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# Interaction of a plant pseudo-response regulator with a calmodulin-like protein

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ABSTRACT

Calmodulin (CaM) plays a crucial role in the regulation of diverse cellular processes by modulating the activities of numerous target proteins. Plants possess an extended CaM family including numerous CaM-like proteins (CMLs), most of which appear to be unique to plants. We previously demonstrated a role for CML9 in abiotic stress tolerance and seed germination in *Arabidopsis thaliana*. We report here the isolation of PRR2, a pseudo-response regulator as a CML9 interacting protein by screening an expression library prepared from *Arabidopsis* seedlings with CML9 as bait in a yeast two-hybrid system. PRR2 is similar to the response regulators of the two-component system, but lacks the invariant residue required for phosphorylation by which response regulators switch their output response, suggesting the existence of alternative regulatory mechanisms. PRR2 was found to bind CML9 and closely related CMLs but not a canonical CaM. Mapping analyses indicate that an almost complete form of PRR2 is required for interaction with CML9, suggesting a recognition mode different from the classical CaM-target peptide complex. PRR2 contains several features that are typical of transcription factors, including a GARP DNA recognition domain, a Pro-rich region and a Golden C-terminal box. PRR2 and CML9 as fusion proteins with fluorescent tags co-localized in the nucleus of plant cells, and their interaction in the nuclear compartment was validated *in planta* by using a fluorophore-tagged protein interaction assay. These findings suggest that binding of PRR2 to CML9 may be an important mechanism to modulate the physiological role of this transcription factor in plants.



## 1. Introduction

Plants use calcium ($Ca^{2+}$) as a secondary messenger in defining cellular responses to a wide range of developmental signals and environmental stress factors [1]. $Ca^{2+}$ pulses that occur in response to many stimuli modulate diverse cellular functions *via* $Ca^{2+}$ binding proteins [2]. Plants have evolved a large set of $Ca^{2+}$ sensors with distinct output functions to transduce $Ca^{2+}$ signals into various biological responses [3]. Calmodulin (CaM), a highly conserved $Ca^{2+}$ sensor in eukaryotes, plays an essential role in many aspects of plant growth and development including environmental stress responses by modulating the activities of diverse enzymes and proteins [4]. An intriguing aspect of CaM-mediated signalling in plants is the presence of numerous CaM-like proteins (CMLs) whose functions have just begun to be elucidated [5,6]. Reverse genetic or ectopic expression analyses have provided direct evidence for a role of CMLs in abiotic stress responses, pathogen defence and some aspects of plant development such as induction of flowering, seed germination and seedling growth [7]. To better understand $Ca^{2+}$ mediated signalling pathways in plants, it is essential to isolate and identify CML interacting proteins. To date, searches for CML targets have resulted in isolation of a limited number of CML binding proteins [8]. Interestingly, some CML targets were found to be specific to one or closely related CMLs, indicating that members of the CML family in plants can exert specialized functions.

We previously examined the role of CML9 in *Arabidopsis*, and showed its implication in plant tolerance to abiotic stress and the regulation of seed germination by abscisic acid [9]. In this study, we isolated PRR2, a pseudo-response regulator as a CML9 interacting protein by using a yeast two-hybrid screening procedure. PRR2 resembles the authentic response regulators of the two-component system, a regulatory mechanism involved in plant hormone signal transduction [10]. The two-component system typically consists of a sensor His kinase that senses a signal input and a response regulator that mediates the output [11]. In response to a ligand, the sensor kinase autophosphorylates its own specific His residue and then transfers the phosphoryl group to the

*Abbreviations:* ABA, abscisic acid; AD, activating domain; ARR, authentic response regulator; 3-AT, 3-aminotriazole; BD, binding domain; CaM, calmodulin; CFP, cyan fluorescent protein; CML, calmodulin-like; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GST, glutathione-S-transferase; PRR, pseudo-response regulator; YFP, yellow fluorescent protein.

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response regulator. The response regulators are composed of two functional domains, an N-terminal receiver domain with a conserved Asp residue which serves to accept the phosphoryl group, and a C-terminal output region which confers the ability to bind DNA and to activate the transcription of target genes [12]. Despite their high structural resemblance to response regulators, PRRs are missing essential residues required for the phospho-accepting activity, suggesting that they use a different signal-responding mechanism; but so far, no alternative mechanisms have been proposed. We showed here that PRR2 is a target of CML9, a calmodulin-like protein, and we validated the protein interaction in plant cells. The physical interaction of PRR2 to CML9 may be an important key in determining the role of PRR2 in plants.

## 2. Materials and methods

### 2.1. Yeast two-hybrid screening and assay

Screening of a random-primed cDNA expression library prepared from 3-week-old *Arabidopsis* seedlings was done by Hybrigenics (http://www.hybrigenics-services.com) using a LexA-based two-hybrid system and CML9 as bait. Briefly, 106 million clones were screened using a mating approach, and positive interactions were selected on a medium lacking Leu, Trp and His, and supplemented with 100 mM 3-aminotriazole (3-AT) to prevent bait auto-activation. The prey fragments of the positive clones were sequenced at their 5′ and 3′ junctions, and the resulting sequences used to identify the corresponding proteins in the GenBank database. Further analysis of observed interactions was performed by using Gal4 two-hybrid assays. Full-length ORFs for *Arabidopsis* CaM1, CMLs and interacting proteins were amplified by PCR using gene-specific primers containing Gateway-compatible extensions, introduced into entry vectors by the Gateway cloning technology (Invitrogen), and verified by sequencing before recombination into bait and prey vectors derived from *pGADT7* and *pGBKT7* plasmids (Clontech). Yeast cells (AH109 strain) carrying both plasmids were selected on Trp and Leu drop-out medium (-TL) and then on Trp/Leu/His/Ade drop-out medium (-TLHA) in the presence of 3-AT to determine the expression of HIS3 and ADE2 reporter genes. Detection of the fusion proteins in yeast cell extracts were performed by Western blot analysis using c-Myc or hemagglutinin epitope tag antibodies.

### 2.2. Expression of recombinant proteins in Escherichia coli and in vitro CML9-binding assay

Constructs for bacterial production of glutathione-S-transferase (GST) fusion proteins were obtained by cloning PCR-amplified DNA fragments in pGEX vectors and introduced into *E.coli* BL21 strain. Production of recombinant proteins, purification of fusion proteins, and labelling of GST-CML9 using commercially available protein kinase and [$\gamma$-$^{33}$P] ATP were performed according to the manufacturer's instructions. *In vitro* binding assay of proteins with radiolabelled CML9 was done as previously described [13].

### 2.3. Subcellular localization of fluorescent fusion proteins and protein interaction assay in planta

Fusion proteins tagged with the cyan fluorescent protein (CFP) or the yellow fluorescent protein (YFP) were constructed by recombination of the corresponding entry clones with the *pAM-PAT-P35S-GW-CFP* and the *pAM-PAT-P35S-GW-YFP* plasmids respectively. For transient expression, the leaves of 4-week-old *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* cells carrying either CFP or YFP fusion constructs. For protein interaction assay, equal volumes of *A. tumefaciens* were mixed prior to infiltration. Infiltrated plants were maintained for 48 h in a growth chamber before analysis with a confocal laser scanning microscope (TCS SP2-AOBS, Leica). CFP and YFP excitation was performed with an argon laser at 458 and 514 nm respectively. Emission was detected in the 465 – 505 nm range for CFP and 525 to 600 nm range for YFP. Fluorescence lifetime imaging microscopy (FLIM) measurements were performed using a multiphoton FLIM system [14]. FRET efficiency ($E$) was calculated by comparing the lifetime of the donor in the presence $\tau^{DA}$ or absence $\tau^{D}$ of the acceptor: $E = 1 - (\tau^{DA})/(\tau^{D})$. The lifetimes were estimated by fitting the data with a bi-exponential function using the Levenberg–Marquart non-linear least-squares estimation procedure with Hamamatsu software.

### 2.4. Accession numbers

*Arabidopsis* genome initiative locus identifiers for the genes described in this article are as follows: CaM1 (At5g37780); CML8 (At4g14640); CML9 (At3g51920); CML10 (At2g41090); CML11 (At3g22930); PRR2 (At4g18020); GLK1 (At2g20570); ARR1 (At3g16857); ARR10 (At4g31920).

## 3. Results and discussion

### 3.1. Isolation of a pseudo-response regulator as a CML9 binding protein

The search for CML9 interacting proteins was performed by two-hybrid screening of an *Arabidopsis* cDNA expression library using CML9 as bait and led us to isolate a clone coding for the pseudo-response regulator PRR2. The PRR2 gene is predicted to encode a polypeptide of 535 residues containing an N-terminal receiver-like domain where the phospho-accepting Asp residue is replaced by a Glu, and a C-terminal extension harbouring a GARP DNA recognition domain, a proline-rich region and a GOLDEN2 C-terminal (GCT) box (Fig. 1A). Based on these structural features, PRR2 is related to the type-B response regulators such as ARR1, 10 and 11, and to the golden-like proteins (GLKs), a subgroup of plant-specific transcription factors [15,16]; but it substantially differs from other PRRs, most of which containing the C-motif identified in the CONSTANS transcription factors [17]. To confirm that PRR2 is a CML9 binding protein, the full-length cDNA was cloned in the *pGADT7* vector to produce the Gal4 activating domain-PRR2 fusion protein (AD–PRR2), and subsequently transformed in AH109 yeast strain with the *pGBGT7* vector carrying the CML9 coding sequence fused to the Gal4 binding domain (BD–CML9). When combined with the empty *pGADT7* vector, the BD–CML9 construct was found to autonomously activate the transcription of the HIS3 reporter gene; therefore, the interaction was tested at a high stringency by adding 3-AT, a competitive inhibitor of the HIS3 gene product to growth media. Increasing stringency reduced the level of auto-activation and confirmed the interaction of PRR2 with CML9 (Fig. 1B). We also examined if PRR2-related proteins interact with CML9. *Arabidopsis* ARR10 and GLK1 cDNAs were cloned in the prey vector and introduced into yeast cells together with the BD–CML9 plasmid. The transformants were not able to grow on a selective medium, despite the fact that all proteins were detected in yeast cell extracts by Western blot analysis (Supplementary Fig. S1). Therefore, PRR2 but not the related proteins tested here behaves as a CML9 partner in a yeast two-hybrid system.

### 3.2. Specificity of the interaction between PRR2 and CML9

According to McCormack and Braam [6], CML9 belongs to a subgroup of *Arabidopsis* CaM/CML family which includes CML8, 10, 11