The symbiotic ion channel homolog DMI1 is localized in the nuclear membrane of Medicago truncatula roots

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Introduction

Symbiotic associations between plants and microbes allow many plants to grow under nutrient-limiting conditions. More than 80% of land plants can enter into symbioses with arbuscular mycorrhizal (AM) fungi, thereby facilitating the uptake of inorganic nutrients (Harrison, 2005); whereas legumes can enter into an additional symbiosis with soil bacteria called rhizobia, thereby gaining access to reduced nitrogen through nitrogen fixation (Soltis et al., 1995). Morphologically the two symbioses appear quite distinct, although they share a number of features including microbial infection of the host plant, transcriptional activation of a common subset of plant genes, and formation of an intracellular microbe–plant interface where nutrient exchange occurs. Most notably, both of these plant–microbe collaborations commence with the exchange of molecular signals within the rhizosphere, a situation that is best understood in the case of symbiotic nitrogen fixation.

Legume roots secrete flavonoids, which trigger the synthesis of a bacterial signal molecule known as Nod factor. Nod factors are lipochitooligosaccharidic molecules composed of β-1,4-linked N-acetyl-glucosamine residues harboring various substituents along the sugar backbone (D’Haese and Holsters, 2002). The nature of these substituents varies by bacterial species, and is the basis for the often high degree of host specificity observed in legume–rhizobium interactions. Nod factors from compatible rhizobia reorient root hair growth to entrap bacteria and facilitate their entry into developing root hairs. Nod factors also initiate a developmental program within the host, creating a new meristematic zone in the root cortex from which a nodule develops. Bacterial infection and nodule development converge when the invading bacteria traverse a plant-derived infection thread into the developing nodule, where they are released into the cytoplasm of specialized host cells of the nodule central tissue. Within these host cells the differentiated bacteria, surrounded by a symbiotic membrane of host origin, function as facultative organelles for nitrogen fixation.

Genetic analyses in several legume species have identified mutants that are unable to form root nodules (Nod–),
and which are blocked at various points in the symbiotic interaction (reviewed by Riely et al., 2006). A subset of Nod-mutants are also unable to form symbiotic associations with AM fungi, indicating that these genes participate in a common symbiotic pathway (Catoira et al., 2000; Kistner et al., 2005). The overlap of the two pathways suggests that rhizobia may have co-opted part of the signaling pathway from the more ancient AM symbioses to evolve the younger nitrogen-fixing symbiosis in legumes. Many of these common symbiotic genes have recently been cloned and phylogenetic analyses have identified orthologs in non-legume species. The presence of these genes in non-legume species is consistent with this model (Zhu et al., 2006).

Some of the earliest responses of legume roots to rhizobial Nod factors involve either the transport or the diffusion of ions across root hair membranes. Calcium, potassium and chloride are all rapidly mobilized in root hairs responding to Nod factors (Felle et al., 1998, 1999; Shaw and Long, 2003). Calcium is a known regulator of polarized tip growth and may participate in redirecting root hair growth, thereby leading to the entrapment of bacteria and subsequent infection (Esseling et al., 2003). Following root tip depolarization, the nuclei of activated root hairs commence the periodic release and re-uptake of calcium known as calcium spiking (Ehrhardt et al., 1996). Calcium spikes have been identified and extensively studied in many eukaryotic systems, and the frequency and amplitude of calcium spikes are believed to encode information eliciting changes in cytoskeleton and gene expression (Oldroyd and Downie, 2004). Transcriptional reprogramming is a likely output from Nod-factor-induced calcium spiking. In particular, a calcium/calmodulin-dependant kinase capable of translating calcium oscillations has recently been found to operate genetically upstream of nodulation-specific transcription factors (Kaló et al., 2005; Lévy et al., 2004; Mitra et al., 2004; Smit et al., 2005).

Many Nod– mutants exhibit defects in some or all of these ion movements implying that ion mobilization is required for eliciting symbiotic responses (Shaw and Long, 2003; Wais et al., 2000). It is therefore not surprising that mutants deficient for both fungal and bacterial symbioses contain defects in putative ion channels. The Medicago truncatula DMI1 gene (Doesn’t Make Infections 1) encodes a protein that is homologous with prokaryotic cation channels and is essential for microbial symbioses (Ané et al., 2004). dmi1 mutant root hairs undergo typical growth deformation in response to Nod factors, but exhibit neither the oscillations in intracellular calcium nor the subsequent changes in gene expression and activation of cell division that are characteristic of the host’s response to Nod factors (Catoira et al., 2000; Shaw and Long, 2003; Wais et al., 2002). Despite the overall homology with prokaryotic potassium channels, DMI1 lacks the canonical GYG motif in the putative filter region. As a consequence the nature of the ions mobilized by DMI1, and therefore its role in Nod factor signal transduction is currently unknown (Ané et al., 2004). Lotus japonicus contains two close homologs of DMI1, CASTOR and POL-LUX, both of which are required for infection and nodule development (Imaizumi-Anraku et al., 2005). In a similar manner as dmi1 mutants, castor and pollux mutants undergo root hair depolarization, but do not respond to Nod factors with nuclear calcium spikes, and are also unable to form AM symbioses. Interestingly, CASTOR and POLLUX both contain putative chloroplast transit peptides, which are proposed to target these proteins to plastids. The role of plastids in symbiotic signal transduction is currently unknown (Imaizumi-Anraku et al., 2005).

In order to model Nod factor signaling mechanisms, it is necessary to have a clear understanding of where the various molecules mediating the signal reside. We investigated DMI1 localization in M. truncatula roots and found that in contrast to CASTOR and POLLUX, DMI1 is localized to the nuclei of root cells and root nodules. The N-terminus of DMI1 does not contain a plastid-targeting transit peptide, but instead contains a sequence that is sufficient to direct proteins to the nucleus. The localization of DMI1 to the nucleus, a site of Nod-factor-induced Ca\(^{2+}\) oscillations, suggests DMI1 may play a direct role in conducting ions within the nuclear compartment.

Results

Functional DMI1::GFP is localized to the nuclear periphery

Previous reports indicated that CASTOR and POLLUX, the DMI1 homologs from L. japonicus, contain transit peptides that target these proteins to root plastids. We analyzed the DMI1 amino acid sequence in silico using multiple online prediction programs and each gave conflicting results. Although ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) predicted a plastid localization for DMI1, WoLF PSORT (http://wolfpsort.seq.cbr.jrc.it/) predicted DMI1 resides in the plasma membrane, and the TargetP algorithm (http://www.cbs.dtu.dk/services/TargetP) suggested DMI1 resided in an unidentified non-plastid compartment (data not shown). Given these discrepancies, we sought to determine experimentally where DMI1 was localized in M. truncatula roots. We cloned a DMI1::GFP fusion into the pRedRoot vector under the control of the constitutive 35S promoter, and transformed this construct into M. truncatula roots. We analyzed the DMI1::GFP fluorescence
in transformed, uninoculated roots using a confocal microscope. Fluorescence was primarily localized to the periphery of nuclei in both root epidermal cells and root hairs (Figures 2b, e). The signal from GFP was typically homogeneous although we occasionally observed patches around the nuclear rim. (Supplemental Figure 1). Following inoculation with *Sinorhizobium meliloti*, we did not observe differences in DMI1::GFP localization in root hairs, root epidermal cells (data not shown) or in root nodules (see below). As a marker for plastid localization, we cloned the DNA sequence encoding the transit peptide (TP) from the *M. truncatula* glutamine synthetase 2 (GS2) gene to the 5' end of the GFP coding sequence and transformed this chimeric construct into *M. truncatula* roots (Melo et al., 2003). The pattern of GFP fluorescence in GS2cTP::GFP-expressing roots differed significantly from that generated by DMI1::GFP. In contrast to DMI1::GFP roots, we observed GS2cTP::GFP fluorescence in round structures approximately 1–2 μm in diameter (Figure 2c, f), a pattern consistent with a plastid-localized protein. Plastids were found both as discrete bodies throughout the cytoplasm and were also observed in clusters surrounding nuclei. However, we never observed fluorescence in the nuclear rim itself.

The DMI1 amino terminus is sufficient, but not required, to target DMI1 to the nucleus

If the 5' region of DMI1 encodes a transit peptide, as predicted by ChloroP, then fusing this domain to GFP should result in the accumulation of GFP in plastids as we observed with the GS2cTP::GFP construct. ChloroP predicts that the N-terminal 69 amino acids of DMI1 constitute a plastid transit peptide. We therefore fused the N-terminal 76 amino acids of DMI1 to GFP (NDMI1::GFP, Figure 1a) and expressed this construct from the 35S promoter in *M. truncatula* roots. We observed strong NDMI1::GFP fluorescence in the nucleus and little to no fluorescence in the cytoplasm (Figure 3b). In contrast, cells expressing GFP alone exhibited strong fluorescence throughout the cytoplasm (Figure 3a). In neither case did we observe fluorescence in plastids. To ensure that we did not mistakenly omit key residues from the N-terminus of DMI1, we generated an additional fusion between the GFP and an N-terminal fragment encompassing the first 152 amino acids of DMI1 (DMI1TM1::GFP, Figure 1a). This construct contains both the putative transit peptide as well as the first transmembrane domain of DMI1. In contrast to the NDMI1::GFP construct, which we observed throughout the nucleus, we observed DMI1TM1::GFP primarily at the periphery of nuclei with weak fluorescence observed throughout the cell in a pattern consistent with an endoplasmic reticulum.

Figure 1. Structure and function of constructs used in this study.
(a) Schematic diagram representing the chimeric GFP fusions. White bands in DMI1 (Doesn’t Make Infections 1) represent transmembrane domains. Gray denotes the plastid transit peptide from glutamine synthetase 2 (Melo et al., 2003). Abbreviations indicate the observed localization of GFP fluorescence. (b) Bright-field image of a root nodule on a *dmi1* mutant plant transformed with pRedRoot::35S::DMI1::GFP::oct plasmid. (c) Fluorescent image indicating that the root in (b) is expressing the *DsRED1* reporter gene. Abbreviations: C, cytoplasm; N, nucleus; NE, nuclear envelope; ER, endoplasmic reticulum; P, plastid. Scale bar represents 1 mm.

Figure 2. DMI1 (Doesn’t Make Infections 1) is localized to the periphery of nuclei and not to plastids. *Medicago truncatula* root epidermal cells (a-c) or root hairs (d-f) expressing (a and d) empty pRedRoot, (b and e) 35S::DMI1::GFP or (c and f) GS2cTP::GFP were visualized on a confocal microscope. Images represent merged data from both the red and green channels. Bars represent 10 μm. Red fluorescence indicates propidium iodide staining of cell walls.

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We inoculated plants with the N-terminal 152 amino acids of DMI1 (Doesn’t Make Infections 1) fused to GFP; (c and d) the N-terminal 152 amino acids of DMI1 including the first transmembrane domain fused to GFP; (e) truncated tDMI1::GFP lacking the N-terminal 76 amino acids of DMI1; (f) GS2cTP::tDMI1::GFP on which the amino-terminus of DMI1 has been replaced with the N-terminus of glutamine synthetase 2. Images represent merged data from both the red and green channels. Arrowheads in (d) highlight putative endoplasmic reticulum. Bars represent 10 μm. Red fluorescence indicates propidium iodide staining of cell walls.

The GS2 transit peptide does not restore function to truncated DMI1

In contrast to DMI1, CASTOR and POLLUX are both reported to function in root plastids (Imaidzu-Anraku et al., 2005). Furthermore, ChloroP predicts that DMI1 also contains a transit peptide that should target it to plastids. We therefore wanted to know if we could artificially target DMI1 to plastids and if re-localization could restore the function of the tDMI1::GFP construct. We replaced the N-terminus of DMI1 with the transit peptide from GS2 and expressed the GS2cTP::tDMI1::GFP fusion in M. truncatula roots. The vast majority of GS2cTP::tDMI1::GFP signal was in plastids (Figure 3f), consistent with the activity of the GS2 transit peptide. However, weak signal was also observed around the nucleus, consistent with the previously established capacity of tDMI1::GFP to localize to the nucleus (Figure 3e). As with GS2cTP::GFP, GS2cTP::tDMI1::GFP plastids were observed both adjacent to the nucleus and dispersed throughout the cytoplasm. These results differ from those obtained with the full-length DMI1::GFP, and indicate that when given an appropriate plastid targeting sequence DMI1 can be efficiently targeted to the plastids.

In order to determine if GS2cTP could restore function to tDMI1::GFP, we transformed dmi1 mutants with the GS2cTP::tDMI1::GFP fusion and inoculated transgenic roots with S. meliloti. As with tDMI1::GFP, and in contrast to DMI1::GFP, GS2cTP::tDMI1::GFP did not complement the dmi1 mutation as plants expressing this construct were completely Nod− (n = 10).

DMI1::GFP expressed from its native promoter is also localized to the nucleus

To rule out the possibility that the observed pattern of localization was caused by over-expression from the 35S promoter, we analyzed DMI1::GFP function and localization following expression from its native promoter. As a prelude to subcellular localization of DMI1::GFP, we sought to establish the tissue specificity of DMI1 promoter activity. As shown in Figure 4a, M. truncatula nodules expressing the uidA gene under the control a 1.8-kb DNA DMI1 promoter fragment exhibited GUS activity in the distal regions of the nodule. These results are consistent with in situ hybridizations performed by Limpens et al. (Limpens et al., 2005) using a DMI1 probe. We fused GFP to the C-terminus of a genomic clone of the DMI1 gene that contained the 1.8 kb of DNA upstream from the ATG (gDMI1) and 500 bp downstream of the stop codon. dmi1 mutants expressing this construct formed root nodules upon inoculation with S. meliloti, indicating that the gDMI1::GFP fusion retained biological activity (Figure 4b, c). We examined the distal region of root nodules expressing
either gDMI1 (Figure 4c) or gDMI1::GFP (Figure 4b) corresponding to the region where we observed GUS activity. Despite high levels of auto-fluorescence commonly observed in the central tissue of root nodules (Figure 4b,c), the distal region of the nodule exhibited relatively low auto-fluorescence, enabling detection of DMI::GFP fluorescence. We observed weak, but reproducible, fluorescence in the distal region of gDMI1::GFP nodules, and a lack of fluorescence in the corresponding region of nodules complemented by gDMI1 that lacked GFP. Higher magnification revealed that fluorescence was primarily localized in spherical structures approximately 10 μm in diameter (Figure 4d), consistent with the previously observed nuclear localization of DMI1 (Figure 2b, e). Moreover, we observed similar patterns of fluorescence in the distal region of nodules expressing 35S::DMI1::GFP (Figure 4e). Thus, both DMI-promoter- and 35S-promoter-driven DMI1::GFP yield complementing proteins with similar subcellular distributions, supporting the conclusion that functional DMI1 resides in the periphery of root and nodule nuclei.

Discussion

Here we report that a DMI1::GFP chimeric protein is localized in the nucleus of M. truncatula roots, and not in the plastids as has been reported for the DMI1 homologs CASTOR and POLLUX. Nuclear localization was observed using both a constitutive 35S promoter, as well as the native DMI1 promoter, ruling out the possibility that ectopic expression of DMI1 causes mislocalization. Expression of DMI1::GFP from either promoter restored the ability of dmi1 mutants to nodulate, indicating that the fusion protein is functional. We provide further evidence that DMI1 localization is mediated, in part, by amino acids present in the N-terminal domain of the DMI1 protein. Fusing the predicted DMI1 transit peptide to GFP causes the GFP to be localized almost exclusively in the nucleus and no fluorescence was detected in plastids. Similar results were obtained when NDMI1::GFP was expressed in other legume and non-legume species (G. Lougnon and J.M. Ané, unpublished results), confirming the ability of the N-terminus of DMI1 to direct proteins to the nucleus, but not to plastids. In contrast, expressing a GFP fusion containing the transit peptide of the M. truncatula GS2 protein from the same 35S promoter localized GFP exclusively in plastids, and replacing the N-terminus of DMI1 with the GS2cTP results in clear localization of GS2cTP::tDMI1::GFP in plastids, a pattern not observed when the full-length coding sequence of DMI1 is fused to GFP. These data provide strong evidence that DMI1 functions in the nucleus.

It is likely that additional sequences outside of the DMI1 amino terminus also function in targeting DMI1 to the nucleus, as a truncated DMI1::GFP construct is also localized in the nuclear envelope. There are examples from the literature of proteins that contain multiple, distinct NLSs. For example, the Lamin B receptor (LBR) contains multiple non-overlapping NLSs. LBR nuclear localization is mediated by a bipartite NLS in the amino terminus, as well as amino acids within and adjacent to the first transmembrane domain (Soullam and Worman, 1995). As with DMI1, fusion of the soluble amino terminus of LBR to GFP targets the

Figure 4. DMI1::GFP expressed from the DMI1 (Doesn’t Make Infections 1) promoter is also localized in the nucleus. (a) A 10-μm-thick GUS-stained section of a root nodule expressing the uidA gene under the control of the 1.8-kb DMI1 promoter. Images (b–e) are confocal images of hand-sectioned root nodules. (b) GFP fluorescence is evident in the distal regions of nodules from dmi1 mutant plants expressing gDMI1::GFP (white star). This region corresponds to the GUS-stained regions shown in (a) and is not evident in gDMI1 nodules that lack GFP (c). A higher magnification image of the section in (b) is shown in panel (d) with the nuclei highlighted by arrowheads. 35S::DMI1::GFP is shown in panel (e) for comparison. The fluorescence observed in the nitrogen-fixing zone (asterisks in b and c) is caused by autofluorescence. Scale bars represent 75 μm in (b and c) and 25 μm in (d and e). The blue color in (a) is caused by GUS staining, whereas the pink color is counterstain from ruthenium red.

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chimeric protein to nuclei, whereas the C-terminal domain, containing eight transmembrane domains, is still targeted to the inner nuclear envelope in the absence of its amino terminus (Soullam and Worman, 1995).

Analysis of the DMI1 peptide using PredictNLS (http://cubic.bioc.columbia.edu/predictNLS/) and WoLF PSORT identified three potential sequences within the DMI1 peptide that could function as nuclear localization sequences. Two of these sequences (PLKKTK and SRKRRQ) reside within the N-terminus and may target NDMI1::GFP to the nucleus. Although the predicted molecular weight of the NDMI1::GFP protein (approximately 35 kDa) is below the level that can freely diffuse in and out of the nucleus, the fact that we do not see appreciable accumulation of GFP in the cytosol indicates either that it is being actively targeted to the nucleus, or that it is retained in the nucleus following diffusion. In either case, the observation that the N-terminus of DMI1 contains signals that are sufficient to cause GFP accumulation within the nuclear compartment suggests that DMI1::GFP also resides within the nucleus, but in association with the inner nuclear membrane. We are currently performing electron microscopy to more precisely define the localization of DMI1 within the nuclear envelope.

WoLF PSORT and Prosite (http://www.expasy.org/prosite/) identified an additional bipartite NLS-like sequence (RTESNKEDVPLKRRVA) between the second and third transmembrane domains of DMI1. This third NLS may explain why the truncated DMI1::GFP protein retained nuclear localization, and why we observed weak remnant fluorescence of GS2cTP::dDMI1::GFP in the nuclear rim. Alternatively unidentified sequences within the transmembrane domains may function in nuclear targeting, as has been shown for LBR (Soullam and Worman, 1995). We are currently undertaking additional mutagenesis studies to precisely define, and functionally characterize, the putative NLSs in DMI1. That truncated DMI1 did not retain biological function indicates that the N-terminus contains residues critical for either protein function, or for the maintenance of the appropriate protein structure.

Previous reports indicate that other essential components of the Nod factor signaling pathway, as well as certain physiological responses to Nod factor, are also localized in the nucleus. The putative L. japonicus nucleoporin Nup133, the M. truncatula LysM receptors LYK3 (NFR1 in L. japonicus), NFP (NFR5 in L. japonicus) and LRR receptor kinases DMI2 (SYMRK in L. japonicus) all contain signal peptides, which are likely to target them to the plasma membrane and possibly the infection thread, as was recently demonstrated experimentally for DMI2 (Endre et al., 2002; Limpens et al., 2003; Limpens et al., 2005; Madsen et al., 2003; Radutoiu et al., 2003; Stracke et al., 2002). Nod factor binding by one or more receptor kinases at the host’s plasma membrane is likely to initiate a pathway that activates nuclear responses. Pharmacological studies suggest a G-protein and lipid signaling pathway links Nod factor perception at the plasma membrane to responses manifested in the nucleus (Charron et al., 2004; den Hartog et al., 2001, 2003). The heterotrimeric G-protein agonist Mas7 activates ENOD11 transcription in dmi1 and dmi2 mutants but not in dmi3 mutants, indicating that second messengers operate upstream of dmi3 and either downstream of dmi1 and dmi2 or in a parallel pathway that converges with the pathway upstream of ENOD expression (Charron et al., 2004). Phospholipase and inositol triphosphate (IP3) receptor inhibitors block ENOD11 expression, implicating phospholipases and inositol tri-phosphates as potential second messengers. The authors of this study postulated that Mas7 activates phospholipases leading to the production of either IP3 or IPα and the subsequent release of calcium from the ER (Charron et al., 2004).

Calcium spiking has been intensively studied in animal systems and is likely to utilize a two-step activation mechanism involving both second messengers such as IP3 and localized calcium release, which may also be triggered by one or more of the aforementioned second messengers (Cancela et al., 2000). That Mas7 can elicit calcium spiking in dmi1 mutants precludes DMI1 as a direct conduit for calcium oscillations (Giles Oldroyd, John Innes Centre, Norwich, UK, personal communication). However, DMI1 may yet play a direct role in this response by triggering the initial calcium flux. Although DMI1 shares overall similarity with prokaryotic potassium channels, it lacks the canonical signature motif in the filter region indicative of a potassium channel (Ané et al., 2004; Imaizumi-Anraku et al., 2005) and may instead be mobilizing calcium from the nuclear envelope. Like the ER the nuclear envelope is also a store for intracellular calcium, which is released by IP3 (Gerasimenko et al., 2003; Xiong et al., 2004). Although the localization of DMI1 in the vicinity of calcium spiking is certainly tantalizing, a direct role in calcium mobilization is speculative. Clearly the biochemical activity of this putative ion channel must be experimentally determined and our current model requires rigorous testing.
It is difficult to reconcile the differences in localization between DMI1 and those of CASTOR and POLLUX. The responses of diverse legume hosts to their cognate Nod factors and symbionts, and the proteins that mediate these responses show remarkable conservation in both structure and function. It therefore seems unlikely that DMI1 and CASTOR/POLLUX would be localized in different parts of the cell. Although we cannot rule out the possibility that these proteins do indeed function in distinct subcellular compartments, we provide evidence that the nucleus is the proper site of DMI1 function, and speculate that CASTOR and POLLUX may function there as well. Indeed, WoLF PSORT and PredictNLS identified potential NLSs in the N-terminus of CASTOR (B.K. Riely, unpublished data), and DMI1 does not preclude a role for plastids in Nod factor signaling, does not preclude a role for plastids in Nod factor signaling.

**Experimental procedures**

**Plant material and rhizobium inoculations**

All experiments were performed using either *M. truncatula* cv. Jemalong A17 seedings or the GY15-3F-4 null mutant of *dmi1* (Ané et al., 2004). Seeds were scarified in concentrated sulfuric acid for 8 min, surface sterilized in 12% sodium hypochlorite, imbibed in sterile water and plated on 1% deionized water agar plates. Seeds were subsequently vernalized for 48 h at 4°C and germinated by incubation at 22°C overnight. All nodulation assays were performed using a suspension of approximately 10⁷ colony forming units ml⁻¹ of *S. meliloti* strain ABS7M (pXLG4D).

**Plasmid construction**

To construct the pLP100::pDMI1 plasmid, we amplified the DMI1 promoter from bacterial artificial chromosome (BAC) Mth2-5A424 DNA using primers 5′-CCCGTGATACCCCTGAGAGAGAGGTTTTCTTCACTTTTTTTAA 3′, digested the PCR product with HindIII and XmnI, and ligated the 1.8-kb fragment into the corresponding sites in the pLP100 vector (Boisson-Dernier et al., 2001) to make a transcriptional fusion with the uidA gene. The GFP coding sequence (CDS) was amplified from pSMRSGFP (Davis and Vieris, 1998) and cloned into either the pTEX or the pTEXI(H) vector (Frederick et al., 1998) to generate 35S::GFP. These vectors contain a multiple cloning site flanked by the 35S promoter and octopine synthase terminator, and differ only in that the 35S cassette is flanked by HindIII sites in pTEX(I), whereas it is flanked by EcoRI and HindIII sites in pTEX. DMI1 was amplified from DMI1 cDNA (Ané et al., 2004) and cloned onto the N-terminus of GFP to generate 35S::DMI1::GFP. The NDMI1 fragment used the reverse primer 5′-CCGTTACTTCTTCTGAGGTGCGGCAAC-3′ to amplify the region encoding the N-terminal 76 amino acids of DMI1 cDNA, which was cloned in frame to the N-terminus of GFP to generate 35S::NDM1::GFP. The primer 5′-AGGGTTACCATGCCAAACACCCTTCTCCTCTCC-3′ was used to truncate DMI1 thus removing the N-terminal 76 amino acids. This PCR fragment was cloned in frame to the N-terminus of GFP to generate 35S::DMI1::GFP. The region of GUS encoding the 56 amino acid transit peptide was amplified from an A17 root cDNA library (Ané et al., 2004) using 5′-AAGGTATCATGTCACAGATTGGCTCTCC-3′ and 5′-GTACAGCAGCTGATCCATTAATAC-3′. This fragment was cloned both into 35S::GFP and 35S::DMI1::GFP to generate the 35S::GSS2::TP::GFP and 35S::GSS2::TP::DMI1::GFP constructs, respectively. The 35S::oct cassettes containing the various GFP constructs were either amplified from pTEX using the primers 5′-AAACCTGCAAGCCATGCTAATGAAATACCTCCAGGCTCCTTC-3′ and 5′-AAACCTGCAAGCCATGCTAATGAAATACCTCCAGGCTCCTTC-3′,

**Generation of transgenic hairy roots**

Hairy roots were generated essentially as described by Boisson-Dernier et al. (2001) using either the A. rhizogenes strain ArquaI (pLP100 and pRedRoot) or MSU440 (pKGWRR plasmids), with the exception that inoculated seedlings were sandwiched between filter paper moistened with Fahraeus media (Fs) without nitrate, or to pots containing Turface moistened with liquid Fs. The exception that inoculated seedlings were sandwiched between filter paper moistened with Fahraeus media (Fs). Transgenic hairy roots expressing the DsRED1 marker were identified using a Zeiss fluorescence microscope.

**Histochemical analysis and microscopy**

Staining for GUS activity was performed by incubating tissue in 0.4 M NaPO₄, pH 7.2, 2 mM potassium ferrocyanide, 2 mM potassium ferrocyanide, and 5 mM NaPO₄ at 37°C for 214 h, after which the tissue was briefly washed in distilled water and stained with 0.1% (w/v) X-gal in 0.4 M NaPO₄, pH 7.2, 2 mM potassium ferrocyanide at 37°C.
2 mM potassium ferricyanide, 2 mM X-Gluc at 37°C. GUS-stained tissue was fixed in 5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.2, under vacuum for 2 h before embedding in Technovit (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Sections (10-μm thick) were cut using a Microm HM340 E rotary microtome (Microm, San Leandro, CA, USA) and counterstained in ruthenium red.

Transgenic roots used for confocal microscopy were stained with propidium iodide (Sigma, St Louis, MO, USA) on a Krypton-Argon laser by hand. All samples were excited using the 488- and 568-nm lines on a Krypton-Argon laser and observed using a Bio-Rad MRC 1024 confocal microscope (Zeiss).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Bright patches on a DM11::GFP nucleus. These patches were always found associated with nuclei and, in contrast to plastids, were not observed in the cytoplasm. Bars represent 10 μm. Red fluorescence indicates propidium iodide staining of cell walls. This material is available as part of the online article from http://www.blackwell-synergy.com

References


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