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CLE peptide tri-arabinosylation and peptide domain sequence composition are essential for SUNN-dependent autoregulation of nodulation in *Medicago truncatula*

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Summary

- *MtCLE12* and *MtCLE13* encode CLAVATA3/EMBRYO-SURROUNDING REGION RELATED (CLE) peptides which regulate autoregulation of nodulation (AON) in *Medicago* through the shoot receptor, SUNN (SUPER NUMERIC NODULES). Genetics suggests RDN1 (ROOT-DETERMINED NODULATION1) arabinosylates MtCLE12 to enable SUNN perception. The functional structures of MtCLE12 and MtCLE13 peptides, however, remain elusive.
- We combined genetic and chemical synthesis approaches to determine if glyco-modifications of three nodule-expressed CLE peptides are essential for AON. We also examined how root and shoot applied AON-CLEs inhibit nodulation.
- MtCLE12, MtCLE13 and MtCLE42 peptides were synthesised with hydroxylation, mono-arabinosylation or tri-arabinosylation (TaP) at Proline 7. Only MtCLE12-TaP and MtCLE13-TaP peptides induced AON in WT and *rdn1-1*, but not in *sunn-4*. The application of MtCLE13-TaP to cotyledons 1 d before rhizobial inoculation completely inhibited both rhizobial infection and nodulation. By contrast, MtCLE12-TaP induced significant AON without abolishing rhizobial infection.
- The results indicate that key CLE domain amino acids and TaP modifications to MtCLE12 and MtCLE13 are essential for SUNN-dependent AON. We also show evidence that RDN1 does not tri-arabinosylate MtCLE13. Finally, MtCLE13-TaP can induce a strong AON response in shoots that inhibits the entire symbiotic processes in roots. We present a new model for AON in *Medicago*.

Key words: arabinosylation, autoregulation of nodulation, CLE, *Medicago truncatula*, nodule formation, plant signaling, RDN1, symbiosis.

Introduction

A multi-stepped symbiosis between soil rhizobia and legumes results in the production of new root organs called nodules where inert atmospheric nitrogen (N) is converted into ammonia. This symbiosis sustainably provides the largest source of N to ecosystems (Bullock, 1992; Vitousek *et al.*, 2002; Jensen *et al.*, 2012; Iannetta *et al.*, 2016). Rhizobia infect root hair cells in the model legume *Medicago truncatula* by inducing and colonizing membrane invaginations called infection threads and they also simultaneously trigger cell divisions in several inner root layers which eventually form the nodule (Timmers *et al.*, 1999; Xiao *et al.*, 2014). Rhizobia propagate inside ramifying infection threads and are eventually released intracellularly into nodule cells surrounded by plant membranes where they initiate N-fixation (Oldroyd & Dixon, 2014). Genes of the symbiosis (Sym) pathway, including those involved in Nod Factor perception (*e.g.* NFP), signal amplification (*e.g.* DMI1, DMI2 and DMI3) and transcriptional regulation (*e.g.* NIN, NSP1 and NSP2), are central to *M. truncatula* nodule formation (Oldroyd & Dixon, 2014).

N-fixation is energetically and metabolically costly, however, and nodulation is favorable only when legumes grow in a low-N environment (Crawford *et al.*, 2000). To balance the carbon cost to the host with the benefits of N-fixation, nodule number and activity is carefully regulated by systemic mechanisms that involve the long-distance root-to-shoot movement of peptide hormones. Specific members of the CLE peptide family negatively regulate nodule number by a process called AON (Ferguson *et al.*, 2010; Mortier *et al.*, 2010; Reid *et al.*, 2011; Suzaki *et al.*, 2012; Djordjevic *et al.*, 2015; Shabala *et al.*, 2016; Kassaw *et al.*, 2017) and *C*-TERMINALLY ENCODED PEPTIDE (CEP) family members positively control nodule number (Delay *et al.*, 2013; Imin *et al.*, 2013; Mohd-Radzman *et al.*, 2015; Mohd-Radzman *et al.*, 2016). In CLE-dependent AON, the descending shoot-derived AON signal and the mechanism by which it restricts nodulation have not been conclusively identified. Current models show a gradual ramping up of the production of specific root-to-shoot AON-CLE peptides in maturing nodules, which trigger a return shoot-to-root AON signal that inhibits cortical cell divisions in younger nodules (Ferguson *et al.*, 2010; Reid *et al.*, 2011; Suzaki *et al.*, 2012; Kassaw *et al.*, 2017).

A specific subset of CLE peptides are involved in the induction of AON in *Lotus japonicus* (e.g. LjCLE-RS1, -RS2 and -RS3) (Okamoto *et al.*, 2013; Nishida *et al.*, 2016), *M. truncatula* (e.g. MtCLE12 and MtCLE13) (Mortier *et al.*, 2010; Saur *et al.*, 2011; Mortier *et al.*, 2012;

Kassaw *et al.*, 2017), soybean (e.g. GmRIC1, GmRIC2, GmNIC1 and GmNIC2) (Reid *et al.*, 2011) and common bean (e.g. PvNIC1, PvRIC1 and PvRIC2) (Ferguson *et al.*, 2014). Overexpressing AON-specific CLE genes suppresses nodule formation in wild-type (WT) plants (Mortier *et al.*, 2010; Okamoto *et al.*, 2013) whereas, this effect is abolished in the AON-CLE receptor mutants of *L. japonicus* (HAR1) (Nishimura *et al.*, 2002; Okamoto *et al.*, 2009), *M. truncatula* (SUNN) (Saur *et al.*, 2011; Mortier *et al.*, 2012; Kassaw *et al.*, 2017) and soybean (NARK) (Reid *et al.*, 2011). Tri-arabinosylation of the 13 amino acid LjCLE-RS2 peptide is required for binding to HAR1 (Okamoto *et al.*, 2013) and LjCLE-RS2 joins the xylem stream when overexpressed in soybean hairy roots (Okamoto *et al.*, 2013). Tri-arabinosylation of LjCLE-RS3 (Nishida *et al.*, 2016) or AON-CLE peptides in other organisms has not been biochemically demonstrated.

Genetic evidence supports the need for tri-arabinosylation of AON-CLEs in *M. truncatula*, since mutation of genes encoding a HPAT (Hydroxyproline-*O*-arabinosyl transferase) enzyme, called RDN1, which glycosylates specific hydroxyproline residues, leads to a super nodulation phenotype (Schnabel *et al.*, 2011). Nodulation levels in RDN1 mutants (e.g *rdn1-1* and *rdn1-2*) are intermediate between WT and *sunn-4* levels. In *M. truncatula*, Kassaw *et al*. (2017) presented genetic evidence that the HPAT, RDN1, modifies the MtCLE12 peptide before it travels shootward to interact with SUNN. The situation for MtCLE13, however, was not clear. Overexpression of *MtCLE13* led to a suppression of nodulation in WT and *rdn1-2* but not *sunn-4* mutants suggesting that either MtCLE13 was able to perform AON functions without the need for RDN1-dependent tri-arabinosylation or that another RDN gene was responsible for tri-arabinosylating MtCLE13 (Kassaw *et al.*, 2017). It is also not known if MtCLE12 and MtCLE13 are the only AON-CLEs or if tri-arabinosylation of other nodule-expressed CLE peptides can mediate AON.

Here, we combine the powerful tools of genetics with chemical synthesis to determine if glycosylation of three structurally-related and symbiosis-expressed CLE peptides (MtCLE12, MtCLE13 and MtCLE42) is required to induce AON in *M. truncatula in vivo*. We synthesised a series of MtCLE12, MtCLE13 and MtCLE42 peptides varying in structure only at proline at position 7 (P7) to determine whether chemical modifications of this amino acid was necessary to induce AON. Peptides were added to roots to determine if CLE peptides were capable of long distance movement to shoots. We also devised a plate-based system in which CLE peptides were applied to cotyledons which allowed the peptide to interact with shoot receptors directly to rapidly induce the shoot-to-root AON return signal. This enabled us to vary the time of peptide exposure to cotyledons with respect to addition of rhizobia to

roots to establish a kinetic analysis of AON induction. We examined the effects of AON in the root to determine what stage AON affects nodule development and how long AON inhibits the symbiosis. We also undertook genetic studies to determine if RDN1 and SUNN were required for CLE-induced AON.

Materials and Methods

Plant materials and synthetic peptides

M. truncatula WT (cv Jemalong genotype A17), *sunn-4* and *rdn1-1* seeds were germinated (Kusumawati *et al.*, 2008) and inoculated with *Sinorhizobium meliloti* WSM1022 (Holmes *et al.*, 2008), unless specified. MtCLE12, MtCLE13 and MtCLE42 structural variants were synthesised in-house using solid phase peptide synthesis (Corcilius *et al.*, 2017) with each having hydroxylation of proline 4 (P4) as a fixed modification. P7 was incorporated as either its hydroxylated, mono-arabinosylated or tri-arabinosylated form by using a suitable synthetic *N*-Fmoc-protected arabinosylated amino acid building block as we have reported previously (Corcilius *et al.*, 2017; Patel *et al.*, 2017). MtCLE12 and MtCLE13 peptides devoid of proline hydroxylation were synthesised by GL Biochem, Shanghai with 95% purity. All synthetic peptides were purified by reversed-phase HPLC and validated by analytical HPLC and both low- and high-resolution ESI-MS (Supporting Information Methods S1).

Sequence alignment

Sequence alignment for MtCLE42 and GmCLE32 proteins was done using MAFFT version 6 (Katoh *et al.*, 2005) and viewed by Jalview (mafft.cbrc.jp/alignment/server/).

Bioactivity of CLE peptides on root nodule number

Structural variants of the MtCLE12, MtCLE13 and MtCLE42 peptides were tested for AON by two methods: directly adding peptides to the roots via the growth medium (root assays) or diffusing them into cut cotyledons (cotyledon-feeding) (Okamoto *et al.*, 2013). For root assays, 1 d post germination (dpg) seedlings were transferred to 150 mm plates containing 50 ml of solid N-free Fåhraeus-medium plus or minus the peptides (1 μM) (Kusumawati *et al.*, 2008; Imin *et al.*, 2013). The medium was slanted at a 20° angle during setting. The roots were oriented to grow on the agar surface and the shoots did not contact the agar. At 4 dpg, the roots were inoculated with *S. meliloti* strain WSM1022 (Holmes *et al.*, 2008). Nodule number was scored at 14-d post inoculation (dpi).

For cotyledon-feeding, the distal end of cotyledons of 5-dpg seedlings were cut and dipped in peptide solution (1 µM) or water in a 250 µl Eppendorf tube fixed in place using double-sided tape. Regular topping-up ensured continuous solution-cotyledon contact. Unless specified, the plants were cotyledon-fed for 24 h before WSM1022 inoculation. Nodule number was scored at 14 dpi relative to the position of the root tip at the time of inoculation (Mohd-Radzman *et al.*, 2016).

Assessment of root hair curling and infection threads in the nodulation zone

The position of the root tip at the time of WSM1022 inoculation was marked and nodule initiation and root hair curling was observed in the nodulation zone at 4 dpi (Mohd-Radzman *et al.*, 2016). The addition of MtCLE13-TaP (1 µM) to cotyledons was varied from 1-d pre- to 3 d post-inoculation.

Agrobacterium rhizogenes-mediated hairy root transformation

M. truncatula RNA extraction and cDNA synthesis was performed as described earlier (Kusumawati *et al.*, 2008). The *CLE13* full-length open reading frame was amplified from cDNA and cloned into pK7WG2D (Karimi *et al.*, 2002). The primers used were as described by Saur *et al.* (2011). Transgenic roots were generated using *A. rhizogenes* strain *Arqua1* (Saur *et al.*, 2011) and identified by stereomicroscopic examination for presence of green fluorescent protein (GFP) (Saur *et al.*, 2011). Nodule number was assessed at 14 dpi.

Results and Discussion

AON by CLE peptides is dependent on tri-arabinosylation and key domain amino acid residues

Sequence alignment showed that MtCLE12, MtCLE13 and MtCLE42 encode related CLE domains that are, in turn, related to other legume AON-CLEs (Fig. 1a). These CLE peptide domains show most variability in amino acid composition at positions 5, 9, 10 and 12 (Fig. 1a). In addition, analysis of expression data showed that *MtCLE12*, *MtCLE13* and *MtCLE42* are induced during symbiosis in temporally-distinct patterns (Fig. S1) and therefore could be involved in early (MtCLE13), intermediate (MtCLE12 and MtCLE13) or potentially late AON-related-responses (MtCLE42) (Fig. S1) (de Bang *et al.*, 2017). The predicted domains of MtCLE12, MtCLE13 and MtCLE42 share amino acid similarity in key *N*-terminal positions (Fig. 1b). The amino acids at the *C*-terminal ends of MtCLE12, MtCLE13 and MtCLE42, however are more variable and there are only 2 amino acids (PQ) where MtCLE13 is unique (Fig. 1b). The amino acid differences between MtCLE13 compared to MtCLE12 or MtCLE42 are boxed in Fig. 1b. In addition, the MtCLE42 prepropeptide sequence (Medtr4g087850) is 50% identical to a soybean CLE peptide (GmCLE32; Glyma13g24026.1; Fig. S2), the predicted MtCLE42 and GmCLE32 domains are identical and GmCLE32 has been shown to be modified *in vivo* at P7 by tri-arabinosylation in separate studies in two soybean cultivars (Okamoto *et al.*, 2015; Patel *et al.*, 2017). Therefore, it would be expected that MtCLE42 would also be tri-arabinosylated *in vivo*. Hence, the MtCLE13, MtCLE12 and MtCLE42 domains share similar *N*-terminal amino acid residues and their overall sequence is similar to other known AON-CLEs. In addition, all three *Medicago* CLE genes are expressed during symbiosis and, therefore, may participate in AON.

To probe the effects of chemical modifications on the ability to trigger AON, 12 amino acid derivatives of MtCLE12, MtCLE13 and MtCLE42 were synthesised which were homogeneously hydroxylated at P4 but differentially modified at P7 by either hydroxylation, mono-arabinosylation or tri-arabinosylation. Additionally, unmodified MtCLE12 and MtCLE13 peptides were also tested. To assess AON responses, the peptides were assayed by directly applying them to roots (root assay) or to cotyledons (cotyledon-feeding assay) (Fig. 1c-e). Both MtCLE12-TaP and MtCLE13-TaP significantly inhibited nodule number (Fig. 1c, d), however, all variants of MtCLE42 peptides and all other MtCLE12 and MtCLE13 derivatives had no significant effect on AON (Fig. 1c-e). The fact that root exposure to MtCLE12-TaP or MtCLE13-TaP triggered a significant AON response suggested that these peptides are translocated to the shoot to interact with SUNN. It should be noted that the root assay was designed to prevent the shoots from contacting the medium containing the peptides. This measure prevented the possibility of unwanted direct activation of AON by direct contact of the shoot tissues with the peptides in the medium. The results also conclusively show that peptides with hydroxylation, mono-arabinosylation or no modification to P7 were unable to induce AON.

Both MtCLE12-TaP and MtCLE13-TaP induced AON when cotyledon-fed (Fig. 1c,d), supporting the hypothesis that these peptides interact with shoot-derived SUNN. Interestingly, exposing cotyledon to 1 µM MtCLE13-TaP completely suppressed nodulation on WT roots, whereas MtCLE12-TaP partially suppressed nodulation (Fig. 1f, g). A complete inhibition of nodulation was not observed with tri-arabinosylated *L*jCLE-RS2 peptides, even at 10 µM (Okamoto *et al.*, 2013). Normally, a 100% AON response is only observed when AON-CLE genes are constitutively expressed in WT transgenic roots (Mortier *et al.*, 2010; Saur *et al.*, 2011; Okamoto *et al.*, 2013; Kassaw *et al.*, 2017). Therefore, the results collectively showed that TaP was necessary for both MtCLE12 and MtCLE13 to induce AON. In addition, the variation in amino acids, especially at positions 8-10 and 12 most likely explains why MtCLE13-TaP (D_8PQHN_{12}), MtCLE12-TaP (N_8HIHN_{12}) and MtCLE42-TaP (D_8AHHH_{12}) had strong, weak and no AON activity, respectively (Fig. 1b-e). Therefore, it is likely that amino acids at positions 8-10 and 12, as well as tri-arabinosylation of P7, collectively affect interactions of AON-CLEs with their cognate receptor, SUNN. These results also demonstrated that a 1-d pre-exposure of MtCLE13-TaP to cotyledons was sufficient to induce a complete AON response in WT roots.

MtCLE12-TaP and MtCLE13-TaP induce AON in *rdn1-1* but not in *sunn-4*

We examined the effect of MtCLE12 and MtCLE13 structural variants on AON in a mutant defective in *RDN1*, which encodes a HPAT, and in a mutant defective in *SUNN*, which encodes the AON-CLE receptor. Genetic evidence (Kassaw *et al.*, 2017) supports RDN1 catalysing the transfer of L-arabinose from the sugar donor UDP-β-L-Ara*f* to a hydroxyl group on MtCLE12 (most likely to P7) in the root and that RDN1 acts upstream of SUNN. Therefore, if perception of both MtCLE12 and MtCLE13 by SUNN is TaP-dependent, both peptides would be expected to induce AON in *rdn1-1*, but not in the SUNN null mutant, *sunn-4*. The results showed that both MtCLE12-TaP and MtCLE13-TaP, but not any other structural variants, induced AON in *rdn1-1* (Fig. 2a). Consistent with the results with WT plants (Fig. 1c,d), MtCLE13-TaP abolished nodulation in *rdn1-1* whereas MtCLE12-TaP significantly reduced nodulation (Fig. 2a,b). In addition, both MtCLE12-TaP and MtCLE13-TaP were unable to induce AON in *sunn-4* (Fig. 2c,d). These results demonstrate that TaP is required at P7 for SUNN to perceive both MtCLE12 and MtCLE13 and that these TaP modifications can override the need for a functional RDN1 or other HPATs.

MtCLE13 peptide is likely to be tri-arabinosylated by another RDN

Kassaw *et al.* (2017) demonstrated that *MtCLE12* overexpression did not impart AON in the *rdn1-2* background but *MtCLE13* overexpression was still capable of imparting AON. They concluded that MtCLE13 acted either as a non-TaP peptide or that another RDN was responsible for modifying MtCLE13. To help distinguish between these two possibilities, we overexpressed *MtCLE13* in WT A17, *rdn1-1* and *sunn-4*. The results show that *MtCLE13* overexpression strongly suppressed nodulation in A17 and *rdn1-1*, but not in *sunn-4* (Fig. S3) and, therefore, *MtCLE13* acts independently of *RDN1*. This data is consistent with previous findings where another RDN1 allele, *rdn1-2*, was used (Kassaw *et al.*, 2017). Since MtCLE13-TaP requires SUNN to impart AON, a likely explanation for the collective results is that the TaP modification of MtCLE13 is mediated through the activity of another HPAT. Kassaw *et al.* (2017) showed that MtRDN2, but not MtRDN3, can complement *rdn1-2* when over-expressed using the 35S promoter, but not when expressed from the native RDN1 promoter, suggesting that RDN2 has similar enzymatic activity to RDN1. This also suggests that *RDN1* and *RDN2* have distinct expression patterns/functions in *M. truncatula* or else *rdn1-1* would not have a hyper-nodulation phenotype. Combining our results with those of Kassaw *et al.* (2017), we propose that different AON circuits exist where different HPATs independently tri-arabinosylate MtCLE12 and MtCLE13. Finally, we conclusively show that

perception of MtCLE12 and MtCLE13 peptides by SUNN in *M. truncatula* is entirely dependent on the TaP-modification and the presence of appropriate *C*-terminal amino acid residues (Fig. 1b).

Kinetic analysis of AON induced by cotyledon-fed MtCLE13-TaP

A 1-d pre-exposure of WT or *rdn1-1* cotyledons to MtCLE13-TaP completely inhibited root nodulation by *S. meliloti* WSM1022 (Figs 1d, 2b). To explore this result further, we examined the cellular responses of root cells to rhizobia in the nodulation zone of WT plants when MtCLE13-TaP was applied to cotyledons at different times relative to the position of rhizobial inoculation (white arrows in Fig. 3a). There was no detectable root or cellular response to rhizobia when MtCLE13-TaP exposure to cotyledons started 1 d before rhizobial inoculation (Fig. 3a,c). This observation is contrary to current models suggesting that AON inhibits cortical cell division only (Ferguson *et al.*, 2010; Reid *et al.*, 2011; Suzaki *et al.*, 2012; Kassaw *et al.*, 2017). To gain further insights, we exposed WT cotyledons to MtCLE13-TaP at between 1 before 3 d after rhizobial inoculation and compared this to a water-fed control (Fig. 3b–e). The results showed that in the 0, 1 and 2 dpi samples, MtCLE13-TaP still induced significant AON and very few to no nodules or cortical cell divisions were observed in the nodulation zone, although root hair curling was observed (Fig. 3a, d). With a 3-d delay in MtCLE13-TaP application, however, nodule number returned to near WT levels in the nodulation zone (Fig. 3a,e). By contrast, although CLE12-TaP induced significant AON under the same conditions, it did not completely inhibit root hair curling or nodule formation (Fig. S4). We conclude that the AON response induced by MtCLE13-TaP addition 1 d before rhizobial inoculation completely inhibits the nodulation pathway, not just the progression of cortical cell divisions. In addition, CLE13-TaP-induced AON inhibited nodule development for up to 2 d, but not 3 d, post rhizobial inoculation. At 2 dpi, root hair curling, infection thread formation and the earliest inner cortical, endodermal and pericycle cell divisions would be occurring (Timmers *et al.*, 1999; Xiao *et al.*, 2014). At 3 dpi, infection threads penetrate to the outer cortical cells and nodule primordia are more progressed (Xiao *et al.*, 2014). We propose that 3 dpi nodules pass a critical stage and become AON-insensitive.

An updated model for MtCLE12- and MtCLE13-dependent AON

We present an updated model for AON (Fig. 4). First, *MtCLE12*, *MtCLE13* and MtCLE42 are induced during nodule initiation and formation with distinct temporal patterns. Although *MtCLE12* and *MTCLE13* induction is Sym pathway dependent, it is unclear what role MtCLE42 plays as it has no demonstrable function in AON. *MtCLE13* expression may also have a dependency on CRE1 (Mortier *et al.*, 2012). Second, the data support that RDN1 and another RDN (most likely RDN2) independently tri-arabinosylate MtCLE12 and MtCLE13 peptides, respectively (Kassaw *et al.*, 2017) and this is one prerequisite for AON. The 12-amino-acid MtCLE13-TaP product is a far more potent AON-inducing peptide than the LjCLE-RS2/RS3 (Okamoto *et al.*, 2013) and MtCLE12-TaP peptides. Third, we propose that MtCLE12-TaP and MtCLE13-TaP travel to the shoot via the xylem stream to interact with SUNN. Fourth, particular amino acid residues, especially those at positions 8-10, 12 (and possibly 5) also strongly influence MtAON-CLE peptide interactions with SUNN since MtCLE42-TaP is AON-inactive and MtCLE12-TaP has considerably weaker activity than MtCLE13-TaP. Fifth, the kinetic experiments show that, with sufficient prior triggering of AON, the shoot-to-root AON signal can very rapidly induce root responses that can completely inhibit all symbiotic interactions including rhizobial infection, not just cortical cell divisions. Therefore, we propose that the shoot AON signal is capable of rapidly suppressing all symbiotic processes. We show that a delay in MtCLE13-TaP application to the cotyledons enables progressively more symbiotic interactions to occur but even at 2 d after rhizobial inoculation, cotyledon-fed MtCLE13-TaP can still induce significant AON. Finally, a 3-d delay in MtCLE13-TaP application allows nodules to progress to a developmental point where they become AON-insensitive.

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Author contributions

N.I. initiated the work and wrote manuscript. N.P. conducted experiments, prepared figures and edited manuscript. L.C. synthesised (glyco)peptides and edited manuscript. R.J.P. critically reviewed the manuscript. M.A.D. conceptualised the research and wrote manuscript. N.I. and N.P. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Kinetic analysis of *MtCLE12*, *MtCLE13* and *MtCLE42* expression by RNA-seq.

Fig. S2 Sequence alignment of MtCLE42 and GmCLE32 proteins.

Fig. S3 The inhibitory effect of constitutive expression *MtCLE13* in *rdn1*-1 and *sunn-4* lines.

Fig. S4 Effect of MtCLE12-TaP on root symbiotic responses when added to cotyledons 1 d before rhizobial inoculation**.**

Methods S1 Analytical data for MtCLE12, MtCLE13 and MtCLE42 (glyco)peptides.

Fig. 1 Structure-dependent inhibition of root nodulation by MtCLE peptides in *Medicago truncatula*. (a) The predicted CLE domain sequences and structure-conservation of known autoregulation of nodulation (AON)-CLE peptides: MtCLE12, MtCLE13, MtCLE42, GmNIC1, GmNIC2, LjCLE-RS1/2, GmRIC1, LjCLE-RS3 and LjCLE40. (b) The domain sequences of MtCLE12 and MtCLE42 are compared to the domain sequence of MtCLE13. The differential amino acids are presented in red within the boxes. (c–e) Mean nodule number induced by structural variants of MtCLE12 (c), MtCLE13 (d) and MtCLE42 (e) added using the root or cotyledon-feeding assays ($n \geq 12$). The structural variants of each of the three MtCLE peptides used in this study were uniformly hydroxylated on P4 but differently modified on P7. Hydroxy: hydroxyproline; MaP: mono-arabinosylated hydroxyproline; TaP: tri-arabinosylated hydroxyproline. Unmodified variants of MtCLE12 and MtCLE13 were also used. Statistically significant differences were determined using a Student's *t*-test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (error bars, ± SE). (f, g) Comparison of water control vs MtCLE13-TaP AON response on *M. truncatula* roots.

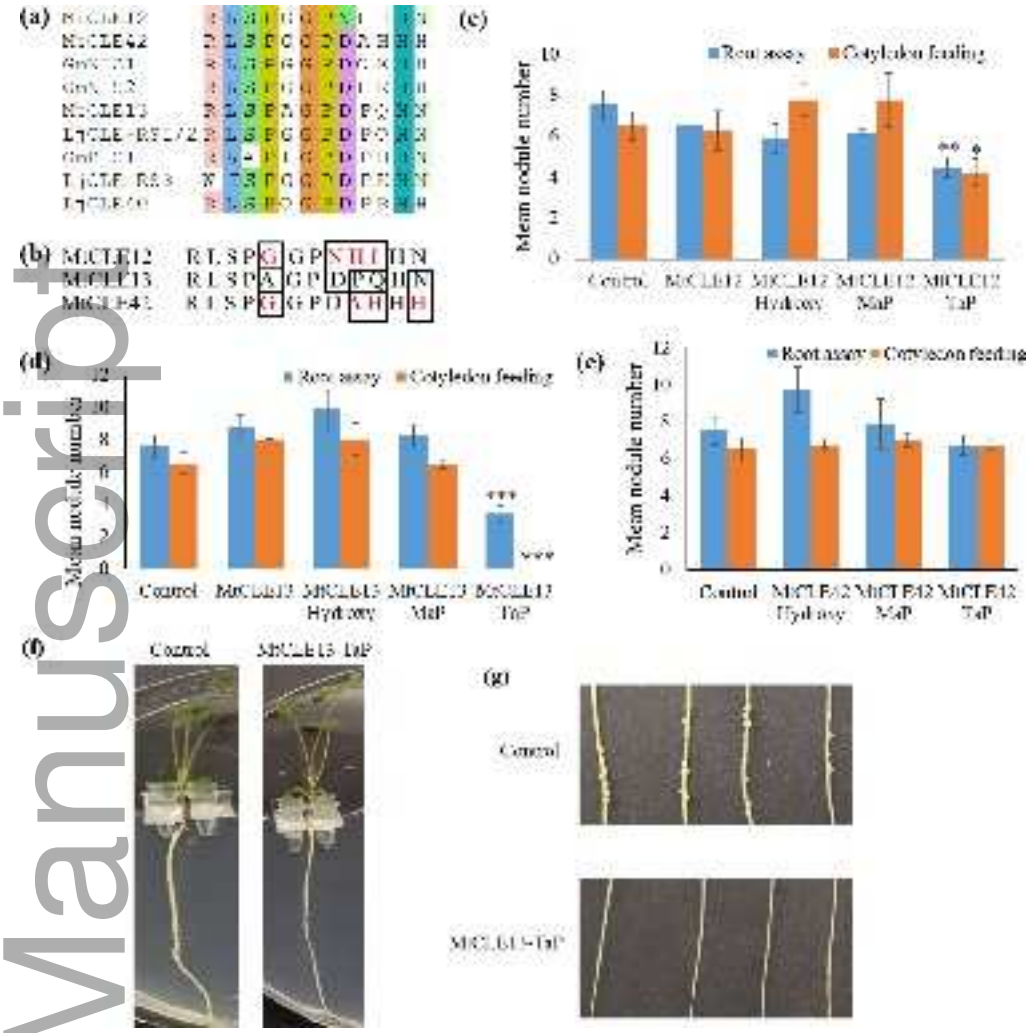
Fig. 2 Autoregulation of nodulation activity of MtCLE12-TaP and MtCLE13-TaP peptides in *Medicago truncatula rdn1-1* and *sunn-4* mutant lines. (a, c) Mean nodule number of (a) *rdn1-1* and (c) *sunn-4* plants root-treated or cotyledon-fed with different MtCLE12 and MtCLE13 peptides. (b, d) Comparison of control and MtCLE13-TaP-treated *rdn1-1* (b) and *sunn-4* (d) roots. Statistically significant differences were determined using a Student's *t*-test: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (error bars, ± SE).

Fig. 3 A kinetic analysis of autoregulation of nodulation activity in the roots of cotyledon-fed MtCLE13-TaP in *Medicago truncatula*. (a) Nodulation is suppressed on roots of plants where MtCLE13-TaP is applied at between 1 before 2 d after rhizobial inoculation. By contrast, a 3-

d delay in MtCLE13-TaP application allowed progression of nodule development in the nodulation zone. White arrows indicates the position of the root tip at the time of inoculation. (b–e) Microscopic examination of methylene blue stained roots at 4 d post inoculation (dpi). (b) Control: cotyledons-fed with water 1-d before rhizobial inoculation have nodule primordia and curled or infected root hairs. (c) Cotyledons were fed with MtCLE13-TaP 1 d prior (-1 d) to rhizobial inoculation. The nodulation zone has no sign of nodule primordia, root hair curling or infections. (d) A representative image of a plant root is shown reflecting the response to MtCLE13-TaP addition to cotyledons at either 0, 1 or 2 d post rhizobial inoculation (actual image is at day 2). Root hair curing and infection thread formation was observed in these samples (red arrows) but there were no or very few nodule primordia. (e) The nodulation zone of plants where MtCLE13-TaP was added to cotyledons 3 d post rhizobial inoculation showed several developing nodule primordia with significant root hair curling and infection activities (red arrows). Bars, 500 µm.

Fig. 4 An updated model for autoregulation of nodulation (AON) control of root nodulation in *Medicago truncatula*. *MtCLE12*, *MtCLE13* and *MtCLE42* are expressed through Sym pathway-dependent circuits and are differentially expressed temporally. In addition, it has been reported that *MtCLE13* is induced in young nodules by a 6-Benzylaminopurine (BAP)-induced and CYTOKININ RESPONSE1/NODULE INCEPTION (CRE1/NIN) dependent circuit (Mortier *et al*., 2012). The biological significance of this observation is not clear and these have not been included in this model. Processing of MtCLE12 and MtCLE13 is likely to occur in the Golgi of nodule primordium cells (Kassaw *et al.,* 2017) but how exactly MtCLE12 and MtCLE13 peptides are processed from the propeptide remains unknown. MtCLE12-TaP is produced depending on ROOT DETERMINED NODULATION1 (RDN1) activity whereas MtCLE13-TaP is modified independently of RDN1 (possibly through RDN2). The putative RDN modifying MtCLE42 is not known. The dissolution of the endodermis during early primordium formation would be a key step to enable MtCLE12-TaP and MtCLE13-TaP to join the xylem stream in the stele or else the Casparian strip would prevent access of the extracellular peptides (Doblas *et al.,* 2017; Nakayama *et al.,* 2017). Once the endodermis is breached early in nodule formation, the MtCLE12-TaP and MtCLE13-TaP peptides would then be able to join the xylem stream to be carried shootward. How long this takes is not known. The TaP modifications and different amino acid composition at positions 8–10, 12 and possibly 5 enable effective but distinct interactions of

MtCLE12-TaP and MtCLE13-TaP with the receptor, SUPER NUMERIC NODULES (SUNN). The actual direct interactions between MtCLE12-TaP and MtCLE13-TaP with SUNN in the shoot remain to be determined experimentally, however, the interaction of MtCLE42-TaP with SUNN appears to be negatively influenced by an inappropriate amino acid composition compared to MtCLE12 and MtCLE13. The shoot-to-root AON signal induced by MtCLE13TaP can inhibit the entire nodule development process between -1 and 2 dpi so that no nodules form, but by 3 dpi nodule development becomes AON-insensitive. NF: Nod Factors; NFP: NOD FACTOR PERCEPTION; NSP: NODULATION SIGNALING PATHWAY; DMI: DOES NOT MAKE INFECTION. Flat ended line, inhibition of further nodulation is mediated through the downward AON signal generated after the interaction of the AON peptide with SUNN; red cross, CLE42-TaP peptide does not interact with SUNN; red question marks, the mechanisms are not fully understood; solid arrows, the pathway is already known; dashed arrows, the pathway is not known.



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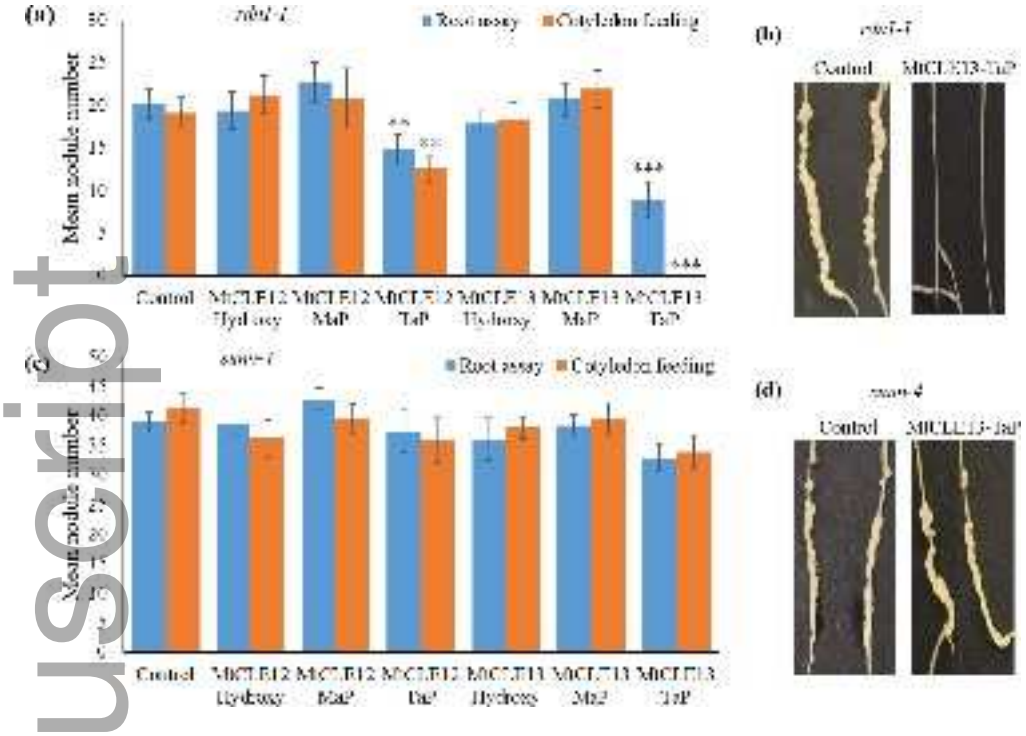

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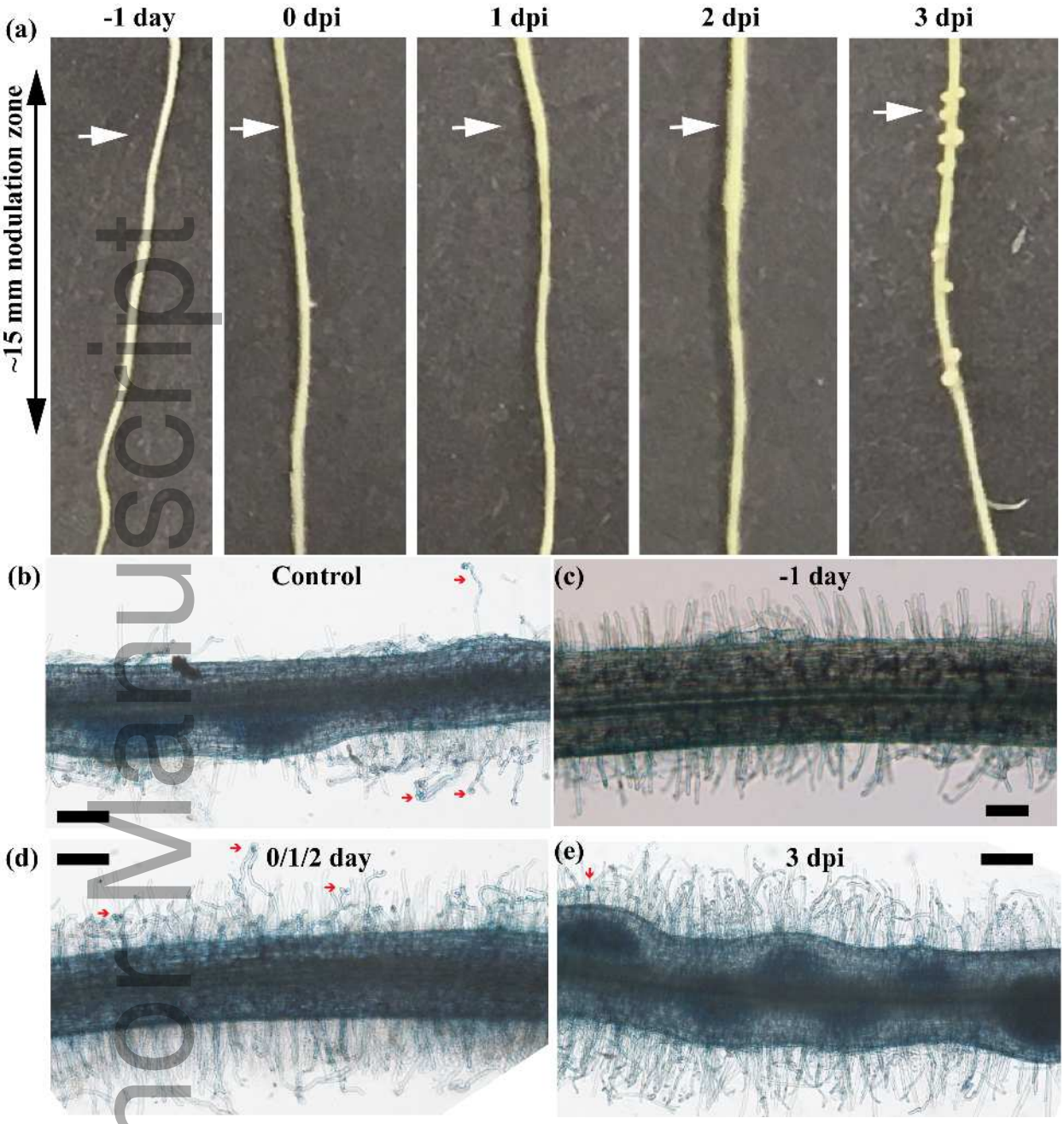

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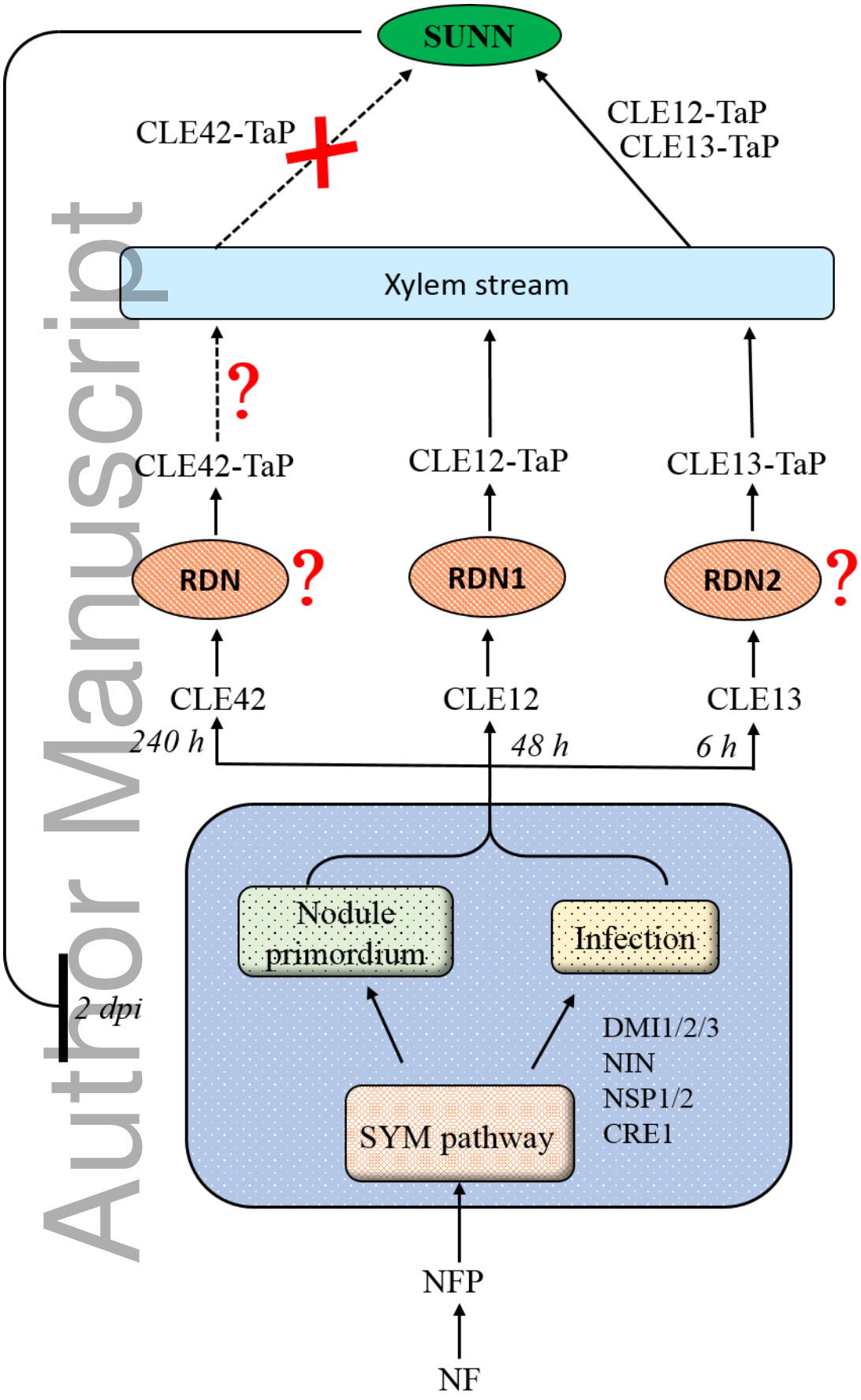

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