A receptor-like kinase from *Arabidopsis thaliana* is a calmodulin-binding protein

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Screening a cDNA expression library with a radiolabelled calmodulin (CaM) probe led to the isolation of AtCaMRLK, a receptor-like kinase (RLK) of *Arabidopsis thaliana*. AtCaMRLK polypeptide sequence shows a modular organization consisting of the four distinctive domains characteristic of receptor kinases: an amino terminal signal sequence, a domain containing seven leucine-rich repeats, a single putative membrane-spanning segment and a protein kinase domain. Using truncated versions of the protein and a synthetic peptide, we demonstrated that a region of 23 amino acids, located near the kinase domain of AtCaMRLK, binds CaM in a calcium-dependent manner. Real-time binding experiments showed that AtCaMRLK interacted *in vitro* with AtCaM1, a canonical CaM, but not with AtCaM8, a divergent isoform of the Ca^{2+} sensor. The bacterially expressed kinase domain of the protein was able to autophosphorylate and to phosphorylate the myelin basic protein, using Mn^{2+} preferentially to Mg^{2+} as an ion activator. Site-directed mutagenesis of the conserved lysine residue (Lys^{423}) to alanine, in the kinase subdomain II, resulted in a complete loss of kinase activity. CaM had no influence on the autophosphorylation activity of AtCaMRLK. AtCaMRLK was expressed in reproductive and vegetative tissues of *A. thaliana*, except in leaves. Disruption in the AtCaMRLK coding sequence by insertion of a *DsG* transposable element in an *Arabidopsis* mutant did not generate a discernible phenotype. The CaM-binding motif of AtCaMRLK was found to be conserved in several other members of the plant RLK family, suggesting a role for Ca^{2+}/CaM in the regulation of RLK-mediated pathways.

Key words: calmodulin (CaM), plant calcium signalling, protein kinase activity, receptor-like kinase (RLK), surface plasmon resonance (SPR).

INTRODUCTION

Plant receptor kinases are a class of transmembrane kinases exhibiting resemblance in domain organization to the animal receptor tyrosine kinases, such the epidermal growth factor (EGF) receptor [1]. Their basic structure is defined by the presence of an extracellular ligand-binding domain, a single membrane-spanning domain and a cytoplasmic kinase domain. Despite conservation in modular organization, plant receptor kinases differ from their animal counterparts in several respects. A prevalence of serine/threonine kinase activity is found in plant receptor kinases, while most of the animal receptors display a tyrosine kinase activity. In addition, the most common extracellular motifs found in plant receptor kinases are the leucine-rich repeat (LRR) and a lectin motif that are involved in protein–protein interactions and carbohydrate binding respectively [2,3]. Plant genomes encode a huge number of putative receptor kinases including at least 610 proteins in *Arabidopsis thaliana* [4]. Although no functional information is available for the majority of them, different members of this family have been shown to act in various aspects of development and plant defence [5]. The interaction between plant receptor kinases and extracellular ligands such as a cysteine-rich protein (SCR) and a secreted protein (CLV3) have been shown to induce receptor oligomerization and autophosphorylation, two critical steps in receptor activation [6]. It has also been demonstrated that plant receptor kinases interact with diverse intracellular proteins, that may either control their phosphorylation status and/or trigger the formation of multi-component complexes to convey information from the cell surface to downstream transduction pathways.

Calcium signalling is one of the best documented pathways in plants; it has been demonstrated to be operative in a series of biological processes from cell division to plant responses to a wide range of stimuli including hormones, light, pathogen elicitors and abiotic stresses [7]. Changes in cytoplasmic Ca^{2+} concentrations induced by these signals occur in a non-stereotypical manner and proceed according to a well-regulated space and time pattern referred to as a 'Ca^{2+} signature' that specifies the nature, intensity and duration of a stimulus. Conversion of information encoded by Ca^{2+} signatures into appropriate responses occurs through Ca^{2+} sensors that activate downstream targets and pathway in a Ca^{2+}-dependent manner. The best known Ca^{2+} sensor is calmodulin (CaM), an ubiquitous and highly conserved protein in all eukaryotes. CaM is a heat-stable protein with four EF-hand motifs (conserved helix–loop–helix structures that can bind a single calcium ion). The active Ca^{2+}/CaM complex interacts with target proteins and regulates their activity. The constantly growing list of CaM targets including metabolic enzymes, structural proteins, transcriptional factors, ion channels and pumps, reveals the ability of CaM to interact with structurally and functionally unrelated proteins and to modulate a wide range of cellular processes [8]. To isolate CaM-binding proteins from *A. thaliana*, we used a cloning strategy based on probing a cDNA expression library

Abbreviations used: AtCaM 1, *Arabidopsis thaliana* calmodulin 1; AtCaMRLK, *Arabidopsis thaliana* calmodulin-binding receptor-like kinase; CaM, calmodulin; EGF, epidermal growth factor; GST, glutathione S-transferase; LRR, leucine-rich repeat; MBP, myelin basic protein; RLK, receptor-like kinase; SPR, surface plasmon resonance.

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with labelled CaM. Here we present evidence demonstrating that CaM binds to a plant receptor-like kinase (RLK). We identified a CaM-binding site in the vicinity of the kinase domain of the protein, and characterized its CaM-binding properties. Binding of CaM had no effect on the kinase activity, and possible roles of CaM in the regulation of plant receptor kinase are discussed.

MATERIALS AND METHODS

Plant materials

A. thaliana ecotypes Columbia and Landsberg erecta were grown in soil under a 16 h:8 h light/dark photoperiod at 20–22 °C in a growth chamber. After 2–4 weeks, plants were transferred to a greenhouse. Rosette leaves and roots were collected from 4-week-old plants, and flowers and siliques were harvested from mature plants. Seedlings were grown on 0.8 % (w/v) agar plates containing half-strength Murashige and Skoog (MS) medium and collected 7 d after sowing. An *Arabidopsis* mutant line carrying the *DsG* element was grown on the same MS medium containing kanamycin (50 μg/ml) as a selective agent, and resistant seedlings were transplanted to soil after 2 weeks. Crosses with homozygous mutants as the male parent and wild-type plants were performed and F1 progeny was selfed. Kanamycin resistance was used to follow the *DsG* element in the progeny.

Expression library screening for CaM-binding proteins

A library from *A. thaliana* cell suspension cultures [9] was screened with [^{33}P]-labelled synthetic CaM (VU-1 CaM) as a probe [10]. The radiolabelled probe was obtained from the *VU-1 CaM* gene subcloned into the pGEX-2TK expression vector (Amersham Biosciences Europe, Sarclay, France) that allows the production of a glutathione S-transferase (GST)-tagged protein and *in vitro* labelling of the fusion product using commercially available protein kinase and [γ-^{33}P]ATP. The bacterially expressed recombinant protein was isolated and radiolabelled following the manufacturer's instructions.

DNA sequencing and computer analysis

Sequencing of cDNAs and PCR-derived products was carried out over both DNA strands. Database searches and comparison with published sequences were performed using the BLAST algorithms. Multiple sequence alignments, prediction of protein sorting signals and transmembrane helices were done using CLUSTAL, PSORT and TMHMM programs. The helical wheel diagram was obtained using the Helical Wheel program (http://marqusee9.berkeley.edu/kael/helical.htm) by Dr Kael Fischer.

Expression of truncated AtCaMRLK (*A. thaliana* calmodulin-binding receptor-like kinase) proteins in *Escherichia coli* and site-directed mutagenesis

GST–AtCaMRLK constructs were obtained by subcloning fragments amplified by PCR using *Pfu* DNA polymerase (Promega, Charbonnières, France) and full-length *AtCaMRLK* cDNA as a template. The PCR products, corresponding either to the protein kinase catalytic domain or to the complete cytoplasmic sequence of AtCaMRLK, were introduced into the plasmid pGEX-6P1 (Amersham Biosciences) at *Bam*H1 and *Sal*1 sites to produce GST–AtCaMRLK (338–666) and GST–AtCaMRLK (315–666) respectively. GST–AtCaMRLK (315–353) was derived from the GST–AtCaMRLK (315–666) construct by deletion of a 944 bp *Eco*R1–*Sal*1 fragment. A kinase-dead mutant was also created by changing Lys^{423} to alanine in GST–AtCaMRLK (338–666) using site-directed PCR-based mutagenesis. After verification by DNA sequencing, the recombinant plasmids were introduced into *E. coli* BL21 cells for protein expression. Expression was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.1 mM and incubation at 22 °C for 4 h. The bacteria were lysed, and the GST-tagged proteins were adsorbed onto glutathione–agarose beads. The proteins were either eluted with 10 mM glutathione in 50 mM Tris/HCl, pH 8.0, or released from the affinity support by proteolytic cleavage of the fusion protein following the manufacturer's instructions. The homogeneity of the purified proteins was evaluated by SDS/PAGE and Coomassie Blue staining. Protein concentration was determined using the Coomassie protein assay reagent (Pierce, Tattenhall, Cheshire, U.K.) with BSA as a standard.

Production of *A. thaliana* CaM and analysis of CaM–peptide interaction

Full-length cDNAs for *A. thaliana* CaM 1 (AtCaM 1) and CaM 8 (AtCaM 8) were isolated from the expression library by PCR cloning. After verification of the CaM-coding regions by DNA sequencing, the PCR products were inserted into the pGEX-6P1 expression vector at *Bam*H1 and *Not*1 sites. The *E. coli* DH5α strain, transformed with the resulting plasmids, was used to produce the GST-tagged proteins. Recombinant proteins were isolated by glutathione affinity chromatography. After binding to glutathione–agarose, the CaM isoforms were recovered by proteolytic cleavage of the fusion proteins. The purity and concentration of the CaM samples were evaluated as described above. Interaction between CaM and peptides was assessed by electrophoretic mobility shift assays. Samples containing 100 pmoles CaM and differing amounts of purified synthetic peptide or melittin in 62.5 mM Tris/HCl, pH 6.8, 15 % glycerol and either 10 mM $CaCl_2$ or 5 mM EGTA in a total volume of 40 μl, were incubated at 22 °C prior to non-denaturing PAGE. Samples were run on a 12 % polyacrylamide gel containing 15 % glycerol. Protein bands were detected by Coomassie Blue staining.

Surface plasmon resonance (SPR) experiments

Real-time binding experiments were performed on a BIAcore X instrument (Pharmacia Biosensor) at 25 °C. Purified GST–AtCaMRLK (315–353) protein was captured on a CM5 sensor chip containing immobilized GST antibodies on its surface, resulting in a permanent change of 1000–1500 resonance units. Defined amounts of recombinant CaM, ranging from nano- to micro-molar concentrations, were injected as the analyte onto the GST–AtCaMRLK-coated surface at a flow rate of 30 μl/min. The running buffer was 10 mM Hepes, pH 7.4, 150 mM NaCl and 0.005 % surfactant P20 containing either 2 mM $CaCl_2$ or 3 mM EDTA, as indicated. The sensorgrams were analysed using BIAevaluation 3.1 software.

Phosphorylation assays

The kinase activity of AtCaMRLK recombinant proteins was assayed by incubating 4 μg of purified protein in 50 mM Hepes pH 7.0, 10 mM $MnCl_2$, 2.5 mM dithiothreitol, 10 μCi [γ-^{33}P]-ATP and 5 μg myelin basic protein as a substrate in a final volume of 40 μl. After incubation for 1 h at 30 °C, the reaction was stopped by the addition of Laemmli SDS/PAGE sample buffer, and the samples were incubated at 95 °C for 2 min and resolved by SDS/PAGE. The gel was stained with Coomassie Blue to verify equal loading and then dried. The products of the reaction were visualized by autoradiography.

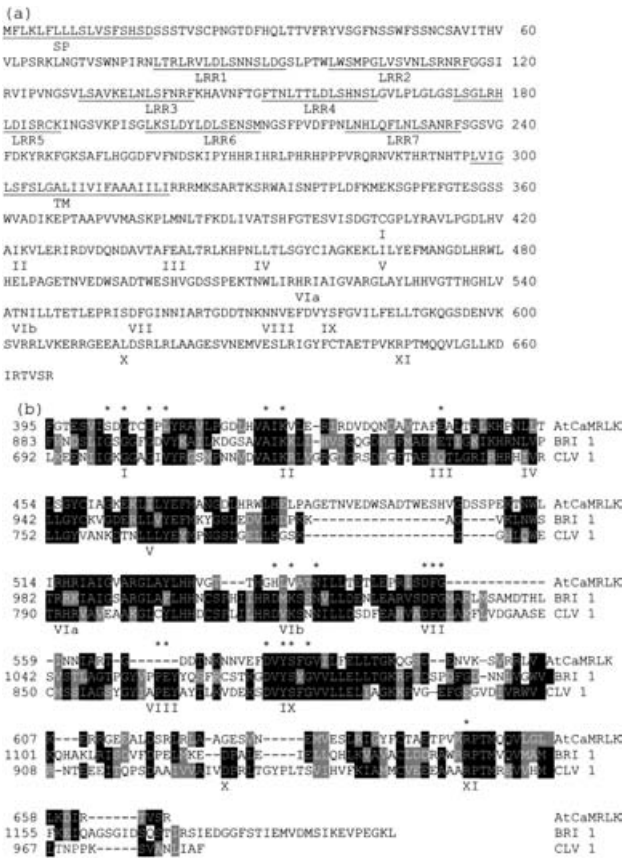

Figure 1 Structural characteristics of the *AtCaMRLK* gene product

(**a**) Deduced amino acid sequence of *AtCaMRLK* cDNA. The predicted signal sequence (SP), the transmembrane segment (TM) and the LRR motifs are underlined. The kinase subdomains are indicated in roman numerals. (**b**) Alignment and comparison of the amino acid sequences of kinase domains from AtCaMRLK, BRI 1 and CLV 1 [19,20]. The black boxes indicate identical residues shared by at least two sequences, and grey boxes indicate conservative substitutions. Highly conserved residues in protein kinase family are indicated with an asterisk.

Northern-blot analysis

Total RNA was isolated from different organs of wild-type plants using the Extract-all reagent (Eurobio, Paris, France). RNA samples were fractionated by electrophoresis in a 1.2 % agarose gel and transferred on to a nylon membrane. DNA fragments encoding the cytoplasmic domain of AtCaMRLK were labelled with [α-^{32}P]-dATP by random priming and used as a probe for Northern hybridization.

RESULTS

Isolation of a cDNA encoding a receptor-like kinase

To identify CaM-binding proteins, we probed an *A. thaliana* cell suspension cDNA expression library with a radiolabelled recombinant CaM. After three rounds of screening, a positive clone bearing a 2.4 kb insert was selected and sequenced. The nucleotide sequence of the cDNA contained a 2001 bp open reading frame encoding a predicted 666 amino acid protein with a calculated molecular mass of 73.9 kDa. As shown in Figure 1(a), the deduced amino acid sequence displays characteristics of a transmembrane receptor protein kinase, including a putative signal peptide, a receptor-like sequence domain with 7 LRR motifs, a predicted transmembrane segment and a protein kinase domain at the C-terminus. The presence of these four prominent domains indicates that we have isolated a full-length cDNA encoding a receptor-like kinase containing LRR motifs (designated AtCaMRLK). The LRR motif is present in over half the *Arabidopsis* RLKs. Substantial variations in the number (from 1 to 32 repeats) and the arrangements of LRRs, as well as in the kinase domain, are observed within this family. As shown in Figure 1(b), the comparison of the kinase domain of AtCaMRLK with those of BRI 1 and CLV 1, two well-characterized members of the LRR-RLK family implicated in brassinosteroid signalling and maintenance of the apical meristem respectively, reveals major differences in the length and the sequence of loops connecting the catalytic sub-domains in these three proteins. AtCaMRLK contains all the subdomain characteristics of eukaryotic protein kinases [11], but a low conservation in the amino acid sequence of subdomains I, VIb and VIII of AtCaMRLK can be seen in a sequence alignment with BRI 1 and CLV 1.

Interaction of AtCaMRLK and CaM

Even if there is no canonical primary sequence characterizing the CaM-binding domain, CaM is known to bind peptide sequences that tend to form a basic amphiphilic α-helix in which hydrophobic residues are segregated from hydrophilic residues along the helix [12]. A search for basic amphiphilic helices in the AtCaMRLK sequence revealed such a structural feature in the cytoplasmic part of the protein. In this region, the peptide sequence between amino acids Leu^{319} and Ile^{335} is rich in basic and hydrophobic residues that concentrate on opposite sides in an helical wheel diagram (Figure 2a). To assess whether this protein segment has the ability to bind CaM, we generated expression constructs bearing partial sequences of AtCaMRLK to produce GST-fusion proteins in *E. coli* cells. As depicted in Figure 2(b), construct 1 comprises the entire intracellular part of AtCaMRLK, construct 2 consists essentially in the kinase domain, and construct 3 only bears the 39 amino-acid residues encompassing the putative CaM-binding site. The different fusion proteins were challenged with radiolabelled CaM in the presence or the absence of calcium, using overlay-blot assays. As shown in Figure 2(b), only constructs 1 and 3, but not construct 2 nor control GST interacted with CaM in a Ca^{2+}-dependent manner, thus confirming the location of a CaM-binding site next to the transmembrane segment of AtCaMRLK.

In *A. thaliana*, CaM is encoded by a multigene family that gives rise to different isoforms of the Ca^{2+} sensor. We took advantage of this diversity to characterize further the interaction between AtCaMRLK and several CaM isoforms from *A. thaliana*. We prepared recombinant proteins corresponding to AtCaM 1, a typical CaM, and AtCaM 8, a divergent isoform sharing only 72 % identity with the AtCaM 1 amino-acid sequence [13]. Binding capabilities of CaM preparations were assessed first by electrophoretic mobility shift assays using melittin, a standard target peptide for CaM. When mixtures of melittin and CaM were fractionated by gel electrophoresis in the presence of calcium, the melittin/Ca^{2+}/CaM complex, whose electrophoretic mobility could be distinguished from that of free CaM, was detected (Figure 3a). At a CaM/melittin ratio of 1:1, trace amounts of free AtCaM 1 were detected, while the melittin/Ca^{2+}/AtCaM 8 complex was readily observed in the presence of an excess of melittin. Importantly, no complex between melittin and CaM isoforms was apparent in the absence of calcium. These data show that both isoforms display the genuine characteristics of CaM. We then addressed the question of whether AtCaMRLK is a common target for AtCaM 1 and AtCaM 8. For this purpose, a

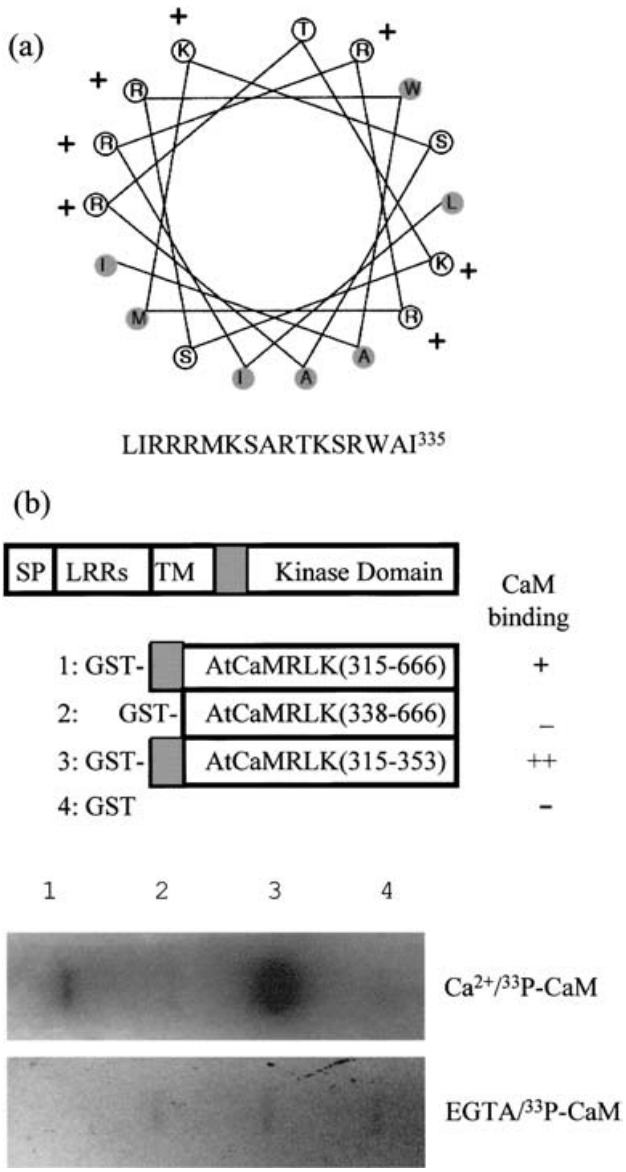

Figure 2 Determination of the CaM-binding domain of AtCaMRLK

(**a**) Helical wheel plot of the amino acid sequence (using the single letter amino acid code) LIRRRMKSARTKSRWAI[335] next to the transmembrane segment of AtCaMRLK. Hydrophobic residues are shaded, and basic residues are depicted with a positive charge symbol. (**b**) Schematic drawing of the AtCaMRLK protein (SP, signal peptide; LRRs, leucine-rich repeats; TM, transmembrane segment), and representation of truncated forms of AtCaMRLK fused in-frame to GST. The putative CaM-binding site is denoted by a grey box. The ability of GST-fusion proteins to bind CaM is represented by plus (+) or minus (−) symbols, and illustrated by blots of fusion proteins probed with ^{33}P-CaM in the presence or absence of calcium. Lane numbers on the blot refer to the GST constructs as described in the Results section. Equal amounts of fusion proteins were spotted in each lane.

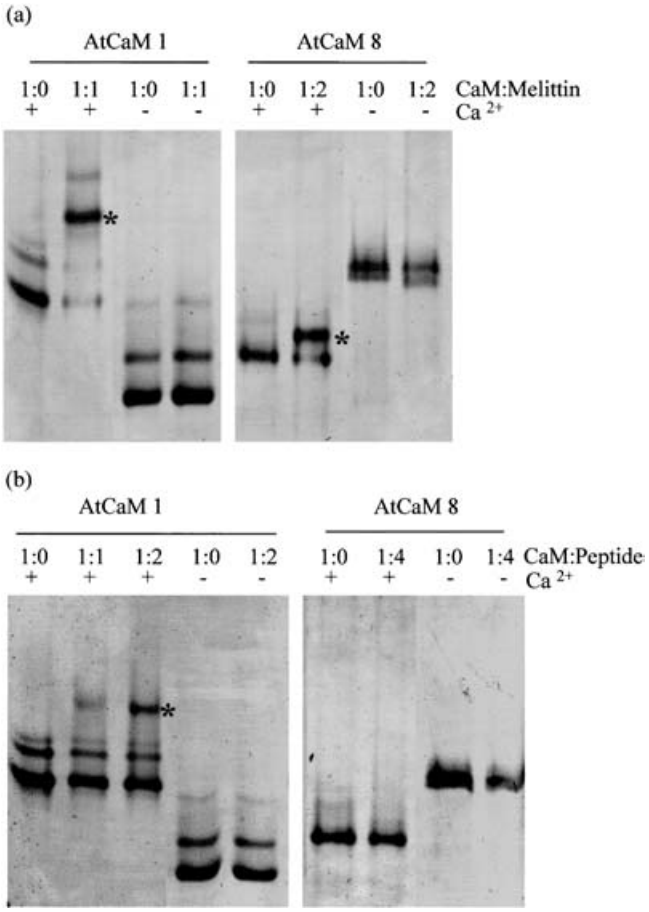

Figure 3 Gel mobility shift analysis of AtCaM interaction with melittin and an AtCaMRLK-derived peptide

AtCaM 1 or AtCaM 8 (100 pmoles) was incubated with either melittin (**a**) or a 23-amino-acid synthetic peptide (AAIILIRRRMKSARTKSRWAISN[337]) derived from AtCaMRLK (**b**) in the presence (lanes designated +) or absence (lanes designated −) of Ca^{2+}. Molar ratios of CaM over melittin or peptide in protein samples are indicated. Protein samples were fractionated by non-denaturing PAGE in a 12% polyacrylamide gel and detected by Coomassie Blue staining. The bands containing CaM/melittin and CaM/peptide complexes are indicated by an asterisk.

synthetic peptide of 23 amino acids (AAIILIRRRMKSARTKSRWAISN[337]), corresponding to the putative CaM-binding site of AtCaMRLK, was prepared, and its ability to bind AtCaM isoforms was analysed by electrophoretic mobility shift assay. Figure 3(b) shows that the peptide interacts with AtCaM 1 in a calcium-dependent manner. In contrast, no AtCaM 8/peptide complex was detected even when the peptide was provided in a large excess relative to CaM. The inability of AtCaM 8 to bind AtCaMRLK was confirmed by real-time binding experiments performed with a BIAcore instrument. As shown in Figure 4(a), no significant response was recorded when AtCaM 8 was injected in the presence of calcium over a sensor chip coated with the GST-fusion protein bearing the CaM-binding site of AtCaMRLK (construct 3 in Figure 2). In contrast, AtCaM 1 rapidly associates with the GST-fusion protein encoded by construct 3 (Figure 4a). At the end of CaM injection, dissociation of the complex was observed during flushing of the flow cell with running buffer. Therefore, AtCaMRLK displays the ability to interact with canonical CaM only. Using BIAevaluation 3.1 software, kinetic data were extracted from sensorgrams recorded at different AtCaM 1 concentrations (Figure 4b). Fitting the data to a single-site ligand-binding model resulted in a K_D value of 120 nM.

Kinase activity of AtCaMRLK

Because AtCaMRLK has several variants in the conserved kinase subdomains, it was important to check if the protein is a functional kinase. As expression of the kinase domain of plant RLKs in *E. coli* usually results in a constitutive kinase activity, the AtCaMRLK constructs described in Figure 2(b) were used as

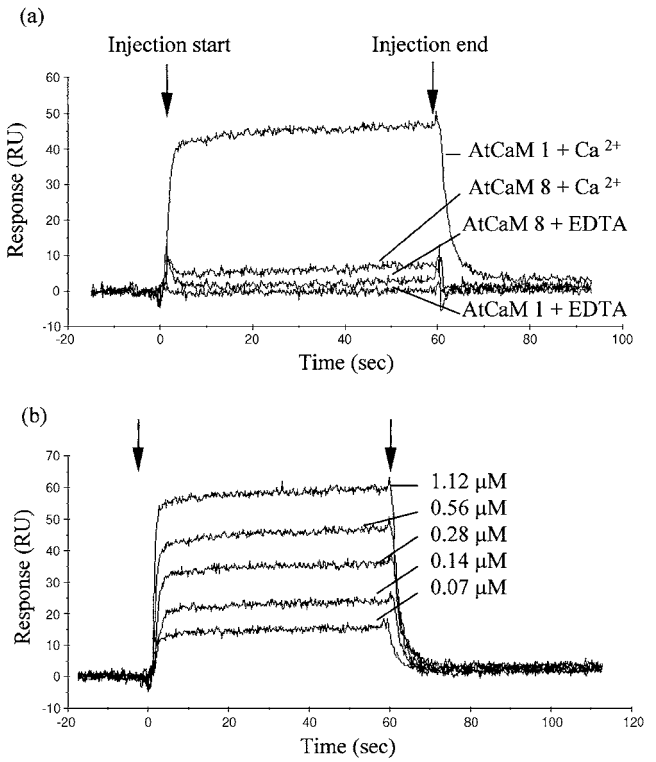

Figure 4 Real-time interaction between AtCaMRLK and CaM analysed by SPR

(**a**) Interaction between AtCaMRLK and two isoforms of CaM in the presence or absence of Ca^{2+}. AtCaM 1 (0.56 μM) or AtCaM 8 (0.6 μM) was injected over the sensor surface coated with GST–AtCaMRLK (315–353) (construct 3 in Figure 2b). Running buffer contained either 2 mM $CaCl_2$ or 3 mM EDTA. Resonance units (RU) are plotted as a function of time. (**b**) Sensorgrams used to determine the affinity constant. Increasing concentrations of AtCaM 1 were applied to the sensor chip coated with GST–AtCaMRLK (315–353) in the presence of 2 mM $CaCl_2$. The response in the control channel coated with GST has been subtracted from the data in (**a**) and (**b**).

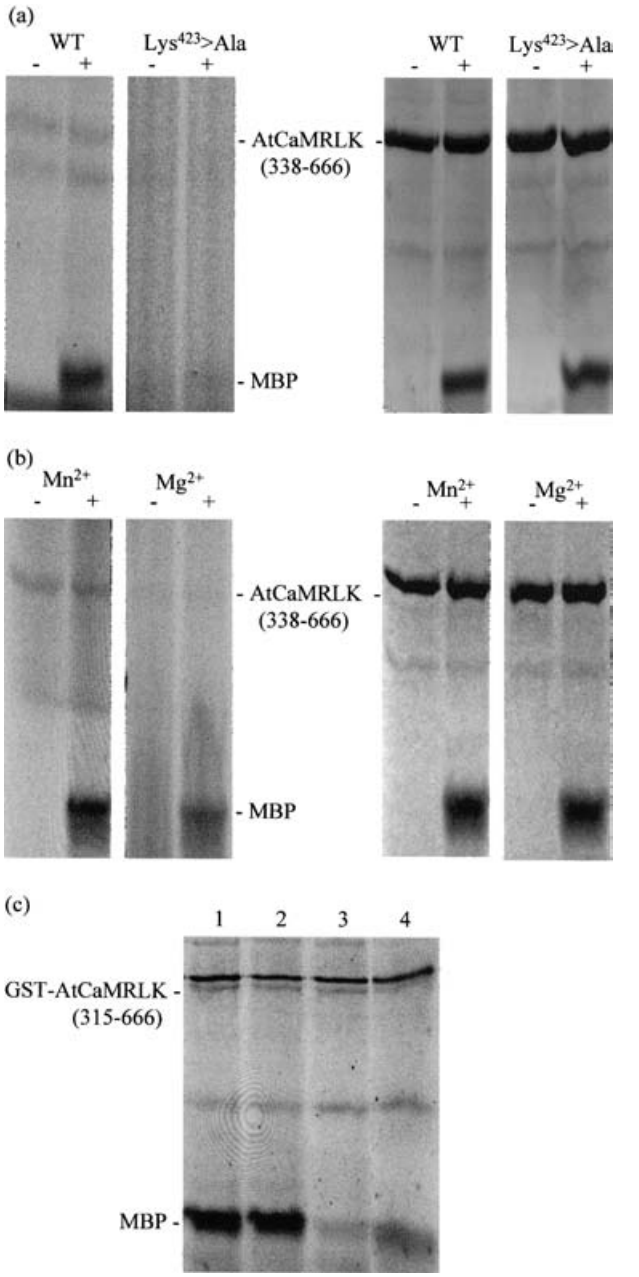

Figure 5 Characterization of the kinase activity of recombinant AtCaMRLK proteins

(**a**) Evidence for phosphorylating activity of the AtCaMRLK kinase domain. Equal amounts (5 μg) of wild-type and Lys^{423} to alanine (Lys^{423} > Ala) mutant proteins were assayed for kinase activity using [γ-^{33}P]ATP and $MnCl_2$ in the presence (+) or absence (−) of MBP, and subjected to SDS/PAGE. The gel was stained with Coomassie Blue (right-hand panels) and the reaction products were visualized by autoradiography (left-hand panels). (**b**) Dependence of kinase activity on divalent metal ions. Kinase activity of AtCaMRLK (338–666) was assayed in the presence of 10 mM $MnCl_2$ or $MgCl_2$. Proteins were visualized by Coomassie stain (right-hand panels) after SDS/PAGE and radiolabelled products were detected by autoradiography (left-hand panels). (**c**) Effect of Ca^{2+} and AtCaM 1 on phosphorylating activity of AtCaMRLK. The kinase activity of GST–AtCaMRLK (315–666) was assayed in the presence of MBP, and Ca^{2+}/AtCaM 1 were added to the reaction mixtures as follows: lane 1, no addition; lane 2, 10 mM $CaCl_2$; lane 3, 10 mM $CaCl_2$ and 4 μg of AtCaM 1; lane 4, 10 mM EGTA and 4 μg of AtCaM 1. Reaction products were visualized by autoradiography after SDS/PAGE.

the enzyme source. In addition, a mutant version of AtCaMRLK (338–666) was generated by changing Lys^{423} to alanine, because substitution of this invariant lysine residue in subdomain II was shown to abolish protein kinase activity of other RLKs [14,15]. As shown in Figures 5(a) and 5(b), AtCaMRLK (338–666) (construct 2 in Figure 2) displayed autokinase and myelin basic protein (MBP) kinase activities in the presence of divalent cations, and Mn^{2+} was more effective than Mg^{2+} in supporting kinase activity. Furthermore, the mutant protein in which alanine replaced Lys^{423}, had no detectable kinase activity, confirming that AtCaMRLK encodes an active protein kinase. When GST–AtCaMRLK (315–666) (construct 1 in Figure 2) was used, kinase activities were also detected (Figure 5c). Therefore, the constitutive kinase activity observed with this construct indicates that the CaM-binding sequence does not act as an auto-inhibitory domain. A low level of autophosphorylation, relative to the MBP kinase activity of the recombinant protein, could be seen and a phosphorylated protein of 70 kDa was also detected (Figure 5c). This additional radiolabelled protein is likely due to the autophosphorylation of DnaK, a bacterial chaperone which often contaminates recombinant proteins expressed in *E. coli* [16]. Addition of CaM and Ca^{2+} to the kinase assay did not produce significant changes in the autokinase activity of AtCaMRLK. In contrast, CaM was found to inhibit the MBP kinase activity, but the inhibitory effect was observed independently of the presence of calcium (Figure 5c). Inhibition of MBP kinase activity by CaM was also observed using AtCaMRLK (338–666), construct 2 in Figure 2 which lacks the CaM-binding sequence (results not

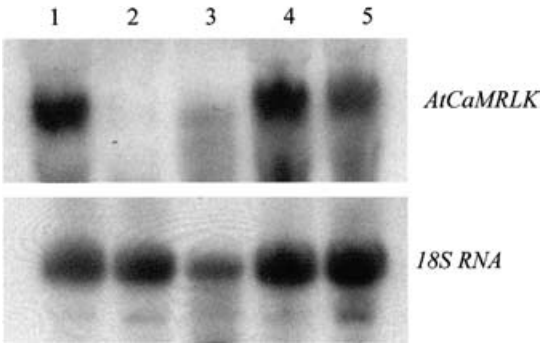

Figure 6 Expression of *AtCaMRLK* gene in different organs of *Arabidopsis*

Total RNA (20 μg) isolated from plant organs was analysed by Northern blot (lane 1, seedlings; lane 2, rosette leaves; lane 3, roots; lane 4, flowers; lane 5, siliques). The blot was first hybridized with a gene-specific probe for *AtCaMRLK* and then with a probe for 18 S rRNA used as a loading control.

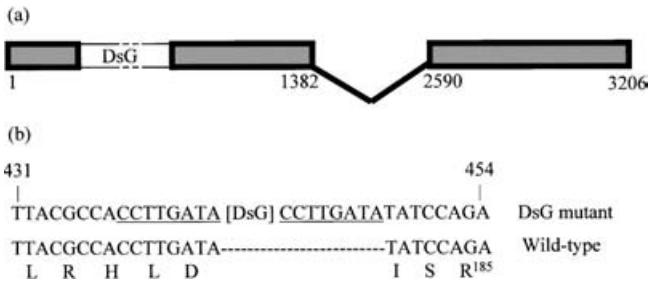

Figure 7 The *DsG* insertion site in *Arabidopsis* mutant line SGT 5600

(**a**) Schematic representation of the *DsG*-disrupted *At5g45800* gene. Grey boxes represent exons. (**b**) DNA sequence flanking the *DsG* element insertion in the mutant. The 8 bp sequence duplicated at the *DsG* insertion site is underlined. Numbers refer to nucleotide positions in the coding sequence of the *At5g45800* gene. Nucleotide and amino acid sequences of *At5g45800* in the wild-type plant are also indicated.

shown). Therefore, these observations are likely to be due to interfering reactions between CaM and MBP. Attempts to avoid these interferences by using other conventional substrates, such as casein and histones, were unsuccessfull because of the occurrence of Mn^{2+}-dependent kinase activities in the commercially available substrates preparations.

Expression profile and functional analysis of AtCaMRLK

As a first step toward a functional analysis, the steady-state trancript level of the *AtCaMRLK* gene in different organs of *Arabidopsis*, and in young seedlings, was examined. Blots of total RNA from plant tissues were hybridized to a specific DNA-probe for *AtCaMRLK*. As shown in Figure 6, *AtCaMRLK* was expressed in all tissues examined except in leaves, and the expression level in seedlings and flowers was substantially higher than in other tissues. Since the *RLK* gene family was reported to be involved in the control of plant development and in stress responses [5], potential regulation of the *AtCaMRLK* gene by various stimuli was also examined. However, no significant induction of *AtCaMRLK* expression was observed in seedlings exposed to wounding, cold or osmotic stress, and during plant–pathogen interaction (results not shown).

To get a deeper insight into the biological role of AtCaMRLK, we took advantage of loss-of-function mutations produced by insertional mutagenesis in *Arabidopsis* [17]. A search in sequence databases identified an *Arabidopsis* mutant (SGT 5600) carrying a *DsG* transposon in the first exon of *At5g45800*, an *RLK* gene predicted to encode a protein identical to AtCaMRLK [18] (Figure 7a). Sequence comparison of the cDNA and genomic sequences in nontranslated regions confirmed that AtCaMRLK cDNA derived from the *At5g45800* gene. Analysis of the nucleotide sequence flanking the transposon indicated that the *DsG* insertion site in the mutant line is located in the LRR domain of the protein, suggesting a loss of function of the *RLK* gene (Figure 7b). In addition, a duplication of 8 bp at the insertion site, which is usually generated by insertion of the *DsG* transposable element, was observed in the mutant. An homozygous line bearing a single *DsG* element in its genome (kindly provided by Dr V. Sundaresan, Institute of Molecular Agrobiology, The National University of Singapore, Singapore) was used for phenotypic analysis. The mutant line showed defects in petal development, leading to a decrease in petal number. However, analysis of the progeny produced by a back-cross between mutant and wild-type plants indicated no linkage between the alteration in flower development and the *DsG* insertion in the *At5g45800* gene. No other discernible phenotype caused by disruption of the *RLK* gene was observed in the progeny, even when mutated plants were grown under stress conditions including drought and salt stress.

DISCUSSION

Signal perception through receptor kinases is a common feature among living organisms. Central to understanding the molecular mechanisms of receptor-kinase signalling are the connections between ligand binding, receptor activation and recruitment of downstream signalling components. Although significant progress on receptor kinase signalling in plants has been made in recent years, a detailed picture of mechanisms and components of receptor kinase-mediated pathways has yet to emerge. Here, we show that AtCaMRLK, a member of the *Arabidopsis* RLK family is a CaM-binding protein. The overall structure of the polypeptide deduced from *AtCaMRLK* cDNA, which consists of a ligand-binding domain, a single membrane-spanning domain and a kinase domain, is very similar to that observed in the family of plant receptor kinases. Furthermore, we show that AtCaMRLK displays a kinase activity towards MBP and is capable of autophosphorylation, as observed for other characterized plant receptor kinases. Interaction between AtCaMRLK and CaM was demonstrated by locating the CaM-binding site in a cytoplasmic domain close to the transmembrane segment of AtCaMRLK. This interaction occurs in a calcium-dependent manner with an apparent K_D of 120 nM, which falls in the range of dissociation constants reported for other CaM-binding proteins [8]. In addition, AtCaMRLK binds selectively to canonical CaM such as AtCaM 1, but not to the divergent AtCaM 8 isoform. A search for conservation of the CaM-binding site of AtCaMRLK in the plant RLK family, revealed that CaM may interact with other receptor kinases including SRK, CLV 1, EMS 1 and a putative RLK encoded by the *At5g43020* gene [20,29,30] (Table 1). Basic and hydrophobic residues are almost conserved in the region next to the transmembrane segment of these receptor kinases, and the propensity of these sequences to adopt an amphiphilic α-helical structure suggests their ability to bind CaM. Interestingly, binding of CaM to SRK and CLV 1 was recently reported [21]. In that study, the CaM-binding site was identified in the subdomain VIa of the kinase domain, and no effect of CaM on the SRK kinase activity was observed. It will be interesting to see if SRK contains an additional CaM-binding site in the vicinity of the kinase domain as suggested by the present study. In addition,

Table 1 Alignment and comparison of the amino acid sequences of the region close to the transmembrane segment of various receptor kinases

Basic residues are shown in bold and hydrophobic residues are in italics; (**.**) denotes identity with the residue of AtCaMRLK.

Protein	Amino acid sequence (single-letter symbols) next to the transmembrane segment																
AtCaMRLK	*L*	*I*	**R**	**R**	**R**	*M*	**K**	S	*A*	**R**	T	**K**	S	**R**	*W*	*A*	*I*
At5g43020	*V*	**K**	**.**	**.**	**.**	S	**.**	T	E	**K**	Q	**.**	G	**K**	E	S	T
SRK	**.**	*W*	**K**	**.**	**K**	Q	**.**	**R**	**.**	**K**	*A*	S	*A*	*I*	S	*I*	*A*
CLV 1	*M*	N	**K**	**K**	**K**	N	Q	**K**	S	*L*	*W*	**.**	*L*	T	*A*	*F*	Q
EMS 1	S	*L*	**.**	**.**	*W*	*A*	*M*	T	**K**	**.**	*V*	**.**	Q	**.**	D	D	P

we demonstrated that the kinase domain of AtCaMRLK was not able to bind CaM (Figure 2). This is consistent with a partial conservation of the subdomain VIa in AtCaMRLK and the lack of predicted amphiphilic structure in this region. We also observed no significant changes in the autophosphorylation of AtCaMRLK in the presence of calcium and CaM. Taken together, these data indicate that diverse members of the plant RLK family interact with CaM either in the kinase domain or in the vicinity of the catalytic domain, but these interactions are not involved in the regulation of the kinase activity.

CaM was shown previously to bind animal receptor kinases such as the insulin receptor and EGF receptor [22,23]. Interaction of CaM and the EGF receptor occurs at a site located next to the transmembrane segment of the receptor, which is reminiscent of that described here for AtCaMRLK [24]. Thus, CaM is a common component of receptor complexes in plants and animals, but the role of CaM in receptor-mediated pathways remains to be established. In this respect, a recent report proposed a role of CaM in intracellular trafficking of the EGF receptor [25]. Internalization and trafficking of transmembrane receptors in animal cells is thought to play an important role in receptor degradation and recycling [26]. Recent findings indicate that internalization of receptor kinases also occurs in plant cells [27], and the possibility remains that CaM-dependent mechanisms of receptor turnover and recycling are conserved in plants.

Although plant RLKs are expected to play crucial roles in all aspects of the plant life-cycle, fewer than 2% of total RLKs identified in the *Arabidopsis* genome have known functions. The availability of gene knockout mutants in *Arabidopsis*, combined with expression studies, were considered here for the functional analysis of AtCaMRLK. AtCaMRLK is expressed in diverse tissues, and prominent levels of transcripts are observed in seedlings and flowers. This contrasts with most of the receptor kinases identified so far, which are expressed in specific tissues or developmental stages. Only a few receptor kinases including RPK 1, ERECTA and BRI 1 were reported to be constitutively expressed in all major tissues [1]. RPK 1 was also found to be rapidly induced by abscissic acid and environmental stresses [28]. Expression studies performed under abiotic stress conditions, or during plant–pathogen interactions, revealed no significant induction of *AtCaMRLK* under any of these treatments. Mutagenesis has facilitated the discovery of the functions of several receptor kinases involved in plant development or growth responses. However, under normal growth conditions, we observed no obvious phenotype in an insertional mutant which is disrupted in the *AtCaMRLK* gene by a transposable element. Although *AtCaMRLK* exists as a single gene in the *Arabidopsis* genome, we cannot exclude the possibility that the lack of observable phenotype in the mutant is due to some degree of functional redundancy in the large family of receptor kinases. The presence of signals perceived by AtCaMRLK under suitable conditions only, is another explanation for the absence of phenotype.

In summary, using a protein–protein-interaction-based screening, we have isolated a novel receptor-like kinase and demonstrated its Ca^{2+}-dependent interaction with CaM. In addition to basic features, such as receptor dimerization and the existence of receptor complexes shared by plant and animal receptor kinases, we show that interaction between receptor kinases and CaM is conserved in plants.

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