Development of Tools for the Biochemical Characterization of the Symbiotic Receptor-Like Kinase DMI2

Brendan K. Riely,¹ Estíbaliz Larrainzar,¹ Cara H. Haney,² Jeong-Hwan Mun,³ Erena Gil-Quintana,⁴ Esther M. González,⁴ Hee-Ju Yu,⁵ David Tricoli,⁶ David W. Ehrhardt,^{2,7} Sharon R. Long,² and Douglas R. Cook¹

¹Department of Plant Pathology, University of California, One Shields Avenue, Davis, CA 95616, U.S.A.; ²Department of Biology, Stanford University, Stanford, CA 94305, U.S.A.; ³Department of Agricultural Biotechnology, National Academy of Agricultural Science, Rural Development Administration, 150 Suin-ro, Gwonseon-gu, Suwon 441-707 Korea; ⁴Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Navarra, Spain; ⁵Department of Life Sciences, The Catholic University of Korea, 43-1 Yeokgok 2-dong, Wonmi-gu, Bucheon 420-743, Korea; ⁶Ralph M. Parsons Foundation Plant Transformation Facility, University of California-Davis, One Shields Avenue, Davis, CA 95616, U.S.A.; ⁷Department of Plant Biology, Carnegie Institution for Science, 260 Panama St, Stanford, CA 94305, U.S.A.

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The *Medicago truncatula* DMI2 gene encodes a leucine-rich repeat receptor-like kinase that is essential for symbiosis with nitrogen-fixing rhizobia. While phenotypic analyses have provided a description for the host's responses mediated by DMI2, a lack of tools for in vivo biochemical analysis has hampered efforts to elucidate the mechanisms by which DMI2 mediates symbiotic signal transduction. Here, we report stably transformed *M. truncatula* lines that express a genomic DMI2 construct that is fused to a dual-affinity tag containing three copies of the hemagglutinin epitope and a single StrepII tag (*gDMI2:HAST*). *gDMI2:HAST* complements the *dmi2-1* mutation, and transgenic plants expressing this construct behave similarly to wild-type plants. We show that the expression patterns of *gDMI2:HAST* recapitulate those of endogenous DMI2 and that we can detect and purify DMI2:HAST from microsomal root and nodule extracts. Using this line, we show that DMI2 resides in a high-molecular weight complex, which is consistent with our observation that DMI2:GFP localizes to plasma membrane-associated puncta and cytoplasmic vesicles. We further demonstrate that Nod factor (NF) perception increases the abundance of DMI2 vesicles. These tools should be a valuable resource for the *Medicago* community to dissect the biochemical function of DMI2.

Legumes possess the unique ability to form symbioses with a family of gram-negative soil bacteria known as rhizobia in order to acquire fixed nitrogen. This interaction is preceded by a molecular dialog between the host and bacterium that takes place within the rhizosphere. Rhizobia secrete molecules known as Nod factors (NF), which activate a well-defined set

of molecular and physiological changes within the root. The hosts' responses to NF culminate with the infection of the host by the bacteria and the development of a novel organ, known as a root nodule. It is within this highly specialized organ that nitrogen fixation takes place. Symbiotic development is a tightly regulated process for which host specificity is dependent on NF structure (Oldroyd and Downie 2008).

Genetic screens have identified host genes required for NF perception and signal transduction. Among them, *DMI2* is required for nodulation, as a mutation in this gene abolishes both infection and developmental processes (Endre et al. 2002). *DMI2* functions very early in the *M. truncatula*–*Sinorhizobium* interaction and *dmi2* mutants lack many of the initial host responses to cognate symbiotic bacteria, including root hair-associated ion fluxes and calcium spiking, early nodulin gene expression, and cortical cell division (Catoira et al. 2000; Wais et al. 2000). Intriguingly, *DMI2* is also required for symbiosis with mycorrhizal fungi and, thus, functions in the “core” symbiotic pathway that controls recognition of multiple symbiotic microorganisms (Catoira et al. 2000).

DMI2 encodes an extracellular, leucine-rich repeat receptor-like kinase (LRR-RLK). LRR-RLK represent a large gene family in higher plants that function in a range of biological pathways, including pathogen recognition, hormone perception, and plant development (Afzal et al. 2008; Shiu and Bleecker 2001). Very little is known about the biochemical mechanisms by which the vast majority of LRR-RLK, including DMI2, regulate their respective pathways. However, several LRR-RLK have been well characterized in *Arabidopsis*, including BRI1, BAK1, and SERK1 (Karlova et al. 2006; Kim and Wang 2010; Wang et al. 2008). For these receptors, biochemical analyses have identified ligands, substrates, and detailed mechanisms of activation and regulation. Phosphorylation, ubiquitination, and endocytosis of these membrane-bound receptors are common mechanisms for both pathway activation and regulation (Geldner et al. 2007; Kim and Wang 2010; Karlova et al. 2006; Lu et al. 2011; Robatzek et al. 2006; Wang et al. 2008). Stably transformed plants expressing epitope-tagged receptors have greatly facilitated these studies by allowing the purification of the receptor and associated proteins. Until now, such resources have not been available to characterize the receptors that regulate symbiotic plant-microbe interactions.

Corresponding author: Douglas R. Cook; E-mail: drcook@ucdavis.edu; Telephone: +1.530.754.6561; Fax: +1.530.754.6617.

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Identifying and characterizing the proteins that interact with DMI2 *in vivo* would represent a significant step toward elucidating the biochemical mechanism underlying symbiotic signal transduction. While several interacting proteins have been identified in yeast two-hybrid screens, these interactions have yet to be demonstrated in their native plant environment (Chen et al. 2012; Den Herder et al. 2012; Kevei et al. 2007; Lefebvre et al. 2010; Zhu et al. 2008). Furthermore, it is unknown how NF perception affects DMI2 kinase activity, phosphorylation status, or protein interactions. With substantial expressed sequence tag and genome sequence data within the public domain, proteins isolated from *M. truncatula* cv. Jemalong A17 can be readily identified using tandem mass spectrometry analyses (MS/MS). Numerous laboratories have utilized this resource to inventory the *M. truncatula* proteome from various tissues or under different conditions (Gallardo et al. 2003; Grimsrud et al. 2010; Larrainzar et al. 2007; Watson et al. 2003; Zhang et al. 2006). Therefore, proteins that interact and copurify with DMI2 may also be identified using MS/MS. Purifying quantities of DMI2 from its native cellular environment that are sufficient for biochemical characterization is a prerequisite for these and other related studies, and the inability to do so has impeded functional characterization of this important signaling pathway.

In contrast to *Arabidopsis*, in which stable transformation is routine, many of the recent complementation and transgenic gene expression studies in *M. truncatula* have relied upon “hairy root transformation” systems (Boisson-Dernier et al. 2001). The ease and speed with which this protocol can generate transformed tissue has proven invaluable for interrogating the molecular underpinnings of NF perception in *M. truncatula*. However, a major shortcoming of hairy-root transformation is that it does not transform germline cells, and experimental material must, therefore, be perpetually generated. While this is not a significant liability when limited quantities of transgenic tissue are required, large-scale production of protein for applications such as MS/MS analysis is impractical using the hairy-root transformation system. Although highly embryogenic *M. truncatula* lines (e.g., R108, 2HA) can be used for stable transformation, the vast majority of genetic and genomic infrastructure (including symbiotic mutants such as *dmi2-1*) has been developed in *M. truncatula* cv. Jemalong A17. We recently developed an improved protocol for stably transforming *M. truncatula* cv. Jemalong A17, which we have used to transform recombinant, symbiotic receptors for cell biological analyses (Haney et al. 2011).

Here, we describe a novel transgenic *M. truncatula* line that expresses recombinant genomic (*g*)DMI2 translationally fused with a tandem-affinity tag for protein isolation. The recombinant gene is regulated by its native promoter and has been stably transformed into the nodulation-deficient, *dmi2-1* mutant background in *M. truncatula* cv. Jemalong A17. Recombinant DMI2 restores the ability of *dmi2-1* to form nitrogen-fixing nodules with *Sinorhizobium meliloti*. To facilitate the generation of tissue expressing the recombinant protein, we crossed the transgenic line with a *sunni dmi2-1* double mutant, resulting in a super nodulating plant expressing affinity-tagged DMI2. We show that our transgenic lines are equivalent to wild-type plants with regard to symbiotic development and that recombinant DMI2 can be detected, isolated, and identified by MS/MS. We further demonstrate that DMI2:HAST resides in high molecular weight (HMW) complexes and observe DMI2:GFP in plasma membrane associated puncta and cytosolic vesicles in *gDMI2:GFP* expressing hairy roots. The *gDMI2:HAST* and *sunni DMI2:HAST* transgenic lines we describe represent valuable, new resources for characterizing the molecular and biochemical function of DMI2.

RESULTS

Construction and characterization of epitope-tagged DMI2.

The *DMI2* gene from *M. truncatula* cv. Jemalong A17 was polymerase chain reaction (PCR)-amplified and was cloned into pENTR D-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) such that a unique *XmaI* site was engineered immediately preceding the stop codon. The resulting *gDMI2* clone, which serves as the foundation for C-terminal modification of DMI2, contains 2 kb of sequence upstream from the start codon, introns, and 500 bp downstream from the stop codon and should, therefore, contain all native regulatory elements. To confirm the cell and tissue specificity of the 2-kb *dmi2* promoter region, transcriptional fusions were prepared using the glucuronidase (*GUS*) reporter gene and introduced into roots of genotype A17 by means of hairy root transformation and were visualized following staining with X-Gluc. *pDMI2:GUS* yielded patterns of *GUS* staining that are consistent with prior observations of *DMI2* expression (Bersoult et al. 2005), with expression in epidermal cells of the infection zone, transitioning to developing nodule primordia, and ultimately in emergent nodules (Supplementary Fig. 1).

The unique *XmaI* introduced into the *gDMI2* foundation vector simplifies the introduction of epitope tags and fluorescent proteins onto DMI2, while the Gateway compatibility of the pENTR vector can be utilized to clone the recombinant genes into binary vectors. To facilitate detection and purification of DMI2, a dual-affinity tag containing three copies of the hemagglutinin (HA) epitope and a single StrepII affinity tag (HAST tag) were introduced into the *XmaI* site (Fig. 1A). HAST modifies the C-terminus of DMI2 so that two different affinity matrices can be used to isolate recombinant protein. As a control in the protein purification experiments and to enable subcellular localization of fluorescent DMI2 protein, an additional construct was made in which green fluorescent protein (GFP) was introduced into the *XmaI* site to generate *gDMI2:GFP*.

As a preliminary test of these constructs, we recombined both *gDMI2:HAST* and *gDMI2:GFP* into the DsRED-expressing binary vector pKGW-RR (Smit et al. 2005) and transformed them into *Agrobacterium* sp. strain MSU440. When transformed into the *dmi2-1* mutant background by hairy-root transformation, both GFP- and HAST-tagged constructs efficiently restored the ability of the *dmi2-1* mutant to nodulate (28 of 31 nod+, 30 of 33 nod+, and 0 of 15 nod+ for *gDMI2:GFP*, *gDMI2:HAST* and control vector, respectively) (Fig. 1B and C). To test the functionality of the HAST affinity tag, total protein extracts from *gDMI2:GFP* and *gDMI2:HAST* nodulated roots were precipitated, using either magnetic streptactin beads or anti-HA agarose beads and were analyzed on Western blots with α -HA antibodies. Both affinity matrices readily purified an approximately 109-kDa protein in *gDMI2:HAST* roots, consistent with the predicted molecular weight of DMI2:HAST. No such band was isolated from *gDMI2:GFP* control extracts (Fig. 1D).

Generation of stable transgenic lines expressing *gDMI2:HAST*.

For purposes of stable transformation, the pENTR:*gDMI2:HAST* construct was recombined with the binary vector pMDC123, which contains a marker gene conferring bioalaphos resistance. Following *Agrobacterium tumefaciens* transformation into a *dmi2-1* non-nodulating mutant background, five independent bioalaphos-resistant lines were regenerated, one of which produced T1 plants that were efficiently nodulated by *S. meliloti*. Screening of T2 progeny identified two siblings whose progeny were completely nod+ (35 of 35),

one of which (hereafter referred to as *gDMI2:HAST*) was chosen for further analyses. Southern blot analyses were performed on genomic DNA isolated from transgenic and nontransgenic plants using either the *DMI2* 3' untranslated region (UTR) or the HAST tag as probe (Fig. 2). Hybridization with the *DMI2* 3' UTR revealed two bands, only one of which was also present in the A17 and *dmi2-1* controls. The 4.9-kb band in A17, *dmi2-1*, and transgenic backgrounds is consistent with the predicted size of the *Hind*III fragment containing the endogenous *DMI2*, based on available genomic sequence (Supplementary Fig. 2). Hybridization of DNA blots using the HAST tag as a probe detected a single band specifically in the transgenic lines. Similar results were obtained when *Bam*HI was used to restrict genomic DNA (Supplementary Fig. 3). These results confirm the presence of the transgene and indicate that *gDMI2:HAST* contains a single copy of the T-DNA.

Expression of recombinant DMI2:HAST.

According to the *M. truncatula* Gene Expression Atlas (MtGEA) (Benedito et al. 2008), *DMI2* is transcribed in uninoculated roots and nodules but not in aerial tissues (Supplementary Fig. 4). Western blot analyses of a range of tissues using anti-HA antibodies confirmed the presence of DMI2:HAST protein specifically in roots and nodules, with higher levels observed in 10-day-old nodules relative to roots (Fig. 3A), consistent with the MtGEA expression data.

Genetic crosses were used to introduce *gDMI2:HAST* into the supernodulating background of *sun*n (Figs. 2 and 3B). The *SUN*N gene encodes an LRR-RLK that functions in the shoot

to negatively regulate root-nodule number (Schnabel et al. 2005), and *sun*n mutants produce approximately 10-fold more nodules than wild-type roots (Penmetsa et al. 2003; Schnabel et al. 2005). Western blot analysis of *sun*n *gDMI2:HAST* plants revealed a tissue distribution pattern for *gDMI2:HAST* that was indistinguishable from that of *gDMI2:HAST* in wild-type backgrounds (Fig. 3A and C). Moreover, although the level of DMI2:HAST protein was similar between wild-type and supernodulating backgrounds when corrected for fresh weight or total protein, as in Figure 3A and C, we observed a dramatic increase in DMI2:HAST on a per plant basis in the *sun*n genetic background that correlated with the increase nodule biomass (Fig. 3B, discussed below).

Quantitative assessment of *gDMI2:HAST* expression.

Introduction of a transgene can lead to its overexpression, causing one or more artifacts such as nonspecific protein interactions, mislocalization, or ectopic activation of a signaling pathway. To determine the level of *gDMI2:HAST* expression relative to wild-type *DMI2*, we performed real-time quantitative (q)PCR using cDNA isolated from uninoculated roots, which were previously shown to be a primary site of *dmi2* expression (Fig. 3). Genotypes under analysis included A17 (wild type), *sun*n, *dmi2-1* mutant, *gDMI2:HAST/dmi2-1*, and *sun*n/*gDMI2:HAST/dmi2-1*. In a first experiment, we used PCR primers that amplify identical sequences from all *dmi2* isoforms (i.e., wild type, HAST-tagged, and *dmi2-1*). As shown in Figure 4, *DMI2* transcript in the *gDMI2:HAST* lines were approximately 60% lower than in nontransformed con-

Fig. 1. Structure and characterization of the *gDMI2:HAST* construct. **A**, Diagram of the *gDMI2:HAST* construct and hemagglutinin (HA) epitope and a single StrepII affinity (HAST) tag sequence. The three copies of the HA epitope are highlighted in bold and the StrepII tag is underlined. **B** and **C**, Darkfield image (upper) and fluorescent image (lower) demonstrating complementation of *dmi2-1* mutant by *pKGW-RR:gDMI2:GFP* (**B**) and *pKGW-RR:gDMI2:HAST* (**C**). Red fluorescence indicates that the root contains the transgene. Note that only transformed roots are nodulated. **D**, Extraction and detection of DMI2:HAST from root extracts. Total protein extracts from *gDMI2:GFP* or *gDMI2:HAST* hairy-root tissue was precipitated with Streptactin magnetic beads or anti-HA agarose beads, and the precipitated proteins were subjected to protein blot analyses with α-HA antibodies. A gel with identical “input” proteins was run concurrently and was stained with Coomassie brilliant blue (CBB) to show equal loading of proteins. Scale bar = 2 mm.

trols. Similar analysis of *sun* and *sun/gDMI2:HAST* revealed comparable transcript levels between the two genotypes. *DMI2* transcripts were detected in *dmi2-1* lines, but at levels approximately 25% of the levels observed in wild-type lines. This result mirrors the results of Mitra and associates (2004), in which *dmi2-1* showed reduced transcript levels presumably due to nonsense-mediated transcript decay. An additional set of PCR primers were designed to flank the HAST insertion site and, thus, distinguish between transcription of ectopic *gDMI2:HAST* and endogenous *dmi2-1* transcript. The results confirm that the significant majority of *DMI2* transcript originates from the *gDMI2:HAST* transgene (Supplementary Fig. 5).

Physiological characterization of *gDMI2:HAST* and *sun gDMI2:HAST*.

To assess the impact of *gDMI2:HAST* on plant growth and symbiotic performance, plants were inoculated with *S. meliloti* and were grown in the absence of inorganic nitrogen under controlled environmental conditions. After 10 weeks, the biomass of shoots, roots, and nodules of transgenic and nontransgenic plants was determined. *gDMI2:HAST* plants showed no significant differences compared with wild-type plants for root (Student's *t*, $\alpha < 0.05$) and nodule biomass (0.46 ± 0.08 g for A17, 0.39 ± 0.04 g for *gDMI2:HAST*), although they exhibited

a slight reduction in shoot biomass (Fig. 5A). Similar analyses with *sun gDMI2:HAST* plants revealed no significant differences for biomass in any of these tissues. As a direct test of nitrogen fixation rates, we quantified apparent nitrogenase activity (ANA) by measuring H_2 evolution from intact plants (Witty and Minchin 1998). Both *gDMI2:HAST* and *sun gDMI2:HAST* lines exhibited ANA equivalent to that of their respective parental lines (Fig. 5B). Taken together, these results indicate that the *gDMI2:HAST* transgene restores the symbiotic phenotypes of *dmi2-1* plants.

Isolation of recombinant *DMI2:HAST* by affinity chromatography.

Our goal in developing *gDMI2:HAST* transgenic lines was to create a tool to facilitate the rapid isolation of sufficient pro-

Fig. 2. Southern blot analyses show that *gDMI2:HAST* transgenic lines contain a single T-DNA insertion. Genomic DNA was isolated from the indicated lines and digested with *HindIII* and Southern blot analysis was performed. Blots were hybridized with probes corresponding to either the 3' untranslated region (UTR) of *DMI2* (left) or to the *HAST* tag (right).

Fig. 3. *DMI2:HAST* protein is only present in roots and nodules in wild-type and supernodulating backgrounds. Approximately 60 μ g of total protein from the indicated tissues were isolated from **A**, *gDMI2:HAST* plants or **C**, *sunn gDMI2:HAST* plants and were subjected to protein blot analyses using anti-hemagglutinin (HA) antibodies. Upper panels depict the results of a Western blot using anti-HA antibodies. Lower panel shows an identically loaded gel that was stained with Coomassie brilliant blue, indicating that comparable amounts of protein were loaded in each lane. **B**, The supernodulation phenotype of *sunn gDMI2:HAST* in comparison with *gDMI2:HAST* 10 days after inoculation. L = leaves; Pe = petioles; S = stems; R = roots; N = nodules; F = flowers; and Po = pods.

Fig. 4. *gDMI2:HAST* expression is comparable to endogenous *DMI2* expression. Quantitative polymerase chain reaction analysis of *DMI2* expression in 2-week-old wild-type (A17), *sunn*, *dmi2-1* mutant, *gDMI2:HAST*, and *sunn gDMI2:HAST* uninoculated roots. Primers were designed to anneal to a region within the coding region of *DMI2*, thus the levels of endogenous and recombinant *DMI2* transcripts are monitored. * and ** denote statistically significant differences relative to wild type (Student's *t*, $\alpha < 0.05$ and $\alpha < 0.001$, respectively).

tein for MS/MS and biochemical characterization. DMI2 is predicted to reside in the plasma membrane based on its putative signal peptide and single transmembrane domain (Endre et al. 2002). We isolated membrane microsomes from nodulated *sun* and *sun* *gDMI2:HAST* roots and used Western blot analysis with the anti-HA antibody to compare microsomal and soluble fractions. The results confirm that the vast majority of DMI2:HAST is associated with membrane microsomes (Fig. 6A). DMI2:HAST was readily solubilized with detergent from microsomal pellets and was subsequently bound to and eluted from Streptactin affinity columns, resulting a substantial enrichment in DMI2:HAST (Fig. 6B). The dual-affinity HAST tag permitted a rapid, second purification using anti-HA antibodies conjugated to agarose beads. Western blot analyses of 5% (vol/vol) of the bound and unbound proteins again demonstrated a substantial further enrichment in DMI2 by the HA-beads (Fig. 7A). Silver staining the remaining 95% (vol/vol) of the eluted protein revealed a discrete band of a size corresponding to DMI2:HAST (Fig. 7B). MS/MS analyses of the excised band identified three unique peptides corresponding to DMI2, thus confirming that we had, in fact, isolated DMI2 (Table 1).

Dynamic localization of DMI2 in *M. truncatula* root hairs.

The LRR-RLK DMI2 contains both a signal peptide and a single pass transmembrane domain and is predicted to localize in the plasma membrane. In order to investigate the subcellular localization of DMI2 in its native root-hair environment, we expressed *gDMI2:GFP* in a *dmi2-1* background by hairy-root transformation and examined transformed root hairs by spinning-disk confocal microscopy. In uninoculated root hairs, the majority of DMI2:GFP exhibited a punctate distribution in the extreme periphery of the root-hair cell (Fig. 8A) that was clearly distinct from autofluorescence in root hairs expressing *gDMI2:HAST* (Fig. 8C and D). The majority of the DMI2:GFP signal was peripheral to a cytoplasmic DsRED marker, consistent with its localization in the plasma membrane (Fig. 8B). However, we occasionally observed DMI2:GFP-labeled organelles that codistributed with the cytosolic DsRED marker that likely correspond to cytoplasmic vesicles. When we treated DMI2:GFP roots with 10 nM NF, we observed a marked increase in the number of cytoplasmic vesicles (Fig 9; Supplementary Videos 1 and 2). Quantifying vesicle abundance before and after NF treatment indicated a 6.5-fold increase in DMI2:GFP-labeled cytoplasmic organelles in NF-treated root hairs

(0.2 ± 0.1 vesicles per $100 \mu\text{m}^2$ of cytoplasm in buffer-treated cells to 1.3 ± 0.3 vesicles per $100 \mu\text{m}^2$ by 30 min after NF treatment [$P < 0.001$ by two-tailed *t*-test]).

Gel filtration analyses suggest that DMI2 is present in HMW complexes in roots.

The discrete puncta and cytoplasmic DMI2:GFP vesicles observed by microscopy suggest that the DMI2 receptor is organized into HMW structures. To further test this hypothesis using the stably transformed DMI2:HAST lines, we extracted total protein from either A17 plants or DMI2:HAST plants treated with either buffer or 10 nM NF and fractionated these extracts using gel-filtration chromatography (Fig. 10). Consistent with our previous results, we did not observe a band corresponding to DMI2:HAST in any of the fractions from A17

Fig. 6. Enrichment and purification of DMI2:HAST from solubilized microsomes using Streptactin affinity matrix. **A**, Protein blot analyses indicate that DMI2:HAST is enriched in microsomes. Total root extracts (T) were subjected to high-speed ultracentrifugation to separate soluble (S) and microsomal (M) fractions. **B**, Microsomes from *sunn* and *sunn gDMI2:HAST* root extracts were solubilized with Triton X-100 and equal amounts of soluble microsomal proteins were used as input (I) on a Streptactin sepharose affinity column. Flow-through (FT) represents the portion of DMI2:HAST that did not bind to the column. Eluted proteins (E) were incubated overnight at 4°C with Strataresin to concentrate the sample, and unbound (UB) and bound (B) proteins were sampled. Equal volumes of all samples were loaded and the relative percentage of sample volume is indicated below the blot. DMI2:HAST was detected using horseradish peroxidase-conjugated anti-hemagglutinin antibodies. Approximate molecular weight is indicated in kDa based on comigrating protein standards. Results are representative of at least three purifications.

Fig. 5. *gDMI2:HAST* transgenic plants grow and fix nitrogen to similar levels as nontransgenic plants. **A**, Biomass content of 10-week-old plants grown under symbiotic conditions. Shoot, root, and nodule biomass values are represented in white, hatched, and black, respectively. **B**, Apparent nitrogenase activity values estimated as H_2 evolution (Witty and Minchin 1998). Asterisk denotes a statistically significant difference (Student's *t*, $\alpha < 0.05$). Error bars represent standard error. FW = fresh weight.

protein extracts. In buffer-treated roots, the peak of DMI2:HAST elution occurred in the void volume (Vo). Vo for the Sephacryl HR-300 contains structures and complexes with an apparent MW >1 MDa, which suggests that DMI2:HAST is present in HMW complexes in the root. Treatment with 10 nM NF for 30 min, however, did not alter the elution profile of DMI2:HAST within the resolution of our column.

DISCUSSION

The ability to monitor and purify sufficient quantities of DMI2 during an active symbiotic host response is essential, if we are to elucidate the mechanism by which this receptor effects symbiotic signal transduction. Efforts to characterize DMI2 at the biochemical level have been hampered by a lack of tools to detect and purify DMI2 from its native root and nodule environment. We have addressed this need by developing stably transformed *M. truncatula* expressing an engineered DMI2 that is fused to the HAST dual-affinity tag. We demonstrate that this *gDMI2:HAST* transgenic line is functionally similar to wild-type plants and that we are able to efficiently detect and purify DMI2:HAST from root and nodule extracts. We have used this line in conjunction with *gDMI2:GFP* expressing hairy roots to show that DMI2 localizes in HMW complexes in the plasma membrane as well as in DMI2-con-

taining cytoplasmic vesicles whose abundance increases following NF treatment.

In designing our *gDMI2:HAST* construct, we included a large, native promoter region, introns, and a native 3' UTR in an attempt to faithfully reproduce the expression patterns and levels of DMI2, avoiding possible artifacts that can arise from the use of constitutive promoters such as *Cauliflower mosaic virus* 35S. Indeed, several reports indicate that 35S is weakly expressed in *M. truncatula* root nodules and constructs expressed under 35S do not fully complement the *dmi2* mutation (Auriac and Timmers 2007; Limpens et al. 2005). Our *gDMI2:HAST* and *sunni gDMI2:HAST* transgenic lines both contain a single copy of the transgene. We demonstrate that DMI2:HAST protein is present specifically in root and nodules, in agreement with previous gene-expression studies (Bersoult et al. 2005) and Affymetrix data available in the MtGEA. Although our qPCR analyses indicate that the expression levels of *gDMI2:HAST* are slightly reduced relative to the level of DMI2 transcript present in wild-type plants, our physiological analyses show that this level of expression is sufficient to fully complement the *dmi2-1* mutation. Importantly, nodule biomass and nitrogen-fixation efficiencies in *gDMI2:HAST* and *sunni gDMI2:HAST* plants are comparable to non-transgenic control plants, indicating that the recombinant protein is fully functional at the expression levels achieved. Interestingly, on a fresh weight basis, the ANA of *sunni* DMI2:HAST is approximately half that of A17 nodules but is comparable to *sunni* nodules. This phenomenon of decreased nitrogen fixation rates in plants with excessively high nodule biomass has been well described in different legume species and is likely a consequence of host regulation (Carroll et al. 1985; Penmetsa and Cook 1997; Penmetsa et al. 2003; Wopereis et al. 2000).

The DMI2 promoter fragment used to drive *gDMI2:HAST* transcription is expressed both early, during the *M. truncatula*-*S. meliloti* interaction in the infection zone of the root, and later, during formation of the nodule primordia and development of mature nodules. RNA interference experiments indicate that DMI2 is involved in the release of bacteria from infection threads within differentiated nodules (Limpens et al. 2005), but the biochemical and cell biological mechanisms by which DMI2 achieves this are not clear. Interestingly, DMI2 expression in nodule primordia precedes the penetration of cortical cells by infection threads, suggesting that DMI2 may have additional functions during nodule development (Bersoult et al. 2005). This speculation is further supported by the findings that DMI2 interacts with HMGR, a gene that is expressed primarily in the epidermal layer and in root hairs and whose expression is substantially reduced in root nodules (Kevei et al. 2007). The fact that *gDMI2:HAST* is expressed in the relevant cell and tissue types during infection and nodule development and at near wild-type levels increases the likelihood that DMI2:HAST will form native protein complexes appropriate to each developmental stage.

We observed DMI2:GFP in discrete, plasma membrane-localized puncta and in cytosolic vesicles, while gel filtration

Fig. 7. Enrichment of DMI2:HAST by anti-hemagglutinin (HA) immunoprecipitation. **A**, Protein blot analyses of anti-HA immunoprecipitation. Eluate from Streptactin affinity column was incubated overnight with anti-HA conjugated agarose beads. Approximately 0.5% of Streptactin eluate (E) was analyzed with 10% of the anti-HA bound protein (B) An equal volume of the immunoprecipitation supernatant representing the unbound protein (UB) was run to analyze binding efficiency of the anti-HA resin. **B**, The remaining 90% of the anti-HA bound protein was run on a separate gel and was stained with silver. Approximate molecular weight is indicated in kDa based on comigrating protein standards.

Table 1. Identification of DMI2 peptides by tandem mass spectrometry following tandem-affinity purification of DMI2:HAST from nodulated root extracts

Peptide sequence ^a	X! Tandem-log(e) score	Calculated peptide mass ^b	Charge state	Observed m/z	Ions ^c
(R)GLAYLHTFGR(S)	5	1232.4364	2	616.18	12(20)
(R)SWIEETNQKDLEVIQK(M)	3.8	1961.1924	2	980.62	13(30)
(R)SWIEETNQKDLEVIQK(M)	5.12	1961.1924	2	980.82	13(30)
(K)YAPQEGDSYVSLEVR(G)	5.6	1713.8526	2	857.16	15(28)
(K)YAPQEGDSYVSLEVR(G)	6.8	1713.8526	2	857.2	16(28)

^a First and last amino acids in parentheses correspond to the residues preceding and following the DMI2 protein sequence.

^b Average calculated peptide mass determined using Protein Prospector.

^c Total number of b- and y- ions identified; observed (theoretical).

analyses suggest that DMI2 is contained in large, >1-MDa complexes. NF application increased the number of DMI2:GFP-containing cytosolic vesicles, indicating that NF perception regulates DMI2 localization. This increase in vesicles may be the result of endocytosis of DMI2 or DMI2-receptor complexes from the membrane or the result of new DMI2 synthesis and secretion. Although we cannot preclude secretion as the source of DMI2:GFP vesicles, ligand activated endocytosis of a plasma membrane localized DMI2 is consistent with current paradigms of receptor regulation and with recent results from Den Herder and colleagues (2012) (discussed below).

In contrast to FL2S and BRI1, which directly bind flg22 and brassinosteroids, respectively (Chinchilla et al. 2006; Kinoshita et al. 2005), DMI2 is not predicted to directly bind NF. Molecular and genetic evidence instead suggest that the LYSM receptors NFP and LYK3 are the Nod-factor receptors (Arrighi et al. 2006; Bensmihen et al. 2011; Limpens et al. 2003; Smit et al. 2007). The rapid increase in DMI2:GFP signal in vesicles upon NF addition suggests that this response is likely dependent on either NFP or LYK3. Intriguingly, the punctate pattern of DMI2 distribution is reminiscent of the LYSM receptor kinase LYK3 and the microdomain associated FLOTILLIN4 (FLOT4), both of which are required for rhizobial infection (Haney and Long 2010; Smit et al. 2007). While LYK3 and FLOT4 have little colocalization in nonsymbiotic root hairs, they show a marked increase in colocalization fol-

lowing inoculation with *S. meliloti* in a NF-dependent manner (Haney et al. 2011). It is possible that the large DMI2 complex we observe by gel filtration represents a microdomain-associated signaling complex containing multiple symbiotic receptors, but biochemical and cell biological evidence for such a structure is currently lacking. Purification and proteomic analysis of DMI2:HAST protein complexes will help identify the constituents of the HMW complex.

The well-supported paradigm of receptor-mediated signal transduction is that ligand binding by transmembrane receptor kinases induces receptor homo- or heterodimerization, auto- and transphosphorylation, and subsequent activation of downstream signaling components. Cells can further modulate receptor activity via endocytosis to quickly regulate the amount of both ligand and receptor in the plasma membrane. Endocytosed receptors are either recycled back to the plasma membrane or alternatively can be directed to the vacuole for degradation (Robatzek et al., 2006; Russinova et al. 2004; Schulze et al. 2010; Wang et al. 2005, 2008). We observed DMI2 vesicles both in the presence and absence of NF ligand similar to the brassinosteroid receptor BRI1. BRI1 exhibits a constitutive endocytosis and recycling of plasma membrane-derived vesicles, and BRI1 signaling takes place from these endosomes (Geldner et al. 2007). In contrast to BRI1, in which ligand activation does not affect BRI1 endocytosis, activation of the symbiotic pathway by NF treatment markedly increased the number

Fig. 8. DMI2:GFP has a punctate localization at the extreme cell periphery. **A**, Single optical plane through a root hair expressing *gDMI2:GFP* (green). DsRED marks the cytoplasm and nucleus (red). **B**, Plot of fluorescence intensity vs. position of blue arrow in (A). Note that the peak intensity of the green signal is peripheral to the red cytoplasmic signal. **C**, Maximum intensity projection of *gDMI2:GFP*-expressing root hair in (A). **D**, Root hair expressing *gDMI2:HAST*. Some autofluorescence is evident in the green channel but does not appear punctate. Scale bars = 10 μ m. Projections in C and D are 15 optical planes taken at 0.2- μ m increments.

of DMI2 vesicles. In this regard DMI2 behavior is reminiscent of the FLS2 receptor, which mediates perception of bacterial flagellin. FLS2 localizes in the plasma membrane and specifically undergoes endocytosis following perception of the flg22 peptide (Robatzek et al. 2006). FLS2 endocytosis is believed to carefully restrict the plant defense response by removing both activated receptors and elicitors from the plasma membrane. Likewise, DMI2 endocytosis may serve as a regulatory mechanism to limit the number of root hairs that respond to rhizobium or perhaps to attenuate the DMI2 signal once pathway activation has been achieved.

Ubiquitination has been shown to elicit the endocytosis of plant receptors (Den Herder et al. 2012; Lu et al. 2011;

Robatzek et al. 2006). Recently, the *Lotus japonicus* 'Seven In Absentia 4' (SINA4) E3 ligase was shown to interact with the *L. japonicus* DMI2 ortholog SYMRK (Den Herder et al. 2012). Coexpression of SINA4 with SYMRK in *N. benthamiana* cells induces a relocalization of SYMRK from the plasma membrane to cytosolic vesicles, which also contain SINA4 (Den Herder et al. 2012). Furthermore, ectopic expression of SINA4 reduced both the amount of SYMRK in transgenic *L. japonicus* roots as well as the number of infections in root hairs. The authors propose that SINA4 mediates ubiquitination, endocytosis, and degradation of SYMRK as a means to regulate symbiotic infection. These data are consistent with our observations in intact *M. truncatula* root hairs in which NF

Fig. 9. Nod factor (NF) treatment results in an increase in DMI2:GFP-labeled cytoplasmic vesicles. **A**, A single optical section of a buffer-treated *gDMI2:GFP*-expressing root hair. Typically most of the DMI2:GFP (green) signal is peripheral to the cytoplasm (red) with little signal showing codistribution with the cytoplasmic dsRED marker ($n = 10$ from three plants). **B**, By 30 min posttreatment with 10 nM NF, cytoplasmic vesicles labeled with DMI2:GFP were evident in most root hairs observed (15 or 19 root hairs [from four plants]). Scale bars = 10 μ m.

Fig. 10. DMI2:HAST migrates in a gel filtration column as a high molecular weight structure. Total proteins were extracted from the roots of either *M. edicago truncatula* 'A17' (wild type) or DMI2:HAST plants treated with either buffer (MOCK) or 10 nM Nod factor (NF) for 30 min and were separated by gel-filtration chromatography. Each 4-ml fraction was concentrated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for protein blot analysis with α -hemagglutinin antibodies. Numbers above each lane refer to fraction number and the migration of size standards is indicated at the top. Total protein extracts were run on the left for reference.

treatment increases the number of DMI2-containing vesicles. Parallel gel filtration analysis of DMI2 reveals HMW complexes both preceding and following NF treatment, although it is unclear if such HMW complexes are representative of vesicle-associated DMI2:HAST protein or simply of residual plasma membrane-associated DMI2:HAST. The availability of the tagged DMI2:HAST protein should facilitate the biochemical analysis of DMI2 at various stages during NF signaling, including direct analysis of ubiquitination and degradation.

In summary, we have developed stable transgenic lines for the biochemical characterization of the symbiotic receptor DMI2. We demonstrate that both the HA and StrepII epitopes are effective tags for purifying DMI2, either individually or as part of a tandem-affinity purification protocol. In these proof of concept experiments, we were able to monitor DMI2:HAST throughout plant development by Western blot analysis, to document protein association with membrane fractions and to identify DMI2 peptides by means of MS/MS. DMI2 localizes in plasma membrane-associated puncta and cytosolic vesicles and we provide evidence that DMI2 functions in a HMW complex both in the presence and absence of NF. The transgenic lines described in the present work will now allow us to characterize the nature of these protein complexes and the biochemical mechanism underlying DMI2 regulation in their native root environment.

MATERIALS AND METHODS

Construction of vectors.

Standard molecular biology techniques were used to generate vectors for transformation. Details on vector construction can be found in the Supplementary Methods published online.

Plant materials.

sun1 dmi2-1 double mutants were created by crossing *sun1* (nod++) pollen onto *dmi2-1* (nod-) recipients and scoring the F1 for complementation of the nod- phenotype. The genotype at the *sun1* locus for nod+ was confirmed using a cleaved amplified polymorphic sequence (CAPS) marker that detects the *sun1-4* allele. Forward 5' ATGGAAATGTTGAATGGGTG 3' and reverse 5' CATTTGTCTACCATCTATCC 3' primers amplify a 787-bp *sun1* fragment from wild-type A17 that is cleavable with *StuI*. The *sun1-4* mutation disrupts the *StuI* site. F1 plants were allowed to self-pollinate, nod- individuals (i.e., homozygous *dmi2* mutants) were identified, and lines homozygous for the *sun1-4* allele were subsequently identified, using the CAPS marker described above.

To create *sun1 gDMI2:HAST*, pollen from a *gDMI2:HAST* homozygous line was crossed onto *sun1 dmi2-1* double mutant plants and nod+ F1 plants (*SUN1/sun1*, *dmi2/dmi2*, *gDMI2:HAST/-*) were identified by inoculating with rhizobium and were scored for complementation of the nod- *dmi2-1* mutation. Nod+ individuals were allowed to self-pollinate, and F2 seed were collected. This generation segregated for the *sun1* mutation and the *gDMI2:HAST* transgene. F2 plants were therefore grown in a cassion and were inoculated with *Sinorhizobium meliloti*. Individuals that exhibited the supernodulation phenotype (i.e., *sun1/sun1*, *gDMI2:HAST/-*) were collected, and their homozygous *sun1-4/sun1-4* genotype was confirmed using CAPS analyses. In order to identify *sun1/sun1* individuals that were homozygous for the *gDMI2:HAST* transgene, F3 pods from individual plants were collected and their nodulation phenotype assessed. More than 30 plants for each F3 line were tested for nodulation phenotype, and one line (*sun1 gDMI2:HAST*) was identified for which all progeny were nod++ (*sun1/sun1*, *dmi2/dmi2*, *gDMI2:HAST/gDMI2:HAST*).

Methods for propagating plants in aeroponic cassions (Cook et al. 1995), hairy-root transformation (Riely et al. 2007), and

generation of stable transgenic plants (Haney et al. 2011) have been previously described.

Confocal microscopy.

Spinning-disk confocal microscopy was performed on a system described previously by Gutierrez and associates (2009) using a 63 $\times$ 1.3NA glycerol immersion objective. GFP and DsRed were excited at 491 and 561 nm, respectively, by solid-state lasers; emission filtering was accomplished with band pass filters (530/50nm for GFP, and 640/50 nm for DsRED; Chroma Technology, Bellows Falls, VT, U.S.A.). Maximum intensity z-projections of root hairs are from approximately 15 images taken at 0.2- μ m increments (Mad City Labs NanoDrive, Madison, WI, U.S.A.). Time-lapse images were taken at 2-s increments. Stacks were processed using ImageJ software. Typical exposure times were 1,000 ms for GFP and 300 ms for DsRED.

Physiological analyses.

For the measurement of plant biomass and nitrogen fixation rates, *M. truncatula* plants were grown under controlled environmental conditions (14-h day and 10-h night; 600 μ mol m⁻² s⁻¹ light intensity; 22°C-day and 16°C-night temperature; 70 to 60% relative humidity) in 1-liter pots with a mixture of vermiculite and perlite (5:2, vol/vol) as substrate. When plants were 10 weeks old, ANA was measured as H₂ evolution of intact plants in an open flow-through system under N₂:O₂ (79:21%, vol/vol) according to Witty and Minchin (1998), using an electrochemical H₂ sensor (Qubit System Inc., Ontario, Canada). At least three plants per genotype were measured. Subsequently, plant shoots, roots, and nodules were individually collected and their fresh weight was determined.

Precipitation and immunodetection analysis.

For protein extraction, tissues were ground to a fine powder in liquid nitrogen and were then thawed in extraction buffer consisting of 100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.5% (vol/vol) Triton X-100, 1% (vol/vol) plant protease inhibitor cocktail (Sigma, St. Louis). Extracts were clarified by centrifugation, the protein concentrations in the resulting supernatants were determined using a Bio Rad RC/DC protein assay (BioRad, Hercules, CA, U.S.A.), and extracts were normalized using extraction buffer. Equal amounts of protein were electrophoresed on a precast 5 to 14% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gradient gel (Bio-Rad) and were transferred to a polyvinylidene fluoride membrane. Membranes were incubated overnight in blocking buffer (phosphate-buffered saline containing 0.1% [vol/vol] Tween-20 [PBST], 5% [wt/vol] nonfat dry milk), before incubating with horseradish peroxidase-conjugated, anti-HA high affinity, monoclonal antibody (clone 3F10, Roche, Indianapolis, IN, U.S.A.) diluted 1:1,000 in blocking buffer. Membranes were washed several times with PBST and membrane was developed using an ECL chemiluminescent Western blotting detection kit (GE Healthcare, Piscataway, NJ, U.S.A.) according to manufacturer's instructions. For precipitation and immunoprecipitation experiments, protein extracts were incubated overnight with either Strep-Tactin magnetic beads (Qiagen, Valencia, CA, U.S.A.) or anti-HA affinity matrix (clone 3F10, Roche) at 4°C with rotation. Affinity resin was subsequently washed three times and bound proteins were eluted by boiling in SDS sample buffer prior to electrophoresis and immunodetection.

DMI2:HAST purification.

For large-scale protein purification, *sun1 gDMI2:HAST* or *sun1* plants were grown in aeroponic cassions for 2 weeks before inoculating with *S. meliloti* 1021. Ten days after inoculation, approximately 40 g of nodulated root systems were har-

vested and were frozen in liquid nitrogen. To isolate microsomes, roots were homogenized in grinding buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 330 mM sucrose, 0.6% (wt/vol) polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg of leupeptin per milliliter, and 1 µg of pepstatin per milliliter) using a blender (Smart Stick, Cuisinart, East Windsor NJ, U.S.A.). Homogenate was filtered through two layers of Miracloth, and extracts were further clarified by centrifugation at $1,000 \times g$ for 10 min. Microsomes were isolated from clarified extracts by high-speed ultracentrifugation for 1 h at $100,000 \times g$. The microsomal pellet was resuspended in resuspension buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% [vol/vol] plant protease inhibitors [Sigma], 0.1 mg of avidin per milliliter). In order to solubilize microsomes, Triton X-100 was added to resuspended microsomes to a final concentration of 0.2% (vol/vol) before incubation in a 4°C room for 30 min with rotation. Unsolubilized material was removed by ultracentrifugation (30 min at $100,000 \times g$), and the resulting supernatant was subsequently passed through a Durapore low-protein binding syringe filter (0.45 µm pore size, Millipore, Bedford, MA, U.S.A.). Extracts were normalized for volume and protein concentration prior to running them over a prepacked 1-ml Streptactin Sepharose column (IBA, Göttingen, Germany). Columns were washed five times with 1 ml of wash buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% [vol/vol] Triton X-100) before elution with 6×0.5 -ml aliquots of elution buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% [vol/vol] Triton X-100, 2.5 mM desthiobiotin). The first two fractions represented Vo and were discarded. The subsequent four fractions were pooled for immunoprecipitation.

MS.

Purified proteins from *sunm gDMI2:HAST* or *sunm* plants were electrophoresed on precast 5 to 14% polyacrylamide gels and were stained with silver, using a Silverquest kit (Invitrogen) according to manufacturer's protocols. Gel slices corresponding to DMI2:HAST and a control slice corresponding to the same MW from the *sunm* purification were excised and destained according to the Silverquest protocol. The gel slices were washed twice with 50 mM ammonium bicarbonate (AMBIC) before dehydration with acetonitrile (ACN). Samples were rehydrated and reduced by incubating in 10 mM dithiothreitol, 50 mM AMBIC for 30 min at 56°C. Following dehydration with ACN, samples were alkylated with 55 mM iodoacetamide in 50 mM AMBIC for 20 min at room temperature. Gel pieces were subsequently washed in 50 mM AMBIC, were dehydrated with ACN, and were dried in a speedvac. To digest the proteins, the gel pieces were rehydrated with 50 mM AMBIC, pH 8.0, supplemented with 250 ng of trypsin and were incubated overnight. The following day, the supernatant from the digest was collected and reserved. Peptides were then extracted from the remaining gel particles by sonication in the presence of 60% ACN (vol/vol) and 0.1% trifluoroacetic acid (TFA) (vol/vol). Supernatant was collected from the sonicated gel particles and were pooled with the previous supernatant. The pooled mixture was concentrated in a speedvac and was resuspended in 2% ACN (vol/vol), 0.1% TFA (vol/vol) before loading onto an autosampler.

Samples were resolved on a paradigm high-pressure liquid chromatography system (Michrom Bioresources, Auburn, CA, U.S.A.) coupled to a Thermo Finnigan LTQ ion trap mass spectrometer (Thermo Fisher, Waltham, MA, U.S.A.) equipped with a Michrom advance captive spray ionization source. Each digested sample was loaded onto an Agilent trap (Zorbax 300SB-C18, 5 µm, 5×0.3 -mm; Agilent Technologies, Santa Clara, CA, U.S.A.) and was desalted online. Peptides were

then eluted from the trap, were separated on a reverse-phase Michrom Magic C18AQ (200 µm $\times$ 150 mm) capillary column at a flow rate of 500 nl/min. Peptides were eluted using a 45-min gradient. The raw data was analyzed using X!Tandem and was viewed using the Scaffold Viewer (Proteome Software, Portland, OR, U.S.A.).

Gel filtration chromatography.

For gel filtration experiments, samples were ground in liquid nitrogen and were thawed for 30 min on ice in extraction buffer containing 0.1 M Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, 2.5 mM NaF, 50 mM Na-pyrophosphate, 0.6% octylglucoside, and 1% plant protease inhibitor cocktail (Sigma). Samples were clarified at 14,000 rpm in a microcentrifuge before being filtered through an Acrodisc 0.2-µm Supor Membrane syringe filter (PALL Life Sciences, Port Washington, NY, U.S.A.). Samples were normalized to 4 mg/ml and a 1-ml sample was loaded onto a HiPrep 16/60 Sephacryl S300 HR column (GE Healthcare) using an AKTA fast protein liquid chromatography system. Extracts were eluted at a flow rate of 0.5 ml/min in 120 ml of running buffer (0.1M Tris, pH 8.0, 150 mM NaCl, 10m M EDTA, 0.06% octylglucoside) and were collected in 4-ml fractions. Fractions were concentrated using Strataresin (Stratagene, La Jolla, CA, U.S.A.) prior to Western blot analysis, which was performed as described above. The column was calibrated with the gel filtration MW markers ribonuclease A (13.7 kDa) carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), ferritin (443 kDa), thyroglobulin (667 kDa), and blue dextran (2,000 kDa) (GE Healthcare).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

ImageJ software: rsb.info.nih.gov/ij
 Protein Prospector server: prospector.ucsf.edu/prospector/tmshome.htm