Running head: *MtIRE*, a Medicago AGC kinase with a role in nodulation

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An *IRE*-like AGC kinase gene, *MtIRE*, has unique expression in the invasion zone of developing root nodules in *Medicago truncatula*.

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Abstract

The AGC protein kinase family (cAMP-dependent protein kinases A (PKA), cGMP-dependent protein kinases G (PKG), and phospholipid-dependent protein kinases C (PKC)) have important roles regulating growth and development in animals and fungi. They are activated via lipid second messengers by 3-phosphoinositide-dependent protein kinase (PDK1) coupling lipid signals to phosphorylation of the AGC kinases. These phosphorylate downstream signal transduction protein targets. AGC kinases are becoming better studied in plants, especially in Arabidopsis, where specific AGC kinases have been shown to have key roles in regulating growth signal pathways. We report here the isolation and characterization of the first AGC kinase gene identified in Medicago truncatula, MtIRE. It was cloned by homology with the Arabidopsis incomplete root hair elongation IRE gene. Semi-quantitative RT-PCR analysis shows that unlike its Arabidopsis counterpart, MtIRE is not expressed in uninoculated roots, but is expressed in root systems that have been inoculated with Sinorhizobium meliloti and are developing root nodules. MtIRE expression is also found in flowers. Expression analysis of a time course of nodule development and of nodulating root systems of many Medicago nodulation mutants shows MtIRE expression correlates with infected cell maturation during nodule development. During the course of these experiments, nine Medicago nodulation mutants including sli and dnf1-7 mutants were evaluated for the first time for their microscopic nodule phenotype using S. meliloti constitutively expressing lacZ. Spatial localization of a pMtIRE-gusA transgene in transformed roots of composite plants showed that MtIRE expression is confined to the proximal part of the invasion zone, zone II, found in indeterminate nodules. This suggests MtIRE is useful as an expression marker for this region of the invasion zone.
Introduction

Nitrogen-fixing root nodules are the result of a complex and unique interaction between leguminous plants and soil rhizobia. During nodule development, rhizobia are brought into roots through infection threads that originate in deformed root hairs that curl to form a so-called “shepherd’s crook”. Infection thread initiation and growth require living rhizobia that are synthesizing specific Nod factors (Ardourel et al., 1994; Limpens et al., 2003). Infection threads bring rhizobia past several root cell layers and deposit them into newly divided nodule cells within a membrane-bounded compartment called the symbiosome, in a process resembling endocytosis. Within the infected cells, an environment is established where the rhizobia and plant express new proteins that enable biochemical support of nitrogen fixation and assimilation. In indeterminate nodulators such as Medicago truncatula, new infections occur continuously over the lifetime of the nodule, with new infections starting below the meristematic distal end of the nodule at the beginning of the infection zone, zone II. In the proximal end of zone II, both rhizobia and plant cells expand and mature. Exit from zone II is marked by starch accumulation, which the rhizobia apparently use as a carbon source as they continue their maturation to nitrogen fixation capability in zone III (Vasse et al., 1990). Reviews on legume root nodule development are available (Brewin, 1991; Kijne, 1992; Gage and Margolin, 2000; Brewin, 2004; Gage, 2004).

Nodule-specific (nodulin) and nodule-enhanced genes are expressed exclusively and primarily in nodules, respectively. Most nodulin genes have homologs in non-legumes, suggesting that nodule-specific genes have been recruited from other developmental pathways. It has been noted that a number of nodulins are also expressed in non-symbiotic tissue, including in floral tissues (Szczyglowski and Amyot, 2003). In some cases, genes expressed in both nodules and in floral tissue may have a role in tip growth during infection thread and pollen tube growth respectively (Rodriguez-Llorente et al., 2004). Other tissues with tip-growth, a type of cell expansion, include root hair cells, which have been used as a model system to study tip growth, especially in Arabidopsis (Schiefelbein, 2000).

The AGC protein kinases contain the PKA, PKG and PKC regulatory kinases. In animals and fungi, members of this kinase family act in protein phosphorylation
cascades. In animals, a key AGC kinase regulator is the 3-phosphoinositide-dependent
protein kinase (PDK1), a central growth regulator that integrates signaling events from
receptors that stimulate the synthesis of phosphatidylinositol 3,4,5-trisphosphate (Bogre
et al., 2003; Mora et al., 2004). Far less is known about AGC kinases in plants. In
Arabidopsis, at least 39 AGC kinase genes have been identified (Bogre et al., 2003), but
only seven have known functions. *IRE*, *incomplete root hair elongation* (referred to as
*AtIRE* here) controls the duration of root hair growth in Arabidopsis (Oyama et al.,
2002). *AGC2-1/OXI1* also regulates root hair development (Anthony et al., 2004;
Rentel et al., 2004) and mediates stress signaling (Rentel et al., 2004; Anthony et al.,
2006). *PINOID (PIN)* plays a role in asymmetrical localization of membrane proteins
during polar auxin transport (Christensen et al., 2000). Phototropins 1 and 2 (*PHOT1*
and *PHOT2*) mediate blue light signaling (Huala et al., 1997; Briggs and Christie, 2002;
Takemiya et al., 2005). *ADJ3* and *PDK1* both regulate plant cell death (Devarenne et
al., 2006). The Arabidopsis AGC kinases group phylogenetically into five subfamilies
(Bogre et al., 2003). A sixth subfamily, called “AGC Other”, according to the Hanks
classification (Hanks and Hunter, 1995), contains *AtIRE* and homologous *IRE* genes.

The Medicago genome project has identified many potential genes with roles in
nodule development by virtue of their being found as expressed tag sequences (ESTs)
only in cDNA libraries prepared from tissue that includes nodules (Fedorova et al.,
2002; El Yahyaoui et al., 2004; Lohar et al., 2006). One of the genes identified in this
manner is *MtIRE*, a Medicago homolog of the *AtIRE* gene. In this work, we present
results showing the cloning of the complete *MtIRE* cDNA and its relationship to *AtIRE*
and *IRE*-like genes. We investigate *MtIRE* expression in plant tissues, during nodule
development and in symbiotically defective Medicago mutants. Our findings show that
*MtIRE* does not have a role in root hair growth in Medicago, and suggest the role of
*MtIRE* is likely to be in maturation of infected nodule cells in zone II, before effective
symbiotic nitrogen fixation occurs.
Results

Isolation and sequence of the complete *M. truncatula* IRE-like (*MtIRE*) gene

In 2002, Fedorova et al. reported 340 tentative consensus sequences (TCs) comprising expressed sequence tags (ESTs) found only in cDNA libraries that were made from *M. truncatula* nodule-containing tissue (Fedorova et al., 2002). One of these was TC33166, currently annotated as TC103185 in the TIGR MtGI 8.0 release (www.tigr.org). TC103185, 1383 nucleotides long, consists of 4 expressed sequence tags (ESTs); its strongest BLASTX hit is the *Arabidopsis thaliana* incomplete root hair elongation (*AtIRE*) protein kinase-like gene (Genbank Accession no. AB037133) (Oyama et al., 2002). Using the working draft of overlapping BAC clones mth2-13b8 and mth1-8d23 (Genbank Accession nos. AC122727 and AC133139 respectively) containing the genomic copy of TC103185, sequence from TC103185, and the *AtIRE* gene, primers were chosen to reverse transcribe and amplify *M. truncatula* A17 nodule mRNA corresponding to *MtIRE* of progressively increasing sizes. These efforts resulted in a cDNA containing the complete *MtIRE* coding region of 3504 nucleotides. Subsequently, 5’- and 3’-RACE were carried out to identify the 5’ and 3’ ends of the *MtIRE* transcript. 5’ RACE identified three different 5’ ends corresponding to untranslated 5’ regions of 174, 120, and 115 bases upstream of the predicted translational start codon. 3’-RACE yielded a single 3’ end that contained 296 nucleotides 3’ to the predicted translational stop. The complete *MtIRE* cDNA of 3974 nucleotides corresponding to the longest transcript detected is available in the Genbank database (Accession no. AY770392).

Characterization of *MtIRE* gene structure and its encoded protein

The Medicago genome project has mapped BACs mth2-13b8 and mth1-8d23 containing *MtIRE* to chromosome 5 (www.medicago.org-genome). Comparison of the *MtIRE* genomic and cDNAs revealed that the *MtIRE* gene spans a genomic region of 9.1 kbp and consists of 17 exons and 16 introns (Fig. 1A). This organization is almost identical to the *AtIRE* gene, which is shown for comparison in Fig. 1B. Both genes have 17 exons of similar sizes, but in the Medicago *MtIRE* gene, the introns are significantly larger than in the *AtIRE* gene. The Arabidopsis genome has four other
IRE-like genes. Their genome organization is similar to AtIRE and to MtIRE although slight differences are noted, especially extra exons in AtIRE_2 and the lack of the first 3 exons in AtIRE_3 (Fig. 1C).

The third start codon (ATG) from the 5’ end of the transcript is the presumed start site for the longest ORF of the MtIRE gene. MtIRE encodes a deduced protein of 1168 amino acid residues. ExPASy (www.expasy.org/tools/scanprosite) (Gattiker et al., 2002) and BLAST (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990) tools were used to analyze the deduced MtIRE protein (Fig. 1D). It contains a Glu-rich region at its N-terminal end, basic-type (Kalderon et al., 1984) and bipartite-type (Robbins et al., 1991) nuclear localization signals, a zinc finger-like sequence (C-X2-C-X11-H-X3-C) (Fig. 1D) (Bohm et al., 1997), and numerous potential phosphorylation sites, including those for casein kinase II, protein kinase C, cAMP-, cGMP- and phosphoinositide-dependent protein kinases. MtIRE contains a putative Ser/Thr protein kinase domain towards its C-terminal end (Fig. 1D). Within the Ser/Thr protein kinase domain is an activation loop motif that is a putative target of PDK1, a 3-phosphoinositide-dependent protein kinase. C-terminal to the kinase domain is the PDK1-interacting fragment (PIF) found in some PDK1 substrates (Fig. 1D). Both the activation loop motif and the PIF are signature sequences of the AGC family (Bogre et al., 2003; Mora et al., 2004).

Relationship with other IRE and AGC protein kinase genes

BLAST (Altschul et al., 1990) was used to search for MtIRE homologs in the Arabidopsis, rice, and tomato genomes as well as the unfinished Medicago genome (Young et al., 2005). In the Medicago genome, twenty other ESTs or TCs (www.tigr.org) were found that had homology to AGC protein kinases (Table 1). Unfortunately, none of these represent a complete cDNA; many contain a recognizable full or partial Ser/Thr protein kinase domain. Of these, three were found by BLAST or ClustalW (Thompson et al., 1994) to be IRE-like ESTs or TCs that belong to the “AGC Other” subfamily of AGC genes (Bogre et al., 2003). The other 17 were more similar to different AGC protein kinases than to the IRE/“AGC Other” family (data not shown).
MtIRE, IRE homologs and the Arabidopsis AGC kinases (Bogre et al., 2003) were subjected to phylogenetic analysis using ClustalW (Thompson et al., 1994) and the neighbor-joining method. The Medicago IRE genes were found to group as a single clade with the AtIRE, rice OsIRE and tomato LeIRE genes (Fig. 2). This suggests that MtIRE, AtIRE and other IRE-like genes diverged from the other AGC kinases before the monocots and dicots diverged, estimated to be about 170 Mya (Sanderson et al., 2004). MtIRE was found to form a sub-clade with AtIRE within the IRE clade (Fig. 2), suggesting that these two genes are orthologous.

The N-termini of MtIRE, AtIRE, and OsIRE are highly diverged (not shown), while the putative nuclear localization sequences, zinc finger, PIF motif and Ser/Thr protein kinase domain are conserved (Fig. 3). Within the Ser/Thr protein kinase domain, the activation loop signature motif is conserved among the IRE genes, including MtIRE. However, the putative PDK1 kinase target Ser residue of the activation loop was found to be missing in both AtIRE and OsIRE; it is present in MtIRE (Fig. 3D) and also present in the other IRE genes for which full sequence is available (not shown).

**MtIRE is a single copy gene in Medicago**

To experimentally determine the MtIRE copy number in *M. truncatula*, Southern blot analysis was carried out. *M. truncatula* genomic DNA was restricted with several enzymes, blotted and probed with a cDNA probes derived from exon 4 of MtIRE (Fig. 1). The results obtained at low stringency (not shown) are similar to those obtained at high stringency (Fig. 4), showing one or two bands per lane. These results are consistent with MtIRE being a single copy gene in *M. truncatula*, and, with the exception of the pattern obtained with HincII enzyme, consistent with predicted in silico restriction pattern. In some cases, HincII cleavage is affected by DNA methylation, which may account for the inconsistency with the in silico prediction. Probing the blot with a different cDNA derived from the putative Ser/Thr kinase domain (Fig. 1) yielded similar results of one or two bands per lane, also consistent with MtIRE being single copy (not shown).

**MtIRE expression is in nodules and flowers**
To examine MtIRE expression, semi-quantitative RT-PCR was carried out on total RNA extracted from different plant tissues of M. truncatula A17 wild-type plants. RT-PCR primers were chosen to flank introns (Fig. 1). The transcript of the MtIRE gene was detected only in nodules and flowers and not in other organs of the plant, including uninoculated roots (Fig. 5A).

To determine when during nodule development MtIRE is first expressed, a time course of MtIRE expression in roots with developing nodules was carried out. The results show that MtIRE is first expressed at 4 days after inoculation of nitrogen-starved roots with Sinorhizobium meliloti (Fig. 5B). This is well before the onset of nitrogen fixation in growth conditions used for these experiments, at about 8 days post-inoculation (dpi).

MtIRE expression in nodulating roots of M. truncatula nodulation mutants

To further pin down the stage of nodule development with which MtIRE gene expression is associated, M. truncatula nodulation mutants were examined for MtIRE expression in nodulated root systems or root areas susceptible to nodulation 10 dpi after inoculation with S. meliloti, also by semi-quantitative RT-PCR. As shown in Fig. 6A, MtIRE was found to be expressed in both supernodulators tested, sunn (Penmetsa et al., 2003) and skl (Penmetsa and Cook, 1997). Mutants defective in Nod factor signal transduction, like dmi2 (Catoira et al., 2000) fail to express MtIRE. MtIRE gene expression was not detected in nodulated root systems of mutants that have defects in nodule invasion: lin (Kuppusamy et al., 2004), sli (Haynes et al., 2004), nip (Haynes et al., 2004; Veereshlingam et al., 2004), and Mtsym1 (TE7) (Benaben et al., 1995) (Fig. 6A). In lin, sli, and nip nodules, almost all nodules contain rhizobia trapped within infection threads, whereas in Mtsym1 nodules, rhizobia are endocytosed into host cells and undergo limited replication, but fail to elongate into bacteroids. M. truncatula dnf mutants, dnf1-7, (Mitra and Long, 2004; Starker et al., 2006) deficient in nitrogen fixation, were examined in a similar fashion. In comparison to the mutants blocked very early in nodule development or during rhizobial invasion, all of the dnf mutants were found to express MtIRE (Fig. 6B). However, the level of MtIRE expression was found to be lower than in wild-type in some of the dnf mutants. The dnf mutants were
grouped into 3 classes, based on the extent of \textit{MtIRE} expression (Fig. 6B). In the first class are the \textit{dnf1-1}, \textit{dnf1-2} and \textit{dnf5} mutants that had significantly lower expression of \textit{MtIRE} in their nodulated root systems. The second class contains \textit{dnf7}, which has lower expression of \textit{MtIRE}. The third class includes \textit{dnf2}, \textit{dnf3}, \textit{dnf4}, and \textit{dnf6} mutants that have wild-type levels of \textit{MtIRE} expression in their nodulated root systems.

To correlate \textit{MtIRE} expression in the mutant nodules with phenotypes of the mutants, microscopic analyses of mutant nodules were carried out by examining X-Gal stained 50 µm sections of nodules or whole roots of mutant plants inoculated with \textit{S. meliloti/hemA::lacZ} (Boivin et al., 1990), also at 10 dpi (Fig. 7). While similar analyses have been reported for some of the mutants used in this study, it was important to verify the phenotypes under the growth conditions employed in these experiments. For the \textit{sli} (Haynes et al., 2004) and \textit{dnf} (Mitra and Long, 2004; Starker et al., 2006) mutants, this type of analysis has not previously been reported.

As shown in Fig. 7A, for the \textit{skl} mutant, more nodules than wild-type, all staining so darkly blue they almost look black, were detected, as reported previously (Penmetsa and Cook, 1997). For \textit{dmi2}, no invasion was seen; as reported previously, root hairs developed bulbous tips (Catoira et al., 2000) (Fig. 7B). For the four mutants with defects in rhizobial invasion, \textit{lin, sli, nip}, and \textit{Mtsym1}, a gradient of rhizobial infection was observed, with \textit{lin} infections confined to the root hair cell containing the initial infection thread, similar to published observations (Kuppusamy et al., 2004) (Fig. 7C). In the \textit{sli} mutant, infections progressed further, with infection threads penetrating the outer cortical cells and occasionally reaching inner cortical cells (Fig. 7D). The rare invaded nodules seen previously (Haynes et al., 2004) at much later times post-inoculation were not observed in our growth conditions at the 10 dpi time point. In the \textit{nip} mutant, infection threads filled nodule primordia that showed signs of defense responses by accumulating brown pigments, as previously described (Veereshalingam et al., 2004) (Fig. 7E). For \textit{Mtsym1} (Benaben et al., 1995) (Fig. 7F), nodule primordia filled with infection threads were readily observed, as were nodules with rhizobia released into plant cells. Brown pigments were observed, although the accumulation of these pigments was less than in the \textit{nip} mutant and were distributed differently from those in \textit{nip}: mostly around infection threads in \textit{Mtsym1}. These results are similar to
those previously described by some researchers (Benaben et al., 1995). The small
uninvaded dtsym1 bumps seen by others (Benaben et al., 1995; Mitra and Long, 2004)
were only rarely observed in our conditions.

The dnf mutants’ nodules host cells all showed evidence of released rhizobia around
a central vacuole (Fig. 7G-R). Nodules from the dnf1 and dnf5 mutants appeared to
have the most serious defects. dnf1-1 and dnf1-2 nodules were characterized by large
accumulations of brown-orange pigments in the most proximal zone developed in these
nodules. To a lesser extent, these pigments also accumulated in the nodule parenchyma
(Fig. 7G, H). In the dnf5 mutant, the rhizobia occupying infected cells appeared to be
sparser than in wild-type nodules and some accumulation of pigments was observed
(Figure 7N). The dnf2 mutant nodules appeared to have lower rhizobial occupancy but
were clearly invaded by rhizobia. Only a small pale-yellow band that may be
polyphenolic accumulation near the invasion zone was noted (Fig. 7I). For the dnf3,
DNF4, dnf6, and dnf7 mutants, at least two different phenotype nodules were observed in
our growth conditions. One type appeared defective with lower rhizobial occupancy in
the infected cells (Fig. 7J, L, O, Q), while the second type was hard to distinguish from
wild-type (Fig. 7K, M, P, R). For the case of dnf7 mutants, nodulated root systems
displayed only a few of the wild-type phenotype nodules (as in Fig. 7R). The defective
dnf7 nodules look quite abnormal, with rhizobia accumulating in a band toward the
apex of the nodule (Fig. 7Q).

To look more closely at mutant nodules not expressing MtIRE versus those with low
levels of MtIRE expression, higher magnifications were used to compare the phenotypes
of the nip and dtsym1 nodules to those of dnf1 and dnf5 (Fig. 8). Both nip and dtsym1
nodules contain rhizobia mostly confined within infection threads (Fig. 8A, B). At the
subcellular level, both mutants have been shown by electron microscopy to have
rhizobial release into host cells; in nip, this is a rare event, but it is frequent in dtsym1
(Benaben et al., 1995; Veereshlingam et al., 2004). In contrast, the dnf1-1, dnf1-2, and
dnf5 nodules have host plant cells with released rhizobia around a central vacuole, as in
wild-type. In the dnf1 mutants, rhizobia also accumulate as dark blue patches in the
intercellular spaces (Fig. 8C, D). In the dnf5 nodules, a lower rhizobial occupancy than
wild-type was observed with rhizobia accumulating in thinner rings around the central
vacuole than they do in wild-type (compare Fig. 8E with Fig. 8F). Brown pigmentation indicative of polyphenolic accumulation is evident in the nip, dnf1, and dnf5 mutants (Fig. 8B-E).

Spatial localization of nodule MtIRE expression

To examine the spatial localization of MtIRE expression, an MtIRE promoter-GUS reporter construct (pMtIRE-gusA) was made and expressed in transgenic roots in composite M. truncatula wild-type plants. Plants were inoculated with rhizobia carrying a constitutive hemA gene and nodulated roots were harvested at 15 dpi. Nodules formed asynchronously and more slowly in the composite plants than in untransformed roots and nodules were found at various stages of development in the composite plants at 15 dpi in our growth conditions. Fixed tissue was stained with X-Gluc to visualize pMtIRE-gusA. The largest wild-type nodules were co-stained with Red-Gal to visualize the rhizobia. The results (Fig. 9A) show that blue staining indicative of MtIRE expression is confined to a narrow zone toward the apical end of the mature nodule, with red-staining rhizobia both apical and distal to the X-Gluc-staining region. Transgenic nodules from composite plants were also stained with iodide to localize the starch, defining the interzone II-III region of the nodule (Vasse et al., 1990). By comparing the results of iodide staining (Fig. 9B) with that for Red-Gal and X-Gluc staining, it can be seen that pMtIRE-gusA expression localizes apically to the iodide staining, showing that MtIRE expression starts at the proximal end of the infection zone, zone II, but ends before the interzone II-III region. Co-staining with X-Gluc and iodide confirms that MtIRE expression is distal to the iodide staining region (data not shown), demonstrating the MtIRE expression is confined to the zone II invasion region of the nodule. As expected, and in confirmation of the semi-quantitative RT-PCR expression results, no expression was detected in root hairs, even those containing infection threads (Fig. 9C).

Expression of the pMtIRE-gusA construct in composite dnf1-2 and dnf7 plants was evaluated to test the validity of using MtIRE as a marker for invaded nodule cell tissue. The results showed pMtIRE-gusA expression in both mutants as expected. dnf1-2 has smaller nodules than does dnf7 (and wild-type) and pMtIRE-gusA staining was confined
to the proximal end of the largest $dnf1-2$ nodules observed (Fig. 9D). For the largest
observed $dnf7$ nodules, staining was observed in approximately the nodule middle (Fig
9E), suggesting that $dnf7$ nodules are capable of further development than are those of
$dnf1-2$.

A pictoral interpretation of the visualized staining pattern for wild-type nodules is
presented in Fig. 9F. pMtIRE-gusA is confined to the proximal side of zone II, and thus
may be a marker for this developmental zone.
In 2002, Fedorova et al. reported nodule-specific tentative consensus (TCs) sequences found in Medicago. Among these was a TC with high homology for the *AtIRE* gene, with a predicted signal transduction function (Fedorova et al., 2002). Because the *AtIRE* gene has a known role in root hair elongation, and is thought to function in regulating the duration of tip growth (Oyama et al., 2002), we thought that the *MtIRE* gene might have a similar role in Medicago or one regulating infection thread growth, which can be viewed as inward apical growth, similar to root hair tip or pollen tube growth.

*MtIRE* is a member of the AGC family of Ser/Thr protein kinases involved in signal transduction. In many animal AGC kinases, activation depends on sequential phosphorylation at two sites, one within the PIF motif, found C-terminal to the kinase domain, and one within the activation loop of the kinase domain (Mora et al., 2004). Although well-studied in animals, the signaling pathways regulated by AGC kinases in plants are not yet well understood. In Arabidopsis, seven of at least 39 AGC kinases have been partially characterized as having important roles in development (Huala et al., 1997; Christensen et al., 2000; Briggs and Christie, 2002; Oyama et al., 2002; Bogre et al., 2003; Anthony et al., 2004; Takemiya et al., 2005; Anthony et al., 2006; Devarenne et al., 2006; Zegzouti et al., 2006) and a number of other AGC kinases are under study (Zegzouti et al., 2006). *MtIRE* is the first AGC kinase studied in legumes.

The large plant AGC kinase family is subdivided into distinct phylogenetic classes (Bogre et al., 2003), with the *IRE* genes, including *MtIRE*, clustering in a single clade (Fig. 2). Of these genes, *AtIRE* and *AtIRE H1* have been previously studied (Oyama et al., 2002). In addition to sequence motifs characteristic of AGC kinases, *MtIRE* encodes a putative zinc finger-like sequence and nuclear-localization sequences (Fig. 3). This suggests that *MtIRE* protein, like some of the other *IRE* genes, could localize to the nucleus, similar to the *NDR* proteins of the AGC family that also contain nuclear localization signals (Tamaskovic et al., 2003). Unique to this *IRE*-like gene, *MtIRE* encodes a glutamate-rich sequence, near its N-terminal. Other Glu-rich proteins interact with Ca^{2+} (Endo et al., 2004; Jo et al., 2004) and the Glu-rich region of *MtIRE* could have a similar function. Although Ca^{2+} signaling has a well documented role in the Nod
factor signaling between rhizobia and legume roots (Levy et al., 2004; Mitra et al., 2004; Gleason et al., 2006; Tirichine et al., 2006), only a few studies have investigated the role of Ca\textsuperscript{2+} in intermediate and later stages of nodule development. In Medicago, the Ca\textsuperscript{2+}/calmodulin dependent protein kinase DMI3 was recently shown to have a role in infection during rhizobial release from infection threads into host cells (Godfroy et al., 2006). In determinate soybean nodules, two calmodulin genes were found to be expressed in the infection zone and proposed to be essential for Bradyrhizobium release into symbiosomes (Son et al., 2003). In Medicago, calmodulin transcripts are expressed in root nodules (Fedorova et al., 2002) and novel Ca\textsuperscript{2+} binding proteins are found in the symbiosome space (Liu et al., 2006). Ca\textsuperscript{2+} has been found to modulate symbiosome membrane intake and efflux (Udvardi and Day, 1997).

Expression studies in developing nodules showed MtIRE expression is first detectable at 4 days post-inoculation in our growth conditions (Fig. 4). This time coincides with the time when symbiosomes are beginning to develop and is long before the onset of nitrogen fixation, at about 8 dpi in our conditions.

To correlate MtIRE expression with the phenotypes formed by nodulation mutants, it was necessary to define the mutated phenotypes in our growth conditions. For the sli (Haynes et al., 2004) and dnf1-7 (Mitra and Long, 2004; Starker et al., 2006) mutants, we report new information. Our findings show that at 10 dpi sli nodules are blocked during invasion of the nodule primordia by rhizobia, different from the rare invaded nodules that are observed at a later time point (Haynes et al., 2004). The dnf mutant nodules are blocked after the rhizobia have invaded the nodules through infection threads and deposited rhizobia within host cells (Figs. 7 and 8). The extent of dnf mutant nodule development that we observed correlates well with previous plant and bacterial gene expression studies in the dnf mutants (Mitra and Long, 2004; Starker et al., 2006).

Comparing MtIRE expression in mutant nodulating root systems (Fig. 6) with phenotypes of the mutant nodules (Figs. 7 and 8) shows that MtIRE expression is associated only with nodules that are able to achieve successful invasion, release of rhizobia into infected cells, and some development of the resulting symbiosomes; i.e., the dnf mutants. MtIRE expression was not observed in the sli or nip mutants that have
little to no rhizobial release from infection threads (Haynes et al., 2004; Veereshlingam et al., 2004). Nor was MtIRE expression found in Mtsym1 (TE7) mutants that have rhizobial release from infection threads into symbiosomes but no obvious replication of rhizobia inside symbiosomes or elongation of rhizobia into bacteroids (Benaben et al., 1995). The finding that MtIRE expression is lower in dnf1, dnf5 and dnf7 mutants may point to defects in these mutants that affect the physiology of infected nodule cell maturation in zone II.

In attempts to discern a function for MtIRE, we and others (S. Gantt, personal communication) have attempted MtIRE RNAi-induced gene silencing. The results have been inconsistent, with phenotypes that varied from root hair defects to defective nodulation to no detectable effect (data not shown). We have tried three different silencing constructs, with two chosen from MtIRE regions that are apparently unique to MtIRE and one from the putative Ser/Thr kinase domain. The lack of consistent results from any RNAi construct tested suggests possible functional redundancy in one or more of the other Medicago IRE-like genes. We note that another group has also reported similar issues with the RNAi technique (Liu et al., 2006).

Localization experiments using pMtIRE-gusA in transformed hairy root composite plants showed that MtIRE expression localizes to a band in the proximal part of zone II, the infection zone. To date, there are very few details about the biology or biochemistry of the symbiotic interaction in this nodule region. In this area of zone II, rhizobia are already released from infection threads, symbiosomes are starting to develop and the host cells are expanding, but the rhizobia are not capable of nitrogen fixation (Vasse et al., 1990). Localization of pMtIRE-gusA expression in dnf1-2 and dnf7 plants showed that while nodules from both mutants express pMtIRE-gusA, the dnf1-2 plants appeared to halt nodule development after the zone where MtIRE is expressed, while the dnf7 plants progressed further in nodulation. Thus MtIRE may be a good marker for the ability of nodules to progress to proximal zone II development.

MtIRE was found to be a single copy gene in M. truncatula and a member of the IRE clade of the AGC kinase family. The phylogenetic analysis suggests that MtIRE may be the ortholog of the AtIRE gene. We performed the phylogenetic analysis with the available, but partial, cDNA sequence for the 3 other Medicago IRE genes. When
the full sequences of these cDNAs are available, it may turn out that one of them is a
closer homolog to *AtIRE* than is *MtIRE*.

Our results demonstrated *MtIRE* expression in nodules and flowers (Fig. 3), similar
to a number of other nodulins (Allison et al., 1993; Crespi et al., 1994; Zucchero et al.,
2001; Rodriguez-Llorente et al., 2004). The *MtIRE* expression pattern has diverged
from the orthologous *AtIRE* gene that is expressed in every organ of the wild-type plant,
with higher expression in roots (Oyama et al., 2002), suggesting that *MtIRE* has been
recruited to nodule development from another role. Several previous studies have
highlighted recruitment of genes from other plant programs to symbiotic processes as an
evolutionary mechanism of the nitrogen fixing symbiosis (Szczyglowski and Amyot,
2003; Rodriguez-Llorente et al., 2004; Liu et al., 2006). These include the *ENOD*
genes, genes that transduce signals from both the arbuscular mycorrhizal and rhizobial
symbioses, genes that control nodule number and genes for leghemoglobin, all of which
appear to have been derived from other functions in plant ancestors that gave rise to
legumes. *AtIRE* regulates the duration of root hair cell expansion in Arabidopsis
(Oyama et al., 2002). Our data show that the *MtIRE* gene has unique expression during
nodule development in zone II, where the expansion and development of host cells and
symbiosomes take place. Because of this, we propose that the recruited *MtIRE* gene has
a role in these processes and because it apparently encodes an AGC kinase, we propose
that this role is in signal transduction.
Acknowledgements

We thank several Medicago researchers for nodulation mutant seeds: Drs. Douglas Cook, Thierry Huguet, Sharon Long, Giles Oldroyd, Varma Penmetsa, Colby Starker, and Kate VandenBosch. We thank Heath Wessler for help with DNA sequencing, and Etienne Journet and David Barker for providing the pENOD11-gusA construct, used as a control in root transformation and X-Gluc staining experiments (not shown). We thank Ed Braun for helpful discussions about the phylogenetic analysis.
Materials and Methods

Plants, growth conditions and rhizobial strain

*M. truncatula* A17 (wild-type) and nodulation mutants were grown in aeroponic chambers, starved for nitrogen for 5 days and inoculated with *S. meliloti* harboring pXLDG4 (Boivin et al., 1990; Penmetsa and Cook, 1997) as previously described (Veereshlingam et al., 2004).

Cloning *MtIRE*

RT-PCR was used to clone *MtIRE* from total RNA extracted from *M. truncatula* root nodules using standard protocols (Ausubel et al., 1988). Reverse transcription primers and reverse primers for PCR had the following sequences: 2371r: 5’GTATTTCGGGTGCCAAATAATC; 3254r: 5’GGGTGAAAGACATTACAGTGTCTG. Forward PCR primers were: 40f: 5’CCATGTCTTCCAACCCTCC; 130f: 5’GGAGTTAGGCCTTTTCCAGTCT; 2005f: 5’GGAGTACTTAAATGGTGGAGATCTCT. RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) for both the 5’ and 3’ ends of *MtIRE* was performed using the FirstChoice RLM-RACE kit according to the manufacturer’s instructions (Ambion, Austin, TX). DNA sequencing of the *MtIRE* cDNA was performed in the UNT DNA sequencing lab using custom primers. Both strands were sequenced to completion.

Sequence and phylogenetic analysis

ExPASy (www.expasy.org/tools/scanprosite) (Gattiker et al., 2002), BLAST (www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990) and BioEdit (Hall, 1999) tools were used to analyze the deduced *MtIRE* protein. Phylogenetic analysis using ClustalW (Thompson et al., 1994) and the neighbor joining method were done at pir.georgetown.edu/pirwww/search/multalin.html.

Gene expression by semi-quantitative RT-PCR

The *MtIRE* primers were designed to span introns: MtIRE F: 5’CATCCATAAAGACCTAGGGGAAAAAGTTC, MtIRE R:
5’CTCCATGATTTCCTGCCCAAAGGC. The 18S rRNA F is
5’CCAGGTCCAGACATAGTAAG and the 18S rRNA R is
5’GTACAAAGGGCAGGGACGTA. Two µg of total RNA, extracted as previously
described (Veereshlingam et al., 2004), was reverse transcribed using M-MuLV reverse
transcriptase in a 25 µl reaction containing 1x RT buffer (NE Biolabs, Beverly MA), 1
mM dNTPs, 100 nM MtIRE R primer and 50 nM 18S rRNA R primer at 37 C for 1 h.
5 µl of the RT was amplified in a 50 µl PCR reaction consisting of 1x Thermopol buffer
(NE Biolabs, Beverly MA), 200 µM dNTPs, 250 nM MtIRE primers and 50 nM 18S
rRNA primers. After 4 min at 94 C, 30 or 35 cycles of PCR were run using 94 C 30 sec,
61 C for 25 sec, 72 C for 20 sec.

Histochemical staining of wild-type and nodulation mutants
Nodules elicited with S. meliloti/pXLDG4 containing the constitutive hemA::lacZ gene
were stained with X-Gal as previously described (Veereshlingam et al., 2004) by using
standard methods (Boivin et al., 1990). The buffer used in our protocols was 80 mM
PIPES, pH 7.2, instead of cacodylate. For sectioning, nodules were embedded in 5 %
low melting point agarose and 50 µm-thick sections were obtained with a 1000 Plus
Vibratome (Vibratome, St. Louis, MO). Sections were observed with an Olympus
BX50 microscope (Olympus, Melville, NY) using bright field or dark field (for dmi2
roots). Digital micrographs were processed using Adobe Photoshop.

MtIRE promoter-GUS reporter construct and M. truncatula root transformation
pRD022 was created by subcloning the SphI fragment from pCAMBIA2301
(Hajdukiewicz et al., 1994) containing the CaMV promoter-GUS coding region into
pUC18. A large region upstream (3458 bps) of the MtIRE gene was amplified from the
mth2-13b8 BAC clone, (obtained from the Clemson stock center,
www.genome.clemson.edu). The forward primer MtIREp F has an EcoRI site at its 5’
end: 5’TGGAATTCTCTGCATGGGCGCGAGCAAATGT, and the reverse primer
MtIREp R has an Ncol site at its 5’ end:
5’ACCATGGTGAGAGATGAAAGGAAGAGAG. After PCR amplification (94 C 2
min, followed by 35 cycles of 94 C 30 sec, 60 C 30 sec, 72 C 3 min) the resulting
product was digested with EcoRI and NcoI and ligated to EcoRI, NcoI digested
pRD022, replacing the CaMV promoter with the MtIRE promoter. This plasmid was
then digested with EcoRI and BstEII and ligated with EcoRI, BstEII digested
pCAMBIA2301 to create pCIP005. pCIP005 was transformed into Agrobacterium
rhizogenes strain ARqua1 (Quandt et al., 1993) using the freeze-thaw method (Hofgen
and Willmitzer, 1988). *M. truncatula* roots were transformed *A. rhizogenes* strains
(Boisson-Dernier et al., 2001) containing pCIP005 or a positive control containing the
p*ENOD11-gusA* construct (Journet et al., 2001).

**Histochemical staining of root nodules on transformed roots**

*GUS* activity in whole roots was detected after fixation in 0.3% paraformaldehyde and
infiltration with X-Gluc, as described by Jefferson (Jefferson et al., 1987). Composite
plants transformed with the p*ENOD11-gusA* construct served as control and yielded
results similar to published ones (Boisson-Dernier et al., 2001; Journet et al., 2001).
For dual staining with Red-Gal, roots were stained first with X-Gluc, then the X-Gluc
was replaced with 1 mM Red-Gal (6-Chloro-3-indoyl-β-D-galactoside, Research
Organics, Cleveland, OH). Staining nodules for starch was done as described (Vasse et
al., 1990), except that 0.1 M PIPES, pH 7.2 was used as the buffer. Sectioning,
visualization and processing of images was done as described above.

All experiments were done in at least duplicate.

Sequence data for the genes discussed in this manuscript can be found in the Genbank
databases under the following accession numbers: *MtIRE*, AAX11214; *AtIRE*,
NP_201037; *AtIRE H1*, NP_188412; *AtIRE2*, BAB02708; *AtIRE3*, NP_001031155;
*AtIRE4*, NP_175130; *OsIRE*, ABA99908; *OsIRE1*, XP_469518; *OsIRE2*, ABF98517;
*OsIRE3*, ABF98518; *OsIRE4*, AK122108; *LeIRE*, BT013855; *AtPDK1_1*, NP_974730;
*AtPDK1_2*, ABF57283; *AtS6K_2*, NP_850543; *AtS6K_1*, NP_187485; *AtNDR1*,
NP_849380; *AtNDR2*, NP_171888; *AtNDR3*, NP_188973; *AtNDR4*, NP_565453;
*AtNDR5*, NP_179637; *AtNDR6*, NP_195034; *AtNDR7*, NP_174352; *AtNDR8*,
NP_568221; *AtAGC1_1*, NP_200402; *AtAGC1_2*, NP_194391; *AtAGC1_3*. 

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NP_850426; AtAGC1_4, NP_198819; AtAGC1_5, AAV85687; AtAGC1_6,
NP_173094; AtAGC1_7, NP_178045; AtAGC1_8, NP_195984; AtAGC1_9,
NP_181176; AtAGC1_10, NP_180238; AtAGC1_11, NP_188054; AtAGC1_12,
NP_190047; AtPK3, NP_175774; AtPK5, NP_199586; AtPK7, AAQ65194; AtKIPK,
NP_850687; AtPID, NP_181012; AtAGC2_1 (OXII), NP_189162; AtAGC2_2,
NP_193036; AtAGC2_3, NP_564584; AtAGC2_4, NP_188719; AtPHOT1, NP_190164;
AtPHOT2, NP_568874
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## Table 1: Medicago ESTs and TCs encoding AGC kinase family members

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<td>TC97389</td>
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<td>OsIRE4 (rice)</td>
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<td>BF643568</td>
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<td>AW774942</td>
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<td>LeIRE (tomato)</td>
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**Figure legends**

**Figure 1.** *MtIRE* genomic organization and coding region. A, B, The *MtIRE* deduced exons (black boxes) and introns (lines) (A) are compared to those of *AtIRE* (B). A, Arrows a and b show positions of primers used for RT-PCR; lines c and d show the positions of the exon 4 cDNA probe and the Ser/Thr kinase domain cDNA probe used for Southern blots. C, Comparison of the *MtIRE* exons to those of all the Arabidopsis *IRE* genes. D, Deduced *MtIRE* amino acid sequence. Dotted underline: Glu-rich region; Boxed shaded type: basic nuclear localization signal; Single underline: zinc finger; Double underline: bipartite nuclear localization signal; Bold type: Ser/Thr protein kinase domain; Boxed bold type: Activation loop signature; Asterisk: Ser residue in activation loop that is putative phosphorylation target; Double dotted underline: PIF motif.

**Figure 2.** Phylogram of Arabidopsis AGC protein kinases and plant *IRE* and *IRE*-like genes. The amino acid sequences used in the alignment were obtained from the Genbank database. The alignment was conducted using the Clustal method with default options. The abbreviations used are as follows: Mt: *Medicago truncatula*; At: *Arabidopsis thaliana*; Os: *Oryza sativa*; Le: *Lycopersicon esculentum*.

**Figure 3.** Alignments of conserved domains in the *MtIRE*, *AtIRE*, and *OsIRE* predicted protein sequences. Amino acid residue positions are indicated on the right of each aligned sequence. A, Zinc finger domain. Asterisks show the conserved Cys and His residues. B, Bipartite nuclear targeting sequence (NLS). Asterisks show the conserved Arg and Lys residues. Note that OsIRE contains two bipartite NLS sequences C, Basic-type nuclear targeting sequence. Asterisks show basic Arg and Lys residues. D, PIF motif. Asterisks show the conserved amino acid residues of the motif. E, Ser/Thr kinase domain. Roman numerals denote the 12 distinct subdomains as described in Hanks and Hunter, 1995. White boxed residues are conserved residues found in Ser/Thr kinase domains and the gray boxed Ser residue in subdomain I is Ser in all three *IRE* sequences, but G in the conserved kinase family (Hanks and Hunter, 1995). The
position of the activation loop is noted with the amino acids in the activation loop
signature motif (Bogre et al., 2003) underlined.

Figure 4. Southern blot analysis of *M. truncatula* genomic DNA. DNA was restricted
with EcoRI (E), HincII (H), Asel (A), or HindIII (Hd), and subjected to Southern
analysis with DNA prepared from exon 4 of *MtIRE*. Markers were run in an adjacent
lane and the blot was subsequently probed with DNA prepared from the marker DNA.
Sizes of the markers are indicated on the left.

Figure 5. *MtIRE* gene expression in plant organs and during nodule development. A,
Total RNA from plant organs was analyzed by semi-quantitative RT-PCR in the
presence of primers specific for *MtIRE* and for *rRNA* as positive control for RNA in the
RT-PCR reaction. M, markers whose sizes are on the left; RT, root tips; R,
uninoculated roots; N, nodules; GS, germinated seeds; S, stems; L, leaves; YF, young
unopened flowers; PF, pollinated flowers; SP, seed pods; (-) no RNA. Below the RT-
PCR results is an ethidium bromide stained agarose gel with 1 µg of total RNA from
each sample used for the RT-PCR. B, Total RNA from nodulating roots was analyzed
at the indicated times after inoculation of roots with *S. meliloti*. M, (-), as in A. Below
the RT-PCR results is a gel with 1 µg of total RNA from each sample used for the RT-
PCR.

Figure 6. *MtIRE* gene expression in nodulation mutants. A, Total RNA from 10 dpi
nodulated root systems of *skl, sunn, dmi2, sli, lin, nip, Mtsym1* (TE7) and wild type
(A17) plants was analyzed by semi-quantitative RT-PCR in the presence of primers
specific for *MtIRE* and for *rRNA* as positive control for RNA in the RT-PCR reaction.
M, markers whose sizes are on the left; Below the RT-PCR results is an ethidium
stained agarose gel with 1 µg of total RNA from each sample used for the RT-PCR. B,
Total RNA from 10 dpi nodulated root systems of *dnf1-1, dnf1-2, dnf2, dnf3, dnf4, dnf5,
dnf6, dnf7* and wild type (A17) plants was analyzed as in A. Below the RT-PCR results
is a gel with 1 µg of total RNA from each sample used for the RT-PCR.
Figure 7. Phenotypes of nodules from nodulation mutants. Plant roots from the indicated mutants or wild type were grown in the presence of S. meliloti/pXLDG4 containing a constitutive hemA::lacZ construct and stained at 10 dpi with X-Gal. A, skl; B, dmi2; C, lin; D, sli; E, nip; F, MtSYM1 (TE7); G, dnf1-1; H, dnf1-2; I, dnf2; J, dnf3; K, dnf3 (similar to wild-type); L, dnf4; M, dnf4 (similar to wild-type); N, dnf5; O, dnf6; P, dnf6 (similar to wild-type); Q, dnf7; R, dnf7 (similar to wild-type); S, A17 (wild type). Bars = 100 µm. A-D are whole mounts and E-S are 50 µm sections.

Figure 8. Higher magnification of phenotypes of nodulation mutants’ nodules. Plant roots from the indicated mutants or wild type were grown in the presence of S. meliloti/pXLDG4 containing a constitutive hemA::lacZ construct and stained at 10 dpi with X-Gal. A, MtSYM1 (TE7); B, nip; C, dnf1-1; D, dnf1-2; E, dnf5; F, A17 (wild-type). Bars = 100 µm. All are 50 µm sections.

Figure 9. Localization of pMtIRE-gusA expression in wild-type and dnf1-2 and dnf7 mutants. Composite M. truncatula plants with transgenic roots were grown in the presence of S. meliloti containing a constitutive lacZ gene. A, Double staining for gusA with X-Gluc (blue), for the localization of MtIRE promoter activity, and lacZ with RedGal (red), for the rhizobia localization. The arrow points to the X-Gluc staining. Bar = 100 µm. B, Staining with iodide reveals the position of interzone II-III. Bar = 100 µm. C, Root hair with an infection thread stained with X-Gluc and RedGal shows rhizobia staining but no pMtIRE-gusA staining. Bar = 20 µm. D, dnf1-2 nodule stained with X-Gluc only shows pMtIRE-gusA staining in proximal part of nodule. Bar = 100 µm. E, dnf7 nodule stained with X-Gluc only shows pMtIRE-gusA staining in middle of nodule. Bar = 100 µm. F, Interpretative diagram of panels A and B showing the position of MtIRE expression in the proximal region of the invasion zone, zone II.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5