Transgenic proteoid roots of white lupin: a vehicle for characterizing and silencing root genes involved in adaptation to P stress

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Summary

White lupin (Lupinus albus L.) has become an illuminating model for the study of plant adaptation to phosphorus (P) deficiency. It adapts to −P stress with a highly coordinated modification of root development and biochemistry resulting in short, densely clustered secondary roots called proteoid (or cluster) roots. In order to characterize genes involved in proteoid root formation and function in a homologous system, we have developed an Agrobacterium rhizogenes-based transformation system for white lupin roots that allows rapid analysis of reporter genes as well as RNA interference (RNAi)-based gene silencing. We used this system to characterize a lupin multidrug and toxin efflux (Lupinus albus MULTIDRUG AND TOXIN EFFLUX, LaMATE) gene previously shown to have enhanced expression under −P stress. Here, we show that LaMATE had high expression in proteoid roots not only under −P, but also under −Fe, −N, −Mn and +Al stress. A portion containing the putative LaMATE promoter was fused to GUS and enhanced green fluorescence protein (EGFP) reporter genes, and a translational LaMATE::EGFP fusion was constructed under control of the LaMATE promoter. The LaMATE promoter directed P-dependent GUS and EGFP expression to proteoid roots. Confocal microscopy in white lupin and Arabidopsis point to the plasma membrane as the likely location of the LaMATE protein. LaMATE displayed homology to FRD3 in Arabidopsis, but did not complement an Arabidopsis ferric reductase defective 3 (FRD3) mutant. RNAi-based gene silencing was shown to effectively reduce LaMATE expression in transformed white lupin roots. LaMATE RNAi-silenced plants displayed an about 20% reduction in dry weight.

Keywords: Agrobacterium rhizogenes, cluster roots, multidrug and toxin efflux (MATE), phosphorus deficiency, proteoid roots, white lupin.

Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development with P concentration ranging from 0.05 to 0.5% plant dry weight. Phosphorus plays a key role in many plant processes such as energy metabolism, photosynthesis, enzyme regulation, and the synthesis of nucleic acids and membranes (Raghothama, 1999). However, P is often unavailable for uptake by plants because it rapidly forms insoluble complexes with cations, particularly Al and Fe under acidic conditions. As a consequence, crop yield on 30 to 40% of the arable land of the world is limited by P availability (Runge-Metzger, 1995).

Plants have developed adaptive mechanisms that aid in the acquisition of P from soil. Compared with many plant species, white lupin (Lupinus albus L.) has been found to display extreme tolerance to P deficiency (Dinkelaker et al., 1989; Gardner et al., 1982). Its adaptation to low P is a highly coordinated modification of root development and biochemistry resulting in proteoid (or cluster) roots, which are densely clustered secondary roots of determinate growth (Johnson et al., 1996b; Massonneau et al., 2001; Neumann and Martinouia, 2002). Biochemical adaptations accompanying proteoid root formation include enhanced synthesis and exudation of acid phosphatase (Gilbert et al., 1999; Neumann et al., 2000) and the organic anions citrate and malate (Dinkelaker et al., 1989; Neumann et al., 1999). At the same time, the expression of a number of genes, including P...
transporters and acid phosphatases, is strikingly enhanced in P-stressed proteoid roots (Liu et al., 2001; Neumann et al., 1999; Penaloza et al., 2002; Uhde-Stone et al., 2003). These adaptations greatly increase P uptake in proteoid root zones.

Because of its extreme tolerance for −P stress, white lupin has become an illuminating model for the study of the adaptation of plants to P deficiency. However, white lupin has been recalcitrant to transformation, though considerable effort has been devoted to the development of a transformation system for this model species. Here, we report the development of an Agrobacterium rhizogenes-based transformation system for white lupin. Transformation via A. rhizogenes led to the development of transgenic roots, including proteoid roots, on composite white lupin plants (plants with transgenic roots and non-transformed shoots) and thus has laid the foundation for the functional characterization of genes in transgenic white lupin roots.

A recent functional genomics approach identified 35 expressed sequence tags (ESTs) or EST contigs that showed enhanced expression of the corresponding genes in proteoid roots of P-deficient white lupin (Uhde-Stone et al., 2003). Of special interest is a highly redundant EST that displayed strong induction in −P proteoid roots, compared with +P and −P normal roots. This contig showed homology to multidrug and toxin efflux (MATE) proteins. Multidrug and toxin efflux proteins are a large family of putative antiporters that are thought to be involved in the excretion of a variety of drugs and toxins (Brown et al., 1999; Debeaujon et al., 2001; Diener et al., 2001; Hvorup et al., 2003; Rogers and Guerinot, 2002). So far only a few MATE genes have been cloned (Debeaujon et al., 2001; Diener et al., 2001; Rogers and Guerinot, 2002) and the function of most MATE proteins in plants remains elusive. In Arabidopsis, a MATE protein (FRD3) is hypothesized to be involved in shoot iron localization, possibly by transport of an iron chelator out of the xylem (Green and Rogers, 2004). We postulate that, in −P stressed proteoid roots of white lupin, Lupinus albus MULTIDrug AND TOXIN EFfLUX (LaMATE) may be involved in the transport of small organic molecules as a response to nutrient stress.

The objectives of this research were: (i) to develop a system for the stable introduction of genes into white lupin; (ii) to isolate the LaMATE gene; (iii) to assess LaMATE promoter fidelity and cellular location of the encoded protein in a homologous system; and (iv) to evaluate RNA interference (RNAi) of the MATE gene as a possible strategy for gene silencing in white lupin roots.

Results

Agrobacterium rhizogenes-mediated transformation of white lupin roots

The functional analysis of P-responsive genes in white lupin roots will be greatly facilitated by rapid introduction of chimeric gene constructs and analysis of roots from transformed plants. To that end, we have developed a protocol for A. rhizogenes-mediated transformation of white lupin. This procedure is based on a protocol developed by Boisson-Dernier et al. (2001) for transformation of Medicago truncatula. In general, transformation protocols that make use of A. rhizogenes involve the cotransfer of the root inducing (Ri) T-DNA and the T-DNA of a binary vector containing the transgene of interest. For the purpose of developing and optimizing the transformation methodology in white lupin, we used A. rhizogenes strain A4TC24, containing LaMATE promoter::GUS and enhanced green fluorescence protein (EGFP) fusions inserted between T-DNA borders of the binary plasmid pBl101.2 (BD Biosciences, Palo Alto, CA, USA). The transformation method involves the inoculation of sectioned seedling radicles (Figure 1a) with A. rhizogenes grown in the presence of 100 mM acetosyringone and 1% glucose to ensure optimal expression of the A. rhizogenes virulence genes (Cangelosi et al., 1990). Inoculated seedlings were grown on slanted agar plates containing the appropriate nutrient solution (Figure 1b,c). Kanamycin (15 mg l⁻¹) was used to select for the cotransformation of transgenic roots (also referred to as ‘hairy roots’) directly with the gene constructs of interest (Figure 1d,e). Transformation efficiency and plant health were evaluated at different temperatures and light conditions. A growth temperature of 18–20°C at slightly dimmed light (200 photosynthetically active radiation, PAR) resulted in the most efficient transgenic root organogenesis and the subsequent development of white lupin ‘composite plants’ (Figure 1d). Mock inoculated control plants did not develop kanamycin-resistant roots at the wounded sections (Figure 1e). The transformation procedure resulted in approximately one out of two seedlings developing transgenic roots from the inoculated cut (Table 1). Transgenic root development started about 2 weeks after inoculation; developed proteoid roots were first found about 5 weeks after inoculation (Figure 1d). Transgenic roots had a similar morphology to normal roots (Figure 1f,g,i,j), except that transformed proteoid roots occasionally displayed secondary proteoid roots (Figure 1g,h, arrows), which were not observed on untransformed control plants.

All plants tested that were transformed with the GUS or EGFP reporter gene fusions and that had developed transgenic roots from inoculated sections displayed GUS or EGFP activity in their roots (Table 1). The generation of transformed roots in white lupin provides a homologous system to directly study gene expression and function in proteoid roots.

LaMATE cDNA and homology to known multidrug and toxin efflux proteins

We sequenced and analyzed the full-length complementary DNA (cDNA) clone corresponding to a previously described...
EST (GenBank no. CA410182; Uhde-Stone et al., 2003) with homology to MATE proteins. The 2.1-kb cDNA inserted in pBluescript contained a 1593 bp open reading frame (ORF) that encodes a deduced protein of 531 AA with an Mr of 56.9 kDa. The phylogram in Figure 2 displays the relationship of LaMATE and selected MATE proteins. The amino acid sequence displayed 55% identity and 75% similarity to the putative MATE protein FRD3 in Arabidopsis (At3g08040, GenBank accession no. NP_187461.1; Rogers and Guerinot, 2002), 51% identity with the FRD3-like protein FRDL.

Figure 1. Agrobacterium rhizogenes-mediated transformation of white lupin roots.
(a) The tips of seedling radicles were removed, the wound inoculated with A. rhizogenes, and placed on slanted agarose plates containing Hoagland nutrient solution and kanamycin 15 (b).
(c) Transformed roots started to develop 3 weeks after inoculation.
(d) Fully developed proteoid roots were found about 5 weeks after inoculation and were clearly distinguishable from mock-inoculated control plants (e).
Transgenic roots (f and i) of white lupin composite plants had a morphology similar to non-transformed roots (g and j, grown without antibiotic) under –P (f and g) and +P (i and j) conditions, respectively; shown are roots of 7 week old lupin plants.
(f, h) Arrows: though proteoid roots were of similar morphology in transgenic and non-transformed control plants, transgenic lupin roots occasionally developed secondary proteoid roots, which were not observed in the non-transformed control plants.
(k) Sections of transformed proteoid roots develop into calli on nutrient plates containing sucrose and a 1:10 ratio of cytokin and auxin.
(l) Calli were transferred onto medium containing cytokinin: differentiation of a developing embryo and roots are visible.
(At1g51340, GenBank accession no. NP_564588.2; Rogers and Guerinot, 2002) and 52% identity with OJ10004_F02.11 from *Oryza sativa* (GenBank accession no. NP_920452.1). In addition, a BLASTn search against ESTs in GenBank identified homologous ESTs in soybean (E-value 2E-75; GenBank accession no. BQ612651.1), *Medicago truncatula* (E-value 9E-47; GenBank accession no. BE997610.1) and *Lotus japonicus* (E-value 5E-36; GenBank accession no. CB829192.1). LaMATE displays higher similarity to the bacterial MATE protein damage-inducible protein F (DinF) (27%) than to enhanced disease susceptibility 5 (EDS5) (16%), TRANSPARENT TESTA 12 (15%) and aberrant lateral root formation 5 (ALF5) (14%) from Arabidopsis (Figure 2).

**Effects of nutrient deficiencies and +Al stress on LaMATE expression**

We have previously shown that LaMATE is highly expressed in −P stressed proteoid roots and to a lesser extend in −P normal roots; no expression was detected in leaves of −P or +P stressed lupin (Uhde-Stone *et al.*, 2003). To investigate whether expression of LaMATE in lupin is influenced by other nutritional stresses besides −P, RNA gel blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR) were performed using RNA samples of normal and proteoid roots from white lupin grown in the absence of P (−P), iron (−Fe), nitrogen (−N), manganese (−Mn) and in the presence of aluminum (+Al), respectively. Figure 3 shows increased LaMATE mRNA in proteoid roots of plants grown under all nutrient stresses tested, as compared with normal roots and nutrient sufficient controls.

### Table 1 Frequency of recovery of transgenic roots

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total plantlets inoculated</th>
<th>Number of plants with root growth from the inoculated cut (%)</th>
<th>Number of GUS/EGFP positive plants out of number tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multidrug and toxin efflux (MATE)-Promoter::GUS</td>
<td>146</td>
<td>71 (49)</td>
<td>42 out of 42 (100)</td>
</tr>
<tr>
<td>MATE-Promoter::EGFP</td>
<td>60</td>
<td>34 (56)</td>
<td>20 out of 20 (100)</td>
</tr>
<tr>
<td>Control (mock-inoculated)</td>
<td>56</td>
<td>0 (0)</td>
<td>0 out of 6 (0)</td>
</tr>
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**LaMATE gene**

To better understand the genetic regulation of LaMATE, we isolated the encoding gene including the 5′ upstream putative promoter region. DNA gel blot analyses were performed to determine gene copy numbers and genomic organization. Under high stringency conditions, two hybridizing bands were obtained when white lupin genomic DNA was digested with the restriction enzyme *EcoR*I and hybridized against LaMATE cDNA (data not shown). The two fragments are due to an internal *EcoR*I site. This finding indicates that LaMATE is present as a single gene in the white lupin genome. In order to isolate the gene, the full-length LaMATE cDNA was used as a probe to screen a lupin genomic library. We identified two overlapping clones, which together contained 17 kb, including the entire coding region as well as approximately 5 kb upstream of the translation start codon.
About 9.3 kb of the 17-kb region was sequenced. A comparison of the nucleotide sequence of cDNA and genomic clones revealed identical coding regions. Eleven introns comprising 4187 bp were located between 12 exons comprising 2054 bp (Figure S1a). In comparison, FRD3 in Arabidopsis displays 13 exons and 12 introns, with an unusually large intron of approx. 2.6 kb in the 5' untranslated region. As shown in Table 2, the LaMATE promoter region contains a number of putative cis-acting elements that have been implicated in nutrient deficiency and general stress response, including a phosphate starvation response 1 (PHR1) element (Rubio et al., 2001), nitrogen regulatory gene 2 (Nit 2) elements (Fu and Marzluf, 1990), a motif similar to iron deficiency responsive cis-acting elements (IDE; Kobayashi et al., 2003) and helix loop-helix elements (Blackwell and Weintraub, 1990). Figure S1(b) shows the distribution of putative cis-acting elements in the LaMATE promoter region.

Reporter gene fusions

To further investigate the spatial and temporal expression pattern of LaMATE, a 5-kb portion upstream of the LaMATE gene containing the putative promoter was ligated to GUS and EGFP reporter genes, respectively. In addition, a translationally fused EGFP fusion under the control of the LaMATE promoter region was constructed to analyze protein localization. These constructs were used to analyze expression and protein localization in transgenic lupin and Arabidopsis roots.

The GUS expression pattern driven by the LaMATE promoter was monitored by histochemical GUS analysis. As demonstrated in Figure 4, GUS staining was found in nutrient stressed proteoid roots and location of GUS activity varied depending on the stage of proteoid root development. The LaMATE promoter drove expression of the GUS reporter gene at sites of newly forming proteoid root meristems (Figure 4a–c). As the proteoid roots elongate, GUS activity disappeared from the base and appeared in the stele of the proteoid rootlets (Figure 4e,f). At this developmental stage, GUS staining was most pronounced in the endodermis and/or pericycle, but not in the vascular system. With further development of the proteoid root, GUS activity continued to increase and eventually stained all tissues of the proteoid rootlets including root hairs (Figure 4g), but remained most pronounced in the stele (Figure 4g insert). A similar pattern of GUS activity was observed under Fe-deficiency (Figure 4h,i) and N-deficiency (Figure 4j). The staining pattern of root tips and adjacent zones varied in white lupin, but often displayed an unstained area at the tip (Figure 4h–i). A comparison of GUS staining intensity under –P, –Fe and –N-stress and +P control showed intense staining in fully developed proteoid roots of nutrient deficient plants, while proteoid roots of plants grown with sufficient nutrients displayed no or only very slight GUS activity (Figure 4k). These findings were confirmed in quantitative 4-methylumbelliferyl-b-D-glucuronide (MUG) assays, which revealed strongest MUG activity under Fe deficient conditions (Figure 5). Transgenic Arabidopsis harboring LaMATE-GUS directed expression in roots; staining was most pronounced at the transition between the differentiation and elongation zone behind the root tip (Figure 4l). In contrast to white lupin, expression of the LaMATE::GUS reporter gene fusion did not respond to nutrient-deficiency in Arabidopsis (data not shown), indicating that reporter gene studies in heterologous systems may lead to spurious conclusions.

Enhanced green fluorescence protein expression driven by the LaMATE promoter was analyzed in white lupin and Arabidopsis, using confocal laser scanning microscopy. Enhanced green fluorescence protein activity patterns corresponded to those observed with histochemical GUS analysis in both species (Figure 6a–d,h,i). A translational LaMATE::EGFP fusion displayed tissue-specific localization of the LaMATE protein (Figure 6c,d,h,i) similar to the expression pattern observed with GUS (Figure 4) and free EGFP (Figure 6a,b,g). Accordingly, translational LaMATE-EGFP directed fluorescence in white lupin was found mainly in proteoid root meristems and in the stele of proteoid rootlets (Figure 6c,d).

### Table 2: Putative cis-acting elements in the promoter region of Lupinus albus MULTIDRUG AND TOXIN EFFLUX (LaMATE)

<table>
<thead>
<tr>
<th>Name of element</th>
<th>Sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate starvation response 1 (PHR 1) element</td>
<td>GNNATATNC</td>
<td>−1833</td>
<td>Rubio et al., 2001</td>
</tr>
<tr>
<td>Vegetative storage protein B (VspB) box II (P1 response)</td>
<td>ATTAATT</td>
<td>−1878, −1559, −1146, −1027, −829, −809, −305</td>
<td>Tang et al., 2001</td>
</tr>
<tr>
<td>DNA binding with one finger (Dof) elements</td>
<td>ACTTTA</td>
<td>−1136</td>
<td>Yanagisawa, 2004</td>
</tr>
<tr>
<td>Nitrogen regulatory protein 2 (Nit2) elements</td>
<td>TATCA/[T/A]/(T)</td>
<td>−748, −41</td>
<td>Fu and Marzluf, 1990</td>
</tr>
<tr>
<td>Similar to iron-deficiency-responsive cis-acting elements (IDE)</td>
<td>CGACATGACTCTTAT LaMATE promoter</td>
<td>−245</td>
<td>Kobayashi et al., 2003</td>
</tr>
<tr>
<td></td>
<td>CAAGCATGCTTTCG IDE consensus</td>
<td></td>
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Figure 4. Histochemical localization of GUS activity directed by a Lupinus albus MULTIDRUG AND TOXIN EFFLUX (LaMATE) promoter::GUS fusion in transgenic roots of lupin (a–k) and Arabidopsis (l).

(a–g) Roots of P-deficient white lupin at different stages of development.
(a–c) The LaMATE promoter drives expression of the GUS reporter gene at newly forming meristems (a, b). Transsections of meristematic tissue displayed GUS activity in the epidermis and cortex surrounding the forming meristem (c). Rootlets just emerging from these meristems do not display GUS activity (a).
(d–g) Developing proteoid roots: GUS activity is first not apparent in the emerging lateral rootlets except at the base (d). During further proteoid root development, blue bases disappear and GUS activity appears in the stele (e, f). Transsection of rootlets that displayed GUS activity in the stele showed GUS staining mainly in endodermis and/or pericycle, but not in the vascular systems (f insert). After further maturation, GUS activity was found in all tissues, including root hairs (g), but remained most pronounced in the stele (g insert).

Similar patterns of GUS activity were observed under Fe-deficiency (h and i) and N-deficiency (j), accumulation in the stele was also found under these deficiencies (data not shown).

(k) Proteoid rootlets of plants grown under sufficient nutrients displayed no or only slight GUS activity.

(l) Transgenic Arabidopsis harboring LaMATE-GUS directed expression in roots, most pronounced at the elongation zone behind the root tip. Expression of the LaMATE::GUS reporter gene fusion did not respond to nutrient-deficiency in Arabidopsis. Bar size = 1 mm, in insert f and g = 0.5 mm.

Confocal oil immersion microscopy was performed to assess subcellular localization of EGFP activity. Epidermal cells showed free EGFP, driven by the LaMATE promoter, in the nucleus and cytoplasm of transgenic white lupin (Figure 6e) and Arabidopsis (Figure 6g), respectively. Propidium iodide staining (red) of cell walls and nucleotide, coupled with plasmolysis experiments of lupin root cells, showed that translationally fused EGFP fluorescence associated with the membrane of plasmolysed cells (green), rather than with the cell wall (yellow–red; Figure 6f). Propidium iodide stained Arabidopsis roots expressing the LaMATE EGFP fusion (Figure 6j), compared with a known plasmamembrane-localized EGFP fusion (Figure 6k; Cutler et al., 2000; available as no. CS84758 at the Arabidopsis Biological Resource Center (ABRC)) and a known tonoplast-localized EGFP fusion (Figure 6l; Cutler et al., 2000; ABRC no. CS84727), respectively, reveals similarity of the GFP pattern between LaMATE:EGFP fusion and the plasmamembrane-located EGFP fusion. This similarity indicates a plasmamembrane location of LaMATE; circular vacuolar membranes that were frequently observed in the tonoplast-located control (Figure 6l) were not observed with the LaMATE:EGFP fusion. In addition, no inclusions of the fluorescent outline at the site of the nucleus (stained red), typical for a tonoplast location of the EGFP fusion, were observed in lupin or Arabidopsis. Taken together, these observations in lupin and Arabidopsis indicate a likely subcellular location of the LaMATE protein in the plasmamembrane.

Analysis of LaMATE in an Arabidopsis T-DNA insertion line of At3g08040

Because LaMATE displayed the highest percentage of identity to FRD3, a T-DNA insertion line of Arabidopsis gene At3g08040 (FRD3) was tested for possible complementation by LaMATE. The T-DNA insertion line SALK_122235 of Arabidopsis gene At3g08040 was identified from the Salk Institute Genomic Analysis Laboratory (SIGnAL) T-DNA insertion collection (http://signal.salk.edu) and obtained from the ABRC. Seeds were grown on germinating soil mix, and heterozygous and homozygous seedlings were identified by PCR. All homozygous seedlings showed significantly delayed growth and development, corresponding to the phenotype previously described for homozygous mutants of this allele (Delhaize, 1996), while heterozygous plants showed no difference compared to the wild type. Two heterozygous plants were transformed with a construct containing the LaMATE cDNA under the control of the LaMATE promoter. PCR was used to identify homozygous SALK_122235 insertion lines carrying the LaMATE cDNA. F1 and F2 seedlings displayed delayed growth and development, compared with heterozygous insertion lines and wild type, indicating that LaMATE does not complement the FRD3 mutation in a SALK_122235 T-DNA insertion line.

RNAi-based silencing of the LaMATE gene

DNA sequences that encode a self-complementary region of hairpin (hp) RNA, separated by an intron, have been implicated with effective RNAi-based gene silencing (Helliwell and Waterhouse, 2003). A 500-bp PCR fragment containing the 5' coding region of LaMATE was inserted via recombination into pHellsgate8, a vector that has been shown to efficiently generate hpRNA constructs (Helliwell and Waterhouse, 2003; Helliwell et al., 2001). Lupin roots were transformed with the MATE RNAi construct and with the empty vector as control, respectively. Kanamycin (15 mg l⁻¹) was used to select for the cotransformation of transgenic roots.

RT-PCR was used to compare LaMATE expression levels in A. rhizogenes transformed roots. Figure 7 shows RT-PCR products derived from proteoid root RNA of four plants independently transformed with LaMATE RNAi (LaMATEi 1–4) and four plants independently transformed with empty vector pHellsgate8 (control 1–4), respectively. All plants were grown under nutrient deficiency to induce LaMATE expression. Plants 1 and 2 were grown under P deficiency and plants 3 and 4 under Fe deficiency. Figure 7a (upper panel) shows no detectable LaMATE amplification in roots of lupin transformed with the LaMATE RNAi construct, but strong signals in plant roots transformed with the empty vector. A parallel PCR using ubiquitin primers indicates similar PCR conditions for all samples (Figure 7a, lower panel).
Inductively coupled plasma (ICP) analysis revealed no significant differences in internal concentrations of P (2 mg ± 0.3 per plant), Fe (0.008 mg ± 0.002 per plant) and Al (0.032 mg ± 0.012 per plant) in LaMATE-silenced plants grown under P and Fe deficiency and +Al stress, respectively, compared to empty-vector-transformed control plants (1.8 mg P ± 0.3, 0.010 mg Fe ± 0.002 and 0.043 mg Al ± 0.027 per plant, respectively). Concentrations are
conditions.

higher root:shoot ratio in LaMATE RNAi compared with empty-vector-transformed controls. How-

an approximately 20% reduction of average dry weight, silenced plants grown under

Figure 7. RNAi-based silencing of LaMATE in white lupin. (a) RT-PCR products derived from proteoid root RNA of four plants independently transformed with Lupinus albus MULTIDRUG AND TOXIN EFFLUX (LaMATE) RNAi (LaMATEi 1–4) and four plants independently transformed with the empty vector pHellsgate 8 (control 1–4), respectively. All plants were grown under nutrient-deficient conditions to induce LaMATE expression. Plants 1 and 2 were grown under P deficiency, plants 3 and 4 under Fe deficiency. The upper panel shows the result of RT-PCR using a primer pair that amplified ubiquitin as control for uniform PCR conditions (lower panel). (b and c) Phenotypic comparison of the LaMATE RNAi-silenced plants grown under –P indicated less vigor and an approximately 20% reduction of average dry weight, compared with the empty vector-transformed controls. However, root weight was not impaired, resulting in a higher root:shoot ratio in RNAi-silenced plants (b), compared with the vector-only control plants (c) under –P conditions.

averages of three independent replicates each consisting of two to three whole plants.

Phenotypic comparison displayed that LaMATE RNAi-silenced plants grown under –P indicated less vigor and an approximately 20% reduction of average dry weight, compared with empty-vector-transformed controls. However, average root weight was not impaired, resulting in a higher root:shoot ratio in LaMATE RNAi-silenced plants under –P conditions (Figure 7b,c).

Discussion

In this report, we have advanced the fundamental understanding of plant adaptation to –P stress by: (i) developing an A. rhizogenes-mediated transformation protocol to generate composite white lupin plants having transgenic roots; (ii) characterizing the full-length LaMATE cDNA and isolating the gene including the promoter region, and defining the sequence and exon-intron structure of LaMATE; (iii) demonstrating that LaMATE transcripts are highly induced in proteoid roots of white lupin under a variety of nutrient stresses; (iv) showing LaMATE is expressed in fully elongated proteoid roots and appears to be localized in the plasma membrane; and (v) demonstrating that RNAi technology effectively silences expression of the LaMATE gene in white lupin roots.

Transformation

A major challenge following the identification of P-responsive ESTs in proteoid roots of white lupin (Penaloza et al., 2002; Uhde-Stone et al., 2003) is to determine the function and regulation of the corresponding genes. White lupin has become an important model organism for the study of plant adaptation to P and Fe-deficiency. However, the use of white lupin as a model organism has been limited by its recalcitrance to transformation. The development of a protocol to generate transgenic white lupin roots provides a system for detailed characterization of P-responsive genes in proteoid roots. The A. rhizogenes-mediated transformation of white lupin allows fast and efficient generation of transgenic roots, with the possibility of antibiotic selection for roots expressing a transgene cotransferred in a binary vector.

The morphology of A. rhizogenes-induced transgenic roots of white lupin composite plants was similar to normal roots and included the formation of proteoid roots. Transgenic proteoid roots appear to have normal proteoid root morphology, allowing the functional analysis of genes in proteoid roots. However, transgenic roots also developed secondary proteoid roots (proteoid roots forming on lateral proteoid rootlets), which are not

Figure 6. Confocal images of lupin (a–f) and Arabidopsis (g–i) roots, expressing either free enhanced green fluorescence protein (EGFP; a, b, e and g) or a translational Lupinus albus MULTIDRUG AND TOXIN EFFLUX (LaMATE)::EGFP fusion (c, d, f, h, i and j).

Both fusions are under the control of the lupin LaMATE promoter:

(a and b) The lupin LaMATE promoter drives expression of free EGFP in lateral root meristems of P-deficient white lupin. (c and d) The translational LaMATE::EGFP fusion indicates the tissue-specific location of the LaMATE protein corresponding to the pattern of LaMATE gene expression (a and b, and Figure 4), namely in proteoid root meristems and in the stele of proteoid rootlets. (e and f) Subcellular location of the transcriptional (e) and translational (f) EGFP fusion. Free EGFP is found in the nucleus and cytoplasm (e), while the translational LaMATE::EGFP fusion associates with the outline of the cell. Propidium iodide staining of cell walls and nucleus (yellow–red), coupled with plasmolysis experiments (f) indicate that EGFP fluorescence associates with the membrane of plasmolysed cells, rather than with the cell wall.

Free EGFP in Arabidopsis roots is found in the nucleus and cytoplasm (g), while the translational LaMATE::EGFP fusion associates with the outline of the cell (h, i and j). The translational LaMATE::EGFP fusion displays fluorescence in roots at the differentiation and elongation zone, but not at the root tip (h). (i) A region of a young root expressing LaMATE::EGFP in comparison to two root sections not displaying GFP activity. Propidium iodide staining of Arabidopsis expressing LaMATE::EGFP (j) and comparison with a known plasmamembrane-located EGFP fusion (k) and a tonoplast-located EGFP fusion (l; Cutler et al., 2000, ABRcno. CS84758 and CS84727, respectively) confirms the likely localization of the LaMATE protein in the plasmamembrane. Bar size = 100 μm.
typically found in white lupin, but are common in Banksia species (Pate and Watt, 2001). These are likely induced by an altered auxin content or sensitivity of A. rhizogenes induced transgenic roots (Lemcke and Schmulling, 1998). In addition to the generation of composite plants (transformed root with non-transformed shoot) presented here, we have started the regeneration of whole transgenic lupin plants from transformed root sections, which will be useful for the study of non root-specific genes. Regeneration of whole plants from A. rhizogenes transformed roots has been shown for a variety of plants, including tomato (Moghaieb et al., 2004). To this end, small sections of transformed root (approximately 5–10 mm) were placed on Murashige and Skoogs (MS) medium containing sucrose, as well as cytokinin and auxin in a 1:10 ratio. Callus formation was visible after 3 weeks (Figure 1k). Upon transfer to sucrose supplemented MS medium containing cytokinin, calli have begun to differentiate into embryos and roots (Figure 1l).

The multidrug and toxin efflux family

To evaluate the potential for using A. rhizogenes mediated root transformation as a tool to study molecular control of proteoid root development and function, we focus on an LaMATE gene, which was previously shown to be highly expressed in P-deficient proteoid roots (Uhde-Stone et al., 2003). Currently, about 200 proteins of the MATE family have been sequenced, including representatives from bacteria, archaea, animals, yeast and plants (Hvorup et al., 2003). Only few members of the MATE family have been characterized functionally: these display highest identity with FRD3 and auxin in a 1:10 ratio. Callus formation was visible after 3 weeks (Figure 1k). Upon transfer to sucrose supplemented MS medium containing cytokinin, calli have begun to differentiate into embryos and roots (Figure 1l).

The functional diversity of MATE proteins is represented by loading an iron chelator out of the xylem or apoplastic space into leaf cells (Green and Rogers, 2004). LaMATE shares an internal addition of about 60 amino acids between transmembrane domains 2 and 3 with only a few other MATE proteins, including FRD3.

LaMATE gene expression

LaMATE displayed several fold enhanced gene expression in proteoid roots of white lupin under a variety of stresses, namely –P, –Fe, –N, –Mn and –Al stress. Computational analysis of the promoter region identified a number of elements that are frequently found in the promoters of putative cis-acting P-responsive genes, including the bindings site recognized by PHR1, a transcriptional factor involved in the regulation of P-responsive genes in Arabidopsis (Rubio et al., 2001), and helix-loop-helix elements (Mukatira et al., 2001). Helix-loop-helix elements were identified in the LaMATE promoter region; helix-loop-helix motifs have been implicated for a variety of stress-responses (Smolen et al., 2002). NIT2 elements have originally been identified as activators of N-regulated genes in Neurospora crassa (Fu and Marzluf, 1990), indicating a possible role in the N-deficiency response of LaMATE. A motif similar to iron-deficiency responsive cis-acting elements, which have been shown to be involved in Fe-responsive expression in transgenic tobacco roots (IDE; Kobayashi et al., 2003), was also present in the LaMATE promoter.

The FRD3 promoter directed gene expression to proteoid roots of nutrient-stressed white lupin. GUS and EGFP reporter gene fusions displayed a complex expression pattern depending on the developmental stage of proteoid roots. In developed proteoid rootlets of nutrient stressed plants, LaMATE expression is found primarily in the stele and, with further development, in all root tissues, except for a zone adjacent to the root tip. The localization of LaMATE expression in lupin resembles that of FRD3 in Arabidopsis, which was found to be expressed in the pericycle and cells internal to the pericycle and surrounding the vascular tissue (Green and Rogers, 2004). High expression at the pericycle/endodermis could indicate involvement of xylem loading, or phloem unloading, of a small organic molecule.
RNA-based gene silencing

We have recently been successful in silencing a lupin acid phosphatase (data not shown; J. Li, C. Uhde-Stone, D. Allan and C. Vance, University of Minnesota, St Paul). Functional characterization of the polyploid white lupin genes would be greatly facilitated by stable silencing of specific genes. RNAi has been demonstrated to suppress the expression of a wide range of genes in a variety of plants (reviewed by Waterhouse and Helliwell, 2003). Here, we have shown that RNAi significantly silenced expression of the LaMATE gene in transgenic roots of white lupin. Phenotypic characterization of the LaMATE RNAi-silenced plants grown under -P indicated less vigor and an approximately 20% reduction of average dry weight, compared with vector-only transformed control plants. However, root dry weight was not reduced, resulting in a higher root:shoot ratio in the LaMATE RNAi-silenced plants. An increased root:shoot ratio is a known response of plants to P-deficiency (Vance et al., 2003). This finding indicates that sensing of -P is not impaired in LaMATE-silenced lupin plants. The observed phenotype in lupin resembles that of the FRD3 phenotype of smaller plants in Arabidopsis (Delhaize, 1996). However, in contrast to the metal accumulating FRD3 mutant, ICP analysis revealed no significant differences in internal concentrations of LaMATE-silenced plants grown under -P, -Fe and +Al, respectively, compared to empty-vector transformed control plant. This finding, together with the observation that LaMATE does not complement the FRD3 phenotype, indicates a function of LaMATE different from that of FRD3. Experiments are ongoing to further elucidate the function of LaMATE in response to nutrient stresses.

Experimental procedures

Plant material

White lupin (Lupinus albus L. var Ultra) was grown in the growth chamber in sand culture under growth conditions as previously described (Gilbert et al., 2000; Johnson et al., 1996a). For transformation experiments, Arabidopsis seedlings ecotype Columbia were grown in germinating soil mix. For expression analysis under nutrient deficiency, transgenic Arabidopsis seeds (F1 generation) were grown for 7 days on Km50; Km-resistant seedlings were transferred and grown for 10 and 20 days, respectively, on slanted 0.6% agarose plates containing 1X Hoagland solution adjusted to +P, -P, lower P (25 μM), -Fe and -N.

Agrobacterium rhizogenes-mediated transformation of white lupin

Surface sterilized seeds (3 min sterilization in 1% bleach, followed by several rinses of sterile water) were germinated in the dark. When emerging radicles reached a length of approximately 10 mm, tip sections of approximately 3 mm were removed with a sterile scalpel. The radicle was inoculated with the appropriate A. rhizogenes strain (derivatives of A. rhizogenes strain A4T; Quandt et al., 1993) and grown on trypton-yeast (TY) plates containing 100 mm acetosyringone and 1% glucose. Six to eight seedlings were placed on 200 ml slanted agarose (0.6% in 1X Hoagland solution, 15 mg l-1 Kanamycin) in clear 22 x 22 mm Q-trays with covers (Genetix USA Inc., Boston, MA, USA). Plates were then placed vertically in a growth chamber at 18 to 20°C for 14 h photoperiods with a light intensity of about 200 PAR (measured in E s-1 m-2). Plants that showed hairy root development after about 3 weeks (about 50%) were transferred to fresh plates and incubated for another 1–4 weeks.

Generation of transgenic Arabidopsis

To analyze the expression profile of LaMATE in Arabidopsis, we generated heterozygous F1 transgenic Arabidopsis carrying transcriptional MATE::GUS and LaMATE::EGFP fusions, and translational MATE::GUS fusion under the control of the LaMATE promoter, respectively. Three independently generated lines were analyzed for each construct. Transgenic Arabidopsis was generated via A. tumefaciens-mediated transformation, using the floral dip/vacuum infiltration method (Bent et al., 1994).

RNA gel blots and DNA gel blots

RNA for gel blot analysis and genomic DNA were isolated as described previously (Liu et al., 2001). RNA gel blots were performed in replicate.

RT-PCR

RNA for RT-PCR was isolated from independently grown plants using the RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). Quantitation of the transcripts was performed using two-step RT-PCR following the manufacturer’s directions (Ambion, Austin, TX, USA, and Invitrogen) using poly thymine deoxynucleotide (dT) primer. The two primer pairs used in the parallel PCR reactions were: LaMATE 5’-GGATTCCAGTTTGGCTTCAAGT-3’ and LaMATE 5’-GTT3CTGTCCATTCCATCTCACAAC-3’. This primer pair amplifies a 470 bp from the 3’ coding region of LaMATE. This region has not been used for the MATE RNAi construct. The primer pair used as control was ubiquitin1 5’-TCTTTGGAAGACCCCTCACC-3’ and ubiquitin2 5’-CTCTGCTGCCGAGGAATG-3’. PCR was performed for 25 cycles (94°C for 1 min, 56°C for 30 sec, 72°C for 30 sec).

Screening of a lupin genomic library

A partially genomic library of white lupin in a γ DASH II vector has been constructed and described previously (Liu et al., 2001). After amplification, plaque filters were hybridized with a random primer-labeled LaMATE cDNA fragment. Single plaques were purified after two rounds of hybridization and the insert was then subcloned in pBluescript vector (Stratagene, La Jolla, CA, USA) for restriction mapping and sequencing.

Sequencing and computational analyses

Sequencing was carried out at the Advanced Genetic Analysis Center (St Paul, MN, USA). Sequences were processed with the unigene program (Devereux and Smithies, 1984).
**Construction of transcriptional and translational reporter gene fusions**

A 2-kb portion of the genomic 5' upstream region of LaMATE was amplified using the following primer pair introducing an upstream HindIII and a downstream SalI restriction site, respectively. The left primer was (HindIII) 5'-TATAAGCTTACAGGCATGTCG-3'; the right primer was (SalI) 5'-TATGTCGACCATGCGAGTAAGCAA-3'. The amplified product was ligated into pGEMTeasy (Promega, Madison, WI, USA) and subcloned as a 2-kb HindIII/SalI fragment into pBl101.2 (BD Biosciences Clontech, Palo Alto, CA, USA) and pBl101.2-EGFP, respectively. The vector pBl101.2 carries a promoter-less GUS cassette in the Agrobacterium binary plasmid pBIN 19 (Bevan, 1984; Jefferson, 1989). Plasmid pBl101.2-EGFP was constructed by amplifying the EGFP gene of plasmid pEGFP (BD Biosciences Clontech) using the following primer pair introducing an upstream Xmal and a downstream SstI restriction site, respectively. The left primer was (Xmal) 5'-TATCGGTCCAGGACATTGGGTGG-3'; the right primer was (SstI) 5'-TATCGAGCTTCAGTGGGAATTCTAGAGTCGCG-3'. The amplified product was ligated into pGEMTeasy, the sequence confirmed by full-length sequencing, and subcloned as a 700-kb Xmal/SstI fragment into pBl101.2., replacing a 1.7-kb Xmal/SstI fragment containing the GUS reporter gene, but not affecting the downstream polyadenylation signal.

For construction of a translational MATE–EGFP fusion, the 1.6-kb coding region of the LaMATE cDNA was amplified except for the stop codon, introducing an upstream SalI and a downstream HindIII restriction site. The left primer was (SalI) 5'-ATAGTCGACCAGGCCAGAATGGGACACTG-3'; the right primer was (Xmal) 5'-ATACCCGGGCAACAGACATTGGGTGGTCCTC-3'. The amplified product was ligated into pGEMTeasy, the sequence confirmed by full-length sequencing, and subcloned as a 700-kb Xmal/SstI fragment into pBl101.2., replacing a 1.7-kb Xmal/SstI fragment containing the GUS reporter gene, but not affecting the downstream polyadenylation signal.

**Histochemical and fluorometric GUS assays**

For histochemical GUS activity detection, samples were incubated overnight at 37°C in GUS assay buffer, using 5-bromo-4-chloro-3-indolyl glucuronide as a substrate (Jefferson, 1989). GUS staining was observed using a stereoscopic zoom microscope (SMZ2000; Nikon, Melville, NY, USA) and images were taken with a DXM1200 digital camera (Nikon).

For quantitative fluorometric MUG assays, proteoid roots of different developmental stages were harvested from independently transformed plants grown under −P, −Fe and −Al, respectively, and quantification was performed as described previously (Trepp et al., 1999).

**Fluorescence microscopy**

Confocal microscopy was performed using an MRC 1024 laser confocal microscope (Bio-Rad, Hercules, CA, USA) with a Krypton/ArIon laser source at the C.B.I. Imaging Center (St Paul, MN, USA), and an LSM-510 META confocal microscope (Zeiss, Jena, Germany) at the College of Natural Resource’s Biological Imaging Facility at the University of California (Berkeley, CA, USA). Optical planes were scanned with the 488-nm laser ray, using a 505–550 nm barrier filter to detect GFP fluorescence. For imaging of GFP and propidium iodide staining, multitracking was performed using a second laser with a 543-nm laser ray and a 570-nm nm barrier filter. Plasmolysis experiments were performed by placing plant material in 0.6 m M glucose for 30 min to 3 h. Roots were stained with propidium iodide in a concentration of 10 μg m l−1 before observation.

**Complementation of an Arabidopsis T-DNA insertion line**

Seeds of T-DNA insertion line SALK_122235 were grown on germinating soil mix, and heterozygous and homozygous seedlings were determined by PCR using the following three primers: left border primer LBa1 5'-GGTTTCAGTGATGGGCCATCG-3'; the right primer was (Bevan, 1984; Jefferson, 1989). Plasmid pBl101.2-EGFP was constructed by amplifying the EGFP gene of plasmid pEGFP (BD Biosciences Clontech) using the following primer pair introducing an upstream Xmal and a downstream SstI restriction site, respectively. The left primer was (Xmal) 5'-TATCGGTCCAGGACATTGGGTGG-3'; the right primer was (SstI) 5'-TATCGAGCTTCAGTGGGAATTCTAGAGTCGCG-3'. The amplified product was ligated into pGEMTeasy, the sequence confirmed by full-length sequencing, and subcloned as a 700-kb Xmal/SstI fragment into pBl101.2., replacing a 1.7-kb Xmal/SstI fragment containing the GUS reporter gene, but not affecting the downstream polyadenylation signal.

**Gene silencing in white lupin**

To amplify a 500-bp PCR product of the 5’ region of LaMATE, flanked by attachment sites (attB1 and attB2), the following following primer pair was used: LaMATE attB1 5'-ATAGTCGACCAGGCCAGAATGGGACACTG-3' and LaMATE attB2 5'-GGTAGCACGTTACAGGTCGCG-3'. The amplified product was confirmed by full-length sequencing and the 1.6-kb SalI/Xmal fragment was inserted in-frame with EGFP in the transcriptional LaMATE-promoter::EGFP fusion in pBl101.2 between the LaMATE-promoter and the EGFP fusion. In-frame transitions were confirmed by sequencing.

**Phenotypic analysis of LaMATE RNAi-silenced mutants**

LaMATE RNAi-silenced white lupin and empty vector-transformed controls were grown under normal nutrient conditions and under −P, −Fe and −Al stress, respectively. Seven weeks after A. rhizogenes inoculation, plants were harvested, fresh and dry weight was determined, and dried plants (after 2 days at 80°C) were pooled in groups of two to three per analysis. Replicates of pooled plants were analyzed by ICP analysis at the University of Minnesota Soil testing laboratory, St Paul campus.

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

**Accession numbers**

The GenBank accession number for LaMATE cDNA is AY631874 and for the LaMATE gene is AY631873.
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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Schematic representation of the intron/exon structure of the Lupinus albus MULTIDRUG AND TOXIN EFFLUX, (LaMATE) gene and location of putative cis-binding elements in the LaMATE promoter region. This material is available as part of the online article from http://www.blackwell-synergy.com

References


