A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning

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In the establishment of the legume–rhizobial symbiosis, bacterial lipochitooligosaccharide signaling molecules termed Nod factors activate the formation of a novel root organ, the nodule. Nod factors elicit several responses in plant root hair cells, including oscillations in cytoplasmic calcium levels (termed calcium spiking) and alterations in root hair growth. A number of plant mutants with defects in the Nod factor signaling pathway have been identified. One such *Medicago truncatula* mutant, *dmi3*, exhibits calcium spiking and root hair swelling in response to Nod factor, but fails to initiate symbiotic gene expression or cell divisions for nodule formation. On the basis of these data, it is thought that the *dmi3* mutant perceives Nod factor but fails to transduce the signal downstream of calcium spiking. Additionally, the *dmi3* mutant is defective in the symbiosis with mycorrhizal fungi, indicating the importance of the encoded protein in multiple symbioses. We report the identification of the *DMI3* gene, using a gene cloning method based on transcript abundance. We show that transcript-based cloning is a valid approach for cloning genes in barley, indicating the value of this technology in crop plants. *DMI3* encodes a calcium/calmodulin-dependent protein kinase. Mutants in pea *sym9* have phenotypes similar to *dmi3* and have alterations in this gene. The DMI3 class of proteins is well conserved among plants that interact with mycorrhizal fungi, but it is less conserved in *Arabidopsis thaliana*, which does not participate in the mycorrhizal symbiosis.

L egumes take part in symbiotic interactions with rhizobial bacteria, which provide the plant with nitrogenous compounds, and with mycorrhizal fungi, which enhance nutrient, particularly phosphate, uptake. The interaction between legumes and rhizobia activates a complex developmental pathway in the plant that leads to the generation of a new organ, the nodule, which hosts the bacteria in an environment optimized for nitrogen fixation. The early stages of this interaction involve molecular communication between the plant and the bacteria: in response to plant-derived flavonoid molecules, bacteria construct a novel lipochitooligosaccharide signaling molecule termed Nod factor that is required for the initiation of the symbiosis (1). Upon exposure to Nod factor, plant root hair cells induce a periodic calcium spiking behavior, which initiates within 10 min (2). Nod factors also induce altered root hair growth (3), gene expression (4, 5), and the mitotic activation of inner cortical cells that leads to the formation of the nodule primordia (6). The mycorrhizal symbiosis involves invasion of the host root by the fungus and the ramification of fungal hyphae within root cortical cells, creating arbuscules that are believed to be the sites of nutrient exchange.

Plant mutants that fail to form nodules (Nod⁻) are typically defective in early responses to Nod factor. Two Nod⁻ plant mutants with the most extreme defects exhibit no measurable responses to Nod factor (7, 8) and have mutations in receptor-like kinases with oligosaccharide binding domains that are hypothesized to function in Nod factor reception. Several *Medi-

cago truncatula* Nod⁻ mutants have defects in the interaction with both rhizobia and mycorrhizal fungi, indicating that the plant employs common pathways for the establishment of both symbioses. Two such mutants, *dmi1* and *dmi2*, exhibit limited responses to Nod factor. In these mutants, Nod factor induces root hair swelling but does not trigger calcium spiking or symbiotic gene expression (9, 10). This phenotype suggests that *dmi1* and *dmi2* perceive Nod factor, but fail to trigger subsequent signaling that leads to gene induction. The *M. truncatula dmi3* mutant shows a phenotype similar to *dmi1* and *dmi2*, but it does induce calcium spiking in response to Nod factor (9), suggesting that the DMI3 protein is required for signal transduction downstream of calcium spiking. Although the *dmi* mutants are defective in mycorrhizal colonization, they induce gene expression in response to diffusible signals from mycorrhizal fungi, indicating that the mutants perceive and mount a limited response to fungal symbionts (11). An analogous Nod factor signaling pathway has been proposed in *Pisum sativum* (pea), in which *Ps-SYM9* functions downstream of calcium spiking (12) at a position parallel to that of *DMI3*.

In several systems, calcium oscillations play a role in signal transduction after ligand perception by a receptor complex. In the guard cells of *Arabidopsis thaliana*, the periodicity of calcium oscillations induced by the hormone abscissic acid regulates the timing of stomatal closure (13). In mammalian systems, the frequency of calcium oscillations defines the level and spectrum of transcriptional induction (14, 15). Information encoded in calcium oscillatory frequency must be decoded, possibly by a protein that modulates its activity on the basis of calcium oscillatory behavior. In mammalian systems, calcium spiking frequency can mediate the activity or localization of protein kinases. For example, the activity of calcium/calmodulin (CaM)-dependent kinase II (CaMKII; ref. 16) and the localization of protein kinase C are both modulated by calcium oscillations (17).

The identification of genes required for a biological process, usually through mutation and positional cloning, is a critical first step in identifying the molecular players in that process. However, positional cloning can be laborious in organisms with sequenced genomes and impractical in organisms, such as crop plants, with large and complex genomes. Here, we use a rapid gene cloning method based on transcript abundance to identify the *DMI3* gene. We show that this method is a viable approach for gene cloning in barley (*Hordeum vulgare*), a crop species with

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a large genome. We report that *DMI3* encodes a calcium/CaM-dependent protein kinase (CCaMK) that functions downstream of calcium spiking and is required for Nod factor signal transduction, nodulation, and mycorrhization. We hypothesize that this kinase is required to transduce the calcium spiking signal to effect changes in plant gene expression.

Materials and Methods

Plant Growth, Treatments, and RNA Preparation. *Medicago truncatula* Gaertner cv. Jemalong A17, *dmi2-1* or *dmi3-1* mutant seeds were prepared as described (18). Plants were grown on buffered nodulation medium, pH 6.5 (19), with 1.2% (wt/vol) agar and 1 mM α-aminoisobutyric acid (Sigma–Aldrich) for 6 days before tissue was harvested. For *M. truncatula* transcript abundance assays root segments beginning 2.5 cm above the point of buffer treatment and ending above the root tip were harvested 24 h later, and RNA was purified by using TRIzol (Invitrogen) (18). Pea seeds were surface sterilized and germinated for 6 days at room temperature on water agar plates. Six root tips ($\approx$1.5 cm) were excised from each mutant or from the wild type and used for RNA purification. Barley seed from lines Sultan5 and M100 (*rar1-2*) were germinated in soil under glasshouse conditions. One gram of leaf tissue was collected from multiple plants per sample at approximately 2 weeks after germination for RNA preparation using TRIzol (18).

Transcript Abundance Assays. The construction of a custom *M. truncatula/Sinorhizobium meliloti* oligonucleotide chip will be described elsewhere (Affymetrix, Santa Clara, CA; R.M.M., Sidney Shaw, Melanie Barnett, Carol Toman, Robert Fisher, and S.R.L., unpublished data). The chip contains 9,935 tentative consensus sequences (TCs; The Institute for Genomic Research, *M. truncatula* Gene Index version 4.0, www.tigr.org/tdb/mtgi/) from *M. truncatula*, represented by 11 probe pairs per gene with 3′ biased probe sets. Oligonucleotide hybridization experiments were performed as described in the Affymetrix technical manual, comparing RNA profiles of buffer-treated wild-type plants to those of buffer-treated *dmi2* or *dmi3* mutant plants. Thirty micrograms of total RNA from $\approx$30 root segments was used for double-stranded cDNA synthesis (SuperScript, Invitrogen). Biotin-labeled complementary RNA (cRNA) was synthesized by using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Biochem). The quality of cDNA and cRNA synthesis was monitored by using agarose gel electrophoresis. Hybridization, washing, staining, and scanning were performed as described in the Affymetrix technical manual at the Stanford Protein and Nucleic Acid Facility. Three independent biological replicates were performed for each genotype. Data were analyzed with DCHIP VERSION 1.3 (www.dchip.org), using data from only perfect-match oligonucleotides and normalizing across 65 independent experiments (20, 21). Model-based analysis was performed by using perfect-match-only analysis, compiling data from three biological replicates for each condition (20). Pairwise comparisons were analyzed for each condition, and a lower 90% confidence bound (LCB) and fold change were determined for each comparison (20). Gene expression changes were considered significant if the LCB was 2-fold or higher and if the intensities between the two conditions differed by >100.

Transcript abundance assays on the barley *rar1-2* mutant and Sultan5 wild type were performed by using standard methods for the Affymetrix barley genome array (Affymetrix). For each genotype, two independent biological replicates were analyzed and pooled for analysis. Data were analyzed as for *M. truncatula* with the exception that the criteria for choosing differentially expressed genes were less stringent for barley experiments: gene expression changes were considered significant if the LCB was 1.4-fold or higher.

Cloning and Sequencing of *DMI3*. A large portion of the *M. truncatula DMI3* gene was amplified by using RT-PCR with primers made to the 5′ end of TC41561 (ATGGGATATGGAA-CAAGAAAACTCTC) and the 3′ end of TC33626 (TGATAT-CAGTTGGAAGACAATCATA). The transcriptional start and end of the gene were defined by using 5′ and 3′ RACE (Invitrogen). The 3′ RACE products generated sequence that contained termination codons indicating identification of the 3′ end of the gene. The 5′ RACE products generated 70 bp of sequence upstream of the start codon; however, no stop codons were in this region. Because upstream sequences did not contain methionine codons, and because the predicted start codon is in the same position as in *DMI3* orthologs, we chose the initial methionine as the start codon for the ORF. A full-length version of the predicted gene was amplified by using RT-PCR.

Overlapping fragments of the pea *Ps-SYM9* gene were amplified with primers based on the *M. truncatula DMI3* sequence (primer pairs: ATGGGATATGGAACAAGAAAACTCT and TGGCTTTCTCACCTTTGACC, GAAGCTGCAACTGTG-GTTCA and TGATATCAGTTGGAAGACAATCATA), using oligo(dT)-primed cDNA as a template and 45°C annealing temperatures. The PCR products were purified (Qiagen, Valencia, CA) and were sequenced directly by using the same primers used for amplification.

Results

Transcript-Based Gene Cloning Successfully Identifies the *DMI2* Gene. We have developed an alternative method to traditional positional cloning methods to rapidly clone mutated genes. We hypothesized that mutations that abolish transcript production or alter transcript stability may be identified by using methods that examine transcript abundance. Transcript stability can be affected if mutated transcripts include premature nonsense codons. Such transcripts are selectively degraded by a process termed nonsense-mediated mRNA decay to prevent the production of nonfunctional or harmful proteins (22). We predicted that mutations that either delete the gene of interest or introduce a premature stop codon may result in altered transcript abundance, which is detectable by using oligonucleotide microarray analyses.

To test whether this transcript-based cloning method works in *M. truncatula*, we used the known mutant *dmi2* (9). The *DMI2* gene has been identified by positional cloning and encodes the receptor-like kinase NORK (23). The *dmi2-1* allele has a frameshift mutation that results in premature translational termination (23). We examined transcript levels in the *dmi2-1* mutant and in wild-type plants by using an Affymetrix oligonucleotide microarray containing 9,935 *M. truncatula* TCs enriched for root- or nodule-specific expression, which are compiled from expressed sequence tags (ESTs). In comparing basal gene expression profiles in the *dmi2-1* mutant to those of wild-type plants, we identified only four TCs that showed significantly lower gene expression levels in the mutant (Table 1). Two of these TCs encode portions of NORK. This result suggests that transcript-based gene cloning can successfully identify mutated genes in *M. truncatula*.

Transcript-Based Gene Cloning in Barley. This rapid method of gene identification may have significant implications in crop species in which positional cloning is limited by genome size and complexity. To test the applicability of this technique in a plant with a larger and more complex genome than *M. truncatula*, we determined whether transcript-based cloning successfully identifies the barley *RAR1* gene. This gene is required for disease resistance signaling in barley and its homologs encode a novel class of eukaryotic zinc-binding proteins (24). The *rar1-2* mutant allele has a single base pair alteration that affects intron processing. The aberrantly spliced *rar1* transcript contains a frameshift mutation that leads to premature translational termination

Table 1. Transcript-based cloning identifies *DMI2* and candidates for *DMI3*

TC number	Sequence homology	log₂(fold change) wild type vs.	
		dmi2	*dmi3*
37248	FRO2-like	**−1.5**	**−1.4**
39727	Unknown	**−1.2**	0.0
29359	NORK	**−2.2**	−0.1
38936	NORK	**−2.2**	0.0
30957	Unknown	**−1.1**	**−1.6**
36850	Nitrate transporter	**−1.2**	**−1.6**
33626	CCaMK	0.1	**−2.0**
41561	CCaMK	0.1	**−2.1**

The first four genes were chosen on the basis of comparisons of *dmi2* transcript levels to those of the wild type. The next four genes were chosen on the basis of the comparison between *dmi3* and the wild type. Numbers in boldface indicate a 2-fold or greater suppression in the mutant on average.

and mRNA instability (24). We compared basal gene expression profiles in wild-type and mutant plants by using the barley Affymetrix oligonucleotide array that contains >22,000 expressed genes. Because low levels of *RAR1* transcript are observed in the *rar1-2* mutant, we chose a less stringent criterion than was used for *DMI2* to identify differentially expressed genes (see *Materials and Methods* and ref. 24). Eighteen genes have reduced transcript levels in the mutant compared with the wild type (Table 2). Of these genes, *RAR1* (probe set name: Contig8942) ranks 11th on the basis of fold suppression in the mutant. Thus, even if a mutation leads to weakly reduced transcript levels in the mutant, this method allows the generation of a list of candidate genes for further analysis. This list may be further narrowed by the examination of gene expression patterns in allelic mutants.

Transcript-Based Gene Cloning Identifies the *DMI3* Gene. To determine whether transcript-based gene cloning could successfully identify new genes, we used this method to identify the *M. truncatula DMI3* gene. The only defined mutant allele of this gene was generated by γ-ray mutagenesis (25), which can cause

Table 2. Transcript-based cloning identifies 18 candidate genes for barley *RAR1*

| Probe set name	log₂(fold change)
Contig12472	−1.65
rbaa15k15	−1.44
EBem10_SQ002_L14	−1.33
Contig16827	−1.16
Contig12321	−1.06
Contig14203	−1.02
Contig2419	−1.01
Contig3265	−0.96
HU14N23u	−0.93
Contig12007	−0.90
Contig8942	**−0.86**
HD12H12r	−0.81
Contig7012	−0.72
Contig11424	−0.70
Contig2778	−0.62
Contig12969	−0.62
Contig6263	−0.59
baak20o05	−0.58

RAR1 represented by Contig8942 is in boldface.

deletions of various sizes. To identify potential candidate genes for *DMI3*, we compared basal expression profiles in the *dmi3* mutant to that of wild-type plants by using microarray analysis. Four TCs showed significantly lower transcript levels in *dmi3* than in wild-type plants, and two of these showed homology to the same CCaMK (Table 1). These TCs showed reduced transcript levels in the *dmi3* mutant but not in the *dmi2* mutant. We used RT-PCR to verify that these two TCs were components of the same gene. Additionally, using real time RT-PCR, we confirmed that abundance of this transcript was reduced in *dmi3* (data not shown). To isolate the full-length sequence for this gene, we used 5′ and 3′ RACE to define the ends and amplified the predicted ORF by using cDNA from wild-type and *dmi3* plants as a template. Sequencing in the *dmi3-1* mutant revealed a 14-bp deletion (TGAAGAGTTTACTG) in this gene, and it is predicted that this will prematurely terminate translation after adding nine amino acids from one of the other reading frames (Fig. 1). This mutation is predicted to create a protein of 209 aa instead of the full-length 523-aa sequence.

Multiple *Ps-sym9* Mutant Alleles Have Mutations in the *DMI3* Gene. Pea *sym9* mutants have a phenotype similar to that of *M. truncatula dmi3* mutants, as early stages of nodulation signaling are blocked in these mutants, but Nod factor-induced calcium spiking is retained (10, 12). The pea *Ps-SYM9* locus has been mapped onto pea linkage group IV (26) at a location that suggests that *Ps-SYM9* is orthologous to *DMI3*. The *Ps-sym9* complementation group includes five different pea mutants (26): P1, P2, P53, and P54 are derivatives of Frisson (27) and R72 carries the first defined *sym9* allele and is in the Sparkle background (28). Sections of the pea *DMI3* gene were amplified from each of these mutants with RT-PCR using primers derived from the *M. truncatula DMI3* cDNA sequence. The pea *DMI3* sequences were 91% identical to the *Mt-DMI3* cDNA sequence and each of the five mutants had a single nucleotide change in this gene. The mutations in P1 and P2 were identical and generate a translational stop (CAA → TAA) replacing glutamine equivalent to residue 222 of the *M. truncatula* protein (Fig. 1). We conclude that P1 and P2 are siblings. The changes in P53 and P54 were also identical and generate a translational stop (TGG → TGA) replacing tryptophan residue 232 (Fig. 1), implying that P53 and P54 are siblings. These mutants were generated by ethyl methanesulfonate mutagenesis, and the changes are classical G/C → A/T transitions characteristic of this mutagen. The change in the R72 mutant also generated a translational stop (TCA → TGA) replacing serine residue 216 (Fig. 1). The identification of three independent null alleles, all of which cause a phenotype in pea similar to that observed in *M. truncatula dmi3-1*, confirms that *Ps-SYM9* is the ortholog of *DMI3*. Furthermore, the identification of mutations in multiple alleles in the same gene in *M. truncatula* and pea confirms that we have successfully identified the *DMI3* gene.

DMI3 and *Ps-SYM9* Encode Proteins with High Homology to CCaMKs. The DMI3 protein shows high sequence similarity ($E = 0.0$, 69–72% amino acid identity) to CCaMKs from trumpet lily (*Lilium longiflorum*, GenBank accession no. 2113422A), tobacco (*Nicotiana tabacum*, GenBank accession nos. AAD52092 and AAD28791) and putative proteins from rice (*Oryza sativa*, GenBank accession no. AK070533), apple ($E = −161$, *Malus x domestica*, GenBank accession no. Q07250) and the moss *Physcomitrella patens* ($E = −149$, GenBank accession no. AAO06899). However, similarity to the next most related protein, from *A. thaliana*, is more limited ($E = −65$, GenBank accession no. NP_187677), which may indicate a loss of selective pressure for the gene; unlike most land plants, including species of moss (29), *A. thaliana* does not form symbiotic associations with mycorrhizal fungi. On the basis of the trumpet lily sequence, the kinase domain is in the N-terminal region of the protein
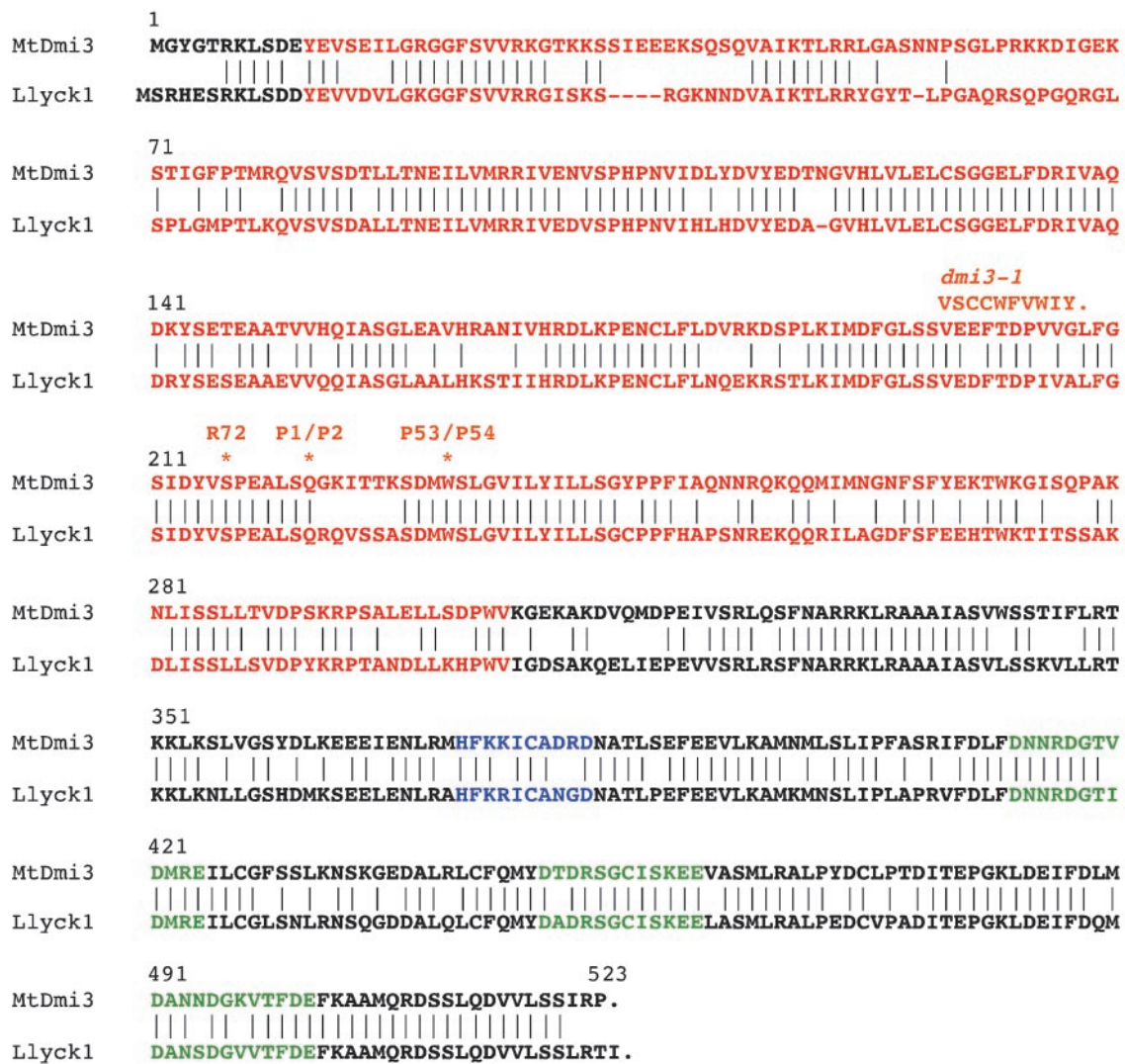
Fig. 1. Sequence of DMI3. Amino acid sequence of *M. truncatula* DMI3 (MtDmi3) aligned with the *Lilium longiflorum* CCaMK 1 (Llyck1) is shown. Amino acids that are identical in the two proteins are connected by vertical bars. Residues shown in red indicate the serine/threonine kinase domain. Residues shown in blue indicate the CaM-binding domain. Residues shown in green indicate EF-hand motifs that specify three different calcium-binding domains. The *dmi3* mutation causes a frameshift resulting in a 9-aa tail before the protein is prematurely terminated (shown in orange above the DMI3 sequence). Mutations in pea result in translation termination marked by orange asterisks above the sequence. Mutation R72 results in termination at amino acid 216, mutations P1 and P2 result in termination at amino acid 222, and mutations P53 and P54 result in termination at amino acid 232.

(amino acids 12–306), followed by the CaM-binding domain (amino acids 373–382) and three calcium-binding domains of the EF-hand type (30). These domains are also present in the apple and tobacco proteins (31). The *dmi3-1* mutation causes translational termination after approximately two-thirds of the kinase domain, presumably resulting in a nonfunctional or a calcium/CaM-independent kinase (Fig. 1).

Although CCaMKs are plant specific, they are distinctly different from plant calcium-dependent protein kinases and other plant serine/threonine kinases (31). CCaMKs have domains that are similar to mammalian proteins, with calcium-binding domains similar to that of neuronal visinin-like calcium-binding proteins and a CaM-binding region similar to that of mammalian CaMKII (32). The activity of CaMKII is modulated by calcium spiking frequency (16). If CCaMKs function in a manner analogous to CaMKII, the DMI3 protein is an excellent candidate for transducing the Nod factor-induced calcium spiking signal.

Discussion

Plants mutated in *DMI3* or *Ps-SYM9* fail to trigger Nod factor-induced gene expression and nodule formation but exhibit Nod factor-induced calcium spiking. This phenotype indicates that the initial signal transduction events downstream of Nod factor recognition are functional in this mutant, but that signal transduction is blocked downstream of calcium spiking. Thus, the DMI3 protein may function to decode and transduce the calcium spiking signal to downstream responses. We show that *DMI3* and *Ps-SYM9* encode proteins with strong homology to chimeric CCaMKs and may fulfill this role. CCaMKs have been identified in a number of plant species, but the role of these proteins in biological processes has been previously unknown.

This class of proteins is multifunctional, with a serine/threonine kinase domain, a CaM-binding domain, and three EF-hand calcium-binding domains that are homologous to that of visinin-like calcium-binding proteins (30). Thus, plant CCaMKs are modulated by calcium in two ways: by free Ca^{2+} and by Ca^{2+} bound to calmodulin (Ca^{2+}/CaM; refs. 30 and 33). By analogy to visinin-like calcium-binding proteins, CCaMKs may bind free Ca^{2+} at nanomolar concentrations (34), which are within the range of calcium concentrations observed during Nod factor-induced calcium spiking (2). The binding of free Ca^{2+}

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promotes autophosphorylation of the lily CCaMK protein (33), which in turn significantly enhances Ca^{2+}/CaM binding (34). The binding of Ca^{2+}/CaM both inhibits autophosphorylation and activates the kinase domain (33). Thus, CCaMKs have the capacity to dramatically increase activity in response to increases in calcium.

CCaMKs such as DMI3 may function in a manner analogous to CaMKII, which modifies its kinase activity in response to calcium oscillation frequency (16). Unlike CCaMKs, CaMKII is modulated solely by Ca^{2+}/CaM and not by free Ca^{2+}. Because CaMKII exists as a multimeric complex, multiple Ca^{2+}/CaM-binding sites are available for regulation and allow frequency-dependent activation of the complex. Ca^{2+}/CaM binding activates kinase activity of this protein and promotes autophosphorylation of neighboring subunits with Ca^{2+}/CaM bound (35). Autophosphorylation significantly increases the affinity of the protein for Ca^{2+}/CaM (36), ensuring that Ca^{2+}/CaM remains bound to this protein even as cytoplasmic calcium levels fall. In response to high-frequency calcium oscillations, Ca^{2+}/CaM will not fully dissociate before the next spike and kinase activity will increase between calcium spikes (35). The timing to maximal kinase activity is directly linked to the frequency of oscillations (35). Although multimeric complexes of CCaMKs have not been described, preliminary data suggest that autophosphorylation of these proteins occurs by means of intermolecular interactions (37). Additionally, the presence of two mechanisms for Ca^{2+} regulation of these proteins suggests that CCaMKs are highly sensitive to changes in calcium levels, possibly integrating multiple calcium-based signals.

We have used a method for cloning the CCaMK encoded by *DMI3* that is based on the instability of a prematurely terminated mutant transcript. We show that oligonucleotide microarray technology can be used to identify mutated genes that encode unstable transcripts; we presume that this technology can also be used to detect deleted genes that fail to produce a transcript. Our results with *dmi2-1* and *rar1-2* mutants indicate that transcript-based gene cloning is viable in both *M. truncatula* and barley. The successful identification of *DMI3* shows that this approach can be used to identify unknown genes. As this genome-wide scanning approach may identify additional mutations unrelated to the mutant phenotype, the analysis of multiple independently generated alleles is important for the success of this approach. We predict that this technique will prove invaluable for rapid gene cloning in plants and possibly other eukaryotes. We believe that this method represents a significant breakthrough in gene cloning, as it transforms a previously laborious process into a rapid and potentially high-throughput method.

Although transcript-based gene cloning may have significant implications in model organisms with sequenced genomes, the greatest impact of this technology is likely to be on plants with large and complex genomes, including most crop species. Because gene identification is independent of gene position, this method does not require the construction of a genetic map, but rather requires a sizable collection of cDNA sequences and microarray technologies. Because transcript-based cloning relies on mRNA instability or absence, the analysis of multiple allelic mutants that are likely to harbor deletions in the gene of interest will increase the chances of successfully identifying mutated genes. Unfortunately, mutations that are pleiotropic will be difficult to identify with this method, as the expression of many genes may be altered with respect to the wild type. In such cases, minimizing phenotypic changes, by altering growth conditions or selecting tissue that is phenotypically similar to the wild type may prove useful. Genes that function in induced pathways, such as those for symbioses, are excellent candidates for this approach because untreated plants are typically indistinguishable from the wild type. We expect that transcript-based cloning will allow the rapid cloning of many important genes in both model and crop plants.

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