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Calmodulin-binding transcription activator (CAMTA) 3 mediates biotic defense responses in *Arabidopsis*

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Abstract Calmodulin-binding transcription activator (CAMTA) 3 (also called SR1) is a calmodulin-binding transcription factor in *Arabidopsis*. Two homozygous T-DNA insertion mutants (*camta3-1*, *camta3-2*) showed enhanced spontaneous lesions. Transcriptome analysis of both mutants revealed 6 genes with attenuated expression and 99 genes with elevated expression. Of the latter, 32 genes are related to defense against pathogens (e.g. WRKY33, PR1 and chitinase). Propagation of a virulent strain of the bacterial pathogen *Pseudomonas syringae* and the fungal pathogen *Botrytis cinerea* were attenuated in both mutants. Moreover, both mutants accumulated high levels of H_2O_2. We suggest that CAMTA3 regulates the expression of a set of genes involved in biotic defense responses.

Keywords: Calcium; Calmodulin; Pathogens; Reactive oxygen species; Signal transduction; Transcriptome

1. Introduction

Ca^{2+} ions play a major role in mediating plant growth and development [1] and in response to biotic and abiotic stresses [2]. In an attempt to elucidate the mechanisms underlying Ca^{2+}-regulated gene expression in plants, we have previously identified a family of six *Arabidopsis* genes encoding calmodulin (CaM)-binding transcription activators (CAMTAs; [3] and reviewed by Finkler et al. [4]) also called SRs [5]. The expression of *Arabidopsis* CAMTAs was found to be mediated by both biotic and abiotic stresses [5]. However, their physiological roles have not yet been revealed. Here we describe physiological, molecular and bioinformatic investigations of *camta3* loss-of-function mutants, revealing a role of CAMTA3 in suppressing biotic stress responses, and a novel link between CAMTAs and WRKY-regulated gene networks.

2. Materials and methods

2.1. Pathogen strains and susceptibility assays

Pseudomonas syringae pathovar *tomato* strain DC3000 is one of the two virulent strains most widely used. *Botrytis cinerea* strain B05.10 is a haploid strain, originally obtained after benomyl treatment of strain SAS56 [6]. For the *B. cinerea* susceptibility assay, the technique of Muckenschnabel et al. [7] was used with some alterations (detailed in Supplementary Material). For the *Pseudomonas* growth assay, the protocol of Katagiri et al. [8] was used with minor adjustments.

2.2. Detection of H_2O_2

Staining with 3-diaminobenzidine (DAB; Sigma #D-8001; 25 mg ml^{-1} stock solution in 1 M HCl, pH 3.8; titrated with 10 M NaOH, kept at −20 °C) was performed based on [9].

2.3. Plant material, microarray analysis, polymerase chain reaction (PCR), reverse PCR, bioinformatics and statistics

These are described in Supplementary Material.

3. Results

3.1. Molecular-genetic and phenotypic characterization of *camta3* T-DNA insertion mutants

Two *Arabidopsis thaliana* homozygous lines with T-DNA insertions in the *CAMTA3* gene (*At2g22300*) were isolated from transgenic lines obtained from the *Arabidopsis* seed stock centre. Locations of the T-DNAs, 601 bp and 3544 bp downstream of the translation initiation codons (Fig. 1), were verified by nucleotide sequencing of PCR-amplified genomic DNA corresponding to the T-DNA border and to the *CAMTA3* gene, and by gel fractionation of genomic fragments amplified by PCR with *CAMTA3*- and T-DNA border-specific primers (Supplementary Fig. S1A). Primers were also used for RT-PCR analysis to ascertain that the homozygous mutants do not contain the intact *CAMTA3* mRNA (Supplementary Fig. S1B).

The *camta3-1* and *camta3-2* mutants displayed chlorotic lesions at a much higher extent (~36% and ~26% of leaves, respectively) than wild type plants (2% chlorotic leaves) (Fig. 2). In addition, *camta3* plants were also smaller compared to wild type plants. These experiments were repeated

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Abbreviations: CaM, calmodulin; CAMTA, CaM-binding transcription activator; DAB, 3-diaminobenzidine; LMM, lesion mimicking mutant; ROS, reactive oxygen species; TF, transcription factor; cfu, colony forming units

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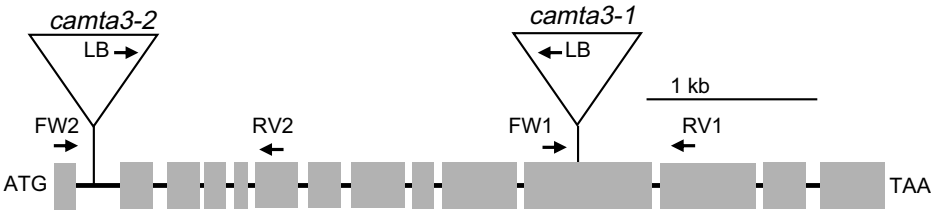

Fig. 1. Genetic and molecular analysis of *camta3*. Genomic organization of *camta3-1* and *camta3-2*. Triangles indicate the insertion site of the T-DNA in each mutant allele within the *CAMTA3* gene sequence. Gene organization in exons (grey boxes) and introns (thick black line) are presented. The primers that were used to isolate *camta3* mutants (Fig. S1) are indicated by arrows.

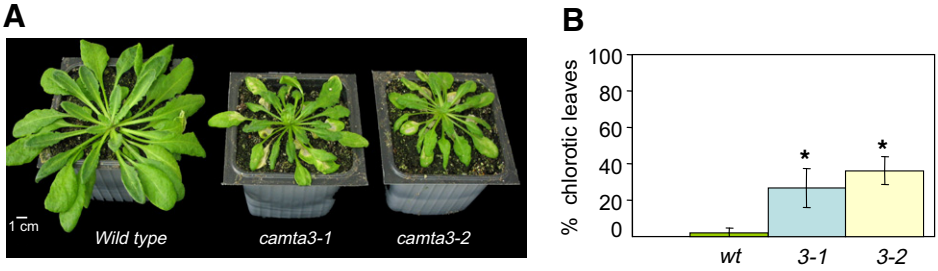

Fig. 2. Enhanced-lesion phenotype of *camta3* mutants. (A) Photographs of 11-week-old wild type and *camta-3* plants grown under short day conditions. (B) Percentage of chlorotic leaves of 11-week-old plants grown under short day conditions (as in A). The two mutant alleles exhibit a higher yet similar percentage of leaves with lesions compared to wild type. Differences between wild type and mutants were statistically significant with ANOVA tests with repeated measures and *t*-test methods in three independent experiments.

independently three times, and the differences between wild type and *camta3* mutants were statistically significant with ANOVA tests with repeated measures and *t*-test methods.

3.2. Defense-associated genes are up-regulated in camta3 mutants

RNA samples isolated from wild type, *camta3-1* and *camta3-2* plants were subjected to transcriptomic analysis using microarrays with the ATH1 *Arabidopsis* probe sets-based chip (two independent biological repeats were performed for each genotype). First, the expression data from the two *camta3* alleles (*camta3-1* and *camta3-2*) were compared to each other in order to find the common genes that are elevated or repressed (see Tables S1 and S2, Supplementary Material). The averages of independent biological repeats were plotted as shown in Fig. S2 (Supplementary Material). In each of the *camta3* alleles there is a substantial effect on gene expression (Fig. S2A and B), and there is a significant linear correlation ($R^2 = 0.9$) between *camta3-1* and *camta3-2* transcriptomes (Fig. S2C). Analysis of genes whose expression was changed by >2.5-fold in both mutants compared to the wild type, revealed 99 common up-regulated genes (Table S1, see Supplementary Material) and 6 common down-regulated genes (fold change >2.5) (Table S2, Supplementary Material).

The *camta3-1* and *camta3-2* common up-regulated genes were classified according to their known or predicted physiological and biochemical functions (Fig. 3 and Tables S3 and S4, Supplementary Material). The highest portion includes 32 known defense-associated genes (listed in Table S5). These genes are not specific to a certain pathogen or elicitor, but rather participate in defense against a wide range of viruses, virulent or avirulent bacterial strains and fungal strains. Amongst these are genes related to defense against different fungal strains (e.g. HR4 against *powdery mildew* and WRKY33 against *B. cinerea* and *Alternaria brassicicola*); bacteria (WRKY-33 and CRK5 against *P. syringae*, PAD4 against virulent and avirulent strains of *P. syringae*); insect (e.g. PAD4 against *Green peach aphid*) and viruses. Furthermore, seven of the up-regulated genes encode disease-resistance proteins with antimicrobial peptide activity (e.g. *At3g04210*, *At2g32680*, *At3g25010* and *At3g11010*). There are also three genes that are involved in hyper-sensitive response, which is the rapid death of plant cells in response to invasion by a pathogen (NDR1, SYP122 and PLP2). Eight genes are known to respond to different bacteria (e.g. WRKY70, WRKY33, NDR1, PAD4 and ACD6; [10]), six genes respond to different fungi (e.g. WRKY70, WRKY33, NDR1, PAD4 and SYP122) and six genes encode proteins that bind/respond/transport chitin, or bind carbohydrate (e.g. chitinase, legume lectin family protein, PR4 and ZAT10) (Table S5). There is also a high portion of genes related to oxygen metabolism, including oxidoreductases, acireductone dioxygenases, glutaredoxins and cytochrome 450 (Table S3, Supplementary Material). Two genes that encode glutathione transferases (ATGSTF6 and ATGSTF3), are involved in toxin catabolic process [11] and respond to oxidative stress [11]. A third gene, which encodes SAG21, responds to oxidative stress as well [12]. In addition, there are eight up-regulated genes with unknown functions (e.g. *At1g13470*, *At5g44820*, *At2g40000* and *At3g18250*).

In the biochemical classification (Fig. 3B and Table S4, Supplementary Material), the up-regulated genes encode proportionally high percentages of kinases, enzymes involved in protein metabolism, hydrolases and signal transduction functions. The kinases include leucine-rich repeat disease resistance proteins, serine/threonine protein kinases and tyrosine protein kinases (e.g. MAPKKK10, WAK1, ARK3, RLK5, RLK6 and disease resistance family proteins). Furthermore, it is particularly interesting that there are high proportions of

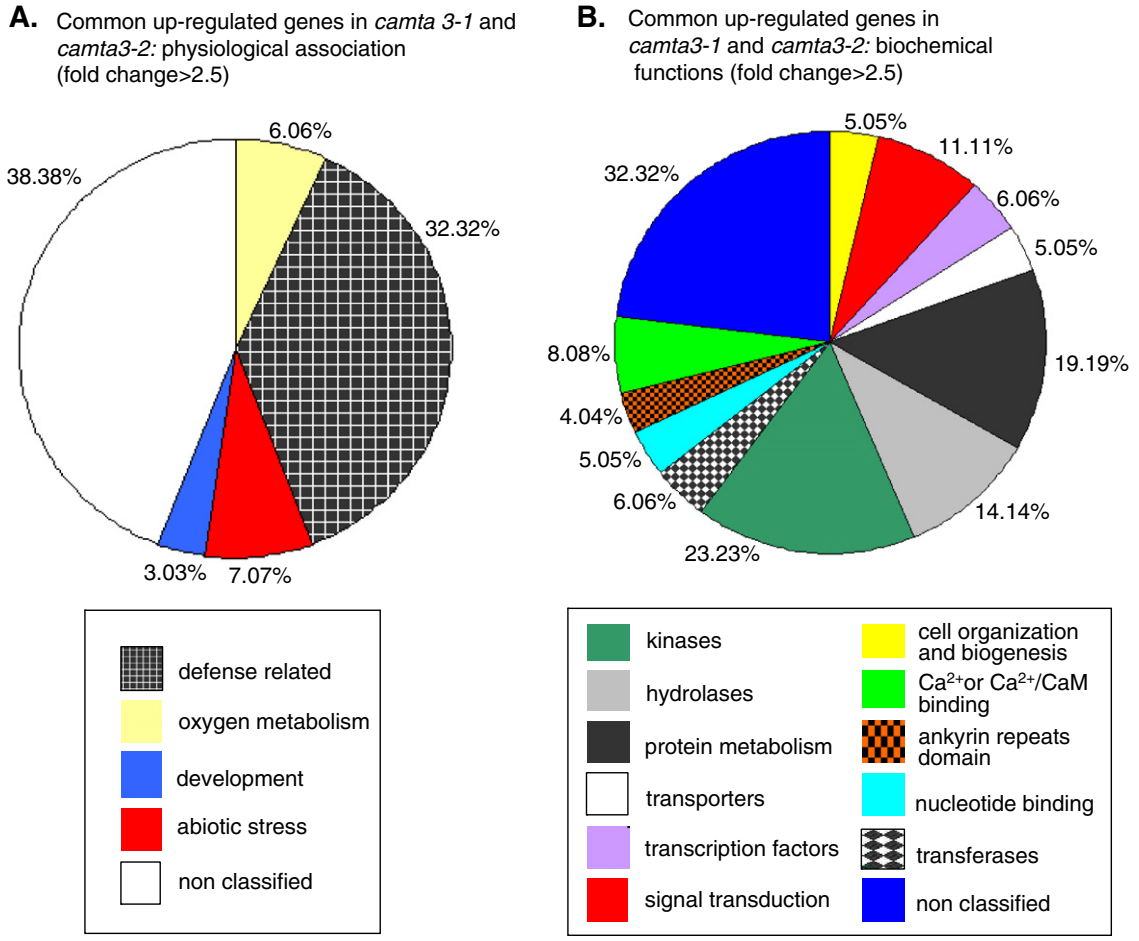

Fig. 3. Pie charts representing the functional classification of *camta3-1* and *camta3-2* up-regulated genes. Up-regulated genes (>2.5-fold) in *camta3-1* and *camta3-2* were classified according to their (A) physiological functions and (B) biochemical functions, and presented as pie charts (see Supplementary Tables S3 and S4 for further details).

calcium- and CaM-binding proteins (e.g. EDA39, CRT3 and TCH3) among the common up-regulated genes. There are six transcription factors (TFs) among the up-regulated genes including WRKYs, zinc finger and NAC-domain proteins. The WRKYs compose the most prominent TF group with four members (detailed in Table S4, Supplementary Material). Furthermore, these WRKYs are all known to be related to defense against pathogens ([13]; Table S5). The elevated expression of three up-regulated pathogen defense-related genes was validated by semi-quantitative RT-PCR (Fig. 4).

3.3. *camta3* mutants exhibit enhanced resistance to bacterial and fungal pathogens

The enhanced expression of defense-related genes in *camta3* mutants and the appearance of leaf lesions suggested the possibility of reduced susceptibility of the mutants to pathogens. To assess this possibility, *camta3* mutants were challenged with bacterial and fungal pathogens. Three-week-old wild type plants and the two *camta3* mutants were infected with the DC3000 virulent strain of *P. syringae* pv. *tomato*. Leaf disks were collected after 2–4 h from the time of infection (day 0) and at 2, 4 and 6 days post-infection. At day 0 both wild type and *camta3* mutants hosted similar numbers of the DC3000 bacteria (data not shown). From this time point to the last tested, there was an increase in the number of bacteria in all genotypes, and the number of bacteria after 2 and 4 days post-infection was similar in the wild type and mutants (data not shown). However, after 6 days, wild type plants hosted 20.6×10^8 colony forming units (cfu) cm^{-2} leaf tissue, whereas *camta3-1* and *camta3-2* hosted only $4.6–5 \times 10^8$ cfu cm^{-2} leaf tissue (Fig. 5A). The same type of experiment was independently repeated three times with consistent results, which were statistically significant using ANOVA with repeated measures and *t*-test methods.

In addition, 3–3.5-week-old wild type and *camta3* plants were inoculated with spores of the necrotrophic fungus *B. cinerea* (10^4 botrytis spores ml^{-1}). Six days post-inoculation the apparent damage in leaves of wild type plants was substantially greater than that in leaves of both *camta3* alleles (Fig. 5B), indicating that *camta3* mutants are less susceptible to the necrotrophic fungus than wild type plants.

3.4. *camta3* mutants accumulate high levels of reactive oxygen species (ROS) during development

Leaf lesions are often associated with enhanced accumulation of ROS (e.g. [14]). Moreover, our microarray results revealed enhanced expression of genes associated with oxygen metabolism in *camta3* mutants (Table S3, Supplementary Material). Subsequently, leaves of 2–4-week-old plants were stained in situ with DAB [9] to detect tissue levels of H_2O_2.

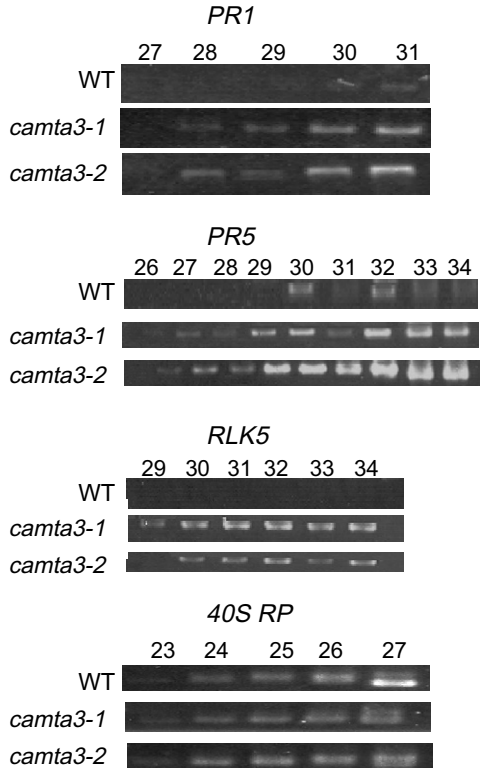

Fig. 4. Expression of defense-related genes in *camta3* mutants. Expression of *PR1* (*At2g14610*, fold changes: in *camta3-1*: 17.43 and in *camta3-2*: 22.87), *PR5* (*At1g75040*, fold changes: in *camta3-1*: 14.13 and in *camta3-2*; 7.48) and *RLK5* (*At4g23140*, fold changes: in *camta3-1*: 14.28 and in *camta3-2*: 8.76) in leaves of 3-week-old *camta3-1*, *camta3-2* and WT was determined by semi-quantitative RT-PCR. The numbers of PCR amplification cycles are indicated above each gel image and the expected sizes of the DNA-fragments are 665, 924 and 634 bp, respectively. As an internal control for the amount of template cDNA in the reactions, gene-specific primers for the 40S ribosomal protein gene were used to amplify a 350 bp DNA-fragment.

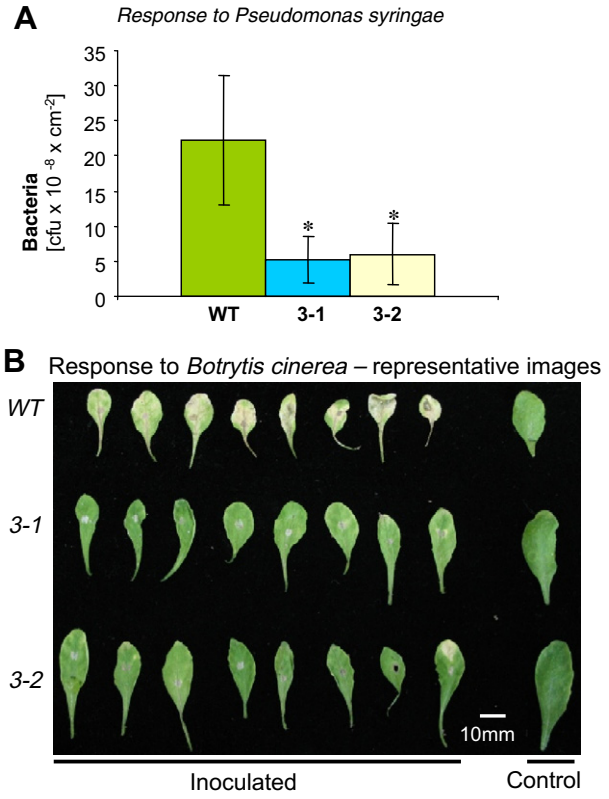

Fig. 5. *camta3* mutants show enhanced resistance to bacterial and fungal pathogens. (A) Growth of the virulent strain of *Pseudomonas syringae* pv. *tomato* (DC3000) at 6 days post-inoculation in wild type Col-0 and in two *camta3* mutant alleles. Spraying was performed with a bacterial suspension of 5×10^7 cfu ml^{-1}. The experiment was independently repeated three times and was found statistically significant with ANOVA with repeated measures and *t*-test methods. The indicated bacteria counts are per cm^2 leaf disk. (B) Leaf damage of 3-week-old wild type and *camta3* plants after 6 days of inoculation with *Botrytis cinerea* spores. The right-end column represents plants that were not inoculated with the fungus. Scale bar represents 10 mm.

The results (Fig. 6) show that *camta3* mutants accumulate high H_2O_2 levels compared to wild type plants by the time they reach 4 weeks of age (Fig. 6). At 2 and 3 weeks of age, H_2O_2 accumulation was similar and barely detected in the wild type and *camta3* mutants (Fig. 6). This experiment was repeated independently three times with similar results.

4. Discussion

4.1. CAMTA3 as a suppressor of defense responses

Molecular and physiological evidence reported here implicate *CAMTA3* in biotic stress responses, particularly in defense against bacterial and fungal pathogens. The enhanced tolerance of *camta3* mutants to these pathogens is correlated with enhanced ROS accumulation and with the appearance of leaf lesions between the third and fourth week. These *camta3* phenotypes are reminiscent of other reported mutants that show spontaneous lesions associated with enhanced tolerance to pathogens such as *hlm1* [15]. Furthermore, these phenotypes are consistent with the fact that an oxidative burst is part of the plant defense against pathogens [16]. Moreover, comparison of the *camta3* transcriptome with that of other lesion mimicking mutants (LMMs) revealed (in the up-regulated gene set) high similarities with that of *cpr5*, less with that of *hlm1*, and weak similarity with a gene set from cell-death induced cells (Supplementary Fig. S4). Thus, *camta3* mutants share both phenotypic and transcriptome characteristics with other LMMs.

The appearance of the *camta3* phenotypes between the third and fourth weeks coincides with the maximal expression levels of the *CAMTA3* gene in leaves, as determined in transgenic plants harboring a *CAMTA3 promoer::GUS* fusion (data not shown). The molecular evidence shows that in loss-of-function mutants of *CAMTA3*, many known defense-associated genes are up-regulated, whilst very few genes are down-regulated. These findings suggest that CAMTA3 normally suppresses biotic defense responses. CAMTA3 may do so by directly binding to the promoters of the suppressed genes, or by activating the expression of a repressor TF. This suppression is likely alleviated when plants are challenged with pathogens. Alleviation of suppression could result from reduced expression levels of *CAMTA3* in response to pathogens, or through modulation of protein activity by cellular signals. Analysis of microarray data sets (by Genevestigator) derived from *P. syringae* and *B. cinerea* infected plants show minor changes in *CAMTA3* gene expression (<1.5-fold in different experiments). Thus, it

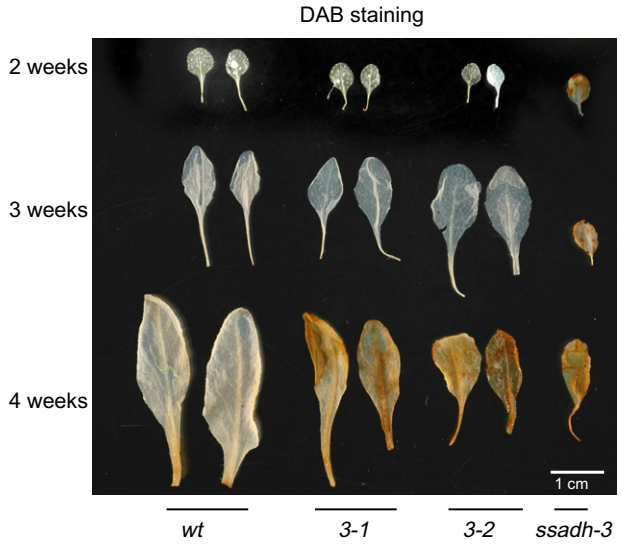

Fig. 6. H_2O_2 accumulation in *camta3* plants. Leaves of 2–4-week-old wild type and *camta3* plants were stained with DAB for the detection of H_2O_2 accumulation as described [14]. The right-end column shows leaves of a succinic semialdehyde dehydrogenase (*ssadh*) mutant as a positive control, since it accumulates high levels of H_2O_2 at all stages of development [14]. The white bar represents 10 mm. The results are from a representative experiment out of three independent experiments. For each line 14 plants and a total of 20–40 leaves were tested.

is possible that modulation of CAMTA3 activity, rather than transcript levels, underlies the alleviation of suppression of defense genes in response to pathogens. Calcium has been shown to be elevated in response to pathogens [17], thus it likely activates CaM and regulates CAMTA3 in this context. Ca^{2+}/CaM could be regulating CAMTA3 negatively, or it could act as a positive regulator of a negative repressor TFs. Interestingly, Choi et al. [18] studied the function of rice CAMTA in *Arabidopsis* protoplasts using synthetic promoters, and found that Ca^{2+}/CaM suppressed CAMTA-mediated transcription activation. In contrast, a *Drosophila* CAMTA that binds target sequences similar to those bound by plant CAMTAs is activated by Ca^{2+}/CaM ([19]; reviewed by Finkler et al. [4]). Therefore, it is possible that Ca^{2+} signaling through CAMTA3 is part of a signaling cascade mediating biotic defense responses.

4.2. Gene networks associated with CAMTA3

An attempt to identify *CAMTA3*-associated gene networks led us to search for common DNA motifs in the putative promoters of the 99 common up-regulated genes in *camta3* mutants, spanning nucleotides 1–500 upstream of their translation start codon. Oligo-analysis of the RSA-tools [20] revealed only four independent and statistically significant DNA motifs (Table S6), which are related to the DNA element 5′-TTGAC(C/T)-3′, known as the W-box. Similar results were found by *MotifSampler* and POBO (Fig. S5, Supplementary Material). The W-box is a *cis*-acting DNA element involved in defense gene activation by the WRKY transcription factors. It is often overrepresented within the promoters of up-regulated *Arabidopsis* defense response genes [10] including the promoters of some *WRKY* genes. In the promoters of parsley *PcWRKY1* and *Arabidopsis AtWRKY33* (*PcWRKY1* orthologue) W-box elements mediate rapid expression of pathogen-mimicking molecules (PAMP) and pathogen-dependent activation of *PcWRKY1* and *AtWRKY33* [13]. Collectively, these results may suggest that CAMTA3 regulates the expression of a TF, such as WRKY, which is responsible for the regulation of many of the up-regulated genes identified in the microarrays of *camta3* mutants.

In summary, our physiological and molecular analyses of *camta3* mutants revealed a role for *CAMTA3* in suppressing biotic defense responses. Although the mechanisms implicating *CAMTA3* in these responses have not been resolved, the molecular and bioinformatic analyses revealed an interesting link between *CAMTA3* and WRKY TFs. A candidate TF that might be regulated by CAMTA3 is WRKY33, whose expression is induced in *camta3* mutants (Table S1) and whose promoter has two putative CAMTA binding sites (not shown). This link may be part of a Ca^{2+} signaling pathways that controls biotic stress responses. However, previous studies revealed enhanced expression of *CAMTA3* (*SR1*) in response to abiotic stresses including heat, cold, NaCl and UV-B [5]. Therefore, we cannot exclude a role for *CAMTA3* in abiotic stress defenses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.02.037. The complete expression data set is available as accession numbers GSM266817 to GSM266822 in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

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