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RESEARCH PAPER

Molecular characterisation of a calmodulin gene, *VcCaM1*, that is differentially expressed under aluminium stress in highbush blueberry

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ABSTRACT

Calmodulin (CaM), a small acidic protein, is one of the best characterised Ca^{2+} sensors in eukaryotes. This Ca^{2+}-regulated protein plays a critical role in decoding and transducing environmental stress signals by activating specific targets. Many environmental stresses elicit changes in intracellular Ca^{2+} activity that could initiate adaptive responses under adverse conditions. We report the first molecular cloning and characterisation of a calmodulin gene, *VcCaM1* (*Vaccinium corymbosum Calmodulin 1*), in the woody shrub, highbush blueberry. *VcCaM1* was first identified as *VCAL19*, a gene induced by aluminium stress in *V. corymbosum* L. A full-length cDNA of *VcCaM1* containing a 766-bp open reading frame (ORF) encoding 149 amino acids was cloned from root RNA. The sequence encodes four Ca^{2+}-binding motifs (EF-hands) and shows high similarity (99%) with the isoform CaM 201 of *Daucus carota*. Expression analyses showed that following Al treatment, *VcCaM1* message level decreased in roots of Brigitta, an Al-resistant cultivar, and after 48 h, was lower than in Bluegold, an Al-sensitive cultivar. *VcCAM1* message also decreased in leaves of both cultivars within 2 h of treatment. Message levels in leaves then increased by 24 h to control levels in Brigitta, but not in Bluegold, but then decreased again by 48 h. In conclusion, *VcCaM1* does not appear to be directly involved in Al resistance, but may be involved in improved plant performance under Al toxicity conditions through regulation of Ca^{2+} homeostasis and antioxidant systems in leaves.

INTRODUCTION

In plants, calcium ions (Ca^{2+}) are important cellular secondary messengers involved in many biological processes, including responses to different stresses (Poutrain *et al.* 2011). Changes in cytosolic Ca^{2+} are sensed by Ca^{2+}-binding proteins, such as Ca^{2+}-dependent protein kinases (CDPKs) and calmodulins (CaMs) (Zhang *et al.* 2002). Calmodulins play key roles in signal transduction pathways, regulating a variety of cellular processes including responses to biotic stresses, gravitropism, phototropism, environmental stresses, as well as growth and development (Yang & Poovaiah 2003; Zhang & Lu 2003; Du & Poovaiah 2005), by modulating the activities of numerous target proteins (Kim *et al.* 2009). Studies with CaM inhibitors have shown that several plant responses to environmental stresses are dependent on CaM. For example, CaM proteins play a role in cold acclimation and freezing tolerance in *Arabidopsis thaliana* (Doherty *et al.* 2009) and tolerance to drought and salinity in *Oryza sativa* (*OsMSR2*) (Xu *et al.* 2011). However, the mechanisms of CaM action at the physiological and molecular levels are not yet well clarified in plants.

Toxicity caused by aluminium (Al) in acid soils is a major environmental stress causing damage to plants (Kochian *et al.* 2005). Aluminium targets multiple cellular sites, resulting in disruption of the structure and function of the cell wall, plasma membrane, cytoskeleton, signal transduction and homeostasis and uptake capability of Ca^{2+} (Rengel & Zhang 2003; Ma 2007; Panda *et al.* 2009). The most obvious symptom is the rapid inhibition of root growth, having a direct effect on the ability of plants to acquire water and nutrients (Pavlovkin *et al.* 2009; Inostroza-Blancheteau *et al.* 2012). Plants have developed mechanisms to cope with Al toxicity, and large differences in tolerance to Al have been reported among genotypes of the same species, as seen in the blueberry, *Vaccinium corymbosum*,

where two cultivars differing in their Al tolerance were found (Reyes-Díaz *et al.* 2009; Inostroza-Blancheteau *et al.* 2011a,b). The current literature characterises plant resistance to Al toxicity as complex and multigenic, and the pathways leading to Al resistance are not well understood in woody plants. In an effort to understand plant differences in Al resistance, we report the cloning and characterisation of a calmodulin gene differentially expressed in two blueberry cultivars, Brigitta and Bluegold, under Al stress. In a previous study employing cDNA-AFLP, Inostroza-Blancheteau *et al.* (2011a) identified a differentially expressed cDNA fragment, *VCAL19*, as having sequence similarity to calmodulin. We report here the first full-length sequence of calmodulin from *Vaccinium*, and characterise its expression in two blueberry cultivars with differing sensitivities to Al.

MATERIAL AND METHODS

Plant material and growth conditions

The *V. corymbosum* cultivars (Brigitta, Al-resistant, and Bluegold, Al-sensitive) and growth conditions were described in Inostroza-Blancheteau *et al.* (2011a,b). Briefly, the plants were conditioned in Hoagland solution for 7 days and then placed in a solution of $CaCl_2$ (0.5 mM) with 0.0 μM or 100 μM Al as $AlCl_3$. The roots were sampled after 0, 2, 6, 24 and 48 h, and quick frozen at −80 °C until use.

Molecular cloning of CaM from *V. corymbosum*

Total RNA was isolated using the protocol of Gambiano *et al.* (2008) as modified in Inostroza-Blancheteau *et al.* (2011a). The 5′- and 3′-untranslated regions of *VcCAM1* were obtained from *V. corymbosum* cv Brigitta root tip total RNA using the GeneRacer Kit (Invitrogen/Life Technologies, Grand Island, NY, USA), essentially as per manufacturer's instructions. The RACE reactions were performed with gene-specific primers designed from the sequence of the *VCAL19* fragment using Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/). The 3′ RACE reaction utilised primer 19 L and the RACE product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. The 5′ RACE reactions were performed using two nested, gene specific primers (Table 1; 19GSP1 and 19GSP2), designed from the *VCAL19* fragment (Accession HO054811). The 5′ RACE product was cloned into the pGEM-T Easy vector and sequenced. Full-length *VcCaM1* was obtained by PCR using *VcCaM1*S and *VcCaM1*R (Table 1), specific primers based on sequences obtained from the RACE reactions. The PCR programme was 95 °C for 5 min, followed by 38 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 40 s for extension. Gene-specific primers used for 3′ RACE and 5′ RACE were designed using Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/).

Table 1. Primer sequences used for this study.

Primer ID	Sequence (5′→3′)
VCAL19-F	TGCTGATGGGAATGGGACTATA
VCAL19-R	CTTGTCGAACACCCGGAAAG
qRT VcCaM1-F	TACCGACGAGGAAGTTGATG
qRT VcCaM1-R	ACTTGGCCATCATGACCTTC
Metallothionein-F[a]	ACCCTGACATGAGCTTCTCG
Metallothionein-R[a]	ACCCAAATCTCTGCTTGCTG
19L	TGAGTTCAAGGAGGCCTTCAGTCT
19GSP1	TCGGGTATCACTTGGCCATCAT
19GSP2	CTTGTCGAACACCCGGAAAG
VcCaM1-F	GATATCTATCGCTCTTGAATTGC
VcCaM1-R	CAGGTTTTACTCAGGACTCATCA

[a]Blueberry metallothionein primer sequences from EST Accession No CF811253, as described in Naik *et al.* (2007).

Multiple alignment and phylogenetic analysis

Sequence homology searches and comparisons were performed using BLAST-X at the National Center for Biotechnology Information (NCBI) network service (http://www.ncbi.nlm.nih.gov/blast). Protein prediction and analysis were performed using the SMART (Simple Modular Architecture Research Tool) domain tool (Schultz *et al.* 1998; Letunic *et al.* 2008). Vector NTI advance 8 was used for multiple sequence alignment. A phylogenetic tree was generated using Clustal X (Larkin *et al.* 2007) and drawn using MEGA 4.1 (Kumar *et al.* 2008). The phylogenetic tree was constructed by the neighbour-joining method.

Quantitative real-time PCR analysis of VcCaM1 transcripts

Total RNA was extracted as above from ca. 250 mg frozen blueberry roots or leaves. RNAse-free DNAase I (Invitrogen) was used to remove contaminating genomic DNA. The integrity of the total RNA was checked by formaldehyde denaturing gel electrophoresis, and the concentration was measured spectrophotometrically using a NanoDrop instrument (NanoDrop TM 1000; ThermoScientific, Wilmington, VA, USA). The purity of the total RNA was assessed using the A260/A280 and A260/A230 ratios. First-strand cDNA was synthesised from 1.0 μg total RNA using 200 U Superscript II reverse transcriptase (Invitrogen) and 1 μl biotinylated oligo-dT25 (700 ng^{-1}).

Real-time RT-PCR analysis of *VcCaM1* expression was carried out using a Stratagene Mx 3000 pTM Real-Time PCR System (Stratagene/Agilent, Santa Clara, CA, USA). The reaction (25 μl) contained 12.5 μl SYBR® Premix Ex Taq (TaKaRa, Shiga, Japan), 0.4 μl forward-specific primer (10 μM), 0.4 μl reverse-specific primer (10 μM) and 2 μl cDNA template. DNA amplification was conducted using the following thermocycling programme: 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, followed by 71 cycles increasing from 57 to 95 °C at increments of 0.5 °C per cycle for 30 s to obtain a melting curve. Blueberry metallothionein (EST Accession CF811253) was used as a reference gene as previously described (Naik *et al.* 2007). Primer pairs (q*VcCaM1*-F, q*VcCaM1*-R and *Metallothionein*-F, *Metallothionein*-R) (Table 1) were designed as above.

Data analysis

The PCR efficiencies of q*VcCaM1* and *Metallothionein* reactions were calculated using MxPro Mx3000 pTM version 3.20 (Stratagene). The values presented are means ± SD of three replicates. Statistical analyses were performed using analysis of variance (ANOVA). Means were compared using Tukey's Test at 95% confidence ($P \leq 0.05$).

RESULTS

Sequence and phylogenetic analysis of *VcCaM1* gene

The sequence of VCAL 19, a 401 bp cDNA fragment previously identified as having homology to calmodulins (Inostroza-Blancheteau *et al.* 2011a), was used to obtain a full-length cDNA clone from the Al-resistant highbush blueberry cultivar, Brigitta. Many different calmodulins have been described in plants, but the structure and size of these proteins is highly conserved at around 148 amino acids. Using 5′ and 3′ RACE, we cloned the full-length cDNA, *VcCaM1*, from roots of plants that had been treated with Al.

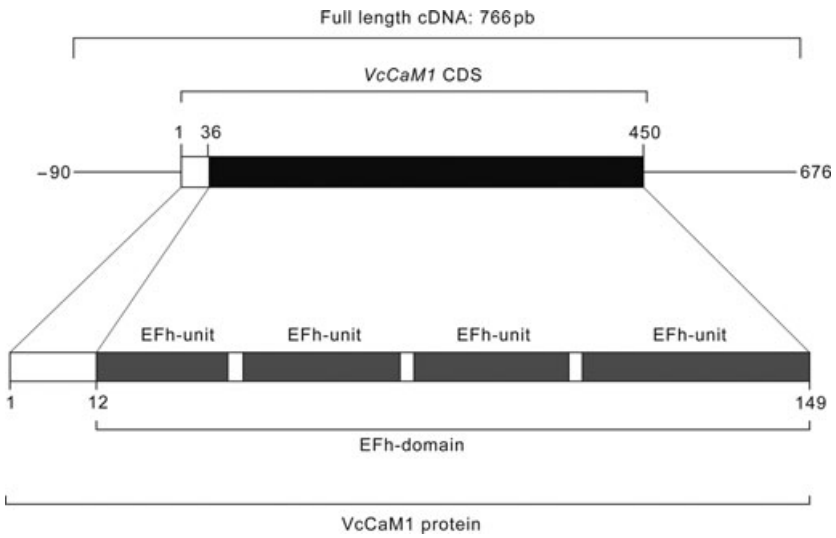

Fig. 1. A schematic representation of *VcCaM1* cDNA sequence (top) and corresponding protein (bottom). The coding sequence (CDS) is shown as white and black boxes and the putative untranslated regions as black lines. The EF-hand units are shown as grey boxes. Numbers refer to a position relative to the first nucleotide (1) of the start codon and numbers of amino acid residues. The full length of *VcCaM1* is 766 bp.

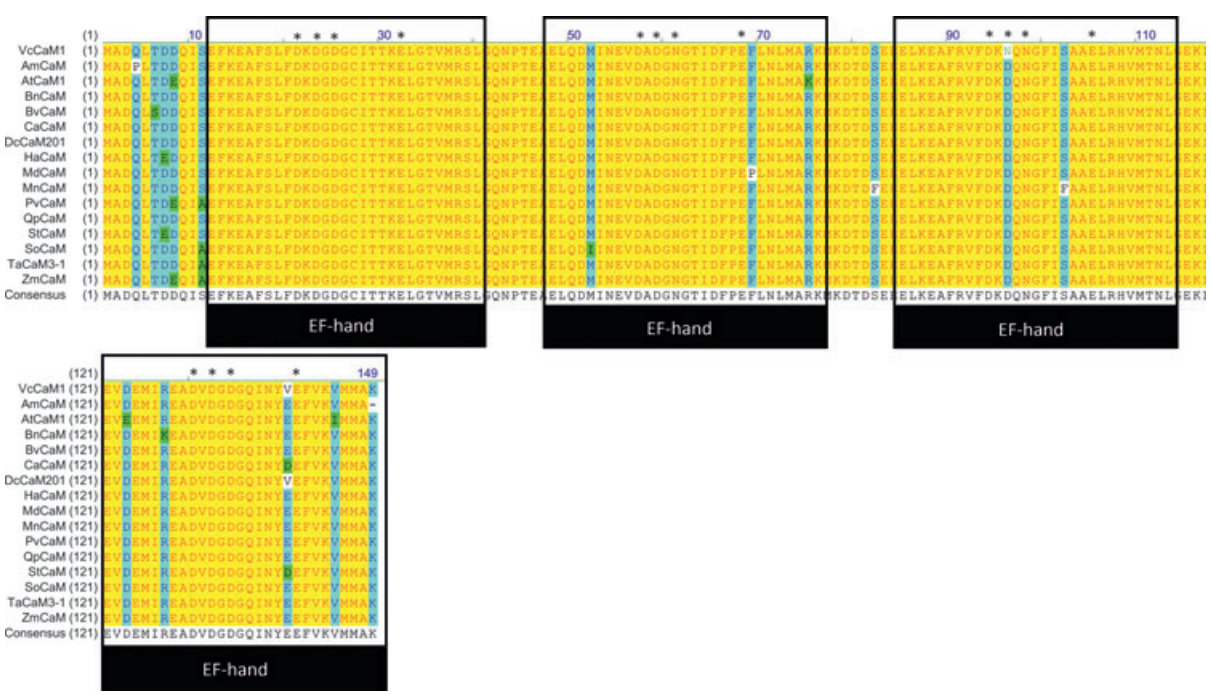

Fig. 2. Alignment of the deduced amino acid sequence of *Vaccinium corymbosum* CaM1 (JX442283) with CaMs from: *Daucus carota* (AAT73614); *Triticum aestivum* (U48689); *Zea mays* (CAA74307); *Saccharum officinarum* (ACT53872); *Capsicum annuum* (AAB46588); *Helianthus annuus* (AAB68399); *Solanum tuberosum* (AAA62351); *Actinidia melliana* (ABR21718); *Beta vulgaris* (ACB32228); *Malus* x *domestica* (CAA43142); *Arabidopsis thaliana* (AAM66012); *Medicago truncatula* (AY649559); *Phaseolus vulgaris* (AAD10246); *Brassica napus* (AAA19571); *Quercus petrea* (CAH57708) and *Morus nigra* (ABS12106). The identical amino acid residues are shaded in yellow. The EF-hand motifs are shown in black boxes and the Ca^{2+} binding sites are indicated by asterisks.

At the protein level, there is a high degree of conservation between animal and plant calmodulins (more than 70% homology; Yang & Poovaiah 2003). A schematic representation of the cDNA sequence of this gene and the encoded protein is shown in Fig. 1. The full-length cDNA is 766 bp in length and the 450 bp ORF encodes a predicted protein of 149 amino acids. *VcCaM1* has four Ca^{2+}-binding EF-hand motifs within the ORF. The 5′-untranslated region of 90 bp upstream from the start codon and a 3′-untranslated region (226 bp) downstream from the stop codon are also shown (Fig. 1). The full-length sequence has been deposited in GenBank with Accession number JX442283.

Multiple sequence alignment of *VcCaM1* shows that the blueberry protein is similar in length and composition to homologous proteins from other species (Fig. 2), and that it shares the same Ca^{2+}-binding domains. *VcCaM1* has 99% identity to homologues in *Daucus carota*, 98% identity to *Solanum tuberosum*, 97% identity to *Morus nigra* and *Actinidia melliana* and 96% identity to *Arabidopsis thaliana*.

A phylogenetic tree of CaM from different plants species was created using neighbour-joining with MEGA 4.1 (Kumar *et al.* 2008). This analysis indicated that *VcCaM1* is most homologous to subgroup I of this conserved family of proteins (Fig. 3). Within this subgroup, *VcCaM1* appears to share more homology with proteins from monocotyledonous than from dicotyledonous species (Fig. 3).

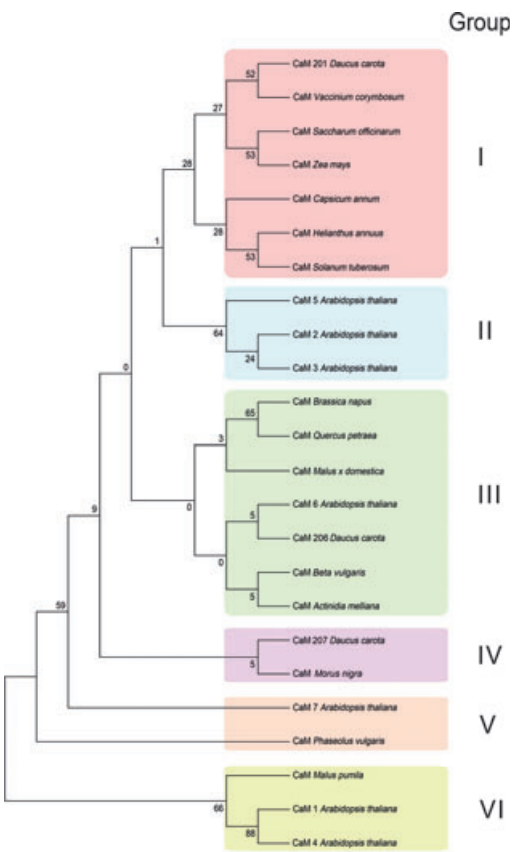

Fig. 3. Phylogenetic tree of *VcCaM1* and CaM proteins from other plant species. The tree was constructed using the neighbour-joining method with MEGA 4.1 (Kumar *et al.* 2008). Branch numbers represent the percentage of bootstrap values in 1000 sampling replicates, and the scale indicates branch lengths. AtCaM1 (AAM66012), AtCaM2 (AAM91152), AtCaM3 (AEE79567), AtCaM4 (AEE34506), AtCaM5 (AEC07926), AtCaM6 (AED92947), AtCaM7 (AEE77831), AmCaM (ABR21718), BnCaM (AAA19571), BvCaM (ACB32228), CaCaM (AAB46588), DcCaM-201 (AAT73614), DcCaM-206 (AAT73619), DcCaM-207 (AAT73620), HaCaM (AAB68399), MdCaM (CAA43142), MnCaM (ABS12106), MpCaM (ADN96172), SoCaM (ACT53872), StCaM (AAA62351), PvCaM (AAD10246), QpCaM (CAH57708), *VcCaM1* (JX442283) and ZmCaM (CAA74307).

Expression of *VcCaM1* in response to Al stress in roots and leaves

Using qRT-PCR analysis, we tested whether the expression of *VcCaM1* in roots and leaves of blueberry was induced by Al stress in two blueberry cultivars with differing Al tolerance, and whether expression correlated with the degree of Al resistance. Unexpectedly, *VcCaM1* message levels decreased in roots within 2 h of Al treatment, and by 6 h, the message levels were lower in Brigitta, the resistant cultivar, than in Bluegold, which is Al-sensitive (Fig. 4A). Message levels remained low in roots throughout the 48h treatment.

The *VcCaM1* message levels in leaves also decreased in response to Al treatment in both Brigitta and Bluegold,

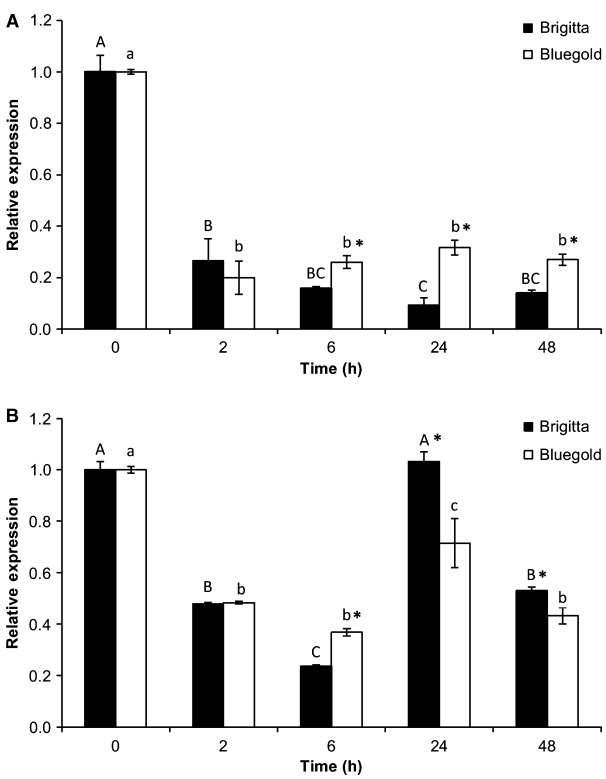

Fig. 4. Real-time PCR (qRT-PCR) analysis of mRNA levels of *VcCaM1* in roots (A) and leaves (B) of blueberry under Al stress. Three independent biological replications were performed. All data were normalised to *metallothionein* expression levels (Naik *et al.* 2007). Capital letters show significant Al treatment differences in the Al-resistant cultivar (Brigitta), and lowercase letters show significant differences in the Al-sensitive (Bluegold) cultivar. The asterisks show significant differences between genotypes at $P \leq 0.05$ according to the Tukey test.

however after 24 h of treatment, message levels had increased (Fig. 4B). In Brigitta the level at 24 h was essentially the same as before treatment; compared to the level in Bluegold, it did not appear to increase as much. After 48 h of treatment, the *VcCaM1* message levels had decreased again in both cultivars.

DISCUSSION

The cloning and characterisation of full-length sequence of the calmodulin gene (*VcCaM1*) differentially expressed in blueberry under Al stress was performed for the first time in this plant species. The major class of Ca^{2+} sensors identified in plants is CaM, which is a ubiquitous Ca^{2+}-binding protein highly conserved in eukaryotes (Yang & Poovaiah 2003). These proteins contain four repeating units, called EF-hands, with a high-affinity Ca^{2+}-binding motif. Once Ca^{2+} binds to the EF-hand domains, CaM alters its structure (Wriggers *et al.* 1998) due to hydrophobic surfaces that serve to interact with target proteins. Protein sequence alignment showed only a few differences in amino acids among the CaM isoforms of different species, and like the other homologues, *VcCaM1* also has the highly conserved calcium-binding EF-hand domains (Fig. 2; see also Nath *et al.* 2010). This motif plays an essential role in eukaryotic cellular signalling (Strynadka & James 1989; Nelson & Chazin 1998) and homeostasis of Ca^{2+}.

Phylogenetic analysis showed that *VcCaM1* had the highest similarity (99%) to carrot *CaM201* gene, and 96% with *CaM7* of *Arabidopsis* (Fig. 2). Similar results have been found in studies describing different isoforms in plants, such as *CAM1* and *CAM2* in *Catharanthus roseus*, each having 149 amino acids and four Ca^{2+}-binding domains and close homology (>91%) with *Arabidopsis* CAM isoforms (Poutrain *et al.* 2011). The dendrogram shows that *VcCaM1* diverges from clusters IV, V and VI, while converging into two big (I, III) and one small cluster (II) with over 95% similarity (Fig. 3). Within cluster I, *VcCaM1*, which is from a woody plant, shows more similarity with monocotyledons that dicotyledons.

Interaction with Al may affect the structure of CaM and its affinity for Ca^{2+}, thereby affecting signalling pathways within the cell (Kurita *et al.* 2005). Some CAM genes show altered temporal and spatial expression in response to environmental stresses, such as NaCl, heat shock, cold, light, pathogens and hormones (Townley & Knight 2002; Park *et al.* 2004; Chang *et al.* 2006), and in this work we describe differential gene expression following Al treatment. In *Arabidopsis*, elevated Ca^{2+} directly activates *AtCaM3*, which in turn stimulates *AtCBK3*, ultimately regulating the phosphorylation and DNA binding activity of heat-shock transcription factors (HSFs) (Liu *et al.* 2008; Zhang *et al.* 2009). These reports point to calmodulin being active in plant response to stress. In *V. corymbosum* leaves, the level of *CaM1* message decreased over the first 6 h of treatment, but by 24 h had increased to control levels in cv Brigitta (Al-resistant; Fig. 4). These results, and the decrease in message levels in roots in response to Al, indicate that Al is affecting gene expression and is not directly involved in Ca^{2+} signalling processes.

Calmodulins exist as a gene family, individual members of which can show different expression patterns. In *Vigna radiata*, *MBCaM-1* and *MBCaM-2* encode essentially identical proteins but show differential expression patterns (Botella & Arteca 1994). Similar findings were reported for *NpCaM-1* and *NpCaM-2 in Nicotiana plumbaginifolia*, which are identical proteins, but gene expression responds to different stimuli (van Der Luit *et al.* 1999). At this time, it is unknown how many CaM genes there are in *V. corymbosum*, and whether other CaM genes will show a different response to Al than *VcCaM1*.

Our results support findings from maize showing that calmodulin is neither relevant to the Al tolerance in maize, nor a primary target for Al toxicity (Jorge *et al.* 2001). Our data show that the expression of *VcCaM1* in response to Al stress is different in leaves as compared to roots, but that the response in roots of the two cultivars did not vary in a manner that could explain their different Al resistance. The expression patterns in leaves, however, do not rule out that *VcCaM1* could be important in various physiological processes and responses to stress and that *VcCaM1* may have indirect participation in Al resistance in shoots of cv Brigitta. Further studies overexpressing this gene in a model plant species may shed light on a role for this protein in response to Al stress.

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