A role for the mevalonate pathway in early plant symbiotic signaling

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Rhizobia and arbuscular mycorrhizal fungi produce signals that are perceived by host legume receptors at the plasma membrane and trigger sustained oscillations of the nuclear and perinuclear Ca^{2+} concentration (Ca^{2+} spiking), which in turn leads to gene expression and downstream symbiotic responses. The activation of Ca^{2+} spiking requires the plasma membrane-localized receptor-like kinase Does not Make Infections 2 (DMI2) as well as the nuclear cation channel DMI1. A key enzyme regulating the mevalonate (MVA) pathway, 3-Hydroxy-3-Methylglutaryl CoA Reductase 1 (HMGR1), interacts with DMI2 and is required for the legume–rhizobium symbiosis. Here, we show that HMGR1 is required to initiate Ca^{2+} spiking and symbiotic gene expression in *Medicago truncatula* roots in response to rhizobial and arbuscular mycorrhizal fungal signals. Furthermore, MVA, the direct product of HMGR1 activity, is sufficient to induce nuclear-associated Ca^{2+} spiking and symbiotic gene expression in both wild-type plants and *dmi2* mutants, but interestingly not in *dmi1* mutants. Finally, MVA induced Ca^{2+} spiking in Human Embryonic Kidney 293 cells expressing DMI1. This demonstrates that the nuclear cation channel DMI1 is sufficient to support MVA-induced Ca^{2+} spiking in this heterologous system.

HMG-CoA reductase | mevalonate | calcium signaling | legume nodulation | arbuscular mycorrhization

The mevalonate (MVA) pathway controls the biosynthesis of hundreds of isoprenoids (sterols, carotenoids, prenyl side chains, etc.) in eukaryotes. These isoprenoids contribute to membrane integrity and development, among many other functions (1). Here, we report that the MVA pathway is also necessary for the earliest responses of plants to symbiotic signals produced by nitrogen-fixing rhizobia and arbuscular mycorrhizal (AM) fungi. These two types of endosymbiotic associations require a common set of genes in host plants to allow successful bacterial and fungal colonization. The molecular mechanisms controlling the establishment of the legume–rhizobium symbiosis have been extensively studied in model legumes such as *Medicago truncatula* and *Lotus japonicus* (2, 3). Rhizobia secrete lipochitooligosaccharides (LCOs) known as nodulation (Nod) factors, which are perceived by LysM-type receptor kinases, such as Nod factor perception (NFP) and LYK3 in *M. truncatula* and are required for both rhizobial infection and nodule organogenesis (4, 5). Similarly, AM fungi release signal molecules, so-called Myc factors, which are likely perceived by other LysM-type receptor kinases in both legumes as well as nonleguminous plants (6–8). The perception of Nod and Myc factors initiates early symbiotic responses in host plants through the activation of the receptor-like kinase Does not Make Infections 2 (DMI2), which is believed to act as a coreceptor (9). Although these signaling components reside on the plasma membrane (10, 11), the perception of symbiotic signals triggers sustained oscillations in Ca^{2+} concentration both within the nucleus (nuclear Ca^{2+} spiking) and around the nucleus (perinuclear Ca^{2+} spiking) (12, 13). As a result, the generation of second messengers transducing the signals from the plasma membrane to the nuclear envelope has long been hypothesized (12–19).

Elegant mathematical models have been developed to explain the mechanism of nuclear Ca^{2+} spiking and the primary role of DMI1 (a nuclear envelope-localized cation channel) in its initiation and maintenance (13, 18, 20–22). Downstream decoding of Ca^{2+} spiking involves the nuclear Ca^{2+}/calmodulin-dependent protein kinase DMI3 (23). In *M. truncatula*, DMI1, DMI2, and DMI3 are essential components of the common symbiosis pathway that is required for establishing both root nodulation and the AM symbiosis, as the respective mutants are defective for both symbioses (2). DMI1 is thus viewed as the first known target of the unidentified second messenger(s) transducing signals from the plasma membrane to the nucleus.

In a previous study, a yeast two-hybrid screen identified a 3-hydroxy 3-methylglutaryl CoA reductase 1 (HMGR1) as strongly interacting with DMI2 (24). HMGRs are well-known regulatory enzymes of the MVA pathway in plants and animals, catalyzing the conversion of HMG-CoA into MVA. Furthermore, the pharmacological inhibition of HMGRs by statin drugs led to decreased nodulation (24). More specifically, silencing *HMGR1* by RNA interference (RNAi) resulted in a drastic reduction in root infection and nodule development (24). However, despite these findings,

Significance

Metabolites of the mevalonate (MVA) pathway play essential roles in the regulation of growth and development in many organisms. In this study, we demonstrate that a key regulatory enzyme of the MVA pathway is directly involved in the signaling pathway that transduces endosymbiotic microbial signals in *Medicago truncatula*. Furthermore, we show that exogenous MVA application is sufficient to activate this transduction pathway. The use of mutants in the signaling pathway and a heterologous expression system provides evidence that the MVA pathway is a missing link between the initial perception of microbial signals at the host plasma membrane and the regulation of symbiotic gene expression in the nucleus.

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the precise role of HMGR1 and MVA in symbiosis remained unclear. We now demonstrate that HMGR1 plays a key role during the initial symbiotic signaling between the host plant and both rhizobia and AM fungi. Using pharmacological, biochemical, and genetic approaches, we show that HMGR1 and the products of the MVA pathway act upstream of DMI1 in the symbiotic signaling cascade, providing the missing link between the perception of symbiotic signals at the plasma membrane and the activation of Ca^{2+} spiking in the nucleus.

Results

***M. truncatula* HMGR1 Possesses HMGR Activity.** Because it was a considerable surprise to discover a metabolic enzyme as an interactor of the symbiotic receptor-like kinase DMI2 (24), we investigated whether MtHMGR1 is indeed a bona fide enzyme with HMG-CoA reductase activity. The catalytic domain of HMGR1 was tagged with a maltose-binding protein (MBP:HMGR1ΔN), expressed in *Escherichia coli*, and purified using amylose resin. Its kinetic properties were studied using spectrophotometry as described in Dale et al. (25). HMG-CoA was used to start the reaction, and the oxidation rate of NADPH was determined before (5 min) and after the addition of HMG-CoA using the absorbance at 340 nm and the initial rate of the reaction was calculated (Fig. S1 *A* and *B*). A Lineweaver–Burk plot was used to calculate the V_{max} and K_m of the reaction (Fig. S1*D*). The apparent V_{max} and $K_{m(\text{HMG-CoA})}$ were 3.62 ± 0.37 μmol/min/mg of protein and 38.88 ± 4.41 μM, respectively. The $K_{m(\text{HMG-CoA})}$ of MBP:HMGR1ΔN was thus within the same range as that of other known plant (e.g., rubber, 13 μM; maize, 10 μM; *Arabidopsis*, 8 μM) and animal (e.g., rat, 29–47 μM) HMGRs, indicating a similar affinity for HMG-CoA (25–28). We also evaluated the effect of lovastatin, a well-characterized competitive inhibitor of HMGRs, on the enzymatic activity of MtHMGR1. The addition of 50 μM lovastatin completely abolished MBP:HMGR1ΔN activity (Fig. S1*C*), thus confirming that HMGR1 has typical HMG-CoA reductase activity. The production of MVA in the HMGR1 reaction mix was determined to be 5 times greater than the control based on the area under the curve corresponding to the extracted ion chromatogram of the MVA standard base peak (Fig. S1*E*).

***HMGR1* Silencing Affects Nod Factor-Induced *ENOD11* Expression and Ca^{2+} Spiking in *M. truncatula*.** *M. truncatula* HMGR1 interacts with DMI2, and RNAi-based silencing of *HMGR1* strongly reduces the ability of transgenic roots to develop nodules when inoculated with *Sinorhizobium meliloti* (24). Because a knockout mutant of *HMGR1* was not available, we used the same RNAi-based silencing strategy to investigate the role of *HMGR1* in early symbiotic signaling. We analyzed the induction of nuclear-associated Ca^{2+} spiking and the expression of the *M. truncatula* early nodulin 11 (*ENOD11*) gene, two early symbiotic responses that have been used extensively to study symbiotic signaling (29–31).

ENOD11 expression was analyzed qualitatively in Jemalong A17 plants expressing *pENOD11*-GUS and also quantitatively using RT-PCR. Control *pENOD11*-GUS–expressing roots transformed with the RNAi vector alone displayed the normal dark blue staining 12 h after 10^{-8} M Nod factor addition, whereas *HMGR1-RNAi* roots were only lightly stained (Fig. S2 *A* and *B*), thus indicating a clear reduction in reporter expression. This was confirmed by quantitative RT-PCR, which revealed that *ENOD11* expression was approximately eightfold lower in *HMGR1*-RNAi transgenic roots compared with that of the control (Fig. S2*C*).

Because actively growing root hairs are used for Ca^{2+} measurements, we tested both the effect of silencing *HMGR1* and the effect of inhibiting the MVA pathway on root hair growth. Silencing *HMGR1* had no significant effect on root hair growth (Fig. S3*A*). Similarly, the application of lovastatin at 0.5 μM, the concentration that reduced nodulation (24), did not affect root hair growth (Fig. S3*B*). It should be noted that the negative effect of lovastatin on root hair growth at the higher 1 μM concentration (Fig. S3*B*) could be due to nonspecific effects. Taken together, these results indicate that the components of the MVA pathway (including *HMGR1*) are not essential for root hair growth.

Nod factor-induced Ca^{2+} spiking was analyzed in *M. truncatula* seedlings expressing both the *HMGR1*-RNAi construct and the Ca^{2+} sensor Yellow Cameleon 3.6 (YC3.6). Transgenic roots expressing YC3.6 alone were used as controls. The application of Nod factors at 10^{-8} M triggered nucleus-associated Ca^{2+} spiking in wild-type control roots 10–15 min after Nod factor application (Fig. 1*A*). Nod factor-induced Ca^{2+} spiking was not observed in the *nfp-1* mutant (Fig. 1*B*), which served as a negative control for this experiment. The application of Nod factors failed to trigger detectable Ca^{2+} spiking in epidermal cells of *HMGR1*-silenced roots (Fig. 1*C*). Ca^{2+} spiking was not detected either in these *HMGR1*-silenced roots in response to germinated spore exudates (GSEs) from the AM fungus *Rhizophagus irregularis* (Fig. S4 *A*

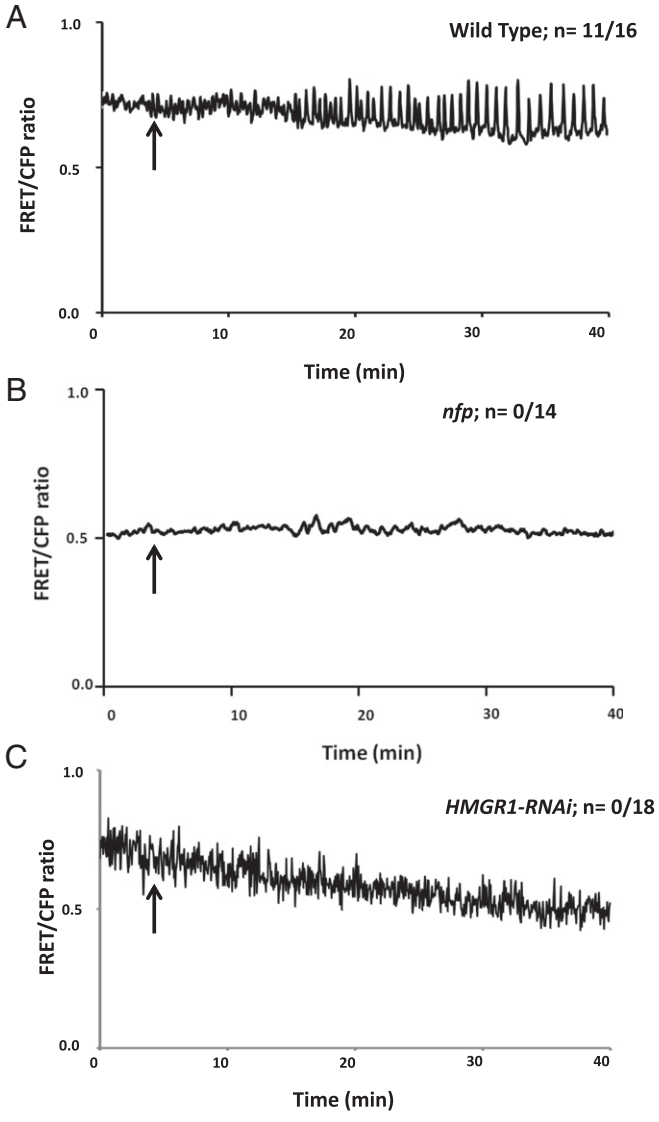

Fig. 1. Effect of silencing *M. truncatula HMGR1* on nuclear-associated Ca^{2+} spiking in root epidermal cells. (*A*) Nod factor-induced Ca^{2+} spiking in *M. truncatula* root hair cells expressing YC3.6. (*B*) Absence of Nod factor-induced Ca^{2+} spiking in a *nfp* mutant expressing YC3.6. (*C*) Silencing *HMGR1* abolishes Nod factor-induced Ca^{2+} spiking in *M. truncatula* root hair cells. Arrows indicate the addition of Nod factors.

and *B*). Altogether, these observations indicate that *HMGR1* plays a role in early symbiotic signaling upstream of Ca^{2+} spiking and *ENOD11* expression. We therefore examined whether products of HMGR1 activity such as MVA are able to elicit Ca^{2+} spiking in root epidermal cells.

MVA Induces *ENOD11* Expression in *M. truncatula* and Ca^{2+} Spiking in Root Epidermal Cells of Legumes and Nonlegumes. To determine whether the product of HMGR1 activity, MVA, is able to elicit *ENOD11* expression in the absence of Nod factors, *M. truncatula* roots stably transformed with *pENOD11*:GUS were treated with a 100 μM solution of MVA, the lowest concentration required to induce signaling events (*SI Materials and Methods*). Although the responses were weaker compared with Nod factor treatment (Fig. S2*D*), clear GUS staining was observed in wild-type roots 24 h after MVA treatment (Fig. S2*E*). However, we were unable to quantify the MVA-induced *ENOD11* expression in *M. truncatula* roots through quantitative RT-PCR, most likely due to the weaker expression of *ENOD11* during MVA treatment compared with Nod factor treatment.

We then investigated whether MVA is also able to activate nuclear-associated Ca^{2+} spiking in the root epidermal cells of *M. truncatula*. The application of the same concentration of MVA triggered sustained Ca^{2+} spiking within 5–10 min in over 50% of cells examined (Fig. 2*A*). Such MVA-induced Ca^{2+} spiking was also observed in roots of the other model legume, *L. japonicus* (Fig. 2*D*). Significantly, 100 μM MVA elicited Ca^{2+} spiking (albeit of lower frequency) in epidermal cells of root organ cultures of *M. truncatula* (Fig. 2*C*). This is particularly interesting, as these root cultures do not respond to rhizobial Nod factors but can be colonized by AM fungi and respond to germinating AM fungal spore exudates (15). Finally, MVA induction of nuclear-associated Ca^{2+} spiking was observed in root organ cultures of carrot, a nonlegume AM host plant (Fig. 2*E*). In contrast, MVA failed to trigger nuclear-associated Ca^{2+} spiking in trichoblast cells of *Arabidopsis thaliana*, which is unable to develop AM associations or to form root nodules (Fig. 2*F*).

Taken together, these results suggest that the agonist activity of MVA in relation to Ca^{2+} spiking is comparable for both the rhizobial and AM symbioses, thus potentially placing this metabolite within the common symbiosis pathway. This hypothesis is consistent with the fact that *HMGR1* silencing abolishes Ca^{2+} spiking in root epidermal cells of *M. truncatula* treated with GSE of *R. irregularis* (Fig. S4*B*). Furthermore, 100 μM MVA restored Ca^{2+} spiking in the same *HMGR1*-silenced roots (Fig. 2*B*), indicating a direct link between *HMGR1* expression and MVA in symbiotic signaling.

Because an array of additional metabolites are synthesized from MVA, it is also possible that some of these (isopentenyl pyrophosphate, geranylgeranyl pyrophosphate, etc.) may be responsible for triggering Ca^{2+} spiking in the root epidermis following the exogenous addition of MVA. The application of isopentenyl pyrophosphate (100 μM) did not induce nuclear Ca^{2+} spiking in root epidermal cells (Fig. S5*C*). Hence, we tested the effect of upstream components of the MVA pathway, such as MVA 5-phosphate and MVA 5-pyrophosphate. Both these phosphorylated versions of MVA triggered nuclear Ca^{2+} spiking in *M. truncatula* root hair cells (Fig. S5 *A* and *B*). To rule out the possibility that MVA and its phosphorylated versions might induce cytoplasmic acidification, which might account for the generation of nuclear-associated Ca^{2+} spiking, we mimicked such an effect by applying sodium propionate. As shown in Fig. S5*D*, sodium propionate did not induce Ca^{2+} spiking at concentrations ranging from 100 μM to 1 mM, thus consistent with a specific role for MVA or its immediate downstream products.

MVA-Induced *ENOD11* Expression and Nuclear-Associated Ca^{2+} Spiking Are Dependent on Upstream Components of the Common Symbiosis Pathway. To determine whether MVA-induced *ENOD11* expression

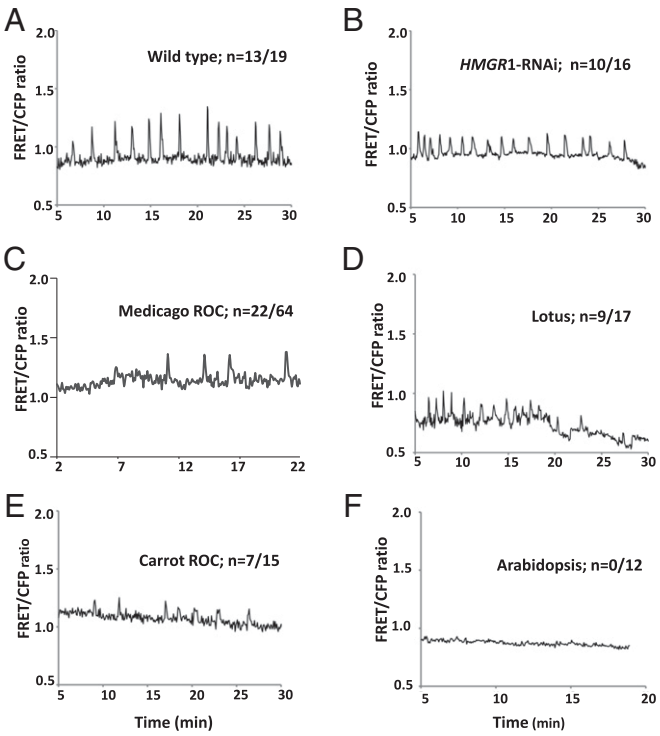

Fig. 2. MVA-induced nuclear-associated Ca^{2+} spiking in legumes and nonlegumes. (*A*) Exogenous application of 100 μM MVA triggers Ca^{2+} spiking in *M. truncatula* root hair cells expressing YC3.6. (*B*) MVA restores Ca^{2+} spiking in roots silenced for *HMGR1*. (*C*) MVA induces nuclear Ca^{2+} spiking in atrichoblast cells of a *M. truncatula* root organ culture expressing nuclear-targeted YC2.1. (*D*) MVA-induced nuclear spiking in root hairs of *L. japonicus* expressing NLS-YC3.6. (*E*) Atrichoblasts of carrot root organ cultures expressing nuclear-targeted YC2.1 also respond to MVA. (*F*) In contrast, MVA does not elicit nuclear-associated Ca^{2+} spiking in trichoblast cells of *Arabidopsis* expressing YC3.6, which is unable to form endosymbiotic associations with either rhizobia or AM fungi.

is dependent on the symbiotic signaling pathway, we treated *M. truncatula nfp-2*, *dmi1*, *dmi2*, and *dmi3* mutants expressing *pENOD11*:GUS with 100 μM MVA and performed GUS staining 24 h after treatment. MVA induces detectable *ENOD11* expression in *nfp-2* and *dmi2* mutants but not in *dmi1* or *dmi3* mutants, suggesting that HMGR1/MVA acts downstream of DMI2 but upstream of DMI1 and DMI3 (Fig. S2 *F–I*).

Parallel experiments were then performed on MVA-elicited Ca^{2+} spiking using *nfp* and various *dmi* mutants expressing the YC3.6 Ca^{2+} sensor. Consistent with the *ENOD11* expression data, 100 μM MVA triggered nuclear-associated Ca^{2+} spiking in both *dmi2* and *dmi3* mutants but not in *dmi1* mutants (Fig. 3 *B–D*). Nevertheless, it should be noted that the spiking profile for the *dmi2* mutant had a noisy background as reported previously (32) and that the percentage of responding cells is significantly lower than in wild-type plants (Fig. 2*A*). On the other hand, MVA-elicited Ca^{2+} spiking was not observed in either trichoblast or atrichoblast cells of two different alleles of *nfp* (*nfp*-1 and *nfp*-2; Fig. 3*A* and Fig. S6). Despite this apparent contradiction with the *ENOD11* expression data for *nfp* (see *Discussion*), we conclude that MVA itself or the products of the MVA pathway are likely to act downstream of DMI2 and upstream of DMI1 in triggering both Ca^{2+} spiking and regulating symbiotic gene expression. This hypothesis is consistent with HMGR1 acting downstream of its interacting protein partner DMI2. To our knowledge, this is the first phenotype that permits the uncoupling of *dmi1* and *dmi2* mutants.

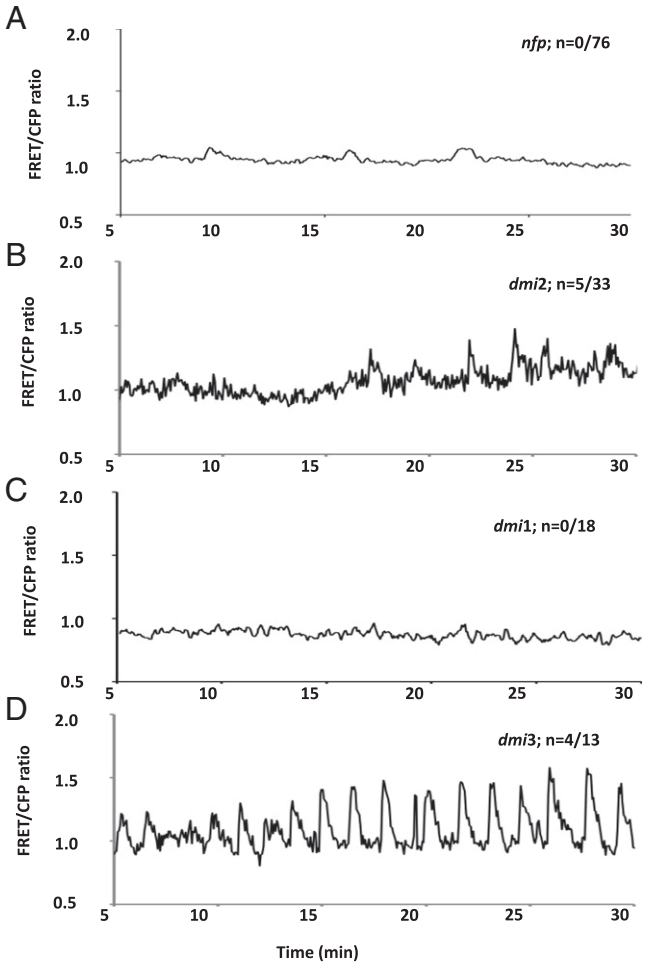

Fig. 3. Analyses of MVA-induced nuclear-associated Ca^{2+} spiking responses in symbiosis-defective mutants of *M. truncatula*. (*A*) MVA-induced Ca^{2+} spiking was not observed in an *nfp* mutant, which acts upstream of DMI2. (*B*) MVA activates Ca^{2+} spiking in the *dmi2* mutant expressing YC3.6, (*C*) but not in the *dmi1* mutant. (*D*) As expected, MVA-elicited Ca^{2+} spiking is not modified in the *dmi3* mutant, as DMI3 acts downstream of the Ca^{2+} spiking machinery.

MVA Elicits Ca^{2+} Spiking in Human Embryonic Kidney 293 Cells Expressing the *M. truncatula* DMI1 Protein. To determine whether MVA and DMI1 can be sufficient to trigger Ca^{2+} spiking, we used Human Embryonic Kidney 293 (HEK-293) cells as a heterologous expression system. We have shown in a previous study (18) that DMI1 maintains its nuclear envelope localization in HEK-293 cells when expressed under the control of the cytomegalovirus (CMV) promoter. Furthermore, following exogenous Ca^{2+} treatment, these cells display perinuclear Ca^{2+} oscillations (18). To determine whether MVA can trigger DMI1-mediated Ca^{2+} spiking in HEK-293, this cell line was transfected with either the Ca^{2+} sensor alone (pIRES2-YC3.6) or the Ca^{2+} sensor along with DMI1 (pIRES2-YC3.6::DMI1). For all experiments, the growth medium was replaced by bath solution, either with or without 100 μm MVA. In the absence of MVA, cells expressing pIRES2-YC3.6 or pIRES2-YC3.6::DMI1 did not exhibit de novo Ca^{2+} spiking (Fig. 4 *A* and *C*). Similarly, the exogenous application of MVA to cells expressing the Ca^{2+} sensor alone did not trigger Ca^{2+} spiking (Fig. 4*B*). By contrast, MVA application to HEK-293 cells expressing DMI1 triggered an intense Ca^{2+} spiking response (Fig. 4*D*). These observations, in which DMI1 is expressed as the sole *M. truncatula* protein in a heterologous system, provide further evidence that DMI1 expression is sufficient to support MVA-induced Ca^{2+} spiking.

Discussion

HMGR1 Is Required in the Early Symbiotic Signaling Cascade. Genetic and genomic approaches have advanced our understanding of the molecular mechanisms of signal transduction during the initial stages of legume nodulation and AM symbioses. This research led to the identification of essential components of the common symbiosis pathway (2, 13). However, the secondary messengers that link the perception of microbial signals at the plasma membrane level to the regulation of ion channels and Ca^{2+} pumps on the nuclear envelope remain unknown (17, 33). With the identification of HMGR1 as both an interactor of the DMI2 coreceptor and a requirement for legume nodulation, we hypothesized that this MVA-producing enzyme might function in the common symbiosis signaling pathway. Metabolites from the MVA pathway play a wide variety of roles in many eukaryotes, including growth, development, and responses to environmental stimuli (34–36), by regulating cell-autonomous transcriptional and posttranscriptional processes (36). The observation that silencing *HMGR1* affects Ca^{2+} spiking in response to Nod factors and GSEs of AM fungi supports the hypothesis that HMGR1 is indeed a component of the common symbiosis pathway (Fig. 5). In addition, the study of MVA-induced *ENOD11* expression and Ca^{2+} spiking in various symbiosis-defective mutants indicated that addition of MVA can partly restore *ENOD11* expression and Ca^{2+} spiking in *dmi2* but not in *dmi1* mutants. To our knowledge, this is the first report clearly uncoupling the phenotypes of *dmi1* and *dmi2* mutants. Because DMI1 and DMI2 belong to the common symbiosis pathway, these results place HMGR1 and its MVA-derived products downstream of DMI2 but upstream of DMI1 in the symbiotic cascade (3).

The conversion of HMG-CoA into MVA by HMGR is the first committed and rate-limiting step of the MVA biosynthetic pathway. The ability of MVA to trigger nuclear-associated Ca^{2+} spiking emphasizes the key role of HMGR1 as a link between signaling proteins residing on the plasma membrane and those located on the nuclear envelope. MVA-activated Ca^{2+} spiking is conserved across diverse AM host plants including legumes and nonlegumes. In contrast, the non-AM host *Arabidopsis* does not respond to either rhizobial or AM signals (2, 14), but does to

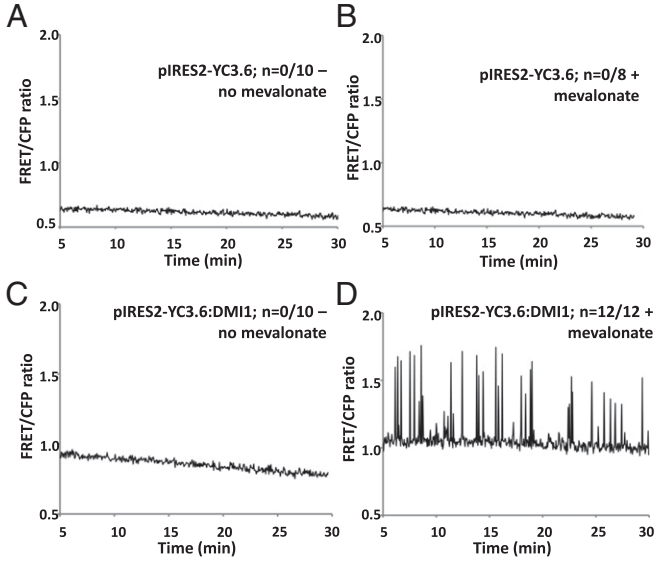

Fig. 4. MVA-induced Ca^{2+} spiking in HEK-293 cells expressing DMI1. (*A* and *B*) Absence of Ca^{2+} spiking in HEK-293 cells expressing the vector control pIRES2-YC3.6 in the absence (*A*) or presence of exogenous MVA (100 μM) (*B*). (*C* and *D*) Ca^{2+} spiking is only observed when MVA is added to HEK-293 cells expressing the pIRES2-YC3.6::DMI1 vector.

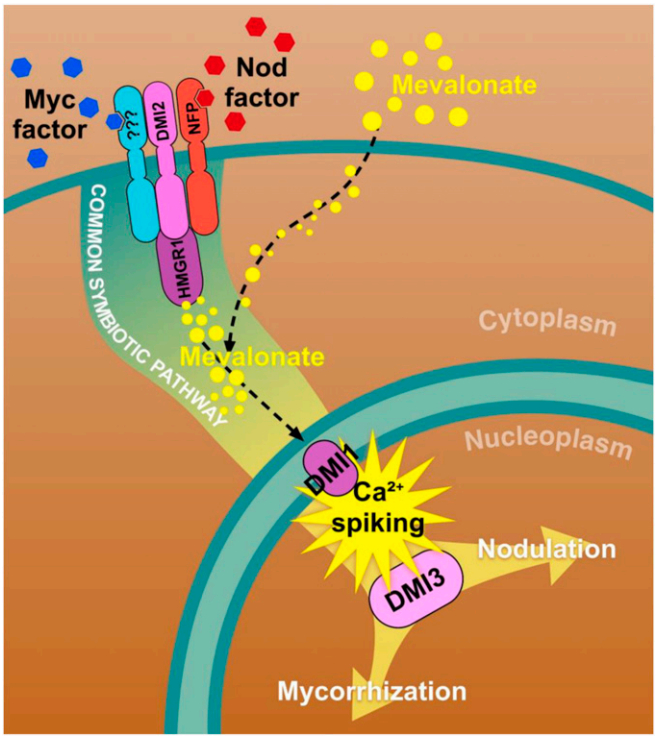

Fig. 5. Model illustrating the proposed role of MVA within the common symbiosis pathway. Nod and Myc factors are perceived at the plasma membrane by a complex including the receptor-like kinase DMI2, interacting with either the Nod factor receptor component NFP or the so far unidentified Myc factor receptor. Based on our observations, we propose that HMGR1—which is known to interact with DMI2—generates MVA as a second messenger, transducing the signal from the plasma membrane to the nuclear compartment where DMI1, the nuclear envelope-localized cation channel, is required for the initiation of nuclear Ca^{2+} spiking. This Ca^{2+} response is then decoded by the Ca^{2+} and calmodulin-dependent kinase DMI3, which in turn leads to downstream endosymbiosis-related gene activation. In our experiments, the exogenous application of MVA is sufficient to activate the common symbiosis pathway and trigger nuclear Ca^{2+} spiking in the absence of receptor activation.

MVA (Fig. 2*F*), suggesting that the targets of MVA (or its derived metabolites) are absent in *Arabidopsis* like many other signaling components required for AM associations.

Do MVA or Other Products of the MVA Pathway Act as Signaling Intermediates Linking the Plasma Membrane to the Nuclear Envelope? Many classical secondary messengers, such as IP_3, NAD^+/NADH, cADP ribose, and Ca^{2+}, have been considered as possible candidates for transducing symbiotic signal perception at the plasma membrane to the activation of Ca^{2+} spiking responses in the nucleus (33, 37, 38). Electrophysiological analyses have ruled out the possibility of IP_3 or Ca^{2+} acting as modulators of Ca^{2+} spiking (33). However, MVA and its immediate phosphorylated derivatives (MVA 5-phosphate and MVA 5-pyrophosphate) elicited nuclear Ca^{2+} spiking, whereas isopentenyl pyrophosphate failed to elicit this response. This negative result for isopentenyl pyrophosphate could be due to restricted diffusion into root epidermal cells. Although of course we do not provide direct evidence that MVA is a second messenger in symbiotic signaling, we consider that the results presented in this article make MVA and its phosphorylated derivatives promising candidates for future studies.

Several isoforms of HMGR1 have been reported in *M. truncatula* (24), and MVA and its derivatives are abundant metabolites in plant cells, including root epidermal cells. Hence, it is interesting to discover that the exogenous application of MVA elicits Ca^{2+} spiking in root epidermal cells. Neither silencing *HMGR1* nor the addition of lovastatin at a concentration that is inhibitory to nodulation (24) affected root hair growth, implying that the MVA pathway is not required for root hair development. We therefore hypothesize that the activation of DMI2 during symbiotic signaling may transiently activate HMGR1, leading to localized production of MVA in root epidermal cells (trichoblasts and atrichoblasts). The transient elevation in the MVA level inside the epidermal cells may in turn be responsible for activating nuclear Ca^{2+} spiking.

Differences clearly exist in the Ca^{2+} spiking patterns elicited by MVA, rhizobial Nod factors, and diffusible signals from AM fungi that were tested in different model plants and in different genetic backgrounds. If MVA activates the common symbiosis pathway by bypassing the receptor-ligand recognition step, it is therefore possible that downstream responses will not necessarily be specific to a particular microbial signal. In addition, it is likely that the exogenous application of MVA to root epidermal cells can only partially mimic an endogenous production of MVA in terms of intracellular concentration or subcellular localization. Thus, exogenous MVA is likely to activate the common symbiosis pathway both nonspecifically and suboptimally, probably explaining the high degree of variability in the observed Ca^{2+} spiking patterns.

If the model presented in Fig. 5 is correct, then it is surprising that *ENOD11* expression was elicited by MVA in *nfp* mutants, whereas Ca^{2+} spiking was not detected in these mutants. The reason for this apparent discrepancy remains unclear. It is known that *nfp* mutants have more severe symbiotic phenotypes compared with *dmi* mutants, as for instance both root hair deformation and Ca^{2+} spiking are blocked in *nfp*, whereas only Ca^{2+} spiking is affected in *dmi*2 mutants in response to Nod factors (4, 10). Thus, it is possible that Ca^{2+} spiking may require not only MVA but also other signaling molecules that are produced in an NFP- but not DMI2-dependent manner. Alternatively, the apparent difference between p*ENOD11*-GUS assays and Ca^{2+} spiking analyses in the *nfp* mutant may simply reflect different sensitivities of the respective techniques. The fact that *ENOD11* expression in response to MVA addition cannot be detected by RT-PCR indicates a much lower level of gene induction with MVA than with Nod factors. This response may be even lower in some mutant backgrounds. Such a lower response was indeed observed for the Ca^{2+} spiking, as lower levels of spiking were observed in response to MVA in the *dmi2* mutant compared with wild-type plants (compare Figs. 2*A* and 3*B*). If we are operating at the detection limit for the Ca^{2+} spiking assay, then this could explain the failure to observe Ca^{2+} spiking in the *nfp* background. However, at this stage, this question remains to be fully clarified.

Finally, the fact that MVA was able to trigger Ca^{2+} spiking in HEK-293 cells expressing *M. truncatula* DMI1 provides preliminary evidence that MVA may act directly on the cellular machinery that controls Ca^{2+} spiking in plants in response to symbiotic microbial signals (Fig. 5). We therefore hypothesize that the perception of symbiotic signals leads to the activation of HMGR1 bound to DMI2 and to the localized production of MVA, which then translocates to the nucleus activating in turn nuclear cation channels (DMI1/POLLUX, CASTOR, or Ca^{2+} channels), and thereby triggering nuclear-associated Ca^{2+} spiking. Although MVA is well known for its essential role in isoprenoid/sterol metabolism in eukaryotes, this study sheds light on a potential new role for this ubiquitous metabolite as a signaling intermediate in intracellular signaling pathways.

Materials and Methods

Ca^{2+} spiking analyses on *M. truncatula* were performed using the wild-type Jemalong A17 line and the symbiosis-defective mutants *nfp*-1, *nfp*-2, *dmi2-1*, *dmi1-4*, *dmi1-2*, and *dmi3*-1 (19, 39), after *Agrobacterium rhizogenes*-dependent transformation with the cytosolic YC3.6 yellow cameleon calcium sensor (40). For *L. japonicus*, the wild-type Gifu line was transformed via *Agrobacterium tumefaciens* with the nuclear-targeted Ca^{2+} sensor NES:YC3.6, and the

Arabidopsis thaliana ecotype Col-0 was transformed with cytosolic YC3.6. GUS assays were performed on Jemalong A17 (*pENOD11:GUS*), *nfp-1* (*pENOD11:GUS*), *nfp*-2 (*pENOD11:GUS*), *dmi2-1* (*pENOD11:GUS*), *dmi1-4* (*pENOD11:GUS*), and *dmi3-1* (*pENOD11:GUS*) lines (38, 41). *M. truncatula* and carrot root organ cultures expressing the nuclear NUP-YC2.1 cameleon sensor were obtained via *A. rhizogenes* transformation (15).

For detailed description of methods pertaining to HMGR1 enzymatic assay, RNAi of *MtHMGR1*, *pENOD11*-GUS assays and RT-PCR, root hair growth assays, Ca^{2+} imaging in root epidermal cells, and Ca^{2+} imaging in HEK-293 cells, see *SI Materials and Methods*.

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1. Osbourn A (1996) Saponins and plant defence—A soap story. *Trends Plant Sci* 1(1):4–9.
2. Venkateshwaran M, Volkening JD, Sussman MR, Ané JM (2013) Symbiosis and the social network of higher plants. *Curr Opin Plant Biol* 16(1):118–127.
3. Kistner C, et al. (2005) Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. *Plant Cell* 17(8): 2217–2229.
4. Amor BB, et al. (2003) The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. *Plant J* 34(4):495–506.
5. Fliegmann J, et al. (2013) Lipo-chitooligosaccharidic symbiotic signals are recognized by LysM receptor-like kinase LYR3 in the legume *Medicago truncatula*. *ACS Chem Biol* 8(9):1900–1906.
6. Maillet F, et al. (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469(7328):58–63.
7. Mukherjee A, Ané JM (2011) Germinating spore exudates from arbuscular mycorrhizal fungi: Molecular and developmental responses in plants and their regulation by ethylene. *Mol Plant Microbe Interact* 24(2):260–270.
8. Genre A, et al. (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytol* 198(1):190–202.
9. Antolín-Llovera M, Ried MK, Parniske M (2014) Cleavage of the SYMBIOSIS RECEPTOR-LIKE KINASE ectodomain promotes complex formation with Nod factor receptor 5. *Curr Biol* 24(4):422–427.
10. Endre G, et al. (2002) A receptor kinase gene regulating symbiotic nodule development. *Nature* 417(6892):962–966.
11. Limpens E, et al. (2005) Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by *DMI2*. *Proc Natl Acad Sci USA* 102(29):10375–10380.
12. Sieberer BJ, et al. (2009) A nuclear-targeted cameleon demonstrates intranuclear Ca^{2+} spiking in *Medicago truncatula* root hairs in response to rhizobial nodulation factors. *Plant Physiol* 151(3):1197–1206.
13. Capoen W, et al. (2011) Nuclear membranes control symbiotic calcium signaling of legumes. *Proc Natl Acad Sci USA* 108(34):14348–14353.
14. Ané JM, et al. (2004) *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. *Science* 303(5662):1364–1367.
15. Chabaud M, et al. (2011) Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis. *New Phytol* 189(1):347–355.
16. Ehrhardt DW, Wais R, Long SR (1996) Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. *Cell* 85(5):673–681.
17. Peiter E, et al. (2007) The *Medicago truncatula* DMI1 protein modulates cytosolic calcium signaling. *Plant Physiol* 145(1):192–203.
18. Venkateshwaran M, et al. (2012) The recent evolution of a symbiotic ion channel in the legume family altered ion conductance and improved functionality in calcium signaling. *Plant Cell* 24(6):2528–2545.
19. Wais RJ, et al. (2000) Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. *Proc Natl Acad Sci USA* 97(24):13407–13412.
20. Granqvist E, et al. (2012) Buffering capacity explains signal variation in symbiotic calcium oscillations. *Plant Physiol* 160(4):2300–2310.
21. Charpentier M, Vaz Martins T, Granqvist E, Oldroyd GE, Morris RJ (2013) The role of DMI1 in establishing Ca^{2+} oscillations in legume symbioses. *Plant Signal Behav* 8(2): e22894.
22. Miller JB, et al. (2013) Calcium/Calmodulin-dependent protein kinase is negatively and positively regulated by calcium, providing a mechanism for decoding calcium responses during symbiosis signaling. *Plant Cell* 25(12):5053–5066.
23. Lévy J, et al. (2004) A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303(5662):1361–1364.
24. Kevei Z, et al. (2007) 3-hydroxy-3-methylglutaryl coenzyme a reductase 1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. *Plant Cell* 19(12): 3974–3989.
25. Dale S, et al. (1995) Bacterial expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase (isoform HMGR1) from *Arabidopsis thaliana*, and its inactivation by phosphorylation at Ser577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase. *Eur J Biochem* 233(2):506–513.
26. Bach TJ, Weber T, Motel A (1990) Some properties of enzymes involved in the biosynthesis and metabolism of 3-hydroxy-3- methylglutaryl coenzyme A reductase in plants. Biochemistry of the Mevalonic Acid Pathway to Terpenoids, Recent Advances in Phytochemistry, eds Towers GHN, Stafford AHA (Plenum, New York.), pp 1–82.
27. Cenedella RJ, Kuszak JR, Al-Ghoul KJ, Qin S, Sexton PS (2003) Discordant expression of the sterol pathway in lens underlies simvastatin-induced cataracts in Chbb: Thom rats. *J Lipid Res* 44(1):198–211.
28. Wititsuwannakul R, Wititsuwannakul D, Suwanmanee P (1990) 3-hydroxy-3-methylglutaryl co-enzyme a reductase from *Hevea brasiliensis*. *Phytochemistry* 29(5):1401–1403.
29. Horváth B, et al. (2011) *Medicago truncatula IPD3* is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. *Mol Plant Microbe Interact* 24(11):1345–1358.
30. Gobbato E, et al. (2012) A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Curr Biol* 22(23):2236–2241.
31. Roberts NJ, et al. (2013) Rhizobial and mycorrhizal symbioses in *Lotus japonicus* require lectin nucleotide phosphohydrolase, which acts upstream of calcium signaling. *Plant Physiol* 161(1):556–567.
32. Shaw SL, Long SR (2003) Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. *Plant Physiol* 131(3):976–984.
33. Charpentier M, et al. (2008) *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. *Plant Cell* 20(12):3467–3479.
34. Stermer BA, Bianchini GM, Korth KL (1994) Regulation of HMG-CoA reductase activity in plants. *J Lipid Res* 35(7):1133–1140.
35. Chappell J (1995) The biochemistry and molecular biology of isoprenoid metabolism. *Plant Physiol* 107(1):1–6.
36. Edwards PA, Ericsson J (1999) Sterols and isoprenoids: Signaling molecules derived from the cholesterol biosynthetic pathway. *Annu Rev Biochem* 68:157–185.
37. Engstrom EM, Ehrhardt DW, Mitra RM, Long SR (2002) Pharmacological analysis of nod factor-induced calcium spiking in *Medicago truncatula*. Evidence for the requirement of type IIA calcium pumps and phosphoinositide signaling. *Plant Physiol* 128(4):1390–1401.
38. Charron D, Pingret JL, Chabaud M, Journet EP, Barker DG (2004) Pharmacological evidence that multiple phospholipid signaling pathways link Rhizobium nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific *ENOD* gene expression. *Plant Physiol* 136(3):3582–3593.
39. Catoira R, et al. (2000) Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. *Plant Cell* 12(9):1647–1666.
40. Boisson-Dernier A, et al. (2001) *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant-Microbe Interactions* 14(6):695–700.
41. Journet EP, et al. (2001) *Medicago truncatula ENOD11*: A novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. *Mol Plant Microbe Interact* 14(6):737–748.
42. Horn MA, Walker JC (1994) Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from Arabidopsis thaliana. *Biochim Biophys Acta* 1208(1):65–74.
43. Benkeblia N, Shinano T, Osaki M (2007) Metabolite profiling and assessment of metabolome compartmentation of soybean leaves using non-aqueous fractionation and GGMS analysis. *Metabolomics* 3(3):297–305.
44. Riely BK, et al. (2011) Identification of legume *RopGEF* gene families and characterization of a *Medicago truncatula* RopGEF mediating polar growth of root hairs. *Plant J* 65(2):230–243.