was elevated compared

to D1A, indicated by spectral-minimum-shift towards higher wavelength. β-hairpin intramolecular bonding may increase in the absence of hydroxyprolination as a result of reduced intermolecular hydrogen-bonding. D1A hydroxy-proline hydroxyl groups are predicted to orient outwards, interacting with the peptide's aqueous environment. The looser, D1A-type conformation may be stabilised via hydroxy-proline-mediated hydrogen-bonding with the hormone's aqueous surroundings, whilst D1C must instead stabilise by increasing intramolecular interactions.

Figure 6: Formation of D1A results from gene-translation followed by cleavage extended peptide-product by endoprotease enzymes in the plant-cell's endoplasmic reticulum (ER), yielding peptide pre-hormones. Hydroxylation of proline residues are required to yield the biologically active D1A peptide, although additional post-translational modifications may occur.



Despite hairpin-structure, no biological activity was observed for D1C (Figure 5), suggesting hairpin-structure is not the only determinant for D1A-receptor-binding.

Hydroxyproline residues may also be required for hormone-receptor-recognition, as previously-observed (Ahearn, Haigis, Bar-Sagi, & Phillips, 2012).

That D1B continues to induce lateral roots and outgrowths without affecting nodulation (Figure 2) suggests D1A may have dual signalling functions, with the nodulation pathway more specific for hydroxy-prolinated peptide-hormone. This proposal would require additional evidence, the most conclusive being identification of D1A's receptor.

Qualitative assessments supported by linear-regression

We further sought to analyse our CD data by fitting to CD spectra of peptides with known structure. Several computational methods exist to estimate protein/peptide structure. These include linear-regression techniques such as LINCOMB (Perczel, et al., 1992), GnF (Greenfield & Fasman, 1969) and MLR (Perczel, et al., 1992), as well as neural network methods such as K2D (Andrade, Chacon, & Merelo, 1993), SELCON and CONTIN (Greenfield, 2007). We commenced our investigation examining the most-commonly-used of these methods.

Neural network processes, K2D, SELCON and CONTIN were dismissed given their reportedly-poor estimation of β-turn and sheet content (structures we anticipated to be most-similar in structure to our peptides) and poor peptide (as opposed to protein) structural-estimation in general (Greenfield, 2007). Turning our attention to linear-regression methods, the venerated GnF program (Greenfield & Fasman, 1969) was rejected given its inability to accept more than 13 spectral data points. This left LINCOMB and MLR, versatile programs, able to fit unknown spectral data to user-specified reference sets. We analysed D1A and its derivatives with LINCOMB and MLR using a poly-L-lysine polypeptide

set of spectra (Reed & Reed, 1993). This set consisted of α-helix, β-sheet and random coil peptide spectra and two β-turn peptide spectra.

Analyses apparently concur with our qualitative observations, indicating biologically-active D1A, D1 MINUS N and D1B all possess considerable β-hairpin structure, whilst inactive D1GtoA and D1 MINUS C are not hairpins. Quantification of β-hairpin character in D1 MINUS N and D1B tends to support the biological importance of the D1A-like hairpin-structure; D1B, which has increased random coil character when analysed by LINCOMB and MLR, is less active. Similar observations were made for D1 MINUS N.

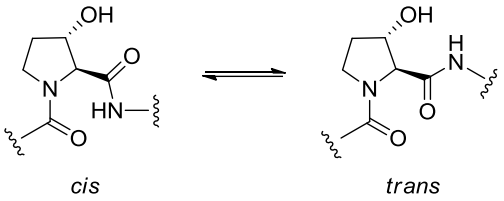
Inconsistencies however, exist between qualitative observation and regression analysis. Whilst the D1GtoA spectrum (Figure 6) would appear super-imposable upon literature spectra of random-structure peptides, both LINCOMB and MLR estimate D1GtoA to be predominately β-hairpin, albeit with less weighting than estimated for D1A.

D1A hydroxyprolination: alternative biological roles

The question also remains, whether D1A's hairpin-structure is biologically-active. This is of particular relevance given our observation hydroxyprolination at positions 4 and 11 are essential for biological activity. Hydroxyproline residues can be oriented *cis* (closer) or *trans* (further away) relative to the preceding peptide bond (Figure 7). Given the steric constraints of typical proteins and peptides, inter-conversion between these configurations triggers significant structural change. Biological systems exist in which hydroxyproline is used as a protein-activity 'switch', with peptidyl-prolyl *cis-trans* isomerase enzymes catalysing *cis*/*trans* inter-conversion of hydroxyproline residue(s). The accompanying conformational shift represents a change between the molecule's "active" and "inactive" forms (Ahearn, et

al., 2012). The evidence of D1A hairpin-structure presented in our study does not rule out peptidyl-prolyl *cis-trans* isomerase-conversion of D1A to its active configuration.

Figure 7: Hydroxyproline residue isomerisation within a peptide chain. As a result of isomerisation, the hydroxyl group's orientation relative to the carbonyl (C=O) functional group



Several observations however, would tend not to support this proposal. It is likely any hydroxyproline-isomerisation in D1A would be structurally-demanding, necessitating complete loss of existing hairpin-structure. Short peptides like D1A are unlikely to possess multiple secondary structures, suggesting the isomerised peptide would be random coil. This would be inconsistent with the specificity displayed by *M. truncatula* for D1A; D1GtoA has the same primary sequence save for one residue and might be expected to display at least some biological activity under this model.

In addition to hydroxy-proline-isomerisation, D1A may undergo additional post-translational modifications prior to receptor-binding. Peptide hormone phosphorylation and sulfation are both known, as is glycosylation (Ohyama, Shinohara, & Matsubayashi, 2009). The effect of such modifications upon the biological activity of D1A and synthesised derivatives is unknown. Further advances will undoubtedly follow isolation of the *in vivo* peptide hormone.

Further Work

Isolation and characterisation of an *in vivo* D1A and its corresponding receptor represent milestones yet to be passed on the road to fuller understanding of D1A's activity. Another question is how the D1A genetic-system might interact with other root hormonal pathways. Strigolactones are known to inhibit lateral-root formation in *A. thaliana*, a phenotype similar with that observed upon exogenous D1A application on *M.truncatula*. D1A-gene over-expression has been found not to be regulated by the phytohormones (unpublished data), suggesting D1A may act upstream of the strigolactone pathway, possibly in the carotenoid biosynthetic pathway, to regulate root development (Cazzonelli & Pogson, 2010).

Concluding Remarks

Using CD spectroscopy, the structures of D1A and selected synthetic derivatives were determined. Spectral data was compared with previously-obtained biological data, allowing inferences of D1A bio-activity structural determinants. Consistent with computational modelling, D1A is a β-hairpin. Hairpin-structure is dependent on the presence of the glycine amino acid, with the steric bulk of other amino acids prohibiting folding. Histidine is also essential for β-hairpin formation, most likely due to a stabilising π-stacking interaction with phenylalanine. Hydroxylation of proline residues 4 and 11 is important for biological activity, although their *in vivo* role is unclear.

Experimental

Peptides

Peptides were synthesised at the Biomolecular Resource Facility, ANU.

Circular Dichroism Measurements

CD spectra of peptides at 1 mM were recorded at 25^{oC} on an Applied Photophysics spectropolarimeter using a 0.1 cm path length cell. CD signals were expressed as the mean residue weight ellipticity values, [θ], in deg.cm^{2}.dmol^{-1}. Samples were scanned in triplicate over the wavelength range 180-260 nm, at a rate of 0.1 nm/s, with data recorded every 0.1 nm. Quantitative evaluation of secondary structure of spectra was carried out using the LINCOMB and MLR programs available at http://www.nature.com/nprot/journal/v1/n6/suppinfo/nprot.2006.202_S1.html

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