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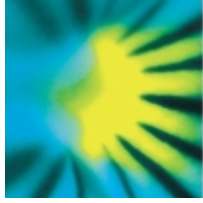
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NLP1 binds the CEP1 signaling peptide promoter to repress its expression in response to nitrate

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Manuscripts

1 **NLP1 binds the CEP1 signaling peptide promoter to repress its expression in response to**
2 **nitrate**

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10

11 Nitrogen (N) is an essential macronutrient for plant growth and development, which is often
12 growth-limiting in agricultural systems. Nitrate is not only the main source of N for most plants, but
13 also acts as a molecular signal to regulate gene expression and root development (Liu *et al.*, 2015).
14 As immobile organisms, plants cannot choose their surrounding environment but have to adapt.
15 Therefore, plants have evolved sophisticated strategies for N acquisition in relation to sources
16 availability, to coordinate plant growth and development in response to a fluctuating external supply
17 (Oldroyd & Leyser, 2020). Regulatory mechanisms for nitrate acquisition comprise both local and
18 systemic long-distance signaling pathways that inform on the intrinsic nutritional status across the
19 whole plant (Alvarez *et al.*, 2012; Bellegarde *et al.*, 2017; Gautrat *et al.*, 2021).

20 Legume plants can symbiotically associate with rhizobia soil bacteria to form root nodules
21 and fix atmospheric N₂ for the benefit of the host plant. This is however an energy-consuming
22 process that is thus inhibited by the presence of mineral N in soils, including nitrate and ammonium
23 (Streeter & Wong, 1988; Carroll & Mathews, 1990). Studies in the model legume *Medicago*
24 *truncatula* and *Lotus japonicus* revealed that the NIN (Nodule Inception)-like proteins, NLP1 and
25 NLP4, are critical for the nitrate inhibition of nodulation (Lin *et al.*, 2018; Nishida *et al.*, 2018;
26 Nishida *et al.*, 2021). Nitrate triggers MtNLP1 translocation from the cytosol to the nucleus, where
27 MtNLP1 interact with MtNIN to repress target gene expression (Lin *et al.*, 2018). In *L. japonicus*,
28 the related LjNLP4 transcription factor binds the promoter of the *LjCLE-RS2* (*CLAVATA-Like Root*
29 *Signal 2*) signaling peptide encoding gene on Nitrate Responsive Elements (NREs) to promote its
30 expression, leading to a systemic repression of nodulation through the shoot LjHAR1
31 (Hypernodulation and Aberrant Roots) receptor (Nishida *et al.*, 2018). A similar mechanism is
32 conserved in *M. truncatula*, where MtNLP1 promotes the expression of the *MtCLE35* related gene,
33 through a direct binding to its promoter on evolutionary conserved NREs (Luo *et al.*, 2021; Mens
34 *et al.*, 2021; Moreau *et al.*, 2021).

35 In response to nitrate deficiency, the expression of another family of signaling peptides is
36 induced, the C-terminally Encoded Peptides (CEPs), which in *Arabidopsis* act systemically from
37 roots to shoots through the CEPR1 and CEPR2 receptors (Tabata *et al.*, 2014). In *M. truncatula*,
38 overexpression of MtCEP1 or exogenous applications of MtCEP1 peptides both negatively
39 regulates lateral root formation and promotes nodulation through the MtCRA2 (Compact Root

40 Architecture 2) receptor, closely related to AtCEPR1/2 (Imin *et al.*, 2013; Huault *et al.*, 2014; Mohd-
 41 Radzman *et al.*, 2016; Laffont *et al.*, 2019; [Gautrat *et al.*, 2020](#)). Knowing that the expression of
 42 most of *MtCEP* genes is promoted by a N deficiency, we hypothesized that, in addition to potential
 43 yet unknown N-deficiency regulations that would promote *CEP* gene expression, MtNLP1 may be
 44 involved in the suppression of their expression in response to nitrate. [In this study, we indeed](#)
 45 [demonstrate using different complementary approaches that MtNLP1 binds to the MtCEP1](#)
 46 [promoter through specific half NRE \(hNRE\) motifs to repress its expression in presence of nitrate.](#)

47 [As a first step](#), we analyzed whether the MtCEP1 promoting effect on nodulation was affected
 48 in the *nlp1* mutant. To this aim, we applied synthetic MtCEP1 peptides in wild-type and *nlp1* mutant
 49 plants and quantified nodule number in the absence or presence of nitrate. [Whereas the *nlp1* mutant](#)
 50 [nodulation was less sensitive to the nitrate inhibition than the wild-type nodulation,](#) MtCEP1
 51 peptides similarly increased nodule number in wild-type and *nlp1* mutant roots either in the absence
 52 or in the presence of nitrate (Fig. 1a and b). [This suggests](#) that MtCEP1 may act downstream of
 53 MtNLP1 to modulate nodule number [according to](#) nitrate availability.

54 To investigate how *MtCEP1* expression is repressed by nitrate ([Imin *et al.*, 2013](#)), we tested
 55 whether the nitrate-induced suppression of *MtCEP1* expression was dependent on MtNLP1. RT-
 56 qPCR analysis of *MtCEP1* transcript levels in *nlp1* mutants grown with or without nitrate first
 57 revealed slightly lower levels in *nlp1* compared to wild-type roots in the absence of nitrate (Fig. 1c).
 58 Interestingly, the nitrate repression of *MtCEP1* in wild-type roots was not observed in *nlp1* mutant
 59 roots (Fig. 1c). Similar results were obtained for the nitrate repression of other *CEP* genes, with
 60 *MtCEP2*, *MtCEP6* and *MtCEP7* being fully dependent on MtNLP1, whereas *MtCEP4* only partially,
 61 [and *MtCEP5* expression being even higher in *nlp1*](#) (Fig. S1). This result suggests that MtNLP1 is
 62 required for the nitrate-induced suppression of the expression of different *CEP* genes, [and that other](#)
 63 [NLPs \(e.g. NLP4\) \(Lin *et al.*, 2018\)](#), or even other unknown pathways, are involved. To further
 64 validate that nitrate represses *CEP1* gene expression [through](#) MtNLP1, *CEP1* transcript levels [were](#)
 65 analyzed in roots overexpressing MtNLP1 (pUb-NLP1) and treated with KCl, as a control, or KNO₃,
 66 10 mM each. First, the expression of *MtCEP1* was increased in pUb-NLP1 control roots in the
 67 absence of nitrate; and second, the repression of *MtCEP1* expression by nitrate was stronger in
 68 MtNLP1 overexpressing roots compared to control roots (Fig. 1d). To independently validate the
 69 link between MtNLP1 and *MtCEP1* expression, a *pCEP1-GUS* transcriptional fusion was expressed
 70 in wild-type or *nlp1* mutant roots. Nitrate suppressed the GUS signal in wild-type roots whereas it
 71 was barely acting in *nlp1* mutant roots (Fig. 1e). These results independently demonstrated that
 72 MtNLP1 is required for the nitrate repression of at least *MtCEP1* expression.

73 [As a next step](#), we wanted to determine if *MtCEP1* could be directly repressed by [the](#) MtNLP1
 74 [transcription factor](#). We first used a dexamethasone (DEX) inducible system, where MtNLP1 is
 75 fused to the glucocorticoid receptor (NLP1-GR) and constitutively expressed thanks to a ubiquitin
 76 promoter, to transiently induce MtNLP1 expression in *M. truncatula* roots. The expression of
 77 *MtCEP1* was reduced within 4 h of dexamethasone (DEX) treatment, followed by a steady reduction
 78 in transcript levels after a 24 h treatment, indicating that MtNLP1 expression is sufficient on its own

79 to repress *MtCEPI* expression. Combining DEX together with the cycloheximide (CHX) protein
 80 synthesis inhibitor did not suppress the DEX-inducible expression (Fig. 2a), demonstrating that *de*
 81 *novo* protein synthesis was not required for the repression of *MtCEPI* expression. This result
 82 suggests that MtNLP1 might indeed directly target the *MtCEPI* promoter to repress its expression.
 83 A dual-luciferase reporter assay was then used in *Nicotiana benthamiana* leaves to further analyze
 84 whether NLP1 was sufficient to repress the *MtCEPI* transcriptional activity. The co-expression of
 85 a *pCEPI-LUC* transcriptional fusion with a *p35S-NLPI* or an EV (empty vector), as a control,
 86 revealed that MtNLP1 can repress the activity of the *MtCEPI* promoter in response to nitrate (Fig.
 87 2b). A *CEPI* promoter deletion analysis was then subsequently carried out using this dual-LUC
 88 assay in *N. benthamiana* leaves. The MtNLP1 repression of *MtCEPI* expression was abolished
 89 when a 220 bp fragment (-1674~ -1426) of the promoter was deleted (Fig. 2c, d).

90 As NLPs were shown to bind NRE *cis*-elements in the promoter of different nitrate-inducible
 91 genes (Konishi & Yanagisawa, 2013; Soyano *et al.*, 2015; Nishida *et al.*, 2018), we then searched
 92 NRE **motifs** in this 220 bp region (-1674 ~ -1426 bp) of the *MtCEPI* promoter. A NRE motif was
 93 identified (Fig. 2e, f), and when comparing its sequence with those previously identified in
 94 promoters activated by NLPs (Nishida *et al.*, 2018; Moreau *et al.*, 2021), we observed that this
 95 *CEPI* promoter NRE motif associated to a MtNLP1 repressive activity was shorter than for
 96 upregulated genes (e.g. NREs in *LjCLE-RS2* and *MtCLE35* promoters; Nishida *et al.*, 2018; Luo *et*
 97 *al.*, 2021), corresponding to only half of the initially described palindromic motif (Fig. 2e). Similar
 98 hNRE **motifs** were present in promoters of other *M. truncatula* *CEP* genes repressed by MtNLP1,
 99 and even in *CEP* gene promoters from *Arabidopsis* (Fig. S2), suggesting an evolutionary
 100 conservation. We then performed a Chromatin Immunoprecipitation (ChIP) assay to test whether
 101 this region is enriched for MtNLP1 binding. A MtNLP1-GFP translational fusion was expressed in
 102 *M. truncatula* roots, and using a GFP antibody, an enrichment for MtNLP1 binding was detected in
 103 the region of the *MtCEPI* promoter containing this hNRE motif (Fig. 2g). We additionally showed
 104 that MtNLP1 binds the *MtCLE35* promoter in two regions containing full size palindromic NREs
 105 (Fig. 2e-g), in agreement with previous results obtained in *L. japonicus*, knowing that *MtCLE35* is
 106 most closely related to *LjCLE-RS2* (Luo *et al.*, 2021; Mens *et al.*, 2021; Moreau *et al.*, 2021). This
 107 *MtCLE35* result can thus be considered as a positive control for the MtNLP1 ChIP assay. The
 108 binding of MtNLP1 to the hNRE within the *MtCEPI* promoter was additionally examined using a
 109 yeast one-hybrid assay. In this heterologous system, MtNLP1 indeed binds to the region containing
 110 the hNRE1 motif, and its deletion (Δ hNRE, -8 bp) is sufficient to reduce this MtNLP1 binding
 111 activity (Fig. 2h). Finally, a deletion of this hNRE *cis*-element in the *pCEPI*:GUS transcriptional
 112 fusion (*pCEPI* $_{\Delta$ hNRE-GUS) revealed in *M. truncatula* roots that the nitrate repression of the
 113 *pCEPI*:GUS activity became barely detectable (Fig. 2i, j).

114 Overall, we can now propose a refined model (Fig.2k) by combining these new results with
 115 previous studies, showing that MtNLP1 not only activates *MtCLE35* expression to mediate the
 116 nitrate inhibition of nodulation (Luo *et al.*, 2021; Moreau *et al.*, 2021) and this study, but also acts
 117 as a repressor by binding the promotor of *CEP* genes to inhibit their expression in the presence of

118 nitrate, at least partially explaining the higher expression levels of *CEP* genes in N deficiency.
 119 MtNLP1 thus acts as a bifunctional transcription factor, and interestingly, instead of the classical
 120 full size NREs found in nitrate/MtNLP1 activated genes, hNRE motifs were retrieved in the
 121 promoter of *MtCEP* genes that are repressed by MtNLP1. Some other transcription factors with
 122 such a bifunctional transcriptional activity exist, like WUSCHEL that acts both as a transcriptional
 123 repressor and as an activator according to the tissues considered (Ikeda *et al.*, 2009). Within the
 124 NLP family, AtNLP7 was proposed to act both as a transcriptional activator and as a repressor,
 125 potentially through the recruitment of different partners (Marchive *et al.*, 2013). Accordingly,
 126 LjNLP1/4 could repress the expression of symbiotic-specific genes such as *NF-YA/B*, *EPR3*, or
 127 *RinRK1* (Nishida *et al.*, 2021). The NIN symbiosis specific transcription factor has both positive
 128 and negative functions in nodulation, and is active in different root tissues required for nodulation
 129 (epidermis, cortex, pericycle), which opens the possibility of a differential transcriptional activity
 130 according to cell types and recruited partners (Marsh *et al.*, 2007; Liu *et al.*, 2019).

131 Half NRE motifs were identified in different *CEP* gene promoters both in *M. truncatula* and
 132 in Arabidopsis, suggesting an evolutionary conservation of the NLP-dependent repression of these
 133 signaling peptide encoding genes. Recently, preferential binding motifs were proposed for nitrate
 134 related NLPs *versus* the symbiosis specific NIN family member (Nishida *et al.*, 2021). We now
 135 highlight here that binding motifs may be different according to the active *versus* repressive action
 136 of the NLP transcriptional complex. Besides such differential activating or repressing NLP binding
 137 motifs, the recruitment of partners and/or of diverse NLP post-translational modifications are likely
 138 involved. Interestingly, the *CEP7* promoter does not contain any hNRE motif, but has instead a NIN
 139 binding site which can be activated by NIN, as previously characterized in (Laffont *et al.*, 2020).
 140 Indeed, *CEP7* is a very specific member of the *CEP* gene family which expression is induced by
 141 rhizobium through the NIN transcription factor. This suggests that the *CEP7* repression by nitrate
 142 is similar as for the regulation of other rhizobia-inducible NIN target genes, e.g. *EPR3*, *NF-YA*, *NF-*
 143 *YB*, which are repressed by nitrate in an NLP1-dependent manner through an interaction with the
 144 rhizobium-induced NIN transcription factor (Lin *et al.*, 2018; Nishida *et al.*, 2021). In Arabidopsis,
 145 it was recently showed that an HB11 (Homolog of Brassinosteroid enhanced expression2 interacting
 146 with IBH1) - TCP20 (Teosinte Branched 1, Cycloidea, PCF (TCP)-domain family protein 20)
 147 transcription factor complex positively regulates *CEP* gene expression in Arabidopsis (Chu *et al.*,
 148 2021), and interestingly, TCP20 can also interact with NLP6/7 to mediate N availability responses
 149 (Guan *et al.*, 2014). It will be thus interesting in the future to identify such additional NLP partners
 150 than NIN, as well as potential post-translational modifications, ideally at a cell-type specific level,
 151 to have a detailed mechanistic understanding of their regulatory functions in legume nitrate
 152 responses, including the regulation of symbiotic nodulation.

153

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162

163 **Authors contributions**

164 FX, ZL and FF designed the work, analyzed data and drafted the manuscript; ZL and JW performed
165 most experiments and analyzed data; CM performed the ChIP-qPCR experiments,
166 contributed to Fig. S1 and Fig. S2, and analyzed the corresponding data.

167

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246

247

248 **Supporting Information**

249

250 **Material and Methods**

251

252 **Fig. S1.** MtNLP1 is required for the nitrate suppression of *M. truncatula* *CEP* gene expression.

253

254 **Fig. S2.** Alignment of putative NRE motifs in the promoter of *CEP* genes.

255

256 **Table S1.** Primer sequences used in this study

257

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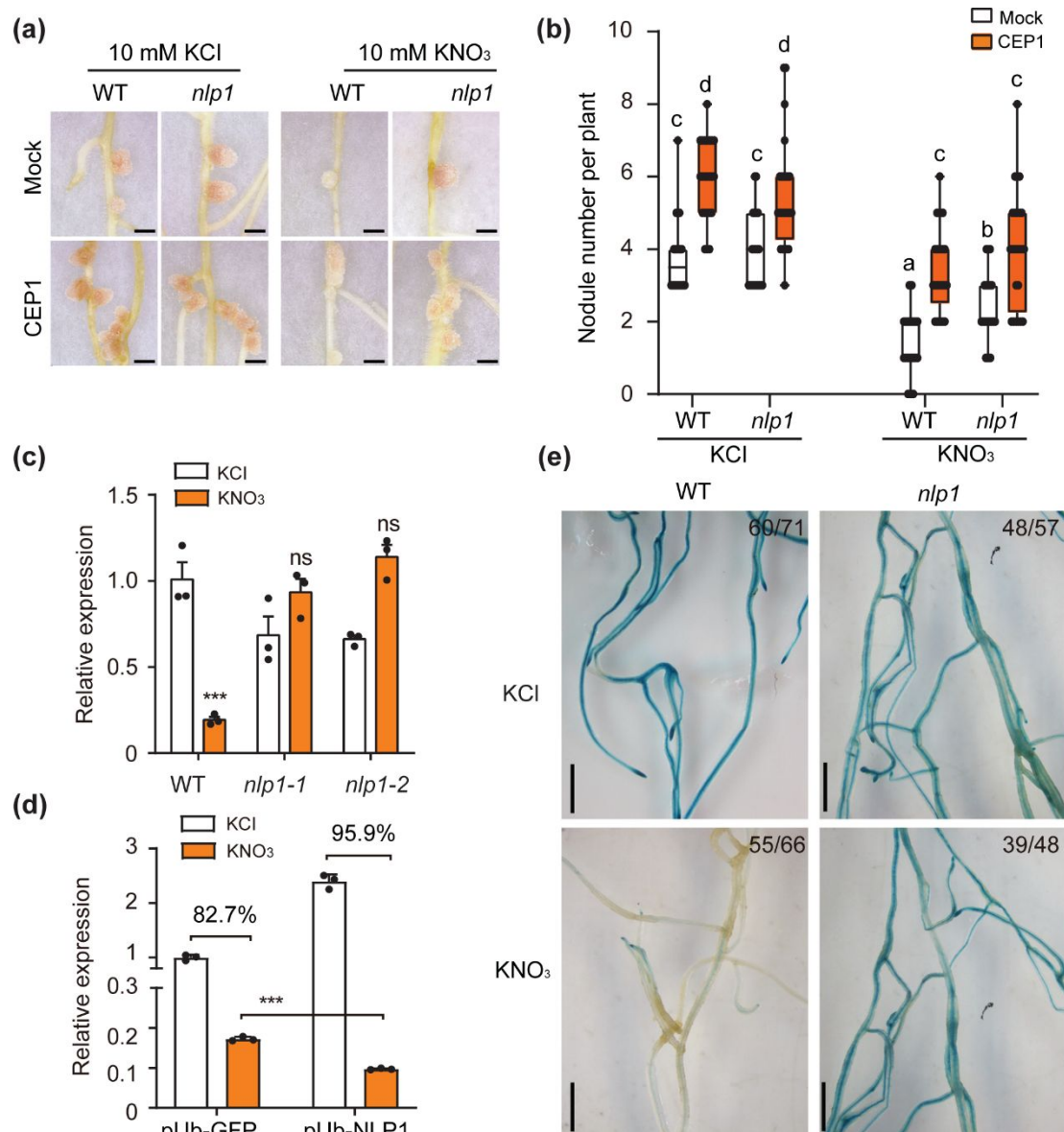


Fig. 1. Nitrate represses *MtCEP1* expression depending on *MtNLP1*. (a and b) Nodule phenotype (a) and total nodule number (b) in wild-type (R108) and *nlp1* mutants treated with *MtCEP1* domain 1 peptides with hydroxyprolines (HyP) 4 and HyP11 modifications, or with deionized water (Mock) in the absence or presence of nitrate (10 mM KNO₃) (n =20–30 plants). Different letters in (b) indicate statistically significant differences (P <0.05, two-way ANOVA followed by a post hoc Tukey's test). (c) Relative expression of *MtCEP1* in roots of wild-type (R108) and *nlp1* mutants in response to nitrate (10 mM KNO₃). (d) Relative expression of *MtCEP1* in Empty Vector (EV) control (pUb-GFP) and NLP1-overexpressing (pUb-NLP1) roots in response to nitrate (10 mM KNO₃ for three days). (n = 3 independent pools of roots from 8-10 plants). Asterisks in (c and d) indicate significant differences (two-tailed *t*-test, ** P<0.01, *** P<0.001; ns, not significant). The decreasing ratio were indicated. Error bars represent Standard Error of the Mean. (e) Bright-field images of the *pCEP1*:GUS reporter expressed in wild-type (R108) or *nlp1-1* roots. Transgenic roots were watered with KCl or KNO₃ (10 mM each), and the material

was harvested at five days after transfer on nitrate and histochemically stained. Numbers in the upper right corners indicate the numbers of roots having a pattern similar to the one shown in the figure as representative, versus the total number of stained roots (n =11-15 plants). Scale bars, 1 mm (a) and 2.5 mm (e). All experiments were repeated at least three times.

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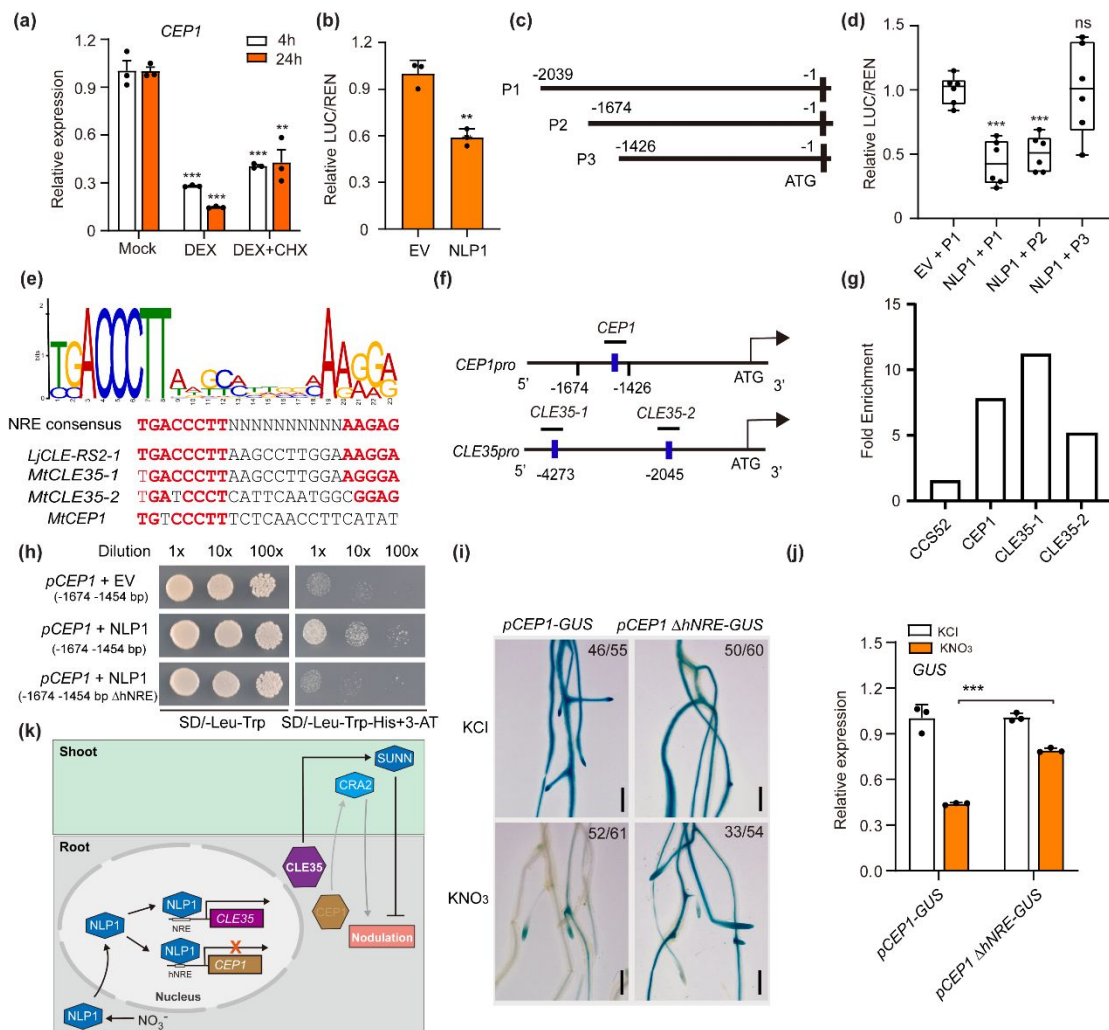


Fig. 2. NLP1 directly binds and suppresses *MtCEP1* expression.

(a) Relative expression of *MtCEP1* in pUb-NLP1-GR roots. Transgenic roots were incubated with mock (DMSO), dexamethasone (DEX), or DEX and cycloheximide (CHX) for 4 or 24 hrs. (**n = 3 independent pools of roots from 6-8 plants**). (b-d) The luciferase activity induced upon the co-expression of an Empty Vector (EV), or of a NLP1 construct, with the *pCEP1*:LUC reporter (b), or with different truncated *pCEP1*:LUC promoters (c, d), was analyzed in *Nicotiana benthamiana* leaves in the presence of nitrate. The luciferase activity was normalized to the Renilla (REN) activity, and fold changes are shown relative to the EV control. (**n = 3-5 biological repeat**). (e) Alignment of NRE motifs previously identified in *LjCLE-RS2*, *MtCLE35* and *MtCEP1* promoters, performed using the MEME algorithm (<http://meme-suite.org/index.html>). Red letters represent conserved nucleotides. (f) Schematic representation of *MtCEP1* and *MtCLE35* genes highlighting promoter regions used for ChIP-qPCR analysis. (g) ChIP-qPCR showing MtnNLP1-GFP binding to the *MtCEP1* or to the *MtCLE35* promoter (**one representative example out of 3 independent experiments; n > 100 transgenic roots**). CCS52 (Cell Cycle Switch 52) was used as a negative control. (h) Yeast-one-hybrid assay of MtnNLP1 binding to the half NRE (hNRE, TGTCCCTT, **corresponding to the conserved domain highlighted in red in (e)**) region of the *MtCEP1* promoter. Binding assays were performed with MtnNLP1 against the synthetic NRE motif, or against the deleted hNRE region. (i and j) Bright-field images (i) and relative expression of *GUS* (j) of roots expressing the *pCEP1*:GUS or the *pCEP1* Δ hNRE:GUS construct in wildtype (R108) transgenic roots.

Numbers in the upper right corners indicate the numbers of roots having a pattern similar to the one shown in the figure as representative, versus the total number of stained roots. The expression of the *DsRed* was used as a reference (j). Scale bars, 1 mm. Asterisks in (a-d and j) indicate significant differences (two-tailed *t*-test, ** $P < 0.01$, *** $P < 0.001$; ns, not significant). Error bars represent Standard Error of the Mean. Data shown are from one representative experiment out of three biological replicates (n = 10-15 plants). (k) A model for the MtNLP1 mediated nitrate inhibition of nodulation through the reciprocal and antagonistic direct regulation of signaling peptides in *M. truncatula*. In the presence of nitrate, MtNLP1 translocation to the nucleus is triggered to activate the expression of *MtCLE35*, leading to the production of peptides systemically inhibiting nodule initiation through the MtSUNN receptor (black lines). At the same time, MtNLP1 also directly suppresses *CEP1* expression, leading to an inhibition of this nodule initiation promoting pathway acting through the MtCRA2 receptor in shoots (grey lines).

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