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Identification of another calmodulin-binding domain at the C-terminal region of AtCBP63

Sun Ho Kim[1] · Yun Hwan Kang[2] · Hay Ju Han[2] · Dong Won Bae[3] · Min Chul Kim[1,2] · Chae Oh Lim[1,2] · Woo Sik Chung[1,2*]

[1]Environmental Biotechnology National Core Research Center and [2]Division of Applied Life Science (BK21 program), Plant Molecular Biology and Biotechnology Research Center, 3Central Instrument Facility, Gyeongsang National University, Jinju 660-701, Korea

ABSTRACT Calcium signals can be transduced by binding calmodulin (CaM), a Ca^{2+} sensor in eukaryotes, is known to be involved in the regulation of diverse cellular functions. We isolated a CaM-binding protein 63 kD (AtCBP63) from the pathogen-treated *Arabidopsis* cDNA expression library. Recently, AtCBP63 was identified as a CaM bining protein. The CaM binding domain of AtCBP63 was reported to be located in its N-terminal region. In this study, however, we showed that ACaM2 could specifically bind to second CaM-binding domain (CaMBD) of AtCBP63 at the C-terminal region. The specific binding of CaM to CaM binding domain was confirmed by a gel mobility shift assay, a split ubiquitin assay, site-directed mutagenesis, and a competition assay using a Ca^{2+}/CaM-dependent enzyme. The gene expression of *AtCBP63* was induced by pathogens and pathogens related second messengers. This result suggests that a CaM binding protein, AtCBP63, may play role in pathogen defense signaling pathway.

Introduction

In both plants and animals, Ca^{2+} mediates stimulus-response coupling in the regulation of diverse cellular functions that are triggered by a variety of biotic and abiotic external stimuli (Knight et al. 1991; Bush et al. 1995; Du et al. 2004). The transient elevation of cytosolic free-calcium concentration ($[Ca^{2+}]_{cyt}$) that occurs in response to specific stimuli differs in amplitude, frequency, and duration depending on the exact nature of the stimulus (Dolmetsch et al. 1997). Calmodulin (CaM), a highly conserved and ubiquitous protein in eukaryotes, binds Ca^{2+} and plays important roles in the transduction of Ca^{2+} mediated signals in eukaryotic organisms (McAinsh and Hetherington 1998, Trewavas and Malho 1998, Reddy et al. 2001). The activated CaM is involved in diverse cellular functions through the regulation of variety CaM-binding proteins (CaMBPs) such as proten kinases, MAPK phosphatases, Ca^{2+} pumps, NAC protein as a transcriptional repressor, and ubiquitin- specific protease (Snedden and Fromm 2001, Hoeflich and Ikura 2002, Kim et al. 2007, Lee et al. 2007, Lee et al. 2008). However, despite the reports showing that Ca^{2+}/CaM participate in Ca^{2-} mediated signaling, little is known about the molecular targets of CaM signaling and the number of CaMBPs is still increasing.

In *Arabidopsis genome*, seven different CaM genes encode four CaM isoform proteins. In addition to CaMs, the *Arabidopsis* genome also encodes 50 CaM-like proteins (CMLs). They contain CaM-like and/or divergent Ca^{2+}-binding domains (McCormack et al. 2005). However, relatively little is known about the biological roles of CaM in *Arabidopsis*, because few CaMBPs was identified and characteriazed yet. Consequently, the isolation and characterization of CaMBPs that are involved in plant signal transduction will understand the role of CaM in *Arabidopsis* and shed further light on this process.

In the present study, to identify and characterization CaMBPs that participate in pathogen defense signaling of plant, we screened a cDNA expression library obtained from *Arabidopsis* plant that had been treated with the bacterial pathogen using horseradish peroxidase (HRP)-conjugated CaM. As a result, we isolated an A. *thaliana* Calmodulin-Binding Protein 63 kD (AtCBP63) that physically interacts with ACaM2. To characterize a CaM-binding domain (CaMBD) of AtCBP63, we performed the a gel mobility shift assay, a split ubiquitin assay, site-directed mutagenesis, and a competition assay using a Ca^{2+}/CaM-dependent enzyme, knew that C-terminus of AtCBP63 physically interacts with ACaM2 in Ca^{2+}-dependent manner *in vitro* and *in vivo*. We showed that expression of the AtCBP63 gene was

*Corresponding author Tel 055-751-6254 Fax 055-759-9363
E-mail: chungws@gnu.ac.kr

induced by biotic and abiotic stresses that relates with pathogen defense signaling. Therefore, we propose that the C-terminal of AtCBP63 protein may be involved in Ca^{2+}/CaM mediated pathogen defense signaling pathway.

Materials and methods

Screening of the *Arabidopsis* cDNA expression library by the CaM-binding assay

An *Arabidopsis* cDNA expression library was constructed in a λ ZAPII vector (Stratagene) from *Arabidopsis* seedlings that had been treated with Pseudomonas syringe DC3000 (*avrRpm1*). SCaM-1 was conjugated to a maleimide-activated HRP by using the EZ-Link maleimide-activated HRP conjugation kit (Pierce) as described (Lee et al. 1999). We screened the *Arabidopsis* cDNA expression library as described by Sambrook and Russell (2001) using the HRP-conjugated SCaM-1 (SCaM-1:HRP) as a probe. A total of 3×10^6 recombinants were screened and 322 positive clones were isolated after three rounds of screening. Positive clones were confirmed by the CaM overlay assay as described above and the cDNA sequences of the resulting positive clones were determined by automatic DNA sequencing (ABI 377, Applied Biosystems Inc.).

CaM-binding assay

To map the CaMBD of AtCBP63, seven variant contructs ($\Delta N\ D_0$, ^{72}M-^{563}L; $\Delta N\ D_1$, ^{72}M-^{410}M; $\Delta N\ D_2$, ^{72}M-^{302}M; $\Delta N\ D_3$, ^{72}M-^{218}S; $\Delta N\ D_4$, ^{405}P-^{563}L; $\Delta N\ D_5$, ^{441}K-^{563}L; $\Delta N\ D_6$, ^{489}D-^{563}L) were generated in a pGEX-5X-2. Recombinant proteins were expressed in E. coli and applied for CaM-binding activity as described in Lee et al. (1999).

CaM mobility shift assay and Phosphodiesterase (PDE) competition assay with a synthetic peptide

A peptide (531-RARWCKVKAAFKVRAAFKEV-550) corresponding to a stretch of 20 amino acids in C-terminus of AtCBP63 was synthesized (Peptron and Peptipharm, Korea) and its CaM-binding ability was detected by observing the relative mobility shift of CaM in its presence (Erickson-Vitanen and DeGrado 1987). The gels were stained with coomassie brilliant blue R-250.

Cyclic nucleotide PDE assays were performed using commercially available bovine heart CaM-deficient PDE (Sigma). PDE competition assay was performed as previously described (Yoo et al. 2005)

Yeast split ubiquitin assay

The yeast split ubiquitin assay was performed as described previously (Laser et al. 2000). *Saccharomyces cerevisiae* strain JD53 was used for all the expreriments. Briefly, ACaM2 cDNA was cloned into pMet-Ste14-Cub-RUra3p, replacing yeast Ste14. AtCBP63 cDNA was also cloned into modified versions of the pCup-Nub-Sec62 vector, replacing yeast Sec62. Interactions between each pair of proteins were tested on selective medium containing 1 mg/mL 5-FOA and selective medium lacking uracil. Plates were incubated at 30℃ for 3-5 days, unless specified otherwise.

Isolation of total RNA and semi-quantitative RT-PCR

Total RNA was isolated from seedlings treated with pathogen (Pseudomonas DC3000 (*Vir* and *avrRpt2*)) or chemicals (2 mM salicylic acid [SA], 200 mM jasmonic acid [JA], or 50 μM methyl jasmonate [MeJA]) for various periods. Five microgram of total RNA was reverse-transcribed in total volume of 50 μL using SuperScript™ II RNase H-Reverse Transcriptase (Invitrogen, USA). RT-PCR was performed in 50 μL solution containing 1 μL the RT product. PCR cycles of 24-27 were performed for *AtCBP63*, 22-25 cycles for *PR1*, 24-27 cycles for *PDF1.2*, and 18-21 cycles for *Tubulin*. The number of PCR cycles chosen was shown to be in the linear range of the amplification reaction (data not shown). *Tubulin* as an internal control was amplified in the same tube as each gene studied. Primers used for the PCR reaction were as follows: 5'-CCTTGATCAGCCAATTCTAG-3' and 5'-GTTGA-GCTTGAAGCTTTGTG-3'for AtCBP63; 5'-ATG AATTTTACTGGCTATTCT-3' and 5'-T-TAGTATGGCTTCTCG TTCA-3' for PR1; 5'-CATCACCCTTATCTTCGCTG-3' and 5'-C AGATACACTTGTGTGCTGG-3' for PDF1.2; 5'-AGGTTCTCA GCAGTACCGTTC-3' and 5'-TCTTGG-TATTGCTGGTACTCT-3' for *Tubulin*

Results

Isolation of the *Arabidopsis* AtCBP63

To identify the signaling components that link CaM to pathogen

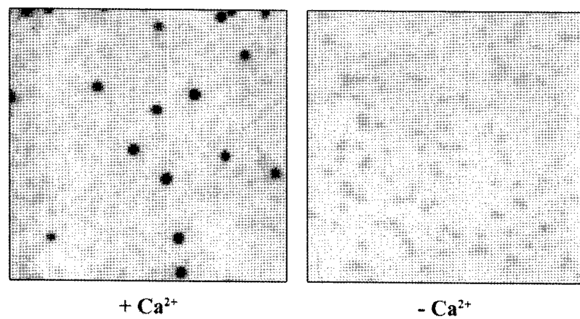

Figure S1. Screening of recombinant phages from λZAPII cDNA expression library using SaCM:HRP. Secondary screening of positive (left panel) and negative (right panel) recombinant phages isolated from the primary screening. Recombinant phage from screening were induced to produce CAMBP and probed with HRP conjugated CaM. Purified positive was tested for its Ca^{2+}-dependent binding in the presence of Ca^{2+} (+ Ca^{2+}) and in the presence EGTA (- Ca^{2+})

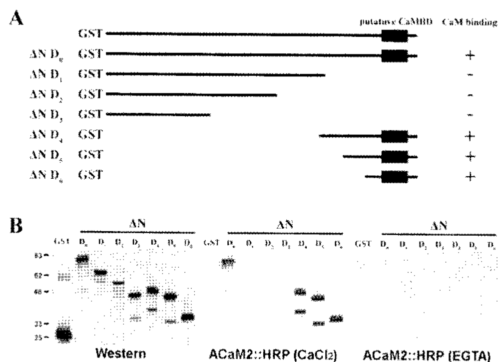

Figure 1. Mapping of a CaMBD in the AtCBP63. (A) Identification of the CaMBD in AtCBP63. Seven AtCBP63 truncates (ΔN D0-D6) were expressed as GST fusion proteins. The putative CaMBD in the C-terminus is shown as a black box. The CaM-binding ability of each fragment is indicated as + (CaM binding) or - (no CaM binding). (B) CaM overlay assays of the ΔN D0-D6 proteins. The expression of the fusion proteins were analyzed with an anti-GST antibody (left) and CaM overlay in the presence ($CaCl_2$) and absence (EGTA) of Ca^{2+} (middle and right)

responses, we screened an *Arabidpsis* cDNA expression library from *A. thaliana* (Col-0) seedlings that had been treated with the *Pseudomonas* DC3000 (*avrRpm1*) using HRP-conjugated ACaM2 (Fig. S1) (Lee et al. 1999). Among the isolated clones, we obtained two cDNA clones of different lengths (1.5 and 2.0 kb) that encode the same CBP gene (GenBank accession number: AY096343). The full-length cDNA of the clone encodes a 63 kD polypeptide composed of 563 amino acid (aa) and has a putative CaMBD according to the basis of comparative analyses with the known CaMBD of many reported CBPs. Therefore, we denoted the protein AtCBP63, for *A. thaliana* Calmodulin-Binding Protein 63 kD. As a prediction, a putative CaMBD was located in the region between R^{531} and V^{550} of AtCBP63. This 20-amino acid stretch contains hydrophobic amino acids at positions 1 (W^{534}), 8 (F^{541}), and 14 (F^{547}), which matches the characteristics of a consensus Ca^{2+}-dependent CaMBD that has been designated "1-8-14" (Rhoads and Friedberg 1997). Recently, AtCBP63 was reported as a CaM binding protein with a CaMBD in the N-terminal (Wang et al. 2009). However, we predicted another CaMBD in the C-terminal region of AtCBP63. So, we characterized the C-terminal CaMBD of AtCBP63.

To define the CaMBD at C-terminal region, we made a series of GST fusion constructs consisting of the serial C-terminal deletion mutants (ΔN D_0-D_6) without the CaMBD of N-terminus (1-71 aa) in AtCBP63 (Fig. 1A). The recombinant proteins were produced in *E. coli*, separated by SDS-PAGE, and transferred to PVDF membranes for western blotting or ACaM2 overlay assays. The four proteins containing putative CaMBD (ΔN D_0 and ΔN D_4-D_6) interacted with ACaM2-HRP in a Ca^{2+}-dependent manner, unlike any of the C-terminal deletion mutants (ΔN D_1-D_3) (Fig. 1B). Thus, ACaM2 binds to AtCBP63 at the predicted CaMBD in a Ca^{2+}-dependent manner.

Confirmation of the interaction between the putative C-terminal CaMBD of AtCAB63 and ACaM2

To confirm that ACaM2 binds to the 20-aa stretch encompassing residues R^{531} to V^{550} in the C-terminal CaMBD of AtCBP63, a peptide corresponding to this region was synthesized and used in a gel shift assay under non-denaturing conditions (Fig. 2A) (Lee et al. 1999). As shown in Fig. 2A, the intensity of a higher molecular mass band representing the peptide-ACaM2 complex increased as the concentration of the synthetic peptide rose. The presence of Ca^{2-} ($CaCl_2$) was necessary for the formation of this complex as the higher molecular weight complex was not detected in the absence of Ca^{2+} (EGTA). The ACaM2 formed a complex with the peptide at a molar ratio of 2:1 (peptide:ACaM2). This result shows that the 20-mer peptide is sufficient for Ca^{2+}-dependent ACaM2 binding. We also analyzed the binding of the synthetic peptide to ACaM2 by a competition assay using PDE, a Ca^{2+}/CaM-dependent enzyme that might compete with the peptide for binding to CaM (Reddy et al. 1999). Thus, we monitored the ACaM2 dose-dependent activation of PDE in the presence (100 nM) or absence of the peptide and determined the change in K_d values induced by the peptide (Fig. 2B). The activation curves shifted to the right in the presence of the peptide, indicating that the peptide competed with PDE for binding to ACaM2. The concentrations of ACaM2 needed to achieve

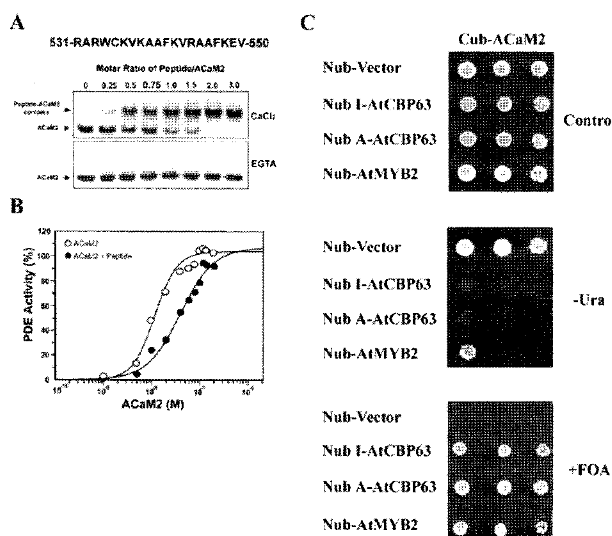

Figure 2. SConfirmation of the interaction between ACaM2 and AtCBP63. (A) Gel mobility shift assay. The synthetic peptide sequence is shown on top. ACaM2 (303 pmol) was incubated with increasing amounts of the peptide in the presence of 0.1 mM CaCl2 (CaCl2) or 2 mM EGTA (EGTA). The samples were separated by non-denaturing PAGE and stained with coomassie brilliant blue R-250. (B) Inhibition of ACaM2-stimulated PDE activity by the synthetic peptide. PDE activity was measured in the presence of varying concentrations of ACaM2 and a fixed amount (100 nM) of the synthetic peptide. The data points represent the means of the results from three independent assays (n=3). (C) Interaction of AtCBP63 with ACaM2 in the split-ubiquitin system. Cells co-expressing the depicted constructs were serially diluted on plates. All proteins were expressed from single-copy vectors

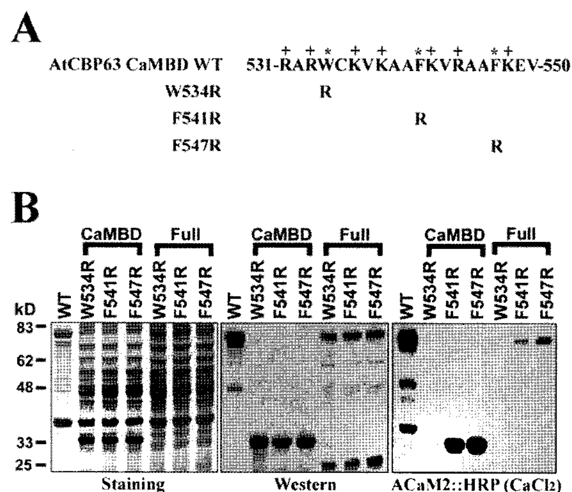

Figure 3. Characterization of the CaMBD in AtCBP63. (A) The key hydrophobic residues are marked with asterisks, while the basic residues within the CaMBD are indicated with +. W534R, F541R, and F547R represent the single substitutions in CaMBD. (B) Effect of the substitutions on the binding of the AtCBP63 to CaM. WT and W534R, F541R, and F547R mutants of the CaMBD and the full-length of AtCBP63 were fused to the C-terminus of GST and expressed in E. coli. The recombinant proteins were then separated by SDS-PAGE and visiualized with coomassie brilliant blue R-250 (left), Western blot with an anti-GST antibody (middle) and CaM overlay (right)

half-maximal activation of PDE in the absence and presence (at 100 nM) of the 20-mer peptide were 11.0 and 37.6 nM, respectively. The Kd value of the peptide for the activation of PDE by ACaM2 was determined to be 30.2 nM.

To further confirm the direct interaction between AtCBP63 and ACaM2 in intracellular conditions, we used the yeast split ubiquitin system, which is based on the reassembly of the N- and C-terminal halves (N_{ub} and C_{ub}) of ubiquitin (ub) (Stagljar et al. 1998; Laser et al. 2000). AtCBP63 and ACaM2 were fused to the C-terminus of N_{ub} and the N-terminus of C_{ub}, respectively, and then were co-introduced into yeast. As shown in Fig. 2C, the cells co-expressing ACaM2-C_{ub} and N_{ub}-AtCBP63 were not able to grow on plates lacking uracil, instead, they grew on plates containing FOA. This indicates that AtCBP63 effectively forms stable complexes with ACaM2. N_{ub} I has a high affinity for C_{ub} and assembles spontaneously to form a split-ubiquitin heterodimer. However, replacement of the I_{13} residue in N_{ub} (N_{ub} I) with alanine (N_{ub} A) decreases the affinity between N_{ub} and C_{ub} (Stagljar et al. 1998). As shown in Fig. 2C, not only Nub I-AtCBP63 but also Nub A-AtCBP63 was able to interact with Cub-ACaM2. This indicates that AtCBP63 binds strongly to CaM within yeast cells.

Identification of the critical residues of the C-terminal CaM-binding motif in AtCBP63

To identify the critical residues of the C-terminal CaMBD in AtCBP63, we used site-directed mutagenesis to introduce single amino acid substitutions into the GST-fused CaMBD of AtCBP63. The hydrophobic residues, which are W^{534}, F^{541} and F^{547} of AtCBP63 that formed the 1-8-14 motif were replaced with an Arg (R) residue, resulting in the W534R, F541R and F547R mutants, respectively (Fig. 3A). The resulting variants were expressed in *E. coli* and examined for their ability to bind to ACaM2 by using a CaM overlay. The expression levels of the full-length mutant fusion proteins in *E. coli* were similar to that of the wild-type full-length protein of AtCBP63, which suggests that these mutations did not affect protein stability (Fig. 3B). We found that the W534R mutant completely abolished the Ca^{2+}-dependent CaM-binding of AtCBP63 while the 5410R and F547R did not affect the ability of AtCBP63 to complex with CaM. Thus, W^{534} is the key residue in the interaction of ACaM2 with AtCBP63.

Induction of AtCBP63 gene expression by the biotic and abiotic

To determine pattern of *AtCBP63* expression in plant tissues, we performed semi-quantitative RT-PCR analysis. The *AtCBP63* transcripts showed higher expression in rosette leaves, cauline leaves, and stems, but not in flower cluster and siliques (data not shown). Because we isolated the *AtCBP63* from cDNA library treated with pathogen, we then investigated whether *AtCBP63* is involved in plant pathigen signaling by examining the expression of *AtCBP63* in wild type seedlings that had been treated with bacterial pathogens, SA, JA, or MeJA. In the Fig. 4, both virulent and avirulent pathogens induced the expression of *AtCBP63* at 1 to 12 hr after exposure (Fig. 4). The expression of *AtCBP63* was also induced by JA and MeJA, but not by SA (Fig. 4). We also examined the pathogen-induced expression of *AtCBP63* in transgenic *NahG* plants, which overexpress bacterial salicylate hydroxylase and therefore are poor producers of SA; this experiment aimed to determine whether SA is involved in the pathogen-responsive expression of AtCBP63. Exposure to a bacterial pathogen also induced *AtCBP63* gene expression in the *NahG* background plant (Fig. 4), indicating that SA is not involved in the pathogen-responsive expression of AtCBP63. Thus, *AtCBP63* gene expression may be strongly induced by response to bacterial pathogens.

Disussion

CaM plays a vital role in transducing Ca^{2+} signals by modulating the activity of numerous target proteins (Lee et al. 2000). To understand the roles CaM plays in biotic and abiotic responses, we isolated *AtCBP63* by screening a cDNA expression library generated from pathogen-treated *Arabidopsis* seedlings. Although, AtCBP63 was reported as a CaM-binding protein (Wang et al. 2009), N-terminal deleted *AtCBP63* clone still could bind CaM, indicating the presence of another CaMBD. The location of CaMBD in *AtCBP63* at the C-terminal region was identified by cDNA expression-deletion mapping (Fig. 1) and was confirmed by a ACaM2 mobility shift assay and PDE enzyme competition assays with a synthetic peptide corresponding to a 20-amino acid stretch from *AtCBP63* (from R^{531} to V^{550}) (Fig. 2A and 2B). Also, the interaction between ACaM2 and *AtCBP63* was confirmed by the split ubiquitin assay in yeast (Fig. 2C), which serves to monitor protein-protein interactions within living cells (Stagljar et al. 1998, Du et al. 2004). We further confirmed the predicted CaM-binding motif by showing that the substitution of a single amino acid residue of the CaMBD of *AtCBP63* (W^{534}) (Fig. 3) abrogated its Ca^{2+}-dependent CaM-binding ability.

Most of the CaMBDs that have been isolated previously are stretches of 16-35 amino acid residues that, in a helical wheel representation, have basic and polar residues on one side and hydrophobic amino acids on the other (James et al. 1995). Recently, the Ca^{2+}-dependent CaMBD have been classified into two major groups, namely, the 1-8-14 and 1-5-10 motifs, in which the numbers indicate the relative positions of conserved hydrophobic residues. The CaMBD of *AtCBP63* belongs to the 1-8-14 group. These observations suggest that the activity of *AtCBP63* may be regulated by CaM in a Ca^{2+}-dependent manner through two CaMBDs.

We showed that the gene expression of *AtCBP63* is induced in response to pathogen (Fig. 4). In wild-type and *NahG* plant, the gene inductions of *AtCBP63* by pathogens were not significantly different. The expression of *AtCBP63* was also strongly induced by JA and MeJA treatment, but weakly by SA. Previously, *AtCBP63* was reported to be induced by SA and MAMP (Wang et al. 2009). These expression patterns of *AtCBP63* suggest that *AtCBP63* may play an important role in the pathogen signaling pathway.

CaM is recognized as a major calcium sensor and orchestrator of regulatory events through its interaction with a diverse group of cellular proteins (Reddy et al. 2001; Reddy and Reddy 2004). Therefore we suggest that environmental change increase cytosolic Ca^{2+} concentration and activate CaM. Aactivated CaM bind to *AtCBP63* that is induced by biotic and abiotic responses. Interaction of *AtCBP63* with ACaM2 may be involved in biotic and abiotic stresses related signal transduction.

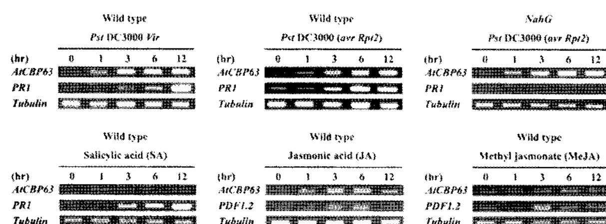

Figure 4. The expression patterns of AtCBP63 gene by semi-quantitative RT-PCR. Total RNA (5 μg) from tissues of mature plants or seedlings treated with pathogen (Pseudomonas DC3000 (Vir or avrRpt2) or chemicals (SA, JA or MeJA) in wild-type or NahG plants was used for RT-PCR. As quantitative controls, Tubulin2 transcripts were amplified

Acknowledgments

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