Plant Signaling & Behavior 7:9, 1121-1124; September 2012; © 2012 Landes Bioscience

CML9, a multifunctional *Arabidopsis thaliana* calmodulin-like protein involved in stress responses and plant growth?

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Keywords: Calmodulin-like protein, *Arabidopsis thaliana*, root growth, flagellin, gibberellins

Plants have evolved complex signaling networks to respond to their fluctuating environment and adapt their growth and development. Calcium-dependent signaling pathways play key role in the onset of these adaptive responses. In plant cells, the intracellular calcium transients are triggered by numerous stimuli and it is supposed that the large repertory of calcium sensors present in higher plants could contribute to integrate these signals in physiological responses. Here, we present data on CML9, a calmodulin-like protein that appears to be involved in plant responses to both biotic and abiotic stress. Using a reverse genetic approach based on gain and loss of function mutants, we present here data indicating that this CML might also be involved in root growth control in response to the flagellin, a pathogen-associated molecular pattern (PAMP) also involved in plant immunity.

Many stimuli such as hormones and stress factors elicit changes in intracellular calcium content that serve to convey information and activate appropriate responses.[1] These Ca^{2+} signals are perceived by different Ca^{2+} sensors, and calmodulin (CaM) is one of the best characterized Ca^{2+} sensors in eukaryotes. Calmodulin-like (CML) proteins extend the Ca^{2+}-toolkit in plants; CMLs share sequence similarity with the ubiquitous and highly conserved CaM, however, except for some of them, their roles at physiological and molecular levels remain largely unknown.[2,3] In our group, we reported data on *Arabidopsis thaliana* CML9 that exhibits 46% amino acid sequence identity with CaM.[4,5] We showed that *CML9* transcripts are found in all organs and that *CML9* gene is rapidly induced by both abiotic and biotic stress. In a recent publication in Plant Journal, we demonstrated that *CML9* expression is also rapidly induced by the phytopathogenic bacteria *Pseudomonas syringae pv tomato* DC3000 (*Pst* DC3000)[4] and that this upregulation belongs to salicylic acid (SA) production and to the flagellin perception receptor FLS2. Moreover, exogenous applications of SA or flg22, the biological active peptide of flagellin, are also able to induce rapid and transient *CML9* gene expression and using a reverse genetic approach, we established that CML9 participates in plant innate immunity through a flagellin-dependent signaling pathway.[4]

In addition to mediate plant innate immunity, flagellin and flg22 are also known to inhibit root elongation and seedling growth.[6] Thus, we explored this facet of flagellin effect and we bring here new informations on plant growth behavior of *CML9* overexpressing and knockout lines upon flg22 treatments and discuss the possible involvement of CML9 in plant growth control through hormonal compounds.

The *cml9* Genotypes Exhibit Altered Responses to Flagellin

As previously showed, *cml9* mutants or *CML9* overexpressors exhibit respectively an enhanced susceptibility and a better resistance against phytopathogenic bacteria.[4] Using the non-host strain of *Pseudomonas syringae pv phaseolicola* mutated in the fliC subunit of the flagellum, we established that plant defense behavior of the *cml9* genotypes (KO and *OE-CC*) mainly depends on the ability of plants to respond to the flagellin perception.[4] This suggests that CML9 is involved in the enhancement of PAMP responses leading to set up faster and/or more robust defense responses. Different physiological effects have been associated to the flagellin-derived peptide flg22 such as the increase of antibacterial resistance[7] but also the inhibition of seedling growth[6] upon perception of flg22 by the FLS2 receptor.

To investigate this possibility, we used a bioassay based on flg22-mediated inhibition of seedling growth to evaluate the involvement of CML9 in this process.[6] Three-day old wild-type Col, knockout *cml9–1*, overexpressing *CML9* lines (*OE-CC-2* and *5*) and *fls2* mutant seedlings were transferred from agar plates to liquid growth media supplemented or not with 1 μM flg22. Untreated *cml9* genotypes (KO and *OE-CCs*) seedlings were indistinguishable from wild type Col (**Fig. 1A**, upper panel). A quantitative analyses based on the measurement of

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Submitted: 06/15/12; Revised: 06/29/12; Accepted: 06/29/12
http://dx.doi.org/10.4161/psb/

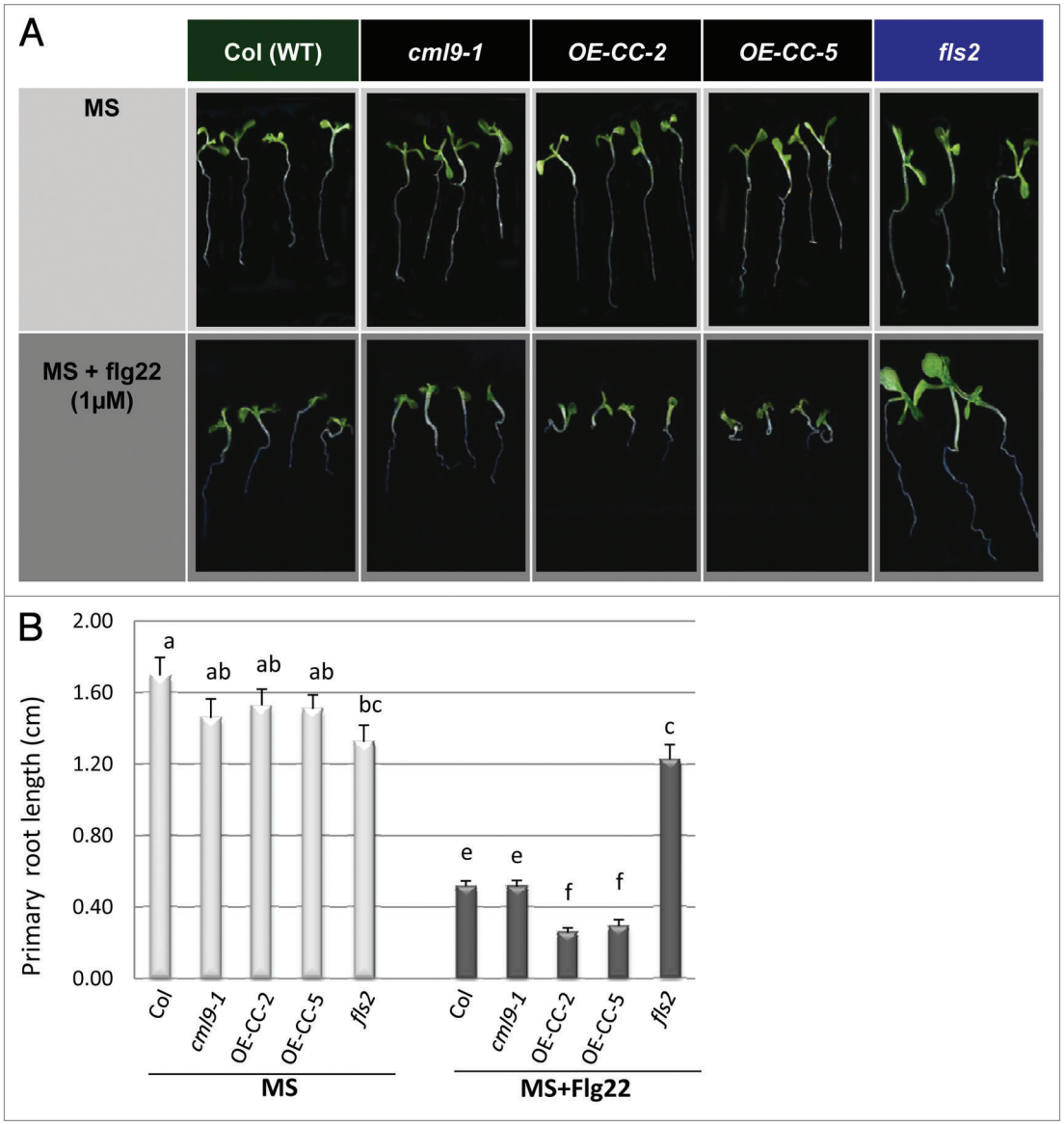

Figure 1. Primary root growth analyses of CML9 genotype in reponse to flagellin treatment (A) Wild-type (Col), *cml9* genotypes (KO *cml9–1 and OE-CCs* lines) and *fls2* mutant seedlings grown for 7 d in MS medium (control, upper panel) in presence or not of flg22 1µM (lower panel). (B) Quantitative analyses of the primary root growth under control condition (MS) or after flagellin treatment (MS + flg22). The experiments were performed using three independent biological replicates and each histogram represents the mean root length (± SEM) analyzed using 20 to 24 independent roots per genotype. Statistical differences between the genotypes treated or not by flg22 are detected by ANOVA analysis followed by Tukey's HSD test, at $p < 0.05$.

the primary root growth confirm these observations because no significative difference was observed between WT and *cml9* genotypes when the plants are grown in MS medium (**Fig. 1B**, MS). Following flg22 treatment, an inhibition of seedlings root growth is observed for all the genotypes except, as expected, for the *fls2* mutant which is unable to perceive the flg22 peptide (**Fig. 1A**, lower panel). Primary root length measurements show a growth inhibition of about 70% for the WT and *cml9–1* KO after flg22 application (**Fig. 1B**, MS+flg22). The *cml9* mutant line display similar enhanced PAMP-induced growth inhibition as in the WT (**Fig. 1A and B**). In contrast, root growth of *OE-CCs* seedlings was severely stunted after 10 d of growth in the presence of flg22 (**Fig. 1A**, + flg22) as compared with plants grown in the absence of the PAMP (**Fig. 1A**, MS). Because CML9 belongs to a multigenic family and although *CML9* is one of the CMLs mostly-induced by flg22 application, we cannot exclude some functional redundancies between CML9 and other CMLs that might explain the absence of visible phenotype in the KO mutant. The transgenic *CML9* overexpressing lines (*OE-CC-2* and *5*) had shorter roots than Col (**Fig. 1A**) and they exhibited a significant enhanced sensitivity (2-fold more important) than Col ecotype or knockout mutant to flg22-mediated inhibition of root growth at 1µM flg22 (**Fig. 1B**).

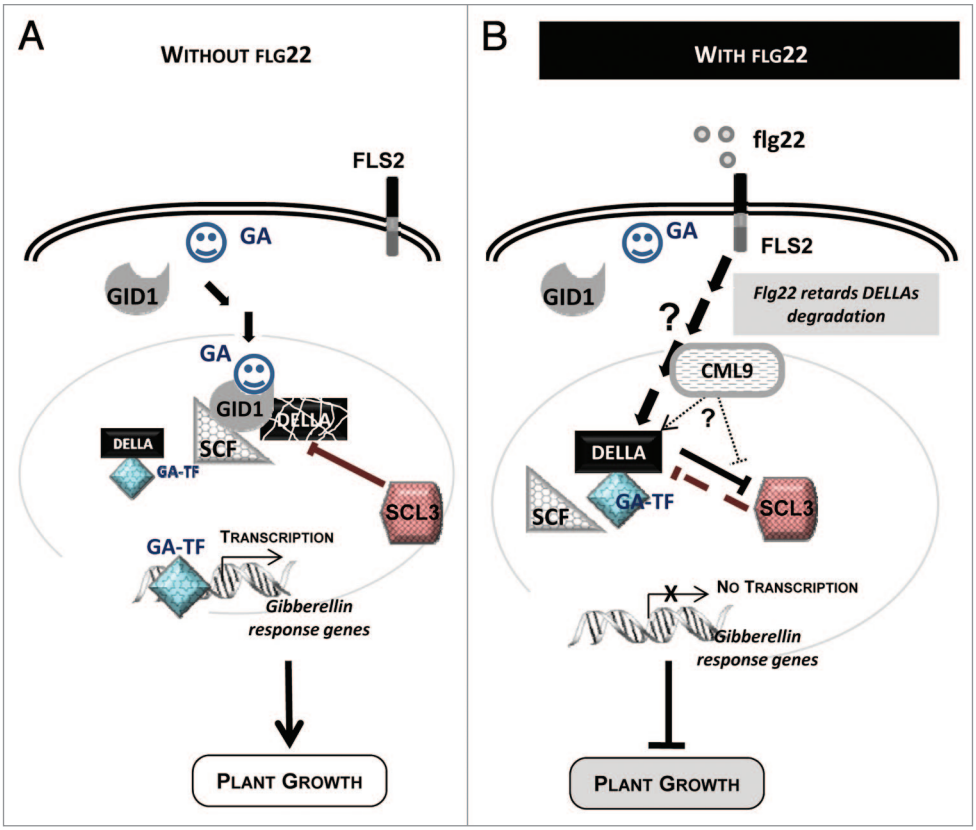

Figure 2. Hypothetical model involving CML9 in flg22-induced plant growth inhibition through GA-dependent signaling pathway. (A) Model of plant growth control through GA-dependent signaling. Upon GA binding to its soluble receptor (GID1), signaling pathway is activated; DELLA proteins are degraded via the ubiquitin-proteasome pathway. The major players of this GA-signaling cascade are the GA receptors (GID1), the DELLA repressor proteins and the F-box protein part of the SCF E3 Ubiquitin ligase complex. DELLAs are nuclear transcriptional regulators, which interact with other transcription factors (*i.e.* GA-TF, GA-dependent transcription factor) to modulate expression of GA-responsive genes. (B) Working model of flg22-induced plant growth inhibition that involve GA signaling cascade components and CML9.

Collectively, these data question about the contribution of CML9 in flg22-induced root growth inhibition process. Interestingly, Navarro et al.[8] established that altered growth inhibition upon fl22 treatments were only detected in mutants affected in gibberellins (GAs) biosynthesis or signaling. These data clearly illustrate the major contribution of both GAs and the key regulatory elements of the GA cascade in growth of plants exposed to flagellin.[8] It is well known that GAs play central roles in the control of plant growth and development by modulating cell division and cell elongation.[9] These past few years, molecular components of GA signaling have been identified and well characterized (**Fig. 2A**). The soluble GA receptors GID1 (*Gibberellin insensitive Dwarf 1*) interact with DELLA proteins (DELLAs), that are considered as major negative regulators of GA signaling.[10] The DELLAs are conserved repressors of GA signaling that act immediately downstream the GA receptor to modulate all aspects of GA-induced growth and development. DELLAs accumulate when bioactive GA levels are low, whereas degradation of DELLA is accelerated when GAs are elevated[9] (**Fig. 2A**). In response to the flg22 treatment, the accumulation and/or stabilization of the DELLA proteins can be observed and this could explain the flagellin-induced root growth inhibition.[8]

What could be the Relationship between CML9 and the Flagellin-inhibition of Root Growth?

To go further in the contribution of AtCML9 to plant immunity and plant growth inhibition by flagellin, the identification of the cellular processes controlled by this CML is needed. It is well known that the typical calmodulin act as Ca^{2+} relay by interacting and modulating the activity of target proteins.[1] Experimental evidences demonstrate the ability of CML9 to bind Ca^{2+} ions[11] and CML9 was shown to fulfil under certain conditions the role of CaM in yeast.[12] More recently, Perochon et al.[13,14] and Popescu et al.[15] identified CML9-interacting proteins suggesting that CML9 participates in Ca^{2+}-regulated processes in plant. It was reported by various approaches (two-hybrid screens, in vitro or *in planta* interactions) that several transcription factors could be the downstream targets of CML9.[14,15] Among these nuclear interacting partners, TGA3, TGA2 and WRKY53 were shown to be involved in plant defense[16,17] and interestingly, others such as the transcription regulators SCL (Scarecrow-like) could be involved in plant growth control.[18] It was shown that CML9 could in vitro interact with SCL3 (identified by an in vitro cDNA library screening),[13] SCL4 and SCL21,[15] three transcription regulators belonging to the GRAS family. *CML9* and *SCL3* exhibit

a similar gene expression pattern in primary root[5,19] and these SCLs exhibit a nuclear localization[18,20] which is consistent with the nucleo-cytoplasmic localization of CML9 protein in plant cells.[14] Functional analyses using *scl3* null mutants indicate that SCL3 acts as a positive regulator of GA signaling that integrate GA signaling in the root to ultimately coordinate cell division and cell expansion.[21] Thus, to explain the contribution of CML9 to plant growth control under flg22 treatment, we can hypothesize that CML9 interacts with SCL3 to negatively regulate its activity. According to this model, the accumulation of DELLA might occur leading to root growth inhibition (**Fig. 2B**).

In the future, the challenge will aim at a better understanding of the biological meaning of the interactions between CML9 and transcription factors associated to plant defenses and to plant growth control. This work will precise the multiple roles played by CML9 and by calcium on the activity of CML9 targets. These researches might lead to new findings that would be integrated into the complex picture of calcium signaling system involved in plant stress responses and plant growth and this will contribute to a better knowledge of CML function in plant physiology.

Material and Methods

Plant material. We used in this study, a T-DNA insertional mutant, *cml9–1* (background Col-8)[5] and two overexpressing lines (***O***ver **e**xpressor of ***C***ML9 in ***C***ol-8 named *OE-CC-2 and OE-CC-5*) harbouring constitutive and stronger expression of the *CML9* gene compared with the WT.[4] Under standard culture conditions, neither the mutation, as reported by Magnan et al. (2008), nor the overexpression of *AtCML9* are responsible for significant effect on growth and morphology of transgenic plants as compared with WTs.[4] The *fls2 Arabidopsis* mutant in Columbia ecotype altered in flagellin perception was purchased from the NASC and used as a control in the in vitro plant growth assay.

Flagellin-induced plant growth inhibition. Surface sterilized seeds of different Arabidopsis genotypes were sown in liquid MS medium (0.5x pH 5.7, 1% sucrose) in microplates (24-well) and cultivated for two days into a growth chamber under 16h light. Once germinated, the seedlings were transferred in MS liquid medium supplemented or not with 1μM flg22. The effects of the flg22 or mock treatment on seedling growth were analyzed after 10 d by a quantitative analysis of primary root growth using ImageJ software. All the experiments were performed three times with three independent biological replicates and statistical analyses were treated by using the software *Statgraphics* Centurion XV (SigmaPlus).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

University Paul Sabatier (Toulouse III), the CNRS supported this work. This work has been done in the lab UMR5546, part of the *"Laboratoire d'Excellence"* (LABEX) entitled *TULIP* (ANR -10-LABX-41).

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