The Recent Evolution of a Symbiotic Ion Channel in the Legume Family Altered Ion Conductance and Improved Functionality in Calcium Signaling

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Arbuscular mycorrhiza and the rhizobia-legume symbiosis are two major root endosymbioses that facilitate plant nutrition. In Lotus japonicus, two symbiotic cation channels, CASTOR and POLLUX, are indispensable for the induction of nuclear calcium spiking, one of the earliest plant responses to symbiotic partner recognition. During recent evolution, a single amino acid substitution in DOES NOT MAKE INFECTIONS1 (DMI1), the POLLUX putative ortholog in the closely related Medicago truncatula, rendered the channel solo sufficient for symbiosis; castor, pollux, and castor pollux double mutants of L. japonicus were rescued by DMI1 alone, while both Lj-CASTOR and Lj-POLLUX were required for rescuing a dmi1 mutant of M. truncatula. Experimental replacement of the critical serine by an alanine in the selectivity filter of Lj-POLLUX conferred a symbiotic performance indistinguishable from DMI1. Electrophysiological characterization of DMI1 and Lj-CASTOR (wild-type and mutants) by planar lipid bilayer experiments combined with calcium imaging in Human Embryonic Kidney-293 cells expressing DMI1 (the wild type and mutants) suggest that the serine-to-alanine substitution conferred reduced conductance with a long open state to DMI1 and improved its efficiency in mediating calcium oscillations. We propose that this single amino acid replacement in the selectivity filter made DMI1 solo sufficient for symbiosis, thus explaining the selective advantage of this allele at the mechanistic level.

INTRODUCTION

The majority of land plants develop arbuscular mycorrhiza (AM) for improved nutrient uptake and protection against stresses (Akiyama et al., 2005). On the other hand, root nodule (RN) symbiosis, established between legume and rhizobia, results in the formation of nitrogen-fixing RNs (Venkateshwaran and Ané, 2011). The development of root endosymbioses is coordinated by signal exchanges between plants and their microbial symbionts. Rhizobia produce diffusible lipochitooligosaccharidic signals, Nod factors (Dénarié et al., 1996). Similarly, AM fungi produce diffusible signals called Myc factors (Akiyama et al., 2005; Maillet et al., 2011; Mukherjee and Ané, 2011). Perception of Nod or Myc factors initiates early symbiotic responses in host plants, such as calcium (Ca2+) spiking and symbiotic gene expression (Ehrhardt et al., 1996; Wais et al., 2000, 2002; Riely et al., 2006; Sieberer et al., 2009; Chabaud et al., 2011). Although RN and AM are distinct symbioses, they require a common set of genes in host plants to support rhizobial and fungal infections. Genetic studies in legumes such as Medicago truncatula, Lotus japonicus, and pea (Pisum sativum) have identified several common symbiosis genes. Several genes that play crucial roles in both RN and AM symbioses have been identified so far in model legumes (Kistner et al., 2005; Groth et al., 2010; Maillet et al., 2011; Murray et al., 2011). Among them, M. truncatula DOES NOT MAKE INFECTIONS1 (DMI1) and L. japonicus CASTOR and POLLUX encode nuclear ion channels that are required for the initiation of Nod and Myc factor-induced Ca2+ spiking (Ané et al., 2002, 2004; Imaizumi-Anraku et al., 2005; Peiter et al., 2007; Riely et al., 2007; Charpentier et al., 2008; Kosuta et al., 2008; Capoen et al., 2011). M. truncatula DM1 and pea DM1 aka SYMBOIATION8 (SYM8) are putative orthologs of Lj-POLLUX and putative paralogs of Lj-CASTOR (Zhu et al., 2006; Edwards et al., 2007). SYM8 can functionally substitute DM11 in M. truncatula (Edwards et al., 2007). These DM1 homologs are present throughout land plants and represent ancient innovations that probably allowed the development of the AM symbiosis and colonization of land by plants (Zhu et al., 2006; Wang et al., 2010). Consistent with the phylogenetically widespread occurrence of common symbiosis genes and AM, rice (Oryza sativa) putative orthologs Os-CASTOR and Os-POLLUX are required for the AM symbiosis (Banba et al., 2008; Gutjahr et al., 2006; Sieberer et al., 2009; Chabaud et al., 2011).

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Some figures in this article are displayed in color online but in black and white in the print edition.
Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.112.098475
et al., 2008; Chen et al., 2009). The presence of a POLLUX putative ortholog in Arabidopsis thaliana is intriguing, as Arabidopsis does not undergo mycorrhization. Therefore, it has been hypothesized that Aт-POLLUX might play a nonsymbiotic role.

L. japonicus CASTOR and POLLUX are nonselective ion channels with a preference for K+ over anions (Charpentier et al., 2008). Competition experiments with Lj-CASTOR reconstituted in planar lipid bilayers revealed a preference for K+ over other cations, such as Na+ and Ca2+. Unfortunately, Lj-POLLUX was not amenable to lipid bilayer analysis but was able to complement a K+ transport-deficient yeast mutant, suggesting its ability to conduct K+ (Charpentier et al., 2008). Although Lj-CASTOR and Lj-POLLUX reside on the nuclear envelope and perform similar roles in symbiotic signaling, these two proteins do not appear to interact and form exclusively homomeric ion channels (Charpentier et al., 2008). While both CASTOR and POLLUX are indispensable for symbiotic signaling in L. japonicus, POLLUX appears to interact and form exclusively homomeric ion channels with a preference for K+ over anions (Charpentier et al., 2008). The pore region, in-cluding a characteristic pore helix, filter, and hinge, is located between the third and fourth transmembrane domains (see Supplemental Figure 1 online). We searched for Mt-CASTOR mutants in the TILLING or Tnt1 insertion libraries without success (Tadege et al., 2005, 2008). Since no mutant was available for functional studies, we used an RNA interference (RNAi)-based gene silencing strategy to investigate its role in RN and AM. As a positive control, we targeted the well-characterized symbiotic gene DMI1. As expected, this resulted in a decline of nodule numbers in DMI1-RNAi—expressing transgenic roots (Figure 1A). The Mt-CASTOR-RNAi transgenic roots showed a significant reduction in Mt-CASTOR expression at the transcript level over control roots transformed with an empty vector (see Supplemental Figure 2A online). However, there was no significant reduction in the nodule number in Mt-CASTOR-RNAi—expressing transgenic roots (Figure 1A). These results indicate that the RNAi-based gene silencing strategy was efficient and that Mt-CASTOR, compared with DMI1, plays a less significant role, if any, in RN symbiosis. In the case of AM symbiosis, Mt-CASTOR-RNAi roots, as well as control roots, showed normal AM associations with intracellular hyphae and arbuscules (Figure 1B). To quantify the abundance of arbuscules in Mt-CASTOR-RNAi and control roots, we performed RT-PCR to monitor the expression level of M. truncatula phosphate transporter4 (Mt-PT4). Mt-PT4 expression is induced only upon arbuscule formation and exclusively in arbuscule-containing cells, and its expression level positively correlates with the degree of AM colonization and arbuscule formation (Harrison et al., 2002). Hence, we used this marker to quantify the arbuscule abundance in Mt-CASTOR-RNAi and control roots. We showed that the expression level of Mt-PT4 upon AM colonization is similar in Mt-CASTOR-RNAi roots to that of control roots (Figure 1C; see Supplemental Figure 2B online), suggesting that silencing Mt-CASTOR does not affect AM colonization. Furthermore, Mt-CASTOR failed to rescue two alleles of Lj-castor mutants; Lj-castor-4 (see Supplemental Figure 3A online; Table 1), Lj-castor-8 (Table 1), Lj-pollux, and the Lj-castor pollux double mutant (see Supplemental Figures 3B and 3C online; Table 1). Taken together, these observations demonstrate that Mt-CASTOR does not play a significant role in RN and AM symbioses.

**RESULTS**

Legume Nodulation and AM Are Independent of CASTOR in M. truncatula

The predicted structure of Mt-CASTOR (824 amino acids) comprises a nuclear localization signal in the N terminus, followed by four transmembrane domains and a soluble region with an RCK (for regulator of conductance of K+) domain, which is highly similar to MthK, a bacterial Ca2+-gated potassium channel (Imaizumi-Anraku et al., 2005). The pore region, in-cluding a characteristic pore helix, filter, and hinge, is located between the third and fourth transmembrane domains (see Supplemental Figure 1 online). We searched for Mt-CASTOR mutants in the TILLING or Tnt1 insertion libraries without success (Tadege et al., 2005, 2008). Since no mutant was available for functional studies, we used an RNA interference (RNAi)-based gene silencing strategy to investigate its role in RN and AM.
The Lj-castor pollux double mutant transformed with DMI1 was also colonized by Glomus intraradices, as indicated by the formation of arbuscules and vesicles (see Supplemental Figure 4A online). Like DMI1, SYM8 was also able to rescue the nodulation phenotype in Lj-castor, Lj-pollux, and Lj-castor pollux mutants (see Supplemental Figures 3M to 3O online). These results indicate that DMI1 putative orthologs in M. truncatula and pea have the capacity to compensate for the loss of both CASTOR and POLLUX ion channels in L. japonicus in both RN and AM symbioses.

Lj-CASTOR and POLLUX Share Their Symbiotic Function Nonredundantly

In agreement with an acquired neofunctionality of DMI1, Lj-CASTOR and POLLUX failed to fully rescue dmi1-4 when expressed individually. This failure was observed regardless of whether their expression was controlled by their respective native promoters (Lj-CASTORpro or Lj-POLLUXpro), constitutive promoters (cauliflower mosaic virus 3SSpro or Lj Ubq1pro), or even the native promoter of DMI1 (DMI1pro). Dmi1-4 roots transformed with Lj-CASTOR showed absolutely no response to inoculation with Sinorhizobium meliloti (pXLGD4) (Figure 3A, Table 2). Lj-POLLUX-transformed dmi1-4 roots showed only bumps (Figure 3B; Table 2). These nodule bumps were small, pale, and devoid of bacteria, as no β-galactosidase activity (due to hemApro-lacZ) was detected (see Supplemental Figures 5A and 5C online). However, when coexpressed, Lj-CASTOR and Lj-POLLUX rescued the dmi1-4 mutant, leading to the formation of colonized and functional nodules in which nifH expression (nifHpro:uidA) was detected (Bright et al., 2005) (Figure 3C, Table 2; Supplemental Figures 6B and 6E online). Expression of Lj-CASTOR and Lj-POLLUX in these double transgenic roots was confirmed at the transcript level by RT-PCR (see Supplemental Figure 2C online). Coexpression of Lj-CASTOR and Lj-POLLUX in dmi1-4 roots also restored mycorrhization, accompanied by the development of arbuscules and vesicles and expression of Mt-PT4 (see Supplemental Figures 4B and 4D online). As a control, DMI1, expressed under the control of 35Spro or its own promoter, was able to rescue fully the dmi1-4 mutant (Figure 3D, Table 2). Collectively, our results indicate that CASTOR and POLLUX are both required in L. japonicus, while DMI1 (from M. truncatula or pea) can compensate for the loss of both CASTOR and POLLUX.

A Ser-to-Ala Substitution in the Filter Region of DMI1 Is Sufficient for Its Integrated Function

We sought to identify the determinants responsible for the neofunctionality of DMI1 and SYM8. We noticed a single amino acid difference in the predicted filter region of DMI1 and SYM8
when compared with the filter region of Lj-CASTOR, Lj-POLLUX, and Mt-CASTOR. The selectivity filters of DMI1 and SYM8 are composed of the amino acid residues ADAGNHA, and these two putative orthologs mutually rescue each other (Edwards et al., 2007). Lj-CASTOR and Lj-POLLUX have ADSGNHA as a putative orthologs mutually rescue each other (Edwards et al., 2007). Lj-CASTOR and Lj-POLLUX have ADSGNHA as a putative orthologs mutually rescue each other (Edwards et al., 2007). Lj-CASTOR and Lj-POLLUX have ADSGNHA as a putative orthologs mutually rescue each other (Edwards et al., 2007).

<table>
<thead>
<tr>
<th>Mutant Line</th>
<th>Construct</th>
<th>Nodulated Plants/Total Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lj-castor-4</td>
<td>35S pro:DMI1</td>
<td>69/70</td>
</tr>
<tr>
<td>Lj-pollux-2</td>
<td>35S pro:DMI1</td>
<td>67/70</td>
</tr>
<tr>
<td>Lj-caspolA</td>
<td>35S pro:DMI1</td>
<td>56/65</td>
</tr>
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</tr>
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<td>Lj-pollux-2</td>
<td>35S pro:Mt-CASTOR</td>
<td>0/13</td>
</tr>
<tr>
<td>Lj-caspol</td>
<td>35S pro:Mt-CASTOR</td>
<td>0/31</td>
</tr>
<tr>
<td>Lj-castor-4</td>
<td>35S pro:Lj-CAStor</td>
<td>0/16</td>
</tr>
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<td>Lj-pollux-2</td>
<td>35S pro:Lj-CAStor</td>
<td>0/18</td>
</tr>
<tr>
<td>35S pro:LI-CAStor</td>
<td>35S pro:Lj-POLLUX</td>
<td>26/29</td>
</tr>
<tr>
<td>Lj-caspol</td>
<td>35S pro:Lj-POLLUX</td>
<td>34/41</td>
</tr>
<tr>
<td>G00532-21b</td>
<td>35S pro:Lj-CAStor</td>
<td>16/19</td>
</tr>
<tr>
<td>Lj-pollux-2</td>
<td>35S pro:Lj-CAStor</td>
<td>0/24 (1/24)</td>
</tr>
<tr>
<td>Lj-caspol</td>
<td>35S pro:Lj-CAStor</td>
<td>0/55 (3/55)</td>
</tr>
<tr>
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<td>35S pro:Lj-POLLUX</td>
<td>0/18 (4/18)</td>
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<td>20/24</td>
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</tr>
<tr>
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<td>42/46</td>
</tr>
<tr>
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<td>35S pro:SYM8 (genome)</td>
<td>52/52</td>
</tr>
<tr>
<td>Lj-castor-4</td>
<td>35S pro:SYM8 (cDNA)</td>
<td>17/17</td>
</tr>
<tr>
<td>Lj-pollux-2</td>
<td>35S pro:SYM8 (cDNA)</td>
<td>18/19</td>
</tr>
<tr>
<td>Lj-caspol</td>
<td>35S pro:SYM8 (cDNA)</td>
<td>22/22</td>
</tr>
</tbody>
</table>

*aLj-castor pollux double mutant is derived from the cross between Lj-castor-5 and Lj-pollux-2 alleles.

*bLj-castor-8 and another Lj-castor allele G00532-21 completely lack the genomic region of Lj CASTOR.

Table 1. Summary of Rescue Assay Performed with Different Constructs Expressing DMI1, Lj-CASTOR, Lj-POLLUX, Lj-CASTORS266A, Lj-POLLUXS329A, and DMI1A2945 in castor, pollux, and castor pollux Double Mutants of L. japonicus.
In the selectivity ADSGNHA with one additional polymorphism, Asn/Ser, in its putative ortholog, which contains the amino acid sequence impact on DMI1 (C) Arabidopsis. The nonmycorrhizal plant, encodes a DMI1 Ortholog. Impact of Ser-to-Ala Substitution on a Nonlegume the neofunctionality of DMI1. These results demonstrate that the Ser/Ala polymorphism in the filter region of these ion channels is the major determinant for the neofunctionality of DMI1. 

Impact of Ser-to-Ala Substitution on a Nonlegume DMI1 Ortholog

The Ser-to-Ala substitution in the filter region also has significant impact on DMI1 putative orthologs of nonleguminous plants. The nonmycorrhizal plant, Arabidopsis, encodes a POLLUX putative ortholog, which contains the amino acid sequence ADSGNHA with one additional polymorphism, Asn/Ser, in its selectivity filter. At-POLLUX fails to rescue the dmi1-4 mutant, while the introduction of an equivalently positioned Ser-to-Ala substitution enables At-POLLUX to rescue the dmi1-4 mutant (Table 2). At-POLLUX, which includes an additional Asn-to-Ser substitution, also rescues the dmi1-4 mutant, similar to At-POLLUX, indicating that the Ser-to-Ala substitution is sufficient to confer improved function to At-POLLUX (Table 2). Considering that the overall sequence similarity between At-POLLUX and DMI1 is only 67%, this result confirms that the Ser/Ala polymorphism in the filter region is the key determinant for the symbiotic capability of the DMI1 and POLLUX channels.

Tracing the Ser-to-Ala Substitution of DMI1 Homologs in Legumes

Amino acid sequences at the pore region of DMI1 homologs were obtained from 20 legume genera largely representing Caesalpinioideae (Cercis), Genistioideae (Lupinus), Dalbergioideae (Arachis), Millettioideae (Cajanus, Glycine, Vigna, and Phaseolus), Robinoideae (Lotus), Galegoidae (Astragalus and Galega), and Vicieae (Cicer, Ononis, Medicago, Trigonella, Melilotus, Trifolium, Pisum, Lathyrus, Vicia, and Lens). The filter sequence ADSGNHA was observed among POLLUX putative orthologs from Caesalpinioideae, Genistioideae, Dalbergioideae, Millettioideae, Robinoideae, and Galegoidae and in a few members of Vicieae (Cicer). The presence of the Ala residue is restricted to the Trifolium and Vicieae tribes among the Vicieae clade and therefore occurred rather recently in legume evolution (Figure 4; see Supplemental Data Set 1 online).

The Ser-to-Ala Substitution in the Filter of DMI1 Reduces Potassium Conductance

We hypothesized that the Ser-to-Ala substitution in the filter region alters the ion permeation of DMI1 and SYM8 when compared with Lj-CASTOR and Lj-POLLUX. To test this hypothesis, the effect of this substitution was analyzed by comparing DMI1 with Lj-CASTOR and Lj-POLLUX carrying the Ser-to-Ala substitution (Lj-CASTORS2266A), which mimics the filter region of DMI1. Lj-CASTOR was chosen for these experiments because its filter region sequence is identical to that
of Lj-POLLUX and its electrophysiological behavior has already been characterized (Charpentier et al., 2008). Unfortunately, Lj-POLLUX has not been amenable to any patch clamp system tested so far. However, since the filter region is the major determinant of ion selectivity, we used Lj-CASTOR for testing the impact of the Ser-to-Ala mutation in the predicted filter region on the channel properties, and we then compared the two Lj-CASTOR variants with the DMI1 channel.

Lj-CASTOR has a positive reversal potential in the presence of KCl, indicating permeability to K⁺, and a negative reversal potential in CaCl₂, revealing permeability for the Cl⁻ anion as well (Charpentier et al., 2008). In this study, we show that in symmetrical KCl solution, Lj-CASTOR (ADSGNHA) exhibited a conductance of around 175 pS (Figure 5A). Interestingly, the DMI1 channel (ADAGNHA) had a much lower conductance of only 64 pS (Figure 5B). Consistent with the idea that the sequence differences in the filter region are responsible for this altered channel behavior, we observed that Lj-CASTOR²⁶⁶⁴ with a filter region identical to DMI1 (ADAGNHA) had a similar low conductance of 61 pS (Figure 5C).

The Ser-to-Ala mutation in the filter region reduces the ion flow without significantly altering the selectivity. Since the filter region is a major determinant of ion permeation, and given that Lj-CASTORS²⁶⁶⁴ and DMI1 carrying the same filter region show the same electrophysiological shift relative to the Lj-CASTOR channel, we hypothesize that Lj-POLLUX has a conductance similar to Lj-CASTOR and that this is higher than that of DMI1. Thus, our results indicate that the better symbiotic performance of DMI1, SYM8, and Lj-POLLUXS²²⁹⁴ is associated with a reduction in K⁺ conductance compared with Lj-CASTOR and Lj-POLLUX.

We also measured the mean open time for DMI1, Lj-CASTOR, and Lj-CASTOR²⁶⁶⁴ by pooling single channel currents obtained in symmetrical 250 mM KCl at various voltages. We were able to record and analyze gating events for DMI1, Lj-CASTOR,
Ser-to-Ala Substitution in the Filter Improves the Ca\textsuperscript{2+}-Induced Ca\textsuperscript{2+} Release Mediated by DMI1

To test the effect of the Ser/Ala polymorphism in modulating Ca\textsuperscript{2+} signaling, we developed a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release assay in Human Embryonic Kidney cells (HEK-293) expressing the Yellow Cameleon 3.6 (YC3.6) Ca\textsuperscript{2+} indicator (see Supplemental Figure 8A online). In these cells, a DMI1:Green fluorescent protein (GFP) fusion was localized to the nuclear envelope (see Supplemental Figures 8B and 8C online). Oscillations in the Förster resonance energy transfer/cyan fluorescent protein (FRET/CFP) ratio resulting from YC3.6 are directly proportional to the free Ca\textsuperscript{2+} available in the cytosol. The addition of 2 mM CaCl\textsubscript{2} to the external bath solution resulted in a drastic increase in the FRET/CFP ratio in HEK-293 cells expressing DMI1. A stepwise increase in the Ca\textsuperscript{2+} concentration of external bath solution from 2 to 10 mM (an increase of 2 mM for every step) resulted in Ca\textsuperscript{2+} transients coupled with periodic oscillations or spikes in the FRET/CFP ratio in HEK-293 cells expressing DMI1 (Figure 7). Such an upward shift, or oscillations, in the FRET/CFP ratio was not observed in control cells expressing only YC3.6, even at a 10 mM Ca\textsuperscript{2+} concentration (Figure 7). Although some control cells shrank in response to a high concentration of CaCl\textsubscript{2} in the external solution, they never displayed any increase in the FRET/CFP ratio or spikes, even at 10 mM CaCl\textsubscript{2} (Figure 7). These results demonstrate that DMI1 has the ability to activate Ca\textsuperscript{2+} channels, thereby modulating a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in animal HEK-293 cells. These Ca\textsuperscript{2+} oscillations seem to originate from internal Ca\textsuperscript{2+} stores, since the application of 10 mM cadmium did not inhibit the spiking pattern initiated by the prior addition of Ca\textsuperscript{2+} to the external bath (see Supplemental Figure 8D online). The spiking continued even 10 min after the application of cadmium to the external bath (see Supplemental Figure 8D online). However, application of cadmium before the Ca\textsuperscript{2+} treatment totally prevented the elicitation of spikes, probably due to the inactivation of Ca\textsuperscript{2+} channels responsible for the induction of Ca\textsuperscript{2+} transients (see Supplemental Figure 8E online).

HEK-293 cells expressing the modified *dmi1* mutant allele *dmi1* (A294S), which mimics the filter of *Lj-CASTOR*/POLLUX, were unable to induce Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} transients and spikes at 2 and 4 mM Ca\textsuperscript{2+} (Figure 7). However,
these cells displayed Ca\textsuperscript{2+} transients and a few spikes when the Ca\textsuperscript{2+} concentration in the external bath reached 6 and 10 mM (Figure 7). Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} transients were absent in cells expressing \textit{dmi1}(A294V), which mimics a null mutant of pea \textit{SYM8} (Edwards et al., 2007). Similarly, cells expressing an empty vector control did not elicit any Ca\textsuperscript{2+} transient either (Figure 7). These results confirmed that the efficiency of \textit{DMI1} in mediating Ca\textsuperscript{2+} spiking in HEK-293 cells is defined by the Ala residue in the filter region.

**DISCUSSION**

In this study, we functionally characterized \textit{M. truncatula} \textit{CASTOR} and \textit{DMI1} and their \textit{L. japonicus} homologs, \textit{CASTOR} and \textit{POLLUX}. The cation channels \textit{DMI1}, \textit{Lj-POLLUX}, and \textit{Lj-CASTOR} are required for Nod factor-induced Ca\textsuperscript{2+} spiking. They probably act either as counter-ion channels to compensate for the positive charge release from the Ca\textsuperscript{2+} store during each spiking event or activate Ca\textsuperscript{2+} channels by changing the membrane potential of nuclear membranes (Charpentier et al., 2008; Matzke et al., 2009). Our data indicate that these ion channels have different functional properties in symbiotic signaling. In our rescue assays, \textit{Lj-CASTOR} and \textit{Lj-POLLUX} together were required to rescue \textit{dmi1}. By contrast, \textit{DMI1} and \textit{SYM8} alone were sufficient to rescue an \textit{Lj-castor pollux} double mutant, indicating an improved ability of \textit{DMI1} and \textit{SYM8} over \textit{Lj-CASTOR} and \textit{Lj-POLLUX}. Unlike \textit{DMI1} and \textit{SYM8}, \textit{Lj-POLLUX} failed to rescue the symbiotic defects of \textit{Lj-castor}, \textit{Lj-castor pollux}, and \textit{dmi1} in our study.
This observation differs from a previous report where Lj-POLLUX was shown to rescue Lj-castor (Charpentier et al., 2008). That may be due to different experimental conditions and Lj-castor alleles used. In the previous study, a single Lj-castor allele with a nonsense mutation (W93*) was used (Charpentier et al., 2008), whereas, in this work, we used three different Lj-castor alleles, including two null mutants (Lj-castor-8 and G00532-21), both of which completely lacked the genomic region. None of the three mutant lines tested in this study were fully rescued for nodulation by Lj-POLLUX (Table 1).

In this study, nodules were categorized into functional nodules and bumps in both M. truncatula and L. japonicus experiments (Tables 1 and 2). The partial rescue phenotypes (presence of nodule bumps) observed in Lj-castor and Lj-castor pollux mutants rescued by Lj-POLLUX were similar to those observed in Lj-pollux and Lj-castor pollux mutants rescued by Lj-CASTOR.

Figure 5. Conductance of Lj-CASTOR, DMI1, and Lj-CASTOR<sup>S266A</sup> in the Presence of Potassium Chloride.

Conductance of Lj-CASTOR (A), DMI1 (B), and Lj-CASTOR<sup>S266A</sup> (C) is based on single-channel open-close events at different voltages, as shown in the traces at the right side of each panel. Conductance in picosiemens was calculated as the ratio between the difference of current (I) measured in the open and closed state and the voltage (V) applied [conductance<sub>pS</sub> = I/pA/V(mV)]. In the presence of KCl, Lj-CASTOR has a significantly higher conductance than DMI1 and Lj-CASTOR<sup>S266A</sup>. These two channels have a similar intermediate conductance.
POLLUX (ADSGHNA) disrupted CASTOR function in the Lj-castor-2 mutant (Charpentier et al., 2008). In this study, the Ser-to-Ala substitution in the ADAGNHA region of these channels due to differences in the regulatory mechanism. This hypothesis is supported by the fact that Lj-POLLUXS329A is able to rescue Lj-CASTOR and Lj-CASTORS266A and likely have similar ion selectivity filter regions and likely have similar ion selectivity and conductivity properties. The failure of Lj-CASTORS266A to rescue Lj-CASTOR and Lj-CASTORS266A, similar to DMI1 (64 pS). In this study, we could not test the electrophysiological properties of Lj-POLLUX and Lj-POLLUXS329A directly. In spite of several attempts, Lj-POLLUX could not be produced through in vitro transcription/translations similar to Lj-CASTOR, Lj-CASTORS266A, and DMI1 and was therefore unavailable for the planar lipid bilayer system. Hence, we relied on Lj-CASTOR, Lj-CASTORS266A, and DMI1 to test our hypotheses. Although Lj-CASTOR and Lj-POLLUX may show differences in their regulatory mechanisms, they have identical ion selectivity filter regions and likely have similar ion selectivity and conductivity properties. The failure of Lj-CASTORS266A to rescue Lj-pollux, Lj-castor pollux, and Lj-dmi1 mutants may be due to differences in the regulatory mechanism. This hypothesis is supported by the fact that Lj-POLLUXS329A is able to rescue the dmi1 mutation, while Lj-CASTORS266A failed to rescue dmi1 in M. truncatula, although Lj-CASTORS266A is still functional and able to rescue the Lj-castor mutation in L. japonicus. This

With the availability of several mutants for rescue assays, we demonstrated that not only Lj-CASTOR, but also Lj-POLLUX, lacks the integrated role of DMI1 and SYM8. These observations are further supported by the inability of rice POLLUX to rescue dmi1 when expressed alone, in which only bumps devoid of bacteria were observed (Chen et al., 2009). By contrast, pea SYM8 could rescue not only dmi1 (Edwards et al., 2007), but also Lj-castor, Lj-pollux, and the Lj-castor pollux double mutant of L. japonicus, just like DMI1 (this study).

DMI1 and SYM8 have an identical selectivity filter ADAGNHA that is absent from CASTOR and POLLUX of L. japonicus and rice. Our data support the current knowledge that specific residues in this region play a key role in the permeation of ion channels. For example, substitutions in the Asp or the Ala residue (ADAGNHA) to Val disrupted the function of SYM8 (Edwards et al., 2007), and substitution in the first Ala residue (ADSGHNA) disrupted CASTOR function in the Lj-castor-2 mutant (Charpentier et al., 2008).

In this study, a Ser-to-Ala substitution in the filter region of POLLUX (ADSGHNA) conferred a superior ability to this channel and has been retained through natural selection in the Trifoliiaceae and Vicieae tribes. POLLUXS329A gained the ability to rescue the nodulation defects of both dmi1 and Lj-castor pollux mutants. Reciprocally, DMI1A294S lost its ability to rescue Lj-castor, Lj-castor pollux, and dmi1 in Mt-CASTOR-RNAi roots. Our conclusions have been validated even outside of the legume family using Arabidopsis POLLUX.

We propose that the Ser-to-Ala substitution might introduce conformational changes at the selectivity filter. Homology modeling of DMI1 and its homologs identified possible conformational differences at the filter region of these channels due to the Ser/Ala polymorphism (Figures 8A and 8B). The Ala (Ala-294) residue in DMI1 interacts only weakly with the Asp (Asp-293) from the adjacent subunit (Figure 8A). By contrast, the hydroxyl residue (Asp-293) interacts only weakly with the Asp (Asp-293) from the adjacent subunit (Figure 8A). By contrast, the hydroxyl residue (Asp-293) interacts only weakly with the Asp (Asp-293) from the adjacent subunit (Figure 8A). By contrast, the hydroxyl residue (Asp-293) interacts only weakly with the Asp (Asp-293) from the adjacent subunit (Figure 8A).

Adapted from Edwards et al. (2007).
explains that Lj-CASTOR, Lj-POLLUX, and DMI1 have distinct states of functionality and that mere conversion to an intermediate ion channel with a long open state in Lj-CASTOR S266A does not rescue dmi1, but a similar modification in Lj-POLLUX S329A rescues dmi1. We used FRET-based assays as a quantitative measure to ascertain the effect of this Ala-to-Ser substitution on the ability of these channels to mediate intracellular Ca2+ oscillations. We showed that the ability of DMI1 to allow Ca2+-induced Ca2+ release in HEK-293 cells is drastically affected when Ala was substituted for Ser to mimic the filter region of Lj-CASTOR and Lj-POLLUX. These observations correlate well with the observed superior functionality of DMI1, which integrates the roles of Lj-CASTOR and Lj-POLLUX. We also observed an increase in the mean open time for DMI1 (fourfold) and Lj-CASTOR S266A (15-fold) over wild-type Lj-CASTOR (461 ms). These data indicate that the Ala residue in DMI1 has a stabilizing effect on the open state of the channel. Our homology-based modeling predicts that the smaller side chain of the Ala compared with Ser allows a more compact packing against the pore helix behind the filter sequence (ADAGNHA). This would be opposite to the stabilization of the filter with the Ser residue (ADSGNHA), as we discussed earlier.

Similarly, point mutations in the pore region of the temperature-activated transient receptor potential cation channel (TRPV1) alter the gating kinetics and duration of the channel open state. It was shown that wild-type TRPV1 channel displayed both short (<1 ms) and long (~10 ms) open states, while the loss-of-function mutants affected in the pore region only displayed short open states (<1 ms) (Grandl et al., 2010). Together with our study, these observations suggest that the duration and stability of the open state of ion channels determine the channel efficiency.

To our knowledge, there is no report of a naturally occurring mutation in the filter region of an ion channel leading to an improvement of function, as seen in this study. Charpentier et al. (2008) proposed a model in which Lj-CASTOR and Lj-POLLUX act as counter-ion channels that compensate for the positive charge associated with Ca2+ release during spiking. This model predicts a significant ion flow through the channels during spiking. This quantitative flux model was also consistent with the idea that two channels (Lj-CASTOR and Lj-POLLUX) are required for calcium spiking. However, the observed conductances of the channels are not simple to reconcile with this model because a higher K+ conductance of the channels would be expected to improve the counter ion permeation capability. By contrast, we observed that DMI1 shows moderate ion conductance, while the Lj-castor-2 channel with high conductance could not support calcium spiking (Charpentier et al., 2008), implying that ion conductance alone is not the only factor determining the functionality of these channels in the generation of

![Figure 7. Influence of DMI1 and Modified Alleles on Ca2+-Induced Ca2+ Release in HEK-293 Cells.](image-url)
the voltage-sensing Ca$^{2+}$ channel(s), the counter-ion channel(s), expected to require at least three types of ion channels/pumps: spike initiation and in limiting the duration of each spike (Golding Ca$^{2+}$ carries the inward current instead of Na$^{+}$. In these cases, carries the outward hyperpolarizing current. In some neurons, action potentials largely by regulating the conductance for Na$^{+}$ Lj-POLLUX. The blue arrow in (A) indicates Ala (DMI1$^{294}$), the black arrow in (B) indicates Ser (Lj-POLLUX$^{329}$ and Lj-CASTOR$^{266}$), and the red arrows in both panels indicate Asp residues (Lj-POLLUX$^{328}$, Lj-CASTOR$^{265}$, and DMI1$^{295}$). (A) The orientation of Asp is affected by the weak interaction with Ala in DMI1. (B) The orientation of Asp is affected by the strong interaction with Ser in Lj-POLLUX and Lj-CASTOR.

calcium spiking. It is likely that the duration of the open state and stability of the pore are also important factors regulating the improved functionality of DMI1 over Lj-CASTOR and Lj-POLLUX.

In mammalian cells, action potentials (spikes) have been more thoroughly characterized. Neurons and muscle cells generate action potentials largely by regulating the conductance for Na$^{+}$ and K$^{+}$. Na$^{+}$ carries the inward depolarizing current and K$^{+}$ carries the outward hyperpolarizing current. In some neurons, Ca$^{2+}$ carries the inward current instead of Na$^{+}$. In these cases, the K$^{+}$ channels are involved in setting the threshold for Ca$^{2+}$ spike initiation and in limiting the duration of each spike (Golding et al., 1999). Reference conductance levels for K$^{+}$ channels mediating action potentials in animal cells are, for example, 200 pS for large conductance BK (big potassium) channels (Lancaster and Nicoll, 1987), 10 pS for small conductance SK (small conductance calcium-activated potassium) channels (Köhler et al., 1996), and 30 to 50 pS for intermediate/moderate conductance ROMK (renal outer medullary potassium) channels (Sackin et al., 2005). Thus, during evolution, the Ser-to-Ala substitution in the filter region converted a large conductance channel (Lj-CASTOR and Lj-POLLUX) to a moderate conductance channel (DMI1) with enhanced functionality in symbiosis.

Based on its analogy to the animal action potential (Hodgkin and Huxley, 1952; Barnett and Larkman, 2007), the generation of nuclear Ca$^{2+}$ spiking during symbiotic signaling in host plants is expected to require at least three types of ion channels/pumps: the voltage-sensing Ca$^{2+}$ channel(s), the counter-ion channel(s), and the Ca$^{2+}$ pump(s). L. japonicus CASTOR and POLLUX, as well as M. truncatula DMI1, are localized to the nuclear envelope (Riely et al., 2007; Charpentier et al., 2008). In a recent study, we demonstrated the role of M. truncatula Ca$^{2+}$ ATPase, MCA8, in Nod factor–induced calcium spiking, and we observed localization of MCA8 to both the inner nuclear membrane (INM) and outer nuclear membrane (ONM), similar to DMI1, suggesting their close proximity on the nuclear membranes and their coordinated role in nuclear calcium spiking (Capoen et al., 2011).

Evidence for the presence of Ca$^{2+}$ pumps that could replenish the nuclear envelope lumen of Ca$^{2+}$ ions against their concentration gradient was initially shown in nuclei prepared from carrot (Daucus carota) cells. It was also shown that Ca$^{2+}$ could be transported across nuclear membranes in an ATP-dependent manner (Bunney et al., 2000), and immunogold labeling identified a homolog of the sarco(endo)plasmic reticulum Ca$^{2+}$ ATPase (SERCA) pump in the nuclear envelope of tomato (Solanum lycopersicum) root cells (Downie et al., 1998).

Nuclear envelope–residing calcium channels mediating calcium spiking have not been reported yet. However, two major types of voltage-gated Ca$^{2+}$ permeable channel activities have been recorded on the plasma membrane by electrophysiological methods. These studies suggest the presence of depolarization-activated Ca$^{2+}$ channels (Thulou et al., 1994; Thion et al., 1998) and hyperpolarization-activated Ca$^{2+}$ channels on the plasma membrane (Shang et al., 2005; Davies and Walker, 2008), although the genes encoding these Ca$^{2+}$-permeable channels have not been identified. A less specific activation mechanism, such as voltage sensing, is more general and includes such Ca$^{2+}$ channels in various signaling pathways. Although no experimental data are currently available to support the polarization state of the INM and ONM, studies indicate the existence of a potential across the nuclear envelope that results in the nucleus having a relatively negative charge than the cytoplasm (Loewenstein and Kanno, 1963). Additionally, a recent study shows periodic oscillations of membrane potential upon release of Ca$^{2+}$ from the nuclear envelope (Yamashita, 2011). Given the lack of experimental values, and since the potential across the plasma membrane is negative toward the cytoplasmic side, it is likely that the INM and ONM are also negatively charged on the cytoplasmic side relative to the perinuclear space.

**Model Depicting the Role of DMI1 and the POLLUX and CASTOR Duo during Nod and Myc Factor-Induced Ca$^{2+}$ Spiking**

Based on our experiments on the channel activities described above, the solo-sufficiency of DMI1 versus the Lj-CASTOR-POLLUX duo, we propose a speculative model explaining the mechanism of nuclear Ca$^{2+}$ spiking (Figure 9). Yet unidentified symbiotic secondary messengers activate the cation channels (Lj-CASTOR and Lj-POLLUX/DMI1), resulting in the flow of K$^{+}$ ions from the cytoplasm into the perinuclear space. This causes the ONM to become hyperpolarized, and because of its close proximity, the INM also becomes hyperpolarized. When a certain degree of hyperpolarization is achieved, the hyperpolarization-gated Ca$^{2+}$ channels are activated, resulting in Ca$^{2+}$ flow out of the perinuclear space to the cytoplasm and nucleoplasm, which leads to Ca$^{2+}$ spiking. The rise in Ca$^{2+}$ concentration is sensed by cation channels and results in Ca$^{2+}$-mediated blockage or inactivation. Simultaneously, the Ca$^{2+}$ flow across the membranes results in depolarization of the nuclear membranes and then closure of Ca$^{2+}$ channels. The Ca$^{2+}$ is pumped back into
the perinuclear space (calcium store) by Ca\(^{2+}\) ATPases such as MCA8.

This model explains the apparent synchronization of the Ca\(^{2+}\) release channels across the nuclear envelope. The Ca\(^{2+}\) release channels must be activated and open simultaneously to generate a rapid spike. A change in the membrane potential is therefore a more likely trigger than the activation of these channels (one by one) by diffusible symbiotic secondary messengers. According to the proposed model, it is possible that an intermediate conductance channel, such as DM1, may be able to outperform two large conductance channels, such as Lj-CASTOR and Lj-POLLUX, through its longer open periods and thus facilitate a bigger net flow of K\(^+\) ions (Figure 6). Considering that the measured conductance of Lj-CASTOR is approximately threefold higher than that of DM1, the fourfold reduction of Lj-CASTOR open time may not be sufficient to explain the integrated role of DM1 versus the Lj-CASTOR/ POLLUX duo. Maybe Lj-CASTOR and Lj-POLLUX have to work together, opening one after the other and therefore creating the change in membrane potential for a certain minimal time period required by activation of the voltage-gated Ca\(^{2+}\) channel.

Finally, from the model described, the justification for the different functionality of CASTOR and POLLUX in the complementation experiments can be partly attributed to a possible offset in activation time required for each channel, to different affinities to Ca\(^{2+}\) as a potential blocker agent or maybe just to the different distribution of the two channels in the nuclear envelope.

Evolution involves the selection of beneficial alleles through improved fitness of the carrier. However, only few examples are documented in which the selective advantage of a novel allele is so well characterized at the mechanistic level. Based on the electrophysiological studies and cross-species rescue assays, we propose that a functional improvement of a symbiotic ion channel DM1 was facilitated by a single amino acid substitution within the selectivity filter region. This substitution was responsible for the conversion of a large conductance channel with a short open state into an intermediate conductance channel with likely a longer open lifetime, which is associated with the capacity of DM1 to act as a more efficient counter ion channel during Ca\(^{2+}\) signaling, and explains why this Ala residue has been positively selected and maintained in the Vicieae and Trifolieae clades.

**METHODS**

**Plant Material**

RNAi and rescue assays in Medicago truncatula were performed in wild-type Jemalong A17 and the dmi1-4 mutant line (GY15-3F-4), respectively.
Rescue assays in *Lotus japonicus* were performed on Lj-castor mutants (Lj-castor-4, Lj-castor-8, and G00532-21fi), the Lj-pollux mutant (pollux-2), and the Lj-castor pollux double mutant. Lj-castor-8 and G00532-21 completely lack the genomic region of Lj-CASTOR. The Lj-castor pollux double mutant was identified among F2 progeny from a cross between Lj-castor-5 and Lj-pollux-2 mutants. A complete list of all the *M. truncatula* and *L. japonicus* mutant lines used in this study with the information on the nature of the mutation is provided in Supplemental Table 1 and Supplemental References 1 online.

**Mt-CASTOR Coding Sequence**

The full-length coding sequence of Mt-CASTOR was obtained by a combination of RT-PCR and 3′ rapid amplification of cDNA ends (RACE). RNA ligase-mediated RACE was performed using GeneRacer (Invitrogen). The nested primers P1, P2, and P3 were used to perform 3′ RACE were designed based on Mt-CASTOR ESTs available on the *M. truncatula* genome project online database. Since the EST sequences obtained from the public database already contained the beginning of the gene, 5′ RACE-PCR was not performed. The full-length product was amplified using the primer set P4 and P5 and cloned into the entry vector pENTR/D-TOPO (Invitrogen) for further use. A list of all the primers used in this study is provided in Supplemental Table 2 online.

**RNAi of Mt-CASTOR and DMI1**

A 451-bp fragment at the 5′ coding region of Mt-CASTOR was amplified using the primers P6 and P7 and cloned into a modified hairpin RNAi-expressing binary vector, pK7GWG2(R)-II, containing the constitutively expressed fluorescent marker DsRed1 (Riely et al., 2011). As a positive control, we performed RNAi-based gene knockdown of DMI1. Primers P8 and P9 were used to amplify a 413-bp fragment of DMI1, which was cloned into pK7GWG2(R)-II. Empty vector was used as the negative control. *Agrobacterium rhizogenes*-mediated root transformation was performed to deliver the constructs into Jemalong A17 as previously described (Boisson-Dernier et al., 2001). Reduction in the Mt-CASTOR expression level was confirmed by RT-PCR using the primers P10 and P11, and Mt-Actin expression was measured using primers P12 and P13. Nodulation and AM assays were performed as previously described (Limpens et al., 2004; Javot et al., 2007). A total of 36 plants were subjected to the nodulation assay for each construct. From three biological replications, a total of 36 plants were subjected to the mycorrhization assay with the Mt-CASTOR RNAi construct and 24 plants with the empty vector control. For the AM assay, *Ceriscus, Lupinus, Arachis, Cajanus, Glycine, Vigna, Phaseolus, Astragalus, Galega, Cicer, Ononis, Medicago, Trigonella, Melilotus, Trifolium, Pismum, Lathyris, Vicia,* and *Lens,* using the primers P26 and P27. The PCR product sequences were aligned using the multiple alignment program T-Coffee (Notredame et al., 2000), and alignment curation was performed using Gblocks (Castresana, 2000). The coding sequence of DMI1 was used as a reference for translation of nucleotide sequences to amino acid sequences at the amino acid level. To mimic the filter region of Mt-CASTOR, a reference gene for loading control.

**Cross-Species Rescue Assays in *M. truncatula* and *L. japonicus***

Lj-CASTOR and Lj-POLLUX were expressed in the dmi1-4 mutant of *M. truncatula*. For constitutive expression of full-length proteins under the control of 35S<sub>ubq</sub>, Lj-CASTOR was cloned into the binary vector pK7WG2D, which has GFP as a visible marker. Lj-POLLUX was cloned into the modified binary vector pK7FWG2-R, which carries DsRed1 as a visible marker. To drive the expression of Lj-CASTOR and Lj-POLLUX under their respective native promoters (2300 and 2800 bp, respectively), the constructs pK7RWG2:Lj-CASTOR<sub>prom</sub>-Lj-CASTOR and pK7RWG2:Lj-POLLUX<sub>prom</sub>-Lj-POLLUX were used. To express Lj-CASTOR and Lj-POLLUX under the influence of the DMI1 promoter (1500 bp), the 35S<sub>ubq</sub> was replaced with the promoter of DMI1. The binary vector pUB-GW-GFP was used to express Lj-CASTOR and Lj-POLLUX under the influence of the Lj-Ubiquitin1 promoter (Maekawa et al., 2008). Nodulation and arbuscular mycorrhization assays in *L. japonicus* were performed as previously described (Banba et al., 2008).

**Site-Directed Mutagenesis**

Modification of the filter region of Lj-POLLUX and Lj-CASTOR was achieved by point mutations in Lj-POLLUX (T985G) and Lj-CASTOR (T979G), both of which mimic the filter region of DMI1 at the amino acid level. The point mutation G887T in DMI1 was performed to mimic the filter region of Lj-CASTOR/Lj-POLLUX at the amino acid level. To mimic the filter region of Mt-DM1 in At-POLLUX point mutations, T709G and G716A were introduced. The QuickChangell site-directed mutagenesis kit (Stratagene) was used to perform point mutations on the entry clones pENTR/D-Lj-CASTOR, pDONR221:Lj-POLLUX, pDONR221:DM1, and pENTR1A:At-POLLUX. Primers used for site-directed mutagenesis were as follows: The primer sets P14–P17, P18–P19, P20–P21, P22–P23, and P24–P25 were used to generate DMI1<sub>T985G</sub>, Lj-POLLUX<sub>G887T</sub>, Lj-CASTOR<sub>G887T</sub>, At-POLLUX<sub>G887T</sub>, and At-POLLUX<sub>G887T</sub> respectively. These mutants were cloned into the Gateway binary vector pK7WG2D by LR recombination (Invitrogen) for rescue assays in *M. truncatula* and *L. japonicas*.

**Phylogenetic Analyses**

A total of 609 bp of nucleotide sequences flanking the filter region of DMI1 homologs was amplified from genomic DNA of legume genera, such as *Cercis, Lupinus, Arachis, Cajanus, Glycine, Vigna, Phaseolus, Astragalus, Galega, Cicer, Ononis, Medicago, Trigonella, Melilotus, Trifolium, Pismum, Lathyris, Vicia,* and *Lens,* using the primers P26 and P27. The PCR product sequences were aligned using the multiple alignment program T-Coffee (Notredame et al., 2000), and alignment curation was performed using Gblocks (Castresana, 2000). The coding sequence of DMI1 was used as a reference for translation of nucleotide sequences to amino acid sequences at the amino acid level. Homologs across legume genera were grouped into DMI1 and POLLUX type, based on amino acid sequence in the filter region, ADAGNHA/ADSGNHA. The amino acid sequence alignment of the pore region of all the legume genera is presented in Supplemental Data Set 1 online. Since the sequence length of the pore region obtained from different legume species was too short (309 bp) for phylogenetic analyses with a high confidence level, a legume phylogeny with selected legume genera was reconstructed based on the existing phylogenetic tree (Wojciechowski et al., 2004).
DM1(A294S), Lj-POLLUX(S329A), and Lj-CASTORS266A were aligned to MthK using the alignment program MUSCLE (http://www.ebi.ac.uk/Tools/muscle/index.html) (see Supplemental Figure 9 online). Then, the pore region of MthK was mutated to the one of candidate proteins in the molecular modeling program SYBYL (Tripos). The alignment was further adjusted by visualizing the mutated filter region for reasonable fits of the side chains. A conserved Gly in the filter region was used to position the remaining amino acids in the alignment. No gaps or insertions were made and the sequences were threaded on the MthK structure. A similar approach was used to model the filter region of DM1 homologs. SYBYL was then used to energy minimize the models for convergence using the Tripos force field and Gasteiger-Huckel charges in the models. Figures of protein models were made with PyMOL (http://www.pymol.org).

In Vitro Expression and Purification of Lj-CASTOR and DM1

Lj-CASTOR and Lj-CASTORS266A were expressed through coupled in vitro transcription/translation and purified as described (Charpentier et al., 2008), with further purification of the hexahistidine-tagged proteins by affinity chromatography on nickel-nitrotriacetic beads. DM1 was expressed using a wheat germ in vitro transcription/translation system (5′ Prime), and the soluble protein was purified via its hexahistidine tag by affinity chromatography on cobalt TALON resin (Clontech).

Reconstitution in Proteoliposomes and Planar Lipid Bilayer Measurements

Two different setups were used for single channel recordings. First, the channels Lj-CASTOR and Lj-CASTORS266A were reconstituted in proteoliposomes and measured in a setup previously described (Charpentier et al., 2008). The conductance was recorded at different voltages in symmetrical conditions (250/250 mM KCl). The linear regression of the data points for each voltage was used to define the conductance. All solutions were buffered with 10 mM MOPS/Tris at pH 7.0 or 10 mM HEPES at pH 7.0.

Second, the conductance of the DM1 channel and the two channels already measured with the first setup were also recorded on bilayers spanning a single micron-sized hole in a glass substrate. This second setup is the automated patch-clamp system Port-a-Patch (Nanion Technologies). The proteins were reconstituted in giant unilamellar vesicles of the lipid 1,2-diphytanoyl-sn-glycero-3-phosphatidylcholine (Avanti Polar Lipids), and conductance was measured in symmetrical potassium chloride solution (250 mM) buffered with 10 mM HEPES at pH 7.0. Single channel electrical recordings were performed with an EPC10 USB patch clamp amplifier (Heka Electronics) using the Patchmaster software (Heka Electronics). Data were plotted using SigmaPlot software.

Ca2+ Imaging Using YC3.6 and Data Analyses

HEK-293 cells were cultured and transfected as described (Johannessen et al., 2009). Thirty-six hours after transfection, HEK-293 cells expressing DM1 or modified alleles dmi1(A294S) or dmi1(A294V) were chosen for confocal microscopy. The complete growth medium in the chamber was replaced with 1 mL of external bath solution (130 mM NaCl, 3 mM KCl, 0.6 mM MgCl2, 10 mM Glc, and 10 mM HEPES, pH 7.4). Transfected cells with bright YC3.6 expression levels were chosen for FRET analyses (see Supplemental Figure 8A online). The CFP emission (473 to 505 nm) and FRET emission (536 to 546 nm) were collected using a 458-nm primary dichroic mirror and the Meta detector of a Zeiss LSM 510 microscope. Known quantities of CaCl2 (2 to 10 mM) were applied to the external solution at the 125th cycle. A stepwise increase of 0–2 mM, 2–4 mM, 4–6 mM, 6–8 mM, and 8–10 mM CaCl2 was required to observe DM1-modulated Ca2+-induced Ca2+ release in HEK-293 cells. We also attempted to trigger Ca2+-induced Ca2+ release in HEK-293 cells expressing DM1 by adding 0–2 mM, 0–4 mM, 0–6 mM, 0–8 mM, and 0–10 mM CaCl2. The addition of CaCl2 at such high concentrations to steady state HEK-293 cells resulted in a mere transient increase in CaCl2 concentration and cell bursting in several instances. Hence, a stepwise increase in Ca2+ concentration was attempted. The objective fields were scanned once every 7.5 s for a total of 250 cycles. Image analyses were performed using LSM Image Browser version 4.2 (Carl Zeiss). A total of 10 cells were observed for all the constructs at all extracellular CaCl2 concentrations tested (2 to 10 mM). Background CFP and FRET signal were subtracted from the signals obtained from test cells. The FRET/CFP ratio was calculated for each time point and plotted in the y axis in a scatter diagram over time (min) in the x axis.

Accession Numbers

Primers used for 3′ RACE to obtain coding sequence of Mt-CASTOR were designed based on ESTs available at GenBank under accession number CX525932. The coding sequences of DM1, Mt-CASTOR, Lj-CASTOR, Lj-POLLUX, Ps-SYM8, and At-POLLUX (At5g49960) can be obtained from GenBank with accession numbers XM-003592883, FJ974130, AB162157, AB162158, EF447277, and NM-124375, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Sequences and Domain Structures of Mt-CASTOR.

Supplemental Figure 2. RT-PCR Analyses to Validate Mt-CASTOR Gene Silencing. Expression of Mt-PT4, and Coexpression of Lj-CASTOR and Lj-POLLUX in dmi1.

Supplemental Figure 3. Rescue of L. japonicus castor, pollux, and castor pollux Double Mutants by Mt-CASTOR, Lj-CASTOR, Lj-POLLUX, DM1, and SYM8.

Supplemental Figure 4. Rescue of the AM Phenotype in the L. japonicus castor pollux Double Mutant and M. truncatula dmi1 Mutant.

Supplemental Figure 5. Characterization of Nodules and Nodule Bumps Formed in dmi1 by Rescue Assays with Lj-POLLUX and DM1 for the Infection Phenotype Using the X-Gal Assay.

Supplemental Figure 6. Characterization of Nodules Formed in the Rescue Assays for Rhizobial Infection Using hemAexpress, lacZ Expression and Nitrogen Fixation Using nitHexpress, lacZ Expression.

Supplemental Figure 7. Traces at Constant Voltage for Lj-CASTOR, Lj-CASTORS266A, and DM1.

Supplemental Figure 8. Subcellular Localization of DM1 in HEK-293 Cells and Investigating the Source of Calcium-Induced Calcium Release.

Supplemental Figure 9. Sequence Alignment of DM1 Homologs with MthK at the Pore Region.

Supplemental Table 1. List of Lj-CASTOR, Lj-POLLUX, and DMI1 Mutant Alleles Used in This Study.

Supplemental Table 2. List of Primers Used in This Study.

Supplemental References 1. References for the Supplemental Data.
Using the Sequence Alignment Program T-Coffee (Notredame et al., 2000).

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AUTHOR CONTRIBUTIONS


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The Recent Evolution of a Symbiotic Ion Channel in the Legume Family Altered Ion Conductance and Improved Functionality in Calcium Signaling

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