Title: **Role of calcium in the defense response induced by brassinosteroids in strawberry plants**

Running title: **Importance of calcium in brassinosteroid-signaling**

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Role of calcium in the defense response induced by brassinosteroids in strawberry plants

Highlights

- Calcium plays a key role in the defense induced by brassinosteroids in plants.
- Calcium-sensor calmodulin-like (CML) proteins were identified in *Fragaria ananassa*.
- Brassinosteroids induce overexpression of *CMLs* and other defense related genes.

Abstract

Brassinosteroids (BRs) are steroidal hormones essential for the normal growth and development of plants. The aim of this work was to study the role of calcium, in the defense response induced by two BRs e.g. BB16 and EP24, and to investigate the expression levels of defense-related genes in strawberry plants treated with these BRs. An increase in the calcium influx in response to treatment with BB16 or EP24 was observed. By comparing *Fragaria vesca* and *Fragaria ananassa* genome sequences 28 nucleotide sequences coding for calcium-sensing proteins called CMLs were predicted. Bioinformatic analyses of the sequences revealed that all of them contained the characteristic EF-hand motif of Ca^{2+} binding, and regulatory *cis*-elements related to stress. qPCR studies showed that both BB16 and EP24 cause the upregulation of selected *CMLs* genes *e.g. FaCML1a*, *FaCML36*, *FaCML42* and *FaCML45*, and defense marker genes, such as *PR1*, *ERF1* and *GLS5*. Results also showed that whereas BB16 induced the upregulation of *MYB30,* EP24 induced the upregulation of *PRX27*. These results provide valuable information of the response mechanisms induced by BRs and demonstrate the role of calcium in the signaling pathways induced by BRs in strawberry plants.

Keywords: Brassinosteroids; Calcium; Calmodulin-like; Defense; *Fragaria ananassa*.

1. Introduction

To defend themselves against pathogens, plants have developed sophisticated mechanisms to perceive the attacker and convert that perception into signals that trigger an effective defense response [1].

It has been demonstrated that Ca^{2+} mediated signaling plays an important role in the response of plants to stress [2]. It has been shown that a transient increase in Ca^{2+} concentration is detected by several Ca^{2+} sensors or Ca^{2+} binding proteins, regulating downstream targets as part of a coordinated cellular response to specific stimuli. It has also been observed that these Ca^{2+} binding proteins usually have helix-loop-helix structures called EF-hand motifs.Three main classes of Ca^{2+} EF-hand containing sensors have been characterized in plants so far, namely: calmodulin (CAM), Ca^{2+}-dependent protein kinase (CDPK) and calcineurin B-like protein (CBL) [3]. Calmodulin (CaM) is considered to be one of the most conserved Ca^{2+} binding proteins in all eukaryotes. The binding of Ca^{2+} to CaM induces a conformational change, which allows the interaction of the CaM/Ca^{2+} complex with target proteins.

In addition to several CAMs, plants also possess another large family of Ca^{2+} binding proteins called calmodulin-like (CML) proteins. These proteins extend the range of potential calcium sensors present in plant cells. The structure of CMLs diverges from the structure of typical CaM proteins, suggesting that these proteins must have particular functions. Gene expression profiles exhibit a wide diversity of expression patterns of CAM and CML genes through the plant development program, or in response to external stimuli [4].

Xu et al. [5], studied the expression pattern of the gene OsMSR2 (*Oryza sativa* L. Multi- Gen-Stress Responsive 2), a new calmodulin-like protein, whose gene was isolated from rice (*Oryza sativa* L.). They observed that *OsMSR2* was strongly upregulated by a wide spectrum of stresses, including cold, drought and heat in different tissues and at different stages of rice development. It was also shown that the expression of *OsMSR2*in Arabidopsis modulates salt tolerance and drought through ABA-mediated pathways, and these transgenic plants exhibited hypersensitivity to ABA during germination of seeds and the stages after germination. Chiasson et al. [6] characterized a tomato gene (*APR134*) encoding a calmodulin-like protein that is induced in leaves of plants resistant to *Pseudomonas syringae* pv. *tomato*. They demonstrated that the suppression of *APR134* expression in tomato (*Solanum lycopersicum*), using virus induced gene silencing (VIGS), compromises the immune response of the plant. By investigating the presence of the *APR134* gene in Arabidopsis they could identify two

orthologous genes called *CML42* and *CML43*. They observed that *CML43* is rapidly induced in Arabidopsis leaves resistant to diseases after inoculation with *Pseudomonas syringae* pv. *tomato*. These data support a role for *CML43* and *APR134* as important mediators of Ca^{2+}-dependent signals during the immune response of plants to bacterial pathogens.

In a previous work, we have shown that the treatment with BB16 induces the stomatal closure in *Fragaria ananassa* [7]. Since the relationship between calcium influx and stomatal closure was clearly demonstrated by Gilroy et al.[8], and Ward and Schroeder [9], in this paper we decided to investigate the participation of Ca^{2+} on the effect observed of the brassinosteroids BB16 (a formulation based on a brassinosteroid spirostanic analogue DI-31) and EP24 (24-epibrasinolide) in strawberry plants.

The defense response is usually associated with the expression of several biochemical and molecular factors. Furio et al. [7] have analyzed the biochemical markers associated with the defense response induced by BRs in strawberry, showing that they induced an oxidative burst, increase in intracellular nitric oxide (NO), stomatal closure, cell wall reinforcement by lignin and callose deposition, and accumulation of calcium oxalate crystals. However, molecular analysis of the genes involved, and calcium participation at the onset of the defense response mediated by BRs were not performed.

The objective of this work was to evaluate the participation of calcium in the activation of the defense response activated by BB16 and EP24, studying the ion influx and the role of CMLs. Since there was scarce information about CMLs proteins in strawberry, a bioinformatics approach was carried out to identify and predict CML sequences of *Fragariaananassa* that have not been characterized yet. The expression of defense-related genes in strawberry plants treated with both BRs was also studied.

2. Materials and methods

2.1. Plant material

Strawberry plants (*Fragaria ananassa*) of the cv. Pájaro were obtained from the strawberry BGA (Strawberry Active Germplasm Bank at Universidad Nacional de Tucumán). Healthy plantlets were obtained from *in vitro* cultures in MS medium (Sigma), rooted in pots with sterilized substrate (humus and perlite, 2:1), and maintained at 28°C, 70% relative humidity (RH), with a light cycle of 16h (white fluorescent, 350 μmol photons m^{-2} s^{-1}).

2.2 Plant treatment

Three months old plants containing three or four fully expanded leaves were used in experiments. Batches of 3 plants constituted the experimental unit, were sprayed to run-off with BB16 (0.1 mgl^{-1}), EP24 (0.1 mg l^{-1}) or water (control), and maintained as indicated above until picking leaf samples. Young totally expanded leaves of treated plants were harvested at different times for RNA extraction and qPCR assays as indicated below. For intracellular calcium determinations leaf discs were obtained from not-treated plants, and treated afterward with BB16 (0.1 mgl^{-1}), EP24 (0.1 mg l^{-1}) or water (control) separately as indicated below.

2.3. Calcium determination

Accumulation of intracellular calcium was measured in leaf discs using the fluorescent probe Fura2-AM (Invitrogen) according to Martos et al. [10]. 5 mm diameter discs were obtained from freshly expanded young strawberry leaves, and 10 leaf discs from 5 plants treated with BB16 or EP24 were used. The discs were placed in test tubes with 1000 μl of W5 buffer and 15 μM Fura2-AM, 5 pulses of vacuum were applied and they were allowed to stand in the dark for 3 hours. The discs were then rinsed twice with distilled water and placed in a multi-well plate with 100 μl of the W5 buffer per well. Immediately before each measurement, BB16 (0.1 mgl^{-1}), EP24 (0.1 mg l^{-1}) or water (control) was added. The fluorescence excitation spectra from 350 nm to 420 nm were obtained at λem = 532 nm using a photon counting spectrofluorometer (ISSAPC1, Owingen, Germany). The calcium level was expressed as the ratio between the relative fluorescence intensities measured at 362 nm and 402 nm. The spectra were obtained every 30 s for 10 min. The spectra did not change in the analyzed time range. Presented values correspond to the first measurement after 30 s.

2.4. Bioinformatic analysis: CML homologous searching and gene prediction

To find *CML* homologous, the Blast algorithm was used, searching by comparison (alignments) of the nucleotide sequences with the sequences deposited in the GenBank NR database. To find *CML* homologous, a search on the genome of *Fragaria ananassa* was carried out using the BlastN homologous search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and taking as reference *CML* sequences already predicted of *Fragaria vesca* and annotated in the GenBank (https://www.ncbi.nlm. Nih.gov/genbank/). The sequence analysis let us find "contigs" that contained sequences with highly similar to the CMLs of *Fragaria vesca* used as a query. Amino acid sequences of FaCML messengers found were *in silico* predicted using the FGenesh gene prediction software [11]. **S**equence assemblies were made with the DNAMAN program [12]. The presence of structural motifs and/or conserved domains was determined by the CDSearch (from NCBI) [13] and SMART [14] programs. Multiple alignments of amino acid sequences were performed by Clustal X [15]. Molecular phylogeny analysis was performed from amino acid alignments with the MEGA 4 software [16], using the nearest neighbor method (Neighbor Joining, NJ) [17]. The reliability of tree branches was evaluated using the Bootstrap method [18].

Bioinformatic analysis of the 5'UTR of the predicted sequences was carried out to identify conserved promoter motifs related to stress responses in plants by using the PLACE (Plant cis-acting regulatory DNA elements) algorithm [19] and Softberry program [20].

2.5. RNA extraction and qPCR analysis

Total RNA was obtained from the youngest totally expanded leaflet of three BRs-treated or control not treated plants (n=3. Leaflets were harvested at 2, 4, 6, 12, 24, 48, and 72 hours post treatments (hpt), pooled, weighted, immediately frozen in liquid nitrogen, pulverized and kept at −80°C, until further use. 80 mg of leaf powder was used for RNA extraction with the RNAqueous-4PCR kit (Ambion, USA). RNA concentration and purity was assessed spectrophotometrically at 230, 260 and 280 nm (BioSpecmini, Shimadzu). Retrotranscription was carried out using the SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer instructions. qPCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) in a 7500 Real-Time PCR System (Applied Biosystems). Values of the gene expression level were normalized using the Elongation Factor-1a (*FaEF1α*) gene (GenBank

152 accession number: XM_004307362) according to [21,22]. PCR primers efficiency and Ct
153 values were calculated using the LinRegPCR software as recommended by Ramakers et al.
154 [23], and profiles were estimated taking the gene*FaEF-1α* as reference and using the
155 fgStatistics software [24], and the algorithms suggested by Pfaffl [25]. Two biological
156 replicates with three technical replicates were used for each treatment. Gene expression was
157 expressed as the ratio between BRs and control expression levels.

158 **2.6. Primers Design and genes analyzed**

159 Primers sequences were obtained from the *F. ananassa* nucleotide sequences freely available
160 at the NCBI GenBank website (https://www.ncbi.nlm.nih.gov/genbank/), and optimized using
161 the Primer Express software (Applied Biosystems, USA) (see Table 1). The primers were used
162 to evaluate the expression level of genes associated to the defense response, and selected
163 Fa*CMLs* in strawberry plants treated or not with BRs. The defense genes studied were: i)
164 *FaPR1*, encoding the Protein Related to Pathogenesis 1; ii) *FaMYB30*, HR marker gene; iii)
165 *ERF1*, transcription factor of the ET signaling pathway iv) *ETR2*, ET receptor, v)
166 *FaPRX27*encoding a plant peroxidase class III, and vi) *FaGSL5* involved in the synthesis of
167 callose. Four *CMLs* genes were selected to study the effect of BRs on their level of expression,
168 one containing 2 EF-hand motif (*FaCML45*), two with 3 EF-hand motifs (*FaCML42*,
169 *FaCML36*) and one exhibiting 4 EF-hand motifs (*FaCML1a*).

170 **2.7. Accession Numbers**

171 The following sequences: *FaCML, FaCML1a, FaCML1b, FaCML2, FaCML3, FaCML7a,*
172 *FaCML7b, FaCML8, FaCML11, FaCML13, FaCML15, FaCML18, FaCML19, FaCML21,*
173 *FaCML23, FaCML24, FaCML25, FaCML27, FaCML29, FaCML36, FaCML38, FaCML41,*
174 *FaCML42, FaCML44, FaCML45, FaCML47, FaCML48* and *FaCML49* are available in the
175 Third Party Annotation Section of the GenBank database under the accession numbers:
176 MK965998-MK966025, respectively.

177 **3. Results**

178 **3.1. Calcium influx**

179 Evaluation of the intracellular calcium content in strawberry leaves treated with
180 brasinosteroids revealed a higher level of accumulation in the strawberry plants treated with

BB16 or EP24 as compared to the control water treated plants (Fig. 1). Nevertheless, the influx of Ca^{2+} was greater when EP24 was used compared to BB16. The intracellular calcium content in plants treated with EP24 or BB16 was 118% and 40% higher to plants treated with water, respectively.

3.2. Analysis of the amino acid sequences of the FaCMLs.

Bioinformatic investigation let us to identify 28 CMLs sequences in *F. ananassa* genome. Both programs used for the identification of structural motifs (e.g. CDSearch and SMART) coincided in the identification of EF-hand motifs for calcium binding in all the FaCML sequences analyzed. The only difference observed among them consisted in the number of EF-hand motifs contained in each protein; FaCMLs containing 2, 3 or 4 EF-hand motifs were found. In figure 2 we present the alignment of the EF-hand motif domains of three FaCML sequences showing 2, 3, or 4 EF-hand motifs, selected at random just to show the structural differences observed. The sequences obtained from *F. ananassa* were then compared with *F. vesca* paralogs, and results showed an identity higher than 95%. The difference observed consisted in few short gaps (up to 22 aa), but the number of FvCMLs and EF-hand motifs contained were conserved (data not shown).

3.3. Molecular phylogenetic analysis

With the amino acid sequences of the CMLs predicted in *F. ananassa*, a molecular phylogenetic analysis was performed. In the phylogenetic tree obtained we observed that the FaCMLs proteins are grouped into three separate clades according to the number of EF-hand motifs, all of which were supported by high bootstrap values (Fig. 3). Sequences that have two EF-hand motifs (red frame) clustered in the phylogenetic clade I. Within this clade, FaCML47 sequence prediction resulted incomplete because the FvCML47 of *F. vesca* permitted only the identification of very small contigs in *F. ananassa* (Fig. 3). Nevertheless, we assumed that it would be a protein with two EF-hand motifs because the *F. vesca* orthologous has two EF-hand motifs. In the case of FaCML21, although it presents four EF-hands it was included in the clade I. The later may be possible due to the statistic weight attributed by the NJ method to the rest of the sequences out of the EF-hand domains.

Sequences that contained mainly three EF-hand motifs (blue frame) were grouped in an heterogeneous phylogenetic clade II. In this clade, although CMLs with only three EF-hands were clearly detected (blue solid-line frame), there were some CMLs that may also present a

possible fourth EF-hand motif (blue dotted-line frame), but with a high E-values suggesting that the amino acid sequences differ from the consensus motif; hence we considered that these proteins contained only 3 EF-hand motifs (Fig. 3). In Figure 3 we can also see that some FaCMLs (*e.g.* FaCML8, FaCML11, FaCML15 and FaCML8) were included in this clade although they exhibit four RF-hands (green frame). In these cases we also assumed that it is due to the same reason mentioned above, e.g. the statistic weight attributed by the NJ method to the rest of the sequences out of the EF-hand domains. The clade III contains only four EF-hand motif CMLs (green frame, Fig. 3). In Table S2 we present the nucleotide (bp) and amino acid (aa) sizes of the FaCMLs predicted according to number of EF-hand exhibited. The position of the EF-hand motifs on the amino acid sequences are also indicated (Table S1).

3.4. Regulatory cis-elements associated to stress response

When analyzing the 5'UTR of all the sequences corresponding to the predicted *FaCML*s, we found some regulatory *cis*-elements directly associated to the response to biotic and abiotic stresses (Table 2). In the supplementary figure (S2), the position of each regulatory element is detailed with respect to the initiation codon of each of the *FaCMLs* genes. Since the 5'UTR sequences suggest that they may be regulated by multiple factors, we decided to investigate whether the BRs studied would also affect the transcription levels of *FaCML*s. In Table S2 we present the location of each regulatory *cis*-element mentioned in Table 2, found at the 5'UTR *FaCML*s sequences. Only 22 *FaCML* were included, excluding the remaining 6 genes (*FaCML, FaCML21, FaCML23, FaCML24, FaCML29, FaCML49*), since the "contigs" of *Fragaria ananassa* that included them were not large enough, to analyze the 5'UTR region and find regulatory elements.

3.5. Expression of FaCMLs in response to treatment with BRs

To evaluate the effect of the BRs on the expression of *CMLs*, only four of them were selected to continue the study because their sequences allowed us to obtain better primers, which in turns increased the reliability of the assay. For the selection of *FaCMLs* we also took into account genes encoding proteins that have different number of EF-hand motifs, namely: FaCML1a, FaCML42, FaCML45, and FaCML36. FaCML1a corresponds to a protein with four EF-hand motifs, FaCML42 with three EF-hand motifs, FaCML45 with two EF-hand motifs, and FaCML36 with three EF-hand motifs plus an altered fourth one (Fig. 3). Results obtained show that the level of expression of each *FaCML* depends on the particular BRs and

the time elapsed after the treatment (Fig. 4). Whereas both BRs induced the upregulation of *FaCML42* between 2 and 6 hpt, BB16 induced the upregulation of *FaCML36* and *FaCML1a* at 4 hpt, and EP24 caused the upregulation of *FaCML45* at 6 hpt (Fig. 4).

3.6. Molecular analysis of defense marker genes in strawberry plants treated with BRs

Taking into account the results obtained in this and previous papers [7,26], and with the aim to assess the effect of BRs on the activation of a defense response, the expression of the genes *PR1*, *MYB30*, *ERF1*, *ETR1*, *FaPRX27* and *FaGSL5* were analyzed at different times post-treatment (hpt). Results showed that the expression level of the genes studied depends on the particular BRs used and the time elapsed after the treatment (Fig. 5). In that figure we can also see that strawberry plants treated with both BRs induce the expression of the *PR1* gene at 72 hpt, *ERF1* at 12 hpt, and *GSL5* at 24 hpt. In addition, BB16 induced an increase of the expression level of the *MYB30* gene at 12 hpt, and only plants treated with EP24 exhibited the upregulation of *PRX27* at 48 hpt, and *ERF1* at 72 hpt (Fig. 5).

4. Discussion

Previously, we have shown that strawberry plants treated with BB16 or EP24 activated a cascade of biochemical and physiological events, such as oxidative burst, production of nitric oxide (NO), stomatal closure, cell wall reinforcement, and accumulation of calcium oxalate crystals [7]. However, calcium involvement and genes associated to a defense response were not evaluated.

It is well known that the change of intracellular calcium concentration is a key signal for many cellular processes, including the activation of plant defenses against biotic or abiotic stresses, and other processes, such as the cell wall strengthening reported by Figueroa et al. [27] which prevents the cell wall degradation of strawberry fruits.

Changes of Ca^{2+} concentration are perceived by different proteins being calmodulins (CaM) one of the most well characterized ones. In plants there is also a family of proteins called calmodulins-like (CMLs), but their physiological functions and regulation are not completely established. It was reported that the protein AtCML24 of Arabidopsis plays an important role in the response to photoperiod, ion-homeostasis, and ABA-mediated inhibition of seed germination and growth [28]. It was also shown that the Arabidopsis genes At*CML37*, At*CML38* and At*CML39* were upregulated in response to various biotic and abiotic stimuli [29], and that the constitutive expression of the soybean *SCaM4*, and *SCaM5* genes in tobacco,

caused the increase of the resistance against a broad spectrum of virulent and non-virulent pathogens [30]. This information stimulated us to investigate whether plants treated with EP24 or BB16 provoked a change of the intracellular concentration of Ca^{2+}.

Results obtained in this paper showed that both BRs induce the increase of the intracellular concentration of Ca^{2+} after the treatment. Thus, we were interested to investigate whether the transcriptional level of the *FaCMLs* would also be affected. However, since *CMLs* genes were not reported in *F. ananassa* a bioinformatic investigation was performed to identify the orthologous genes using as reference sequences of *F. vesca*. Outcomes of this analysis let us to identify 28 genes in *F. ananassa*, and with the amino acid sequence to predict the corresponding FaCMLs. Further structural analysis showed that all FaCMLs predicted presented the characteristic Ca^{2+} binding EF-hand amino acid motif, varying only in the number of EF-hand motifs present in each protein. Each motif consists in a 12-residue loop that undergoes a conformational change when binding Ca^{2+} leading to the activation (or inactivation) of target proteins [31].

With the FaCMLs amino acid sequences we performed a phylogenetic analysis and results showed that they can be grouped in three clear clusters according to their EF-hand motifs. However, few of them, namely: FaCML8, FaCML11, FaCML15, FaCML18, and FaCML21 did not fit into the groups. One possible hypothesis, already mentioned above, is that the NJ method used attributed more statistic weight to the rest of the sequences out of the EF-hands. Another plausible hypothesis would be that some of these CMLs may suffer post-translational modifications (i.e. formation of disulfide bonds) that may affect the formation of an EF-hand, as reported by Khan et al. [32]. This analysis further revealed that although the EF-hand motif is an important structural feature conserved in the molecular evolution of these proteins, the rest of the amino acid sequence is also important and may be implied in the interaction with other molecules or cellular structures participating in its regulatory feature. Some evidence supporting the latter hypotheses can be found by analyzing the information presented in Table S2. In that table we can observe that the positions of the EF-hands motifs on the FaCMLs amino acid sequences are not regular, suggesting that although the EF-hands motifs can be clearly identified, the rest of the protein is not comparable in many cases.

The identification of the *FaCMLs* sequences on the *F. ananassa* genome also allowed us to analyze the 5'UTR sequences that may correspond to putative regulatory *cis*-elements. In plants, there are more than 1500 trancriptional factors (TFs) reported, controlling the

306 expression of genes in complex signaling networks [33]. From the *CMLs* nucleotide sequences
307 predicted in *Fragaria ananassa*, we could identify regulatory elements that participate in the
308 defense response against biotic and abiotic stresses (Table 2). Since these regulatory elements
309 suggested that FaCMLs are regulated by plant defense signals, the effect of BRs on the
310 expression levels of selected *FaCMLs* and defense associated genes were investigated. Results
311 confirmed that EP24 and BB16 are able to regulate the expression of *FaCML1a*, *FaCML36*,
312 *FaCML42* and *FaCML45* in a complex manner depending of the particular BR used and the
313 time elapsed after the treatment (Fig. 4).
314 It has been reported that the CMLs have numerous functions in the perception of stress and in
315 the development of plants [34]. Vanderbeld and Snedden [29] showed that the Arabidopsis
316 genes *AtCML37*, *AtCML38* and *AtCML39* are regulated by biotic and abiotic stresses, as well
317 as by hormonal and chemical treatments. They observed a strong and early upregulation
318 (usually within the first 24 hours) of these *CMLs* in response to wounds, osmotic stress,
319 drought and infections with bacterial pathogens. Similarly, Braam [35] analyzed the
320 expression of three *TCH* genes, encoding for CMLs, and observed a rapid upregulation in
321 response to stimuli such as touch, wind, rain, wounds and darkness. Likewise, Tomas-Grau et
322 al. [36] also reported that a soft mechanical stimulation (SMS) induced in strawberry plants
323 the upregulation of *TCH* genes encoding for CML proteins, demonstrating the importance of
324 these CMLs in signal transduction.
325 By analyzing the information presented in Table 2 and Table S2, we would notice that from
326 the nine regulatory elements detected at the *FaCML*s 5'UTR sequence, three associated to
327 biotic (pathogen) responses (e.g. WRKY71OS, BIHDIOS, WBOXATNPR1) and three
328 associated to abiotic response (e.g. EBOXBNNAPA, MYBIAT, GTICONSENSUS) were
329 present in most of the FaCMLs analyzed, whereas the other regulatory *cis*-elements (e.g.
330 BOXLCOREDCPAL, ASFIMOTIFCAMV, ABRERATCAL) were found in lower frequency.
331 (Table S2). However, all FaCMLs analyzed exhibited regulatory cis-elements of both types.
332 This information let us conclude that the expression of FaCMLs is tightly controlled by many
333 regulatory *cis*-elements and there is no apparent correlation between the number of EF-hand
334 motifs and the regulatory elements found.
335 When evaluating the expression of some defense associated genes in plants treated with EP24
336 or BB16 different expression patterns were observed (Fig. 5).The results show that strawberry

plants treated with any of the BRs tested induce the expression of the *FaPR1* gene at 72 hpt (Fig. 5a), suggesting the participation of SA signaling pathway [37,38,39,1].

The upregulation of *FaMYB30* at 12 hpt (Fig. 5b) in response to treatment with both BRs, suggests its participation during the hypersensitivity response (HR) [40,41]. The relationship between ROS and HR was established many years ago, when Doke [42] reported the production of superoxide previous to the HR caused by *Phytophthora infestans* and the tobacco mosaic virus, in potatoes and tobacco, respectively.

Polymerization of lignin is regulated by class III peroxidases, which are induced by different stimuli, and biotic stress [43,44]. Specifically, the peroxidase PRX27 is a functional enzyme that is required for the polymerization of phenylpropanoids during the formation of lignin in the maturation of strawberry fruits [45]. In this study, however, we observed that the *FaPRX27* gene was induced at 48 hpt, in strawberry leaves after EP24 treatment (Fig. 5e). These data suggest that this peroxidase participates in the defense response induced by EP24 catalyzing the cross-linking of monolignoles in the cytosol, contributing to the final formation and accumulation of lignin in leaves. However, we did not observe this effect in plants treated with BB16. Hence, we may speculate that the induction of this gene occurs at times that were not studied, or that the observed production of lignin in response to BB16 occurs through the action of other peroxidases, or laccases, which were not analyzed in this study [46]. When studying the level of expression of the *FaGSL5* gene, we observed that the callose synthase is upregulated in response to both BRs (Fig. 5f), confirming that the deposition of callose is part of the defense response induced by these compounds.

The role of ET as a signal molecule involved in the activation of defense mechanisms has been confirmed by numerous studies [47]. When studying genes related to the ET signaling pathway, a clear upregulation of *FaERF1* [48] was observed 12 hpt in response to both BRs, and 72 hpt in response to EP24 (Fig.5c). It is well known that the ERF1 factor initiates a transcriptional cascade that results in the activation or repression of many genes involved in the regulated the ET signaling pathway [49]. Additionally, ERF1 is also a key integrator of the signaling pathways of JA involved in the regulation of defense response genes such as *b-CHI* and *PDF1.2* [50]. The upregulation of *FaERF1* may indicate, on one side, a strong activation of genes involved in the synthesis enzymes ACS and ACO, and on the other side, the possible activation of the JA signaling pathway in plants treated with BRs. The downregulation of

368 *ETR2* observed after 48 hpt (Fig. 5d) may be explained by the fact that the ET receptor
369 proteins are a negative regulators of the pathway [51,52].
370 The upregulation of genes related to ET signaling pathway, such as *ERF1*, and genes related to
371 the SA pathway, such as *PR1*, suggest that the BRs may activate both plant defense signaling
372 response but in a time dependent manner confirming that there is a complex cross-talk among
373 hormones modulating plant immunity [53].
374 This work unveils the importance of Ca^{2+} in the defense response induced by EP24 and BB16
375 in strawberry plants opening new ways to investigate the mechanisms by mean of which BRs
376 activates the plant innate immunity.

Notes

Authors' contributions

RNF, SMP and GGM are CONICET fellowship, and MGMZ and JCDR are members of CONICET. RNF performed the experiments, analyzed and interpreted the data. MGMZ collaborated in the bioinformatics analysis, SMP participated in real time assays and GGM collaborated with calcium influx assay. RNF wrote the manuscript, and JCDR and MGMZ critically reviewed the article. All authors approved the final version of the manuscript.

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Compliance with ethical standards

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest: The manuscript has not been published and is not under consideration for publication elsewhere. All authors have approved the manuscript and agreed to submit the paper to Scientia Horticulturae. The research was conducted in the absence of any commercial relationships that could be considered as a potential conflict of interest.

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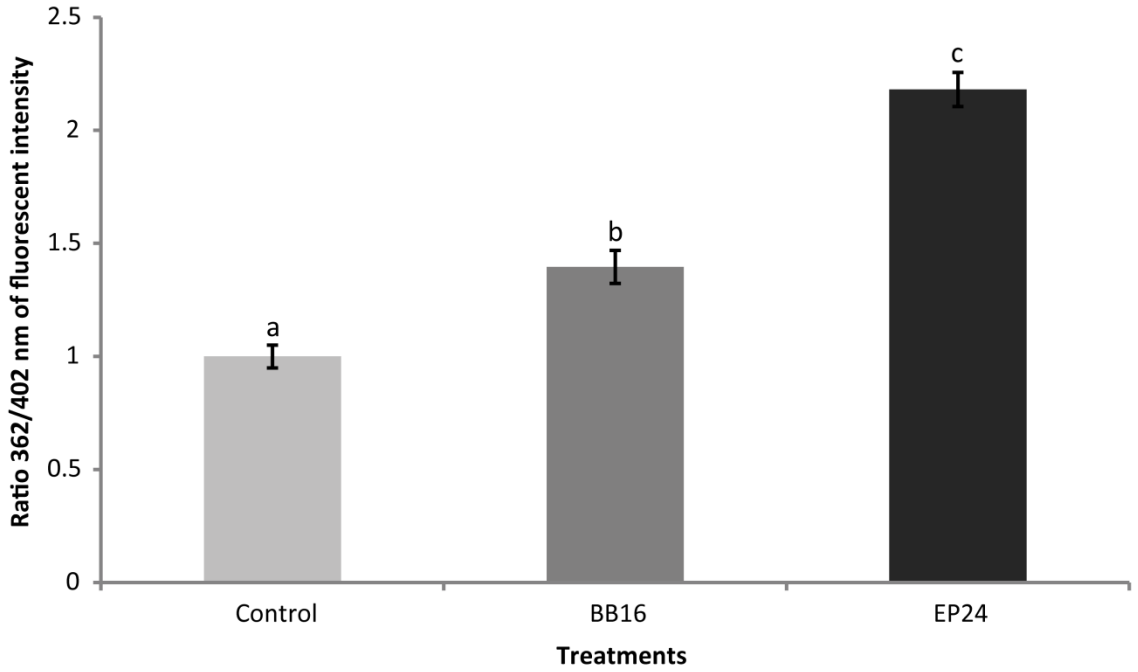
560 **Table 1:** Primers sequences used in real-time expression assays.

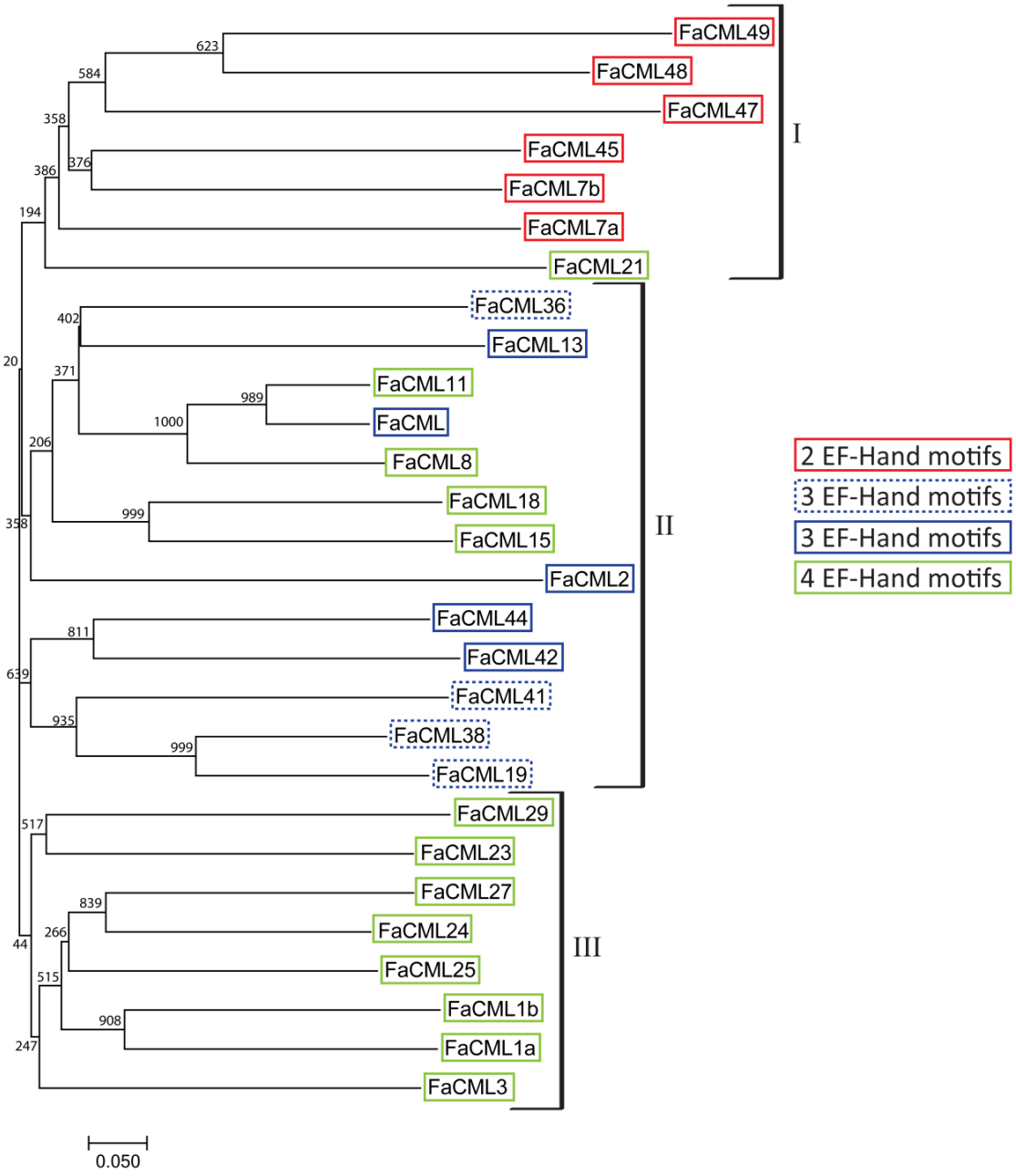
Genes	Names	Primers sequences	Accession no.
FaEF-1α	Elongation factor Tu-1	Fw 5'-GAACTTCCCAGGCTGATT-3' Rv 5'- CTGACCATCCTTCGAGATAC-3'	BK009992
FaPR1	Pathogenesis-relatedprotein 1	Fw 5'-CAAAGAGCTCCGGCGACTT-3' Rv 5'-TCTCCCACCCACAGGTTCAC-3'	AB462752
FaMYB30	HR-marker gene	Fw 5'-TGCCGGCTCAGATGGACTA-3' Rv 5'-TCGGTGAAGTTACCGCGTTT-3'	BK009993
FaERF1	Ethylene response factor 1	Fw 5'- CTCGATGCTGTGGCTAAA-3' Rv 5'- GTGACCTCCTCTTCCTTTAC-3'	MH332903
FaETR2	Ethylene response 2	Fw 5'- GGAGGGTTGGTCGTATTT-3' Rv 5'- ACTATCAGATGAGTTCTGTCTC-3'	KC852067
FaPRX27	Peroxidase27	Fw 5'-CACCAGCTTGCCACAGACAA-3' Rv 5'-CTGGCTTTTGGCCATCTTCT-3'	AFQ36036
FaGSL5	Glucansynthase-like 5	Fw 5'-GGCACTTAAAATGCGGAATCTG-3' Rv 5'-GATCGTAGGCTTCCGGATACC-3'	BK009994
FaCML45	Calmodulin-like 45	Fw 5'-GGAGCAGAGGAGGTTTCAAGAG-3' Rv 5'-TTCCTTCACCTCCTCCAAGCT-3'	MK966022
FaCML42	Calmodulin-like 42	Fw 5'-GGGTTCATATCGGCAAAGGA-3' Rv 5'-CCTTCCGGGAACCCTAGCT-3'	MK966020
FaCML36	Calmodulin-like 36	Fw 5'-CGGATCACGACGGGAAGAT-3' Rv 5'-CCCCTATCGCCGTGAACAC-3'	MK966017
FaCML1a	Calmodulin-like 1a	Fw 5'-TTGGAAAGGGAAGCATGGTT-3' Rv 5'-CGTCCAAATCAACAAGCTTGAA-3'	MK965999



561 **Fig. 1.** Intracellular calcium content in leaf discs pre-incubated with Fura2-AM and subsequently
562 treated with: water (control), BB16 (0.1 mg l^{-1}) and EP24 (0.1 mg l^{-1}) All measurements were taken 30
563 seconds after the treatment. The calcium concentration is expressed as the ratio of the band intensities
564 362 nm/402 nm. The bars represent the standard deviation (mean values ± SE). The comparison
565 between two groups was performed using the Tukey test ($p < 0.05$). Different letters denote significant
566 difference.

567

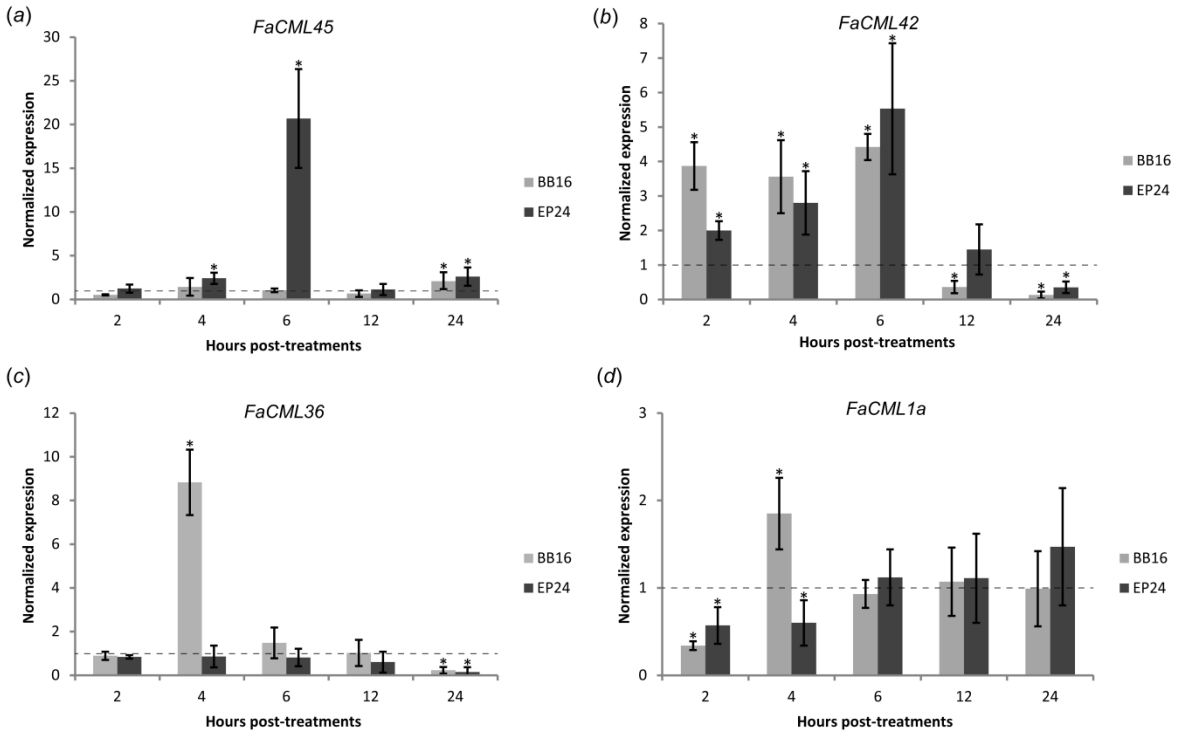
568 **Fig. 2.** Sequences alignment of three FaCMLs obtained with the Clustal program and edited with
569 BOXSHADE. The consensus sequences corresponding to the FaCML42, FaCML23 and FaCML45
570 EF-hand motifs are presented. The number of repetition of EF-hand motif varies in the different
571 FaCMLs studied. FaCML42 presents three EF-hand motifs (blue boxes), FaCML23 presents four EF-
572 hand motifs (yellow boxes), and FaCML45 two EF-hand motifs (red boxes).



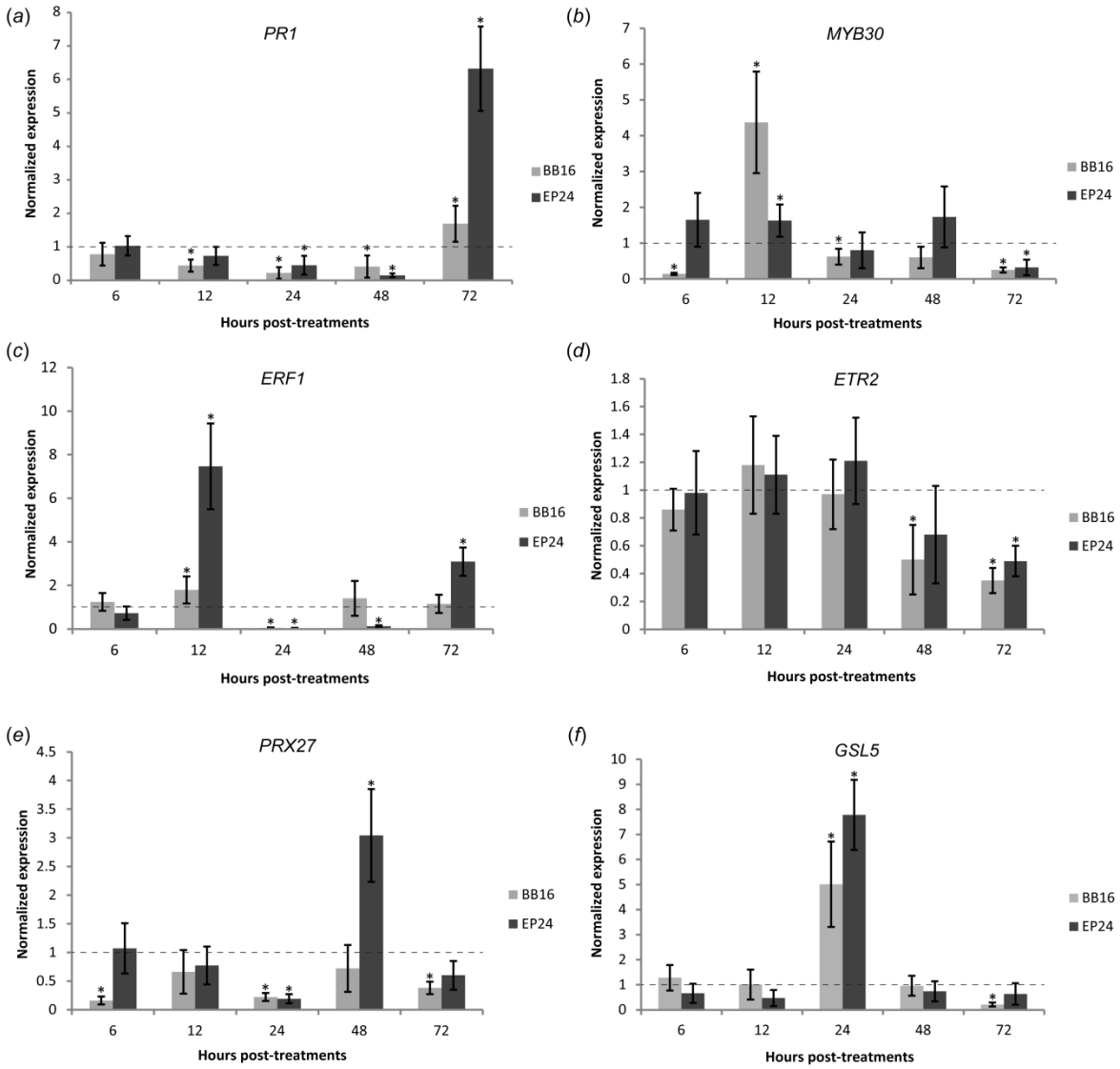
573 **Fig. 3.** NJ phylogenetic tree, created with MEGA 4, from the amino acid sequence of the FaCMLs
574 proteins predicted in *Fragaria ananassa*. The numerals at the branches indicate bootstrap values.
575 FaCMLs framed in red indicate CMLs containing two EF-Hand motifs, in blue (solid or dotted)
576 indicate CMLs containing three EF-Hand motifs, and in green indicate CMLs containing four EF-Hand
577 motifs.

578 **Table 2:** Regulatory *cis* elements associated biotic and abiotic stress responses found at the 5'UTR
579 *CML* sequences predicted in *Fragaria ananassa*. The ambiguity code characters correspond to: W =
580 A/T; Y = C/T; N = A/T/G/C; R = A/G; B = G/T/C; M = A/C.

Response element	cis-regulatory element	Sequence	Functions	References
Inductor/ pathogen response elements	WRKY71OS	TGAC	Defense response to infections or wounds. Induced by SA and JA	[54]
	BIHD1OS	TGTCA	Related to disease responses	[55]
	WBOXATNPR1	TTGAC	Binding site ofWRKY transcription factors, involved in the expression of stress-induced genes	[56,57]
	BOXLCOREDCPAL	ACCWWCC	Recognition sites for DcMYB1 (transcriptional activator of the phenylalanine ammonia-lyase gene). Important role in SA-mediated defense responses.	[58]
Abiotic stress response elements	EBOXBNNAPA	CANNTG	Response to salt stress, water deficit. Induced by ABA	[59]
	MYB1AT	WAACCA	Response to abiotic stresses e.g. low temperatures and water stress	[60]
	ASF1MOTIFCAMV	TGACG	ASF-1 binding site (transcription factor of the bZIP family). Involved in activationof number of genes induced by SA and auxins	[61]
	GT1CONSENSUS	GRWAAW	Binding site of the transcription factor GT-1. Regulates the expression of *PR* genes inducible by SA	[62]
	ABRERATCAL	MACGYGB	Response to water deficit, mediated by ABA	[63]



581 **Fig. 4.** Quantitative analysis by qRT-PCR of genes that code for different *FaCMLs* in strawberry
582 plants treated with BB16 or EP24. All values were normalized with respect to the expression of the
583 internal reference gene FaEF1α and are expressed in relation to the values of the control plants treated
584 with water. Dotted line represents the normalized expression value of control (water treated) plants.
585 Bars represent average values ± standard error of three biological replicas. The asterisks indicate a
586 statistically significant difference calculated by the fgStatistics software ($P \leq 0.05$).



587 **Fig. 5.** Quantitative analysis by qRT-PCR of genes related to the defense response in strawberry plants
588 treated with BRs at different times. All values were normalized with respect to the expression of the
589 internal reference gene FaEF1α and are expressed in relation to the values of the control plants treated
590 with water. Dotted line represents the normalized expression value of control (water treated) plants.
591 Bars represent average values ± standard error of three biological replicas. The asterisks indicate a
592 statistically significant difference calculated by the fgStatistics software ($P \leq 0.05$).

593 **Table S1:** Sizes and position EF-hand motifs on the amino acid sequences of the FaCMLs analyzed

EF-Hand motifs	FaCMLs	Gene length	Number of amino acids	EF-hand motif position in amino acid sequence			
				1°	2°	3°	4°
2 EF-Hand motifs	**FaCML7a**	477 bp	158 aa	37-65	109-137		
	FaCML7b	366 bp	122 aa	56-84	92-120		
	FaCML45	525 bp	174 aa	109-137	147-174		
	FaCML47	378 bp	126 aa	52-80	88-116		
	FaCML48	840 bp	279 aa	108-136	177-205		
	FaCML49	564 bp	187 aa	85-113	126-154		
3 EF-Hand motifs	**FaCML**	342 bp	113 aa	12-40	49-77	85-113	
	FaCML2	477 bp	158 aa	21-49	58-86	91-119	
	FaCML13	444 bp	147 aa	12-40	83-111	119-147	
	FaCML19	576 bp	191 aa	55-83	91-119	165-191	
	FaCML36	633 bp	210 aa	70-98	144-172	181-209	
	FaCML38	423 bp	140 aa	6-34	78-106	114-140	
	FaCML41	576 bp	191 aa	60-88	96-124	165-191	
	FaCML42	657 bp	218 aa	40-68	144-172	182-210	
	FaCML44	480 bp	159 aa	8-36	90-118	128-156	
4 EF-Hand motifs	**FaCML1a**	558 bp	185 aa	46-74	82-110	117-145	153-181
	FaCML1b	576 bp	191 aa	50-78	86-114	122-150	158-186
	FaCML3	660 bp	219 aa	65-93	101-129	147-175	185-213
	FaCML8	444 bp	147 aa	12-40	48-76	84-112	120-146
	FaCML11	453 bp	150 aa	12-40	48-76	85-113	121-149
	FaCML15	483 bp	160 aa	11-39	47-75	86-114	122-150
	FaCML18	513 bp	170 aa	20-48	56-84	99-127	135-163
	FaCML21	687 bp	228 aa	58-86	94-122	149-177	187-215
	FaCML23	588 bp	195 aa	50-78	89-117	129-157	166-194
	FaCML24	411 bp	136 aa	3-31	39-67	72-100	108-136
	FaCML25	486 bp	161 aa	14-42	50-78	87-115	123-151
	FaCML27	504 bp	167 aa	21-49	57-85	93-121	129-157
	FaCML29	474 bp	157 aa	19-47	55-83	92-120	129-157

594

595 **Table S2:** Location of regulatory *cis*-elements found on the 5'UTR of the FaCMLs analyzed

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		Inductor/pathogen response elements				Abiotic stress response elements				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
2 EF-Hand motifs	FaCML7a	607 (-) 329 (+)	235(+)			961 (+) 961 (-)	1016 (-)		60 (+, 533 (+)	980 (+)
	FaCML7b	24 (-)	24 (-)				152 (+)			329 (-)
	FaCML45	112 (+) 247 (-)		112 (+) 1314 (-)		421 (+) 421 (-),	421 (+) 440 (-)			
	FaCML47	33 (-) 303 (+)		32 (-), 130 (+)		173 (+) 173 (-)	201 (-)		16 (-) 159 (-)	
	FaCML48	51 (+) 191 (+)		592 (-)		389 (+) 389 (-)	349 (-)	593 (-)	207 (+) 366 (+)	89 (+), 766 (+)
3 EF-Hand motifs	FaCML2	254 (+) 335 (+)	199 (-) 364 (+)	335 (+) 363 (-)	298 (+)	341 (+) 341 (-)			397 (-)	212 (+)
	FaCML13	557 (+) 613 (-)	556 (-) 586 (-)			589 (+) 589 (-)	198 (+)		152 (+) 279 (-)	
	FaCML19	215 (-) 412 (-)	412 (+) 1379 (-)	411 (-) 1239 (-)	172 (+) 381 (+)	330 (+) 330 (-)	19 (+) 782 (-)	647 (+) 1277 (+)	1103 (-) 1498 (-)	1763 (+)
	FaCML36			687 (+) 882 (+)		937 (+) 937 (-)	436 (+) 580 (+)		487 (+) 583 (+)	
	FaCML38						6 (+)		27 (-)	
	FaCML41	267 (-) 623 (+)	622 (+)	415 (-)		42 (+) 42 (-)	295 (-)	225 (+)	648 (-) 659 (-)	
	FaCML42	328 (-) 555 (+)	554 (-) 1804 (-)	327 (-) 1805 (+)	2582 (+)	196 (+) 196 (-)	219 (-) 1872 (+)	2451 (-) 2674 (+)	304 (-) 336 (-)	117 (+), 557 (-),
	FaCML44	524 (+) 757 (-)	628 (-)	847 (+) 1722 (-)	1680 (-)	527 (+) 527 (-)	27 (+) 48 (+)	1560 (+)	156 (-) 694 (+)	
4 EF-Hand motifs	FaCML1a	265 (-) 317 (-)	265 (+) 317 (+)	395 (+)			581 (+)		54 (-), 108 (-)	
	FaCML1b	356 (-) 567 (-)	274 (-) 573 (+)			771 (+) 771 (-)	687 (+)		496 (-), 780 (-)	
	FaCML3	18 (-)		17 (-)		170 (+) 170 (-)			10 (+), 253 (-),	
	FaCML8	104 (-) 291 (-)	137 (-) 312 (-)	103 (-) 341 (+)		174 (+) 174 (-)		104 (-)	36 (-), 76 (+)	
	FaCML11					341 (+) 341 (-)	121 (-)		34 (+)	
	FaCML15	25 (-) 80 (-)	25 (+) 278 (-)			285 (+) 285 (-)	162 (+) 258 (+)		266 (+) 402 (-)	
	FaCML18					11 (+) 11 (-)				
	FaCML25	474 (-)		640 (+)	682 (+)	352 (+) 352 (-)	92 (+) 318 (+)		548 (-), 742 (-)	
	FaCML27	278 (+) 411 (+)		411 (+) 818 (-)		642 (+) 642 (-)	18 (+)		163 (-), 219 (-)	112 (+), 759 (+)

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Numerals correspond to the following regulatory *cis*-elements: (1), WRKY71O; (2), BIHD1OS; (3), WBOXATNPR1; (4), BOXLCOREDCPAL; (5), EBOXBNNAPA; (6), MYB1AT; (7), ASF1MOTIFCAMV; (8), GT1CONSENSUS; (9), ABRERATCAL. (+) and (-) indicate the location of the first nucleotide of the *cis*-elements found on the positive or negative strands, respectively.