Nitric oxide is the shared signalling molecule in phosphorus- and iron-deficiency-induced formation of cluster roots in white lupin (*Lupinus albus*)

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**Background and Aims** Formation of cluster roots is one of the most specific root adaptations to nutrient deficiency. In white lupin (*Lupinus albus*), cluster roots can be induced by phosphorus (P) or iron (Fe) deficiency. The aim of the present work was to investigate the potential shared signalling pathway in P- and Fe-deficiency-induced cluster root formation.

**Methods** Measurements were made of the internal concentration of nutrients, levels of nitric oxide (NO), citrate exudation and expression of some specific genes under four P × Fe combinations, namely (1) 50 μM P and 10 μM Fe (+P + Fe); (2) 0 P and 10 μM Fe (–P + Fe); (3) 50 μM P and 0 Fe (+P–Fe); and (4) 0 P and 0 Fe (–P–Fe), and these were examined in relation to the formation of cluster roots.

**Key Results** The deficiency of P, Fe or both increased the cluster root number and cluster zones. It also enhanced NO accumulation in pericycle cells and rootlet primordia at various stages of cluster root development. The formation of cluster roots and rootlet primordia, together with the expression of *LaSCR1* and *LaSCR2* which is crucial in cluster root formation, were induced by the exogenous NO donor *S*-nitrosogluthathione (GSNO) under the +P + Fe condition, but were inhibited by the NO-specific endogenous scavenger 2-(4-carboxyphenoxy)-4, 5, 5-tetramethylimidazoline-1-oxyl- 3-oxide (cPTIO) under –P + Fe, +P–Fe and –P–Fe conditions. However, cluster roots induced by an exogenous supply of the NO donor did not secrete citrate, unlike those formed under –P or –Fe conditions.

**Conclusions** NO plays an important role in the shared signalling pathway of the P- and Fe-deficiency-induced formation of cluster roots in white lupin.

**Key words:** Cluster roots, gene expression, iron deficiency, nitric oxide, phosphorus deficiency, *Lupinus albus*.

**INTRODUCTION**

Cluster roots are a section of densely packed rootletls with determinate elongation in a certain region along the lateral roots. Cluster rootlets are initiated opposite every protoxylem pole in a radial direction within the cluster zone. Cluster roots are common in the Proteaceae family and they are also found in other families (Lambers et al., 2006). Cluster roots formed in different species show different physiological functions in response to different nutritional stresses including phosphorus (P), iron (Fe) or nitrogen (N) deficiency (McCluskey et al., 2004; Hawkins et al., 2005; Paungfoo-Lonhienne et al., 2009). As an integrated structural and functional unit, the formation of a cluster root is defined by four key characteristics: initiation, determinate growth, exudative burst and associated physiology (Skene, 2003). Among these four key steps, the exudative burst and associated physiology have been widely studied (Neumann et al., 2000). Exudates from P-starvation-induced cluster roots contain various low molecular weight compounds such as organic acids and phenolics, and protons. They mobilize and increase the bioavailability of insoluble P sources in soil, while P deficiency also enhances expression of P transporters (Neumann and Römhild, 1999; Lambers et al., 2006). It is reported that the physiological changes of cluster roots in response to P or Fe deficiencies are different more in quantity, rather than in qualitative aspects (Hagström et al., 2001; Skene, 2001). For instance, Fe-starvation-induced cluster roots show the same exudative pattern as P-starvation-induced cluster roots, but the former have a higher internal citrate content, exudative rate and related enzyme activities (Hagström et al., 2001; McCluskey et al., 2004). However, it is unknown whether the initiation and development of cluster roots induced by P or Fe deficiency share the same signalling pathway.

White lupin (*Lupinus albus*) is an ideal model plant to study the formation of cluster roots under nutrient deficiencies (Skene, 2001). It readily forms a large number of cluster roots with similar root basic structures despite some differences in rootlet length under P- and Fe-deficient conditions (Hagström et al., 2001). Attempts have been made to illustrate the key factors affecting the formation of P-deficiency-induced cluster...
roots. For example, the concentration of P in shoots is shown to be one of the key factors to induce the signalling cascade regulating the formation of cluster roots (Shane et al., 2003). Sucrose also acts as a signal molecule in cluster root formation and induces the expression of acid phosphatase genes (Liu et al., 2005; Zhou et al., 2008). Moreover, a number of phytohormones are involved in the formation of cluster roots under P or Fe deficiency. For instance, cluster root formation, but not physiological functions, is induced in a dose-dependent manner by foliar or root application of synthetic auxin and inhibited by auxin transport inhibitors (Gilbert et al., 2000; Neumann et al., 2000; Skene and James, 2000). Ethylene is a regulator in cluster root formation in Casuarina glauca under Fe deficiency (Zaid et al., 2003). Cytokinin is a negative regulator of cluster root formation (Gilbert et al., 2000). So far, there is no common signal found in the formation of cluster roots induced by P and Fe deficiency.

Nitric oxide (NO) is a signalling molecule. It is not only involved in various growth and developmental signalling pathways in plants, but also participates in root branching and cell differentiation (Pagnussat et al., 2002; Stohr and Strelau, 2006; Zhao et al., 2007). Nitric oxide plays a central role in the formation of adventitious roots in cucumber (Pagnussat et al., 2003, 2004), crown roots in rice (Xiong et al., 2009a, b), initiation of lateral roots in tomato and maize (Correa-Aragunde et al., 2004; Creus et al., 2005; Zandonadi et al., 2010) and cluster root development under P deficiency (Wang et al., 2010). In addition, NO is involved in the root physiological response to Fe deficiency in tomato (Graziano and Lamattina, 2007) and enhances the citrate exudation in P-deficiency-induced cluster roots in white lupin (Wang et al., 2010). All these studies imply that NO might be a node in the possible shared signalling pathway in the formation of cluster roots under P or Fe deficiency, but direct evidence is lacking.

Primordium formation is established after the activation of cell cycle genes in lateral roots in arabidopsis (Himanen et al., 2002). Encoding members of the GRAS family of transcription factors, SCARECROW (SCR) and SHORTROOT (SHR), are required for the radial root pattern and asymmetric cell division in arabidopsis and rice (Di Laurenzio et al., 1996; Helariutta et al., 2000; Kamiya et al., 2003). They are also the upstream genes related to cell cycling, involving D cyclins and KRP2 genes in root stem cell maintenance in arabidopsis roots (Wildwater et al., 2005). In white lupin, LaSCR1 and LaSCR2 are mainly expressed during early cluster root developmental stages. Their transcripts localize in cluster primordia and quiescent centres. Suppression of LaSCR1 dramatically reduces root number and growth. Thus, functional expression of SCR is responsible for cluster root development (Shabou et al., 2010).

To explore further the molecular mechanism and a possible shared signalling pathway of cluster root formation induced by P and Fe deficiency, the role of the proposed downstream element NO was investigated in white lupin. We conducted in situ measurements of NO in rootlet primordia of cluster roots and examined the effect of an exogenous NO donor and an NO scavenger on cluster root formation.
**Determinations of total P and Fe concentrations**

Dry shoot and root samples (0-1 g) were digested in 4 mL of 11 M HNO₃ for 5 h at 110 °C. The P and Fe concentrations were measured by ICP-AES (Fisons ARL Accuris) (Zheng et al., 2009).

**NO donor treatments**

After 14 d of cultivation in the +P + Fe treatment, GSNO (S-nitrosogluthathione; 0, 50, 100 and 200 μM) was added to the nutrient solution. The solution was continuously aerated and replaced daily for 6 d. S-Nitrosogluthathione, a widely used NO donor, can release NO in aqueous solution and was synthesized as reported previously (Stamler and Loscalzo, 1992; Graziano and Lamattina, 2007; Chen et al., 2010).

**NO scavenger treatments**

After 14 d of cultivation in the +P + Fe, −P + Fe, +P–Fe and −P–Fe treatments, plants were transferred to individual conical flasks containing 100 mL of the described nutrient solution (pH 6.0) with addition of 0 or 500 μM cPTIO [2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide]. The solution was continuously aerated and replaced every 2 d. The plants were held in the neck of the conical flasks by a sponge, leaving the roots submerged in the nutrient solution. The conical flasks were wrapped with black plastic to avoid possible impacts of light on root and nutrient solution.

**Endogenous NO localization**

Root segments (1.5 cm) of cluster roots with rootlet primordia were excised and incubated with 10 μM fluorescent probe DAF-FM DA (3-amino, 4-aminomethyl-2′, 7′-difluorescein, diacetate; Beyotime, China) at 25 °C for 30 min. The root segments were then rinsed with HEPES-NaOH (pH 7.5) three times (2 min each time) to remove excess fluorescence. The root tissue was transversely sectioned. The bright field and epifluorescence images were captured by a Nikon Eclipse 80i (EX460–500, DM505, BA510–560).

**Quantitative RT-PCR analysis**

Total RNA was isolated using the manufacturer’s protocol (Qiagen, Valencia, CA, USA). cDNA was synthesized from total RNA using a PrimeScript RT reagent kit (Takara). The amount of the LaUbiquitin gene in each mRNA sample was used to normalize the LaPEPC3 [AY663387 (Peñaloza et al., 2005)], LaMATE [AY631873 (Uhide-Stone et al., 2005)], LaSCR1 [FJ236985 (Sbabou et al., 2010)] and LaSCR2 [FJ236986 (Sbabou et al., 2010)] genes. Primer sequences used are LaUbiquitin, 5′-ATGCTAAAAGCCAAGATCAGAAG-3′ and 5′-GAACCTTCCCAGAATCTCAAA-3′; LaPEPC3, 5′-GGGACTGCTGATCGGTGCGCGTGC-3′ and 5′-GAGGCCTGTCCCTATGCTACCC-3′; and LaMATE, 5′-CATCATTTGGCATCAAGGTTA-3′ and 5′-TTCTCGTGTAAGGAAACAG-3′. Degenerate primers of LaSCR1 and LaSCR2 were used according to previous studies (Sbabou et al., 2010). In quantitative PCR, a SYBR Premix Ex Taq (Takara) kit was used following the manufacturer’s instruction. A Light Cycler 480 (Roche) was used for the PCR and the detection of the fluorescent signal. Three biological replicate RNA/cDNA samples were generated, and each cDNA PCR was performed with triplicate technical replicates, from which the relative expression was calculated against that of the LaUbiquitin internal control gene.

**Citrates determination**

Cluster root segments were excised, and root exudates were collected and citrate was measured according to the method described by Delhaize et al. (1993).

**Statistics**

Data are shown as means ± s.e. of 4–8 replications. The Tukey test at 5 % was used to analyse the differences.

**RESULTS**

After 20 d of treatment, white lupin had formed about 14 cluster roots per plant at 0 μM P and 10 μM Fe (−P + Fe), 9 at 50 μM P and 0 μM Fe (+P–Fe) and 12 without P and Fe (−P–Fe), while it formed only two cluster roots with an adequate supply of both P and Fe (+P + Fe) (Fig. 1A).

The initiation of rootlet primordia is the first step of cluster root formation. In order to clarify the morphology of cluster roots formed under the above four treatments, an anatomical study was performed. The plants grown in P-deprived medium (−P + Fe) initiated the largest number of cluster roots and the greatest length of cluster zones (Fig. 1A, B). The primordia were also densely and continuously arranged (Fig. 1D, E). Furthermore, under deficiency of both P and Fe (−P–Fe), the cluster roots presented shorter rootlet zones (Fig. 1B). In contrast, under the +P + Fe condition, very few cluster zones were initiated (Fig. 1A, B), and the average length of rootlets was also the shortest (Fig. 1C). Iron deficiency (+P–Fe) enhanced the initiation of cluster roots and also the length of cluster zones (Fig. 1A, B). Despite the quantitative morphological traits, the cluster roots formed under the four different conditions were basically the same in structure, as they all developed from clustered primordia and showed determinate growth after emergence.

As the cluster roots are induced by nutrient starvation, and the nutrient status in the plant might act as a signal, the concentrations of Pi and soluble Fe in shoots, and total P and Fe in shoots and roots were determined. In the presence of 10 μM Fe, P deprivation slightly increased the soluble Fe concentration in shoots and moderately decreased the total Fe, although none of the changes was statistically significant at P < 0.05 (Fig. 2). In the presence of 50 μM P, deprivation of Fe did not increase the concentration of Pi or total P (Fig. 2), suggesting that the internal P concentration was not related to the formation of cluster roots under Fe deficiency condition.

As NO is a downstream signalling molecule in cluster root development under P deficiency (Wang et al., 2010), a possible role for endogenous NO in initiation and development of rootlet primordia was investigated by NO localization in cluster roots. Cross-sections of the primordium zone of cluster roots formed under −P + Fe, +P–Fe and −P–Fe
conditions were labelled with a specific NO fluorescent probe, DAF-FM DA, and were photographed by confocal laser scanning microscopy. The green fluorescence was clearly observed in the pericycle and endodermis cells and primordia. An accumulation of NO occurred first in cell layers which differentiated from opposite xylem poles in pericycle cells (Fig. 3A), and then in the rootlet primordia (Fig. 3B), and in the emerged rootlet (Fig. 3C), suggesting the involvement of NO in the initiation, development and emergence of rootlets in the root cluster.

When the plants were grown in the +P + Fe solution for 14 d and then treated with an NO donor, GSNO, for 6 d, the number of cluster roots was significantly increased. The addition of GSNO at 100 and 200 μM induced 7.5 cluster roots (Fig. 4A). The fluorescence detection showed that the green fluorescence filled the cross-section of the primordium zone of +P + Fe plants treated for 6 d with 200 μM GSNO (Supplementary Data Fig. S1), confirming that the exogenous supply of GSNO increased the endogenous NO level. In contrast, the addition of 500 μM cPTIO, an NO scavenger, to the –P + Fe, +P–Fe and –P–Fe plants decreased the number of cluster roots to 3–6, a number similar to that of the +P + Fe plants (Fig. 4B). Meanwhile, the endogenous NO level was also decreased to a level similar to that of +P + Fe (Supplementary Data Fig. S2). All these findings indicate that NO might be a joint signalling molecule in the P- or Fe-deficiency-induced formation of cluster roots.

The initiation of cluster roots was not evident until 10, 12 and 13 d after +P–Fe, –P–Fe, and –P + Fe treatment, respectively. However, the rootlet primordia of cluster roots could be observed on the third to fourth day, and emerged on the fourth to fifth day after the NO donor was supplied.
in the +P + Fe treatment, suggesting that exogenous NO supply remarkably accelerated the process of cluster root initiation.

Quantitative RT-PCR analysis of LaSCR1 and LaSCR2 was performed to estimate the role of NO in regulating cell division and radial root patterning. Because they are expressed in the very early stages of cluster initiation and are more responsive to cluster root development than P status (Sbabou et al., 2010), LaSCR1 and LaSCR2 showed a similarly quick response to the exogenous supply of GSNO and cPTIO (Fig. 5). After 3 h treatment, expression of both LaSCR1 and LaSCR2 was triggered by GSNO and suppressed by cPTIO. In three different growth conditions (+P + Fe, −P + Fe, +P − Fe and −P − Fe) for 20 d, there was no dramatic difference in transcript accumulation of LaSCR1 and LaSCR2 in primordia zones, except a higher level of expression in +P − Fe (Fig. 6). However, the NO donor enhanced and the NO scavenger inhibited the expression of these two genes in all treatments (Fig. 6).

When the cluster roots became more mature, the transcripts of physiologically related genes began to accumulate. The phosphoenolpyruvate carboxylase gene LaPEPC3 was induced only when P supply was withheld (Fig. 7A). The expression of the LaMATÉ gene, encoding a citrate transporter,
was induced in cluster roots formed under P or Fe deficiency (Fig. 7B). Accordingly, while P deficiency induced a moderate rate of citrate secretion, Fe deficiency induced the highest rate of citrate secretion (Fig. 8). The deficiency of both nutrients did not induce further secretion as compared with Fe deficiency alone (Fig. 8). However, the cluster roots induced by GSNO in \(+P+Fe\) did not produce elevated citrate secretion (Fig. 8). These results indicate that the variation in gene expression may be responsible for the variation in physiological functions under different nutrient stresses.

**DISCUSSION**

It has been reported that the basic structures of cluster roots induced by P and Fe deficiency are similar, although the length or density of clustered zones and rootlets differs, while the rate of citrate exudation from the cluster roots is higher in Fe-deficient plants (Hagström et al., 2001; Skene, 2001; McCluskey et al., 2004). However, none of the studies demonstrates whether there is a shared signalling pathway in cluster root initiation. Our work ruled out the role of the internal Pi or Fe concentration as a shared signal since their concentration differed significantly in \(-P\) and \(-Fe\) plants (Fig. 2), and demonstrated for the first time that NO is a shared signalling molecule to trigger the formation of cluster roots under P or Fe deficiency.

**NO is correlated with the number of cluster roots**

Nitric oxide participates in construction of root morphology by affecting cGMP (Hu et al., 2005), interacting with Ca\(^{2+}\) and protein kinases (Courtois et al., 2008), impacting on phospholipase D to accumulate phosphatidic acid (Lanteri et al., 2008), and modulating cytoskeleton building in root apices (Kasprzowicz et al., 2009). In white lupin, Wang et al.
previously reported that NO plays a role in P-starvation-induced formation of cluster roots, as they found that NO accumulation was correlated with cluster root development under P deficiency. However, in their work, cluster roots were not promoted by application of another NO donor, 50 mM sodium nitroprusside (SNP), in the +P condition. Therefore, they had no convincing evidence that NO is the closest signalling molecule to trigger cluster root formation. In this work, we used a different NO donor, GSNO. As a widely used NO donor in both plants and animals, 50 μM GSNO in 1 L of solution can release NO at a rate of 30 nmol min⁻¹ (Graziano and Lamattina, 2007). We found that the higher concentration of 200 μM GSNO promoted rootlet initiation under the +P + Fe condition, and the initiation of the rootlet is much faster compared with –P or –Fe treatment (Fig. 4A). On the other hand, application of the NO scavenger cPTIO completely inhibited the initiation of the rootlets in –P or –Fe treatments, proving that NO must be the closest signalling molecule to trigger cluster root initiation. The discrepancy may be due to the concentrations of NO employed in the different studies. As a controversial molecule in root development, the function of NO in the formation of both lateral roots and cluster roots seems to depend on its concentration. Supply of NO increases the number of lateral roots in tomato at a concentration of 200 μM SNP but decreases it at higher SNP concentrations (Correa-Aragunde et al., 2004). We also found a slight difference of cluster root number in control (+P + Fe) plants (Figs 4A and 5A), which is due to the different cultivation conditions applied in the two experiments.

**NO is intensively accumulated in the primordium**

As the initiation of cluster rootlets begins with the formation of the primordium, we detected the in vivo NO localization patterns in root cross-sections where primordia had just initiated and rootlets were about to emerge. First of all, we tested the specificity of the probe in NO staining. In a previous study, Wang et al. (2010) employed a DAF probe for NO staining. In the present study, we used DAF-FM DA, a specific NO probe, to react with NO to form a benzotriazole derivative, which can be excited to emit green fluorescence. The green
Nitric oxide, P and Fe deficiency in cluster roots

Shababou et al. (2010) characterized the LaSCR genes in white lupin, and found that the expression of LaSCR genes is clearly localized in the cylinder and primordia cells, suggesting that the SCR genes play a vital role in radial patterning and development in cluster roots. Here we demonstrated for the first time the possible role of NO in regulating LaSCR gene expression. A 3 h treatment with an NO donor significantly increased the transcript level of LaSCR1 and LaSCR2; in contrast, the NO scavenger cPTIO significantly decreased the level in all the P × Fe treatments (Figs 5 and 6), indicating that the expression of LaSCR genes responds to the changes in NO levels. Any differences in the LaSCR transcripts among different treatments would be due to the density and developmental stage of primordia, since the proportion of primordia in different developmental stages and the total primordia number vary. Moreover, cell cycle regulation genes such as KRP2, CYCD3 and E2Fa in plants are reported to be downstream of SCR in stem cell fate in Arabidopsis roots (Wildwater et al., 2005). Nitric oxide is also reported to be a regulator of many cell cycle regulatory genes in pericycle cells, leading to lateral root formation in tomato (Schachtman and Shin, 2007). It is demonstrated that NO targets the G1 to S transition phase through induction of the CYCD3:1 gene and repression of the KRP2 gene in the initiation of primordia in tomato (Correa-Aragunde et al., 2006). Considering the fact that both the cell cycle and LaSCR expression are regulated by NO, therefore, NO would be a very promising signal directly participating in primordium initiation leading to the formation of cluster roots.

NO plays a more important role in the morphology than in the physiology of cluster roots

Nitric oxide accumulation was observed in all the –P or/and –Fe treatments, and the exogenous NO donor also promoted cluster root formation in the present study. However, cluster roots induced by GSNO did not produce higher citrate secretion in +P + Fe (Fig. 8), indicating that NO alone cannot trigger citrate secretion when the plants are grown in normal conditions. However, Wang et al. (2010) reported that the application of exogenous NO enhanced citrate secretion in cluster roots, but their results were only obtained using P-deficient white lupins. The transcript of LaPEPC3, a gene related to organic acid biosynthesis (Johnson et al., 1996; Peñaloza et al., 2005), was accumulated to higher levels in –P plants than in –Fe plants, but the citrate secretion showed the opposite results (Figs 7 and 8). On the other hand, we also found that the transcript of the LaMATE gene, which encodes a citrate transporter, was only induced by –P and/or –Fe treatments (Fig. 7). Therefore, we hypothesize that NO acts more like an enhancer only if the plants are grown under –P or –Fe conditions in which citrate secretion is already induced (Wang et al., 2010).

In conclusion, our results demonstrated that NO is a signalling molecule involved in the shared pathway of P- and Fe-deficiency-induced formation of cluster roots in white lupin.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: effects of an NO donor (GSNO) on endogenous NO accumulation of white lupin in +P + Fe plants. Figure S2: effect of an NO scavenger (cPTIO) on endogenous NO accumulation of white lupin grown at different concentrations of P and Fe.
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LITERATURE CITED


