CML42-Mediated Calcium Signaling Coordinates Responses to *Spodoptera* Herbivory and Abiotic Stresses in Arabidopsis[1][W][OA]

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In the interaction between Arabidopsis (*Arabidopsis thaliana*) and the generalist herbivorous insect *Spodoptera littoralis*, little is known about early events in defense signaling and their link to downstream phytohormone pathways. *S. littoralis* oral secretions induced both Ca^{2+} and phytohormone elevation in Arabidopsis. Plant gene expression induced by oral secretions revealed up-regulation of a gene encoding a calmodulin-like protein, *CML42*. Functional analysis of *cml42* plants revealed more resistance to herbivory than in the wild type, because caterpillars gain less weight on the mutant, indicating that *CML42* negatively regulates plant defense; *cml42* also showed increased aliphatic glucosinolate content and hyperactivated transcript accumulation of the jasmonic acid (JA)-responsive genes *VSP2* and *Thi2.1* upon herbivory, which might contribute to increased resistance. *CML42* up-regulation is negatively regulated by the jasmonate receptor Coronatine Insensitive1 (COI1), as loss of functional COI1 resulted in prolonged *CML42* activation. CML42 thus acts as a negative regulator of plant defense by decreasing COI1-mediated JA sensitivity and the expression of JA-responsive genes and is independent of herbivory-induced JA biosynthesis. JA-induced Ca^{2+} elevation and root growth inhibition were more sensitive in *cml42*, also indicating higher JA perception. Our results indicate that CML42 acts as a crucial signaling component connecting Ca^{2+} and JA signaling. CML42 is localized to cytosol and nucleus. CML42 is also involved in abiotic stress responses, as kaempferol glycosides were down-regulated in *cml42*, and impaired in ultraviolet B resistance. Under drought stress, the level of abscisic acid accumulation was higher in *cml42* plants. Thus, CML42 might serve as a Ca^{2+} sensor having multiple functions in insect herbivory defense and abiotic stress responses.

Plants respond actively to insect herbivory by the production of phytohormones and antiherbivore secondary metabolites and proteins (Mithöfer et al., 2009). Many of these defenses are coordinated by a jasmonic acid (JA)-dependent signaling cascade that is a key component in plant defense against herbivores. However, the recognition process in plant-herbivore interactions and the signal transduction pathways connecting it to downstream defense induction are less well understood. Studied from the perspective of plant-pathogen interaction, plant defense involves two components. One is immunity due to the recognition of general elicitors, like microbe- or pathogen-associated molecular patterns (MAMP/PAMP), by specific pattern recognition receptors that trigger basal resistance or PAMP-triggered immunity. However, during the coevolution of pathogens with host plants, pathogens acquired effector molecules to suppress PAMP-triggered immunity. The plants then counteracted this with a second component of immunity, recognition of effectors by specific resistance proteins, resulting in effector-triggered immunity or classical gene-for-gene resistance (Millet et al., 2010). Downstream of elicitor-receptor and effector-receptor interactions, the chain of events leading to defense-related gene activation consists of ion fluxes at the plasma membrane (Ca^{2+} influxes, K^+/Cl^- effluxes), an oxidative burst, and mitogen-activated protein kinase activation (Ebel and Mithöfer, 1998). One of the earliest signaling events after MAMP/PAMP perception is rapid changes in cytosolic calcium (Ca^{2+}) concentration, and it has been found to be crucial for downstream responses in plant-pathogen recognition (Ranf et al., 2011).

Herbivore-associated molecular patterns (HAMP) are also postulated to be present in insect oral secretions (OS) and are of two kinds: (1) chemical elicitors derived from insect OS and oviposition fluids; and (2) plant-derived self-recognition factors occurring due to a specific pattern of wounding (Mithöfer and Boland, 2008; Heil, 2009). Insect OS contain elicitors that are derived as a result of plant-insect interactions. Notable examples are inceptins, which are peptides formed as proteolytic products of plant chloroplastic ATP synthase, which is

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formed in caterpillar midgut, and fatty acid-amino acid conjugates (FACs) such as volicitin in maize (*Zea mays*). However, in Arabidopsis (*Arabidopsis thaliana*), it was shown that neither volicitin, other FACs, nor inceptins were active in inducing a phytohormone elevation and, hence, that there are further unidentified components in the OS that result in specific ligand-receptor interactions mediating recognition (Schmelz et al., 2009). The identity of many HAMP and knowledge of their perception mechanisms are still lacking.

Ca^{2+} is a universal second messenger, which is activated very early in signaling cascades and holds an important place in plant signaling as a mediator for response against a wide array of biotic and abiotic stimuli (Kudla et al., 2010). Ca^{2+} is a toxic cellular compound at high concentrations because of its ability to form insoluble complexes with proteins, membranes, and organic acids. So a tight spatial and temporal control over Ca^{2+} concentration is maintained in a cell, with the cytosol having a lower concentration than the external medium and subcellular compartments, resulting in a steep gradient. Upon perception of a signal, Ca^{2+} elevations arise in the cytosol due to an influx of Ca^{2+} from different stores, and this subsequently activates signaling (Dodd et al., 2010). The Ca^{2+} signature of a given signal, characterized by its amplitude, duration, frequency, and location, was shown to encode a message that, after decoding by downstream Ca^{2+} sensors, contributes to a specific physiological response (Sanders et al., 2002). Arabidopsis has at least 250 proteins with predicted Ca^{2+}-binding EF hand domains. Such a large number of genes involved in a single pathway point to the importance of Ca^{2+} signaling in biological responses. Plant Ca^{2+} sensor proteins are classified as sensor responders (both Ca^{2+}-binding and kinase activities; e.g. calmodulin-dependent protein kinases [CDPKs]) and sensor relay proteins (which only bind Ca^{2+} and undergo conformational changes). Calmodulin-like proteins (CMLs) are sensor relay proteins that are unique to plants, with 50 members in Arabidopsis. CMLs are defined by the presence of two to six predicted EF hand motifs, by the absence of any other identifiable functional domains, and by at least 15% amino acid identity with calmodulins (McCormack and Braam, 2003). CMLs have varied roles in stress perception and plant development. CML37, -38, and -39 transcripts are regulated by biotic and abiotic stress as well as hormone and chemical treatment (Vanderbeld and Snedden, 2007). CML24 is known to cause alterations in flowering time, abscisic acid (ABA), and ion stress (Delk et al., 2005; Hubbard et al., 2008). CML9 alters plant responses to ABA and abiotic stress (Magnan et al., 2008), and loss of CML42 function leads to aberrant trichomes with increased branching (Dobney et al., 2009).

In insect herbivory, the role of Ca^{2+} in the defense strategies of the plant is less well studied. It has been reported that feeding by *Spodoptera littoralis* on *Phaseolus lunatus* causes a transient increase in cytosolic calcium concentration in cells adjacent to the insect bite (Maffei et al., 2004). A FAC fraction from larval OS results in an immediate Ca^{2+} influx in soybean (*Glycine max*) suspension cells without any obvious lag phase, suggesting a detergent-like effect rather than a receptor-mediated one (Maischak et al., 2007). In contrast, these authors also showed that still unknown compounds are present in the OS of *S. littoralis* that are able to induce Ca^{2+} transients in soybean suspension cells resembling classical elicitor effects. The importance of Ca^{2+} has also been demonstrated in experiments with *P. lunatus*, which in response to attack by the spider mite *Tetranychus urticae* shows activated defense gene expression requiring Ca^{2+} influx and protein phosphorylation (Arimura et al., 2000). In Arabidopsis, the calmodulin-binding protein IQD1 was shown to be involved in plant defense against feeding by the aphid *Myzus persicae* and the lepidopteran *Trichoplusia ni* by affecting glucosinolate (GS) biosynthesis (Levy et al., 2005). In the Arabidopsis response to *S. littoralis*, the role of CDPKs (CDPK3 and CDPK13) in the herbivory-induced signaling network via HsfB2a-mediated regulation of the defense-related transcriptional machinery was recently outlined. This cascade was reported to directly impact transcription factors for defense responses independent of phytohormone-related signaling pathways (Kanchiswamy et al., 2010). In the case of interaction between Arabidopsis with the generalist grasshopper herbivore *Schistocerca gregaria*, application of grasshopper OS to puncture wounds elicited increased cytosolic Ca^{2+} and phytohormone levels (Schäfer et al., 2011). Ca^{2+} has also been implicated in downstream parts of herbivore-triggered signaling cascades, because jasmonates and synthetic analogs induce Ca^{2+} elevations in tobacco (*Nicotiana benthamiana*) BY-2 cell culture (Walter et al., 2007).

In this study, we have addressed the functional role of Ca^{2+} sensor proteins, which are a component of specificity in Ca^{2+} signaling, in the interaction between Arabidopsis and the generalist insect herbivore *S. littoralis*. Understanding the role of Ca^{2+} signaling in defense against *S. littoralis* would provide more insights into early stages of plant-herbivore interactions and their parallels to the conserved PAMP/MAMP perception pathways. We show here that CML42 is an early signaling component activated in plants upon the perception of elicitors in *S. littoralis* OS. Loss of CML42 negatively regulates defense and results in reduced herbivory due to increased JA sensitivity. Apart from its role in herbivory, CML42 also coordinates the perception of abiotic stress responses.

RESULTS

S. littoralis OS Induce Cytosolic Ca^{2+} Elevation and a Phytohormone Burst in Arabidopsis

As they feed, caterpillars use OS to transport the chewed leaf tissues into their mouth. These OS, which include regurgitant and labial and mandibular saliva, provide a milieu of putative elicitors that might be recognized by the plant (Mithöfer and Boland, 2008). In our study, transgenic Arabidopsis plants with the cytosolic Ca^{2+} reporter aequorin were used to measure the

Ca^{2+} elevation by *S. littoralis* OS. We used crude OS from *S. littoralis* previously fed with Arabidopsis to test for elicitors an insect might produce on a natural diet as opposed to an artificial diet. *S. littoralis* OS application to aequorin transgenics induced cytosolic Ca^{2+} elevation in Arabidopsis leaves. After a lag phase of 40 s, the Ca^{2+} levels increased and reached a maximum at 2 min, followed by a slow decline to resting levels in 10 min (Fig. 1A). To analyze whether OS induces downstream defense responses in the leaf, we measured phytohormone changes upon wounding + water and wounding + *S. littoralis* OS (single application) treatments. We found that OS induced a phytohormone burst in Arabidopsis. JA-Ile, the bioactive form of JA, accumulated transiently, reaching a peak at 30 min and declining gradually to resting levels by 90 min (Fig. 1B). JA levels upon OS application increased gradually, reaching a peak at 60 min and then decreasing gradually, but did not reach resting levels after 120 min (Fig. 1C). 12-Oxophytodienoic acid (cis-OPDA) levels also increased drastically upon OS application and stayed at elevated levels for a prolonged period (Fig. 1D). Salicylic acid levels, on the other hand, did not change upon insect OS application, and their levels increased equally upon both treatments (Fig. 1E).

Elicitors in *S. littoralis* OS Induce Transcript Accumulation of *CML42*, a Calmodulin-Like Protein

Downstream of Ca^{2+} elevation, there is an array of Ca^{2+}-binding proteins that detect changes in Ca^{2+} and encode for specificity in the Ca^{2+} response. To identify the Ca^{2+} sensors involved in the interaction between Arabidopsis and the generalist insect *S. littoralis*, an Affymetrix array was used to compare the expression of early-signaling genes between mechanically wounded (W) rosette leaves treated with water (W + W) and with *S. littoralis* OS (W + OS) for 30 min each (H. Vogel, J. Kroymann, and A. Mithöfer, unpublished data). Microarray analysis revealed that the gene encoding CML, *CML42* (At4g20780), is up-regulated by *S. littoralis* OS application. Using quantitative real-time PCR, we confirmed the microarray data and found that application of *S. littoralis* OS elevated *CML42* transcript accumulation in a transient manner, with the levels reaching maximum at 30 min and sustaining for 60 min (Fig. 2A). Plants may respond to two aspects of herbivory: mechanical wounding and application of OS. To test if *CML42* expression is specific to OS and is not due to mechanical wounding, we used the MecWorm robot, which mimics mechanical injury upon insect biting (Mithöfer and Boland, 2008). MecWorm treatment, however, did not up-regulate *CML42* expression (Fig. 2B) at 30 and 60 min and the transcript was down-regulated at 180 min, indicating that the expression of *CML42* is induced by OS only. We also compared the kinetics of *CML42* gene expression upon *S. littoralis* feeding and found that *CML42* is up-regulated transiently, with maximum fold change at 1 h and returning to baseline expression in 2 h (Fig. 2C). Because the kinetics of *CML42* expression (30–60 min) overlaps with *S. littoralis* OS-induced JA biosynthesis, we analyzed if *CML42* up-regulation is a response to phytohormone elevation rather than a direct response to elicitors. However, we found that spaying 50 μM JA did not up-regulate *CML42*, although the marker gene *VSP2* was up-regulated (Fig. 2D). Because there are 50 CMLs in Arabidopsis, there might be functional redundancy in this protein family. CML42 and CML43 are two closely related proteins that display 35% identity to calmodulin, and CML43 expression is constitutive in roots and induced in leaves after pathogen infection (Chiasson et al., 2005). However, we found no significant induction of CML43 upon OS application (Supplemental Fig. S1).

Loss of Function of CML42 Makes Plants More Resistant to *S. littoralis* But Does Not Affect Herbivory-Induced Phytohormone Elevation

Sensor proteins that decode Ca^{2+} signals have not yet been proved to affect insect feeding patterns in Arabidopsis. Because the *CML42* transcript was up-regulated by *S. littoralis* OS, a possible role in early plant defense signaling was hypothesized. To analyze the function of *CML42* in insect herbivory, we compared the growth of *S. littoralis* larvae on wild-type plants and *cml42* lines. We observed that larvae gain significantly less weight on the *CML42* loss-of-function mutant (Dobney et al., 2009) as compared with the wild type after they feed for 8 d on either plant (Fig. 2E). The experiment was repeated four times, showing significant changes by Mann-Whitney rank sum test; larvae feeding on *cml42* gained 20% less weight than larvae feeding on the wild type. A second independent T-DNA insertion line (SALK_040227) yielded similar results (Fig. 2F). This indicates increased defense upon *CML42* loss of function, as opposed to the expected role, where loss of function would increase larval weight due to decreased plant defense. The *cml42* line we used has been shown to result in plants having fewer aberrant trichomes with increased branching (Dobney et al., 2009). Although the mutant trichome-branching pattern is more pronounced in younger leaves, there is no increase in trichome density, so these do not impede the mobility of insects in the larger leaves of the 5-week-old plants used for study (Supplemental Fig. S2). Thus, the increase in defense on *CML42* loss-of-function plants is not related to the trichome pattern. To test if the reduced insect performance on *cml42* plants is due to altered phytohormone levels, both wild-type and *cml42* plants were challenged with *S. littoralis* larvae and OS and phytohormone levels measured at different time points. The levels of JA, JA-Ile, and cis-OPDA were increased upon insect feeding, but there was no difference between the lines at 24 h and 1 week after feeding (Fig. 3). Salicylic acid levels remain unchanged upon insect herbivory. Similar results were obtained by wounding and OS treatment (Supplemental Fig. S3), indicating that increased resistance in *cml42* is not due to an increase in phytohormone production.

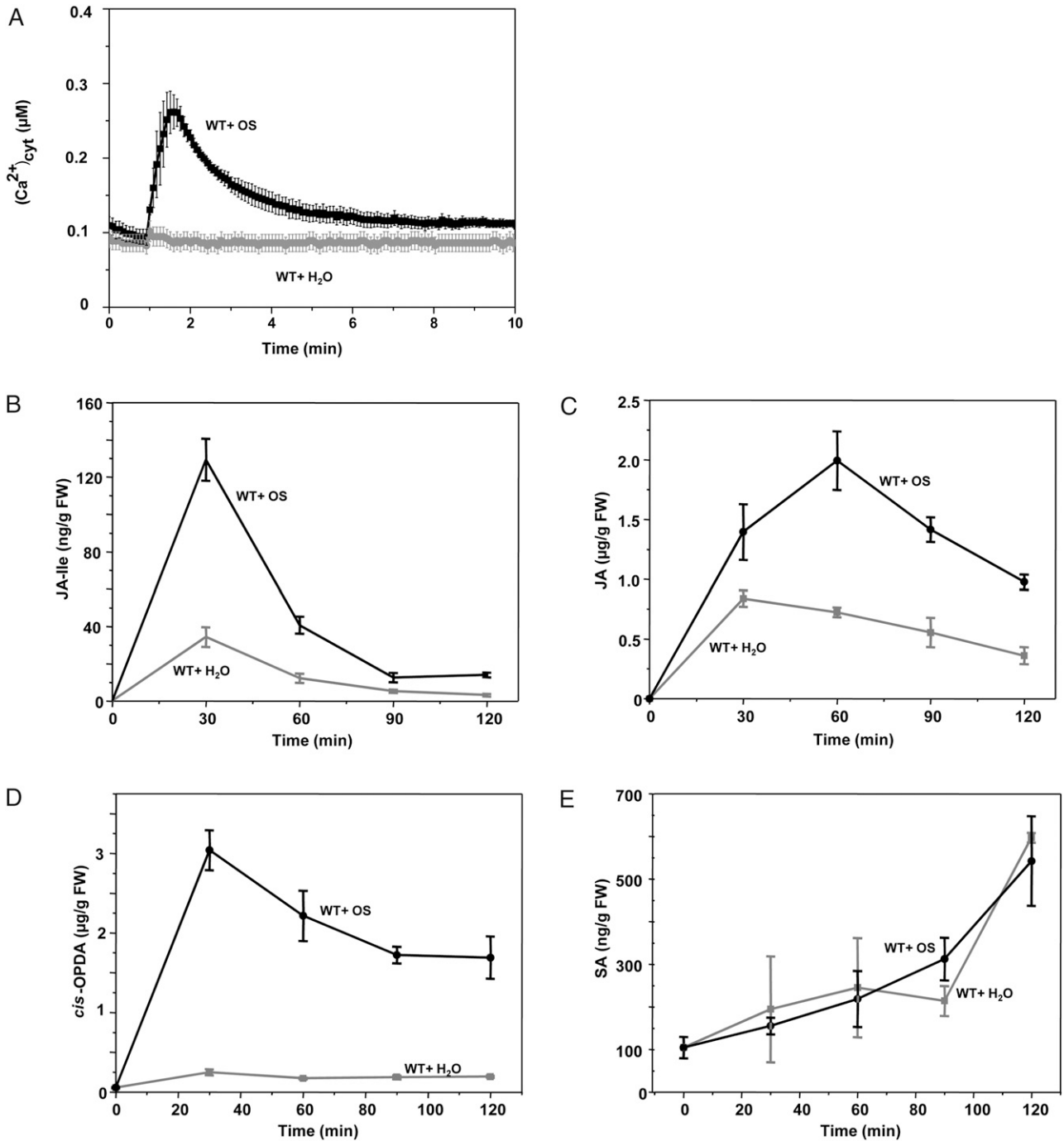

Figure 1. *S. littoralis* OS-induced changes in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) and phytohormones in Arabidopsis. A, Application of 40 μL of *S. littoralis* OS (1:1 diluted) to 5-week-old Arabidopsis leaf discs expressing aequorin. Mean ± SE ($n = 4$) $[Ca^{2+}]_{cyt}$ was calculated from the relative light units measured in leaf discs at 5-s integration time for 10 min. In all the experiments, water was used as a control and gave background readings. B to E, Mean ± SE ($n = 4$) content of JA-Ile (B), JA (C), cis-OPDA (D), and salicylic acid (SA; E) in mechanical wounding + *S. littoralis* OS-elicited leaves and a control with mechanical wounding + water treatment. Phytohormones were measured in locally treated leaves. FW, Fresh weight; WT, wild type.

CML42 Is Targeted to Cytosol and Nucleus

The specificity of a common signal like Ca^{2+} is due to both the spatial and temporal patterns of Ca^{2+} elevation and due to spatially distinct Ca^{2+}-decoding protein complexes. Hence, it was important to study the subcellular localization of CML42, so we generated transgenic Arabidopsis lines expressing CML42-GFP fusion protein under the control of the constitutive 35S cauliflower mosaic virus promoter. The CML42-GFP fusion

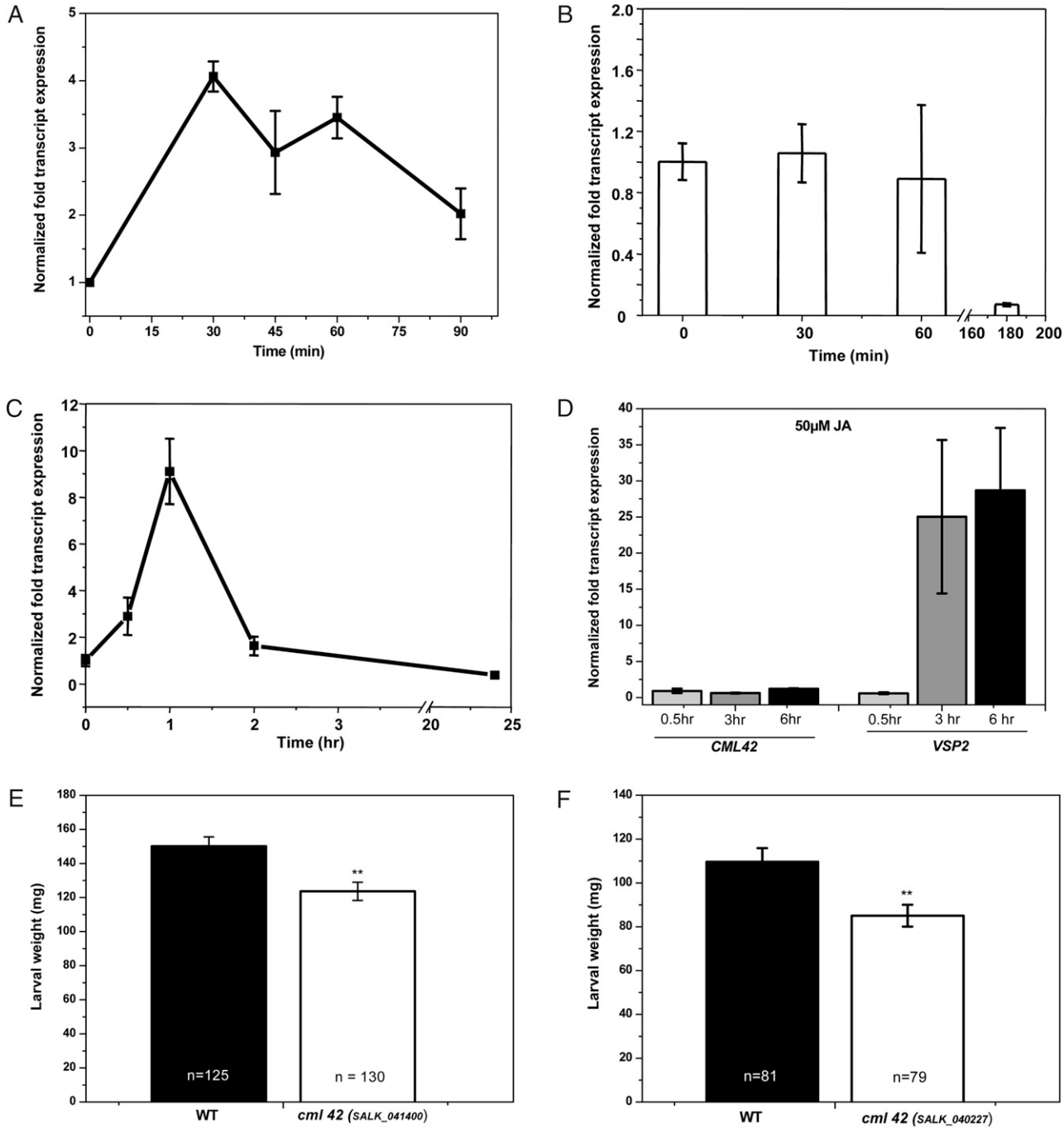

Figure 2. *CML42* elicitation and its functional role in *S. littoralis* herbivory. A, *CML42* transcript levels in OS-treated leaves of Arabidopsis after 30, 45, 60, and 90 min of treatment. Leaves were elicited by pattern wheel wounding and subsequently treating the wound with 20 μL of water or 1:1 diluted OS per leaf. Transcript abundance in leaves was determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The fold change was calculated relative to the control, which underwent mechanical wounding + water. The graph shows fold induction of the mRNA levels by *S. littoralis* OS relative to the levels in the water-treated control leaves. Values are means ± SE ($n = 6$). B, *CML42* transcript levels in Arabidopsis leaves after 30, 60, and 180 min of MecWorm damage. Undamaged leaves were used as controls for quantification. Values are means ± SE ($n = 3$). C, *CML42* transcript levels in Arabidopsis leaves after 30 min and 1, 2, and 24 h of *S. littoralis* feeding. Undamaged leaves were used as controls for quantification. Values are means ± SE ($n = 5$). D, *CML42* and *VSP2* transcript levels in JA-treated (50 μM) Arabidopsis leaves after 0.5, 3, and 6 h of treatment. Elicitation and calculation were as in A. *VSP2* was used as a marker for JA responsiveness in plants. The fold change was calculated relative to the control, which were leaves with 0.015% methanol spray. Values are means ± SE ($n = 6$). E and F, Larval weight after feeding on Col-0 and two different *cml42* lines, SALK_041400 (E) and SALK_040227 (F). *S. littoralis* first instar larvae growing in light for 3 d after hatching were preweighed,

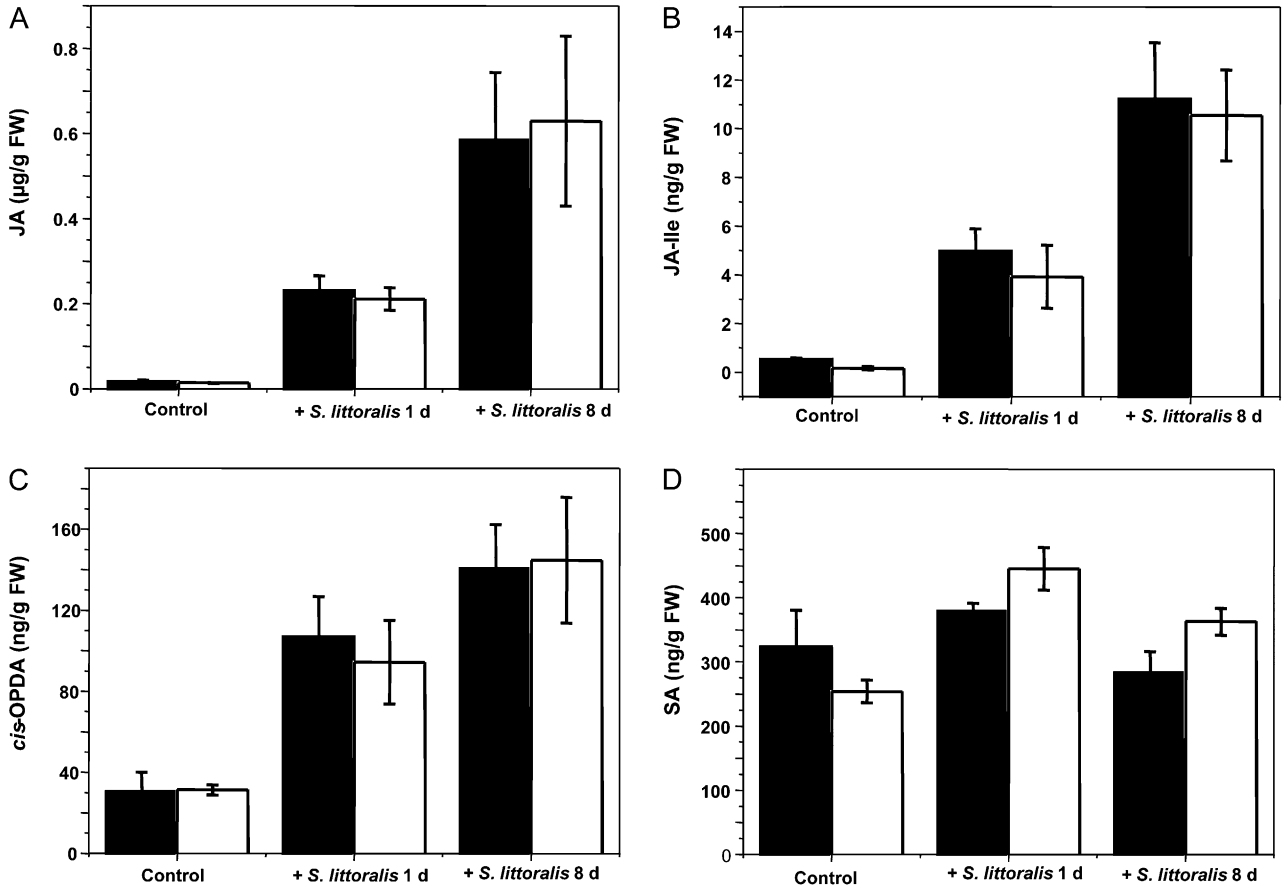

Figure 3. Phytohormone elevation upon *S. littoralis* herbivory in *cml42* plants. Mean ± SE ($n = 5$) levels are shown for JA (A), (+)-JA-Ile (B), cis-OPDA (C), and salicylic acid (SA; D) in Arabidopsis wild-type (white bars) and *cml42* (black bars) plants fed on by *S. littoralis* for 1 and 8 d. The phytohormone levels were measured from the whole plant rosette, which includes both local and systemic leaves. Statistical differences in phytohormone levels among the wild type and *cml42* (ANOVA; $P < 0.05$) were calculated, and no significant changes were determined. FW, Fresh weight.

protein was located within the nucleus and cytosol, as was clear from views of the cytosol surrounding the chloroplast and the presence of cytoplasmic strands (Fig. 4A). To rule out the possibility of plasma membrane localization, we did a colocalization experiment using *N. benthamiana* transiently cotransformed with plasma membrane markers (Fig. 4B). In both cases, CML42 was localized to the cytosol and nucleus. The CML42-GFP fusion protein was of the expected size, 51 kD, and the full-length protein was expressed in Arabidopsis (Supplemental Fig. S4).

JA Sensitivity Is Higher in *cml42* Plants

In Arabidopsis, wounding activates both JA-dependent and independent signaling pathways regulating different sets of target genes at the wound site. The JA-dependent pathway induces the expression of wound-responsive genes such as *VSP*, *PDF1.2*, or *Thi2.1* (Bohlmann et al., 1998; Berger et al., 2002). To assess if the JA-responsive gene induction is affected in the *cml42* plants, we analyzed the gene expression pattern upon *S. littoralis* feeding. *cml42* did up-regulate the JA-responsive gene *VSP2* to a much higher level (550-fold) than the wild type (150-fold) upon *S. littoralis* herbivory. Similar results were obtained for the JA-responsive gene *Thi2.1*. Here, in the *cml42* mutant, the mRNA level was up-regulated 200-fold, in contrast to a 100-fold higher level in the wild type (Fig. 5A). *PDF1.2* showed no differences and was up-regulated 25-fold in the wild type and 30-fold in *cml42* upon *S. littoralis* herbivory. Furthermore, it was observed that *cml42* plants per se

Figure 2. (*Continued.*)
and three larvae were placed on plants of each genotype. The larval weight (mean ± SE) was measured after 7 d of feeding. The total number of larvae weighed is indicated on the bars. Values are means of four and three independent experiments for E and F, respectively. Statistically significant differences between Col-0 and *cml42* plants after feeding of the larvae were analyzed by the Mann-Whitney rank sum test (** $P < 0.001$). WT, Wild type.

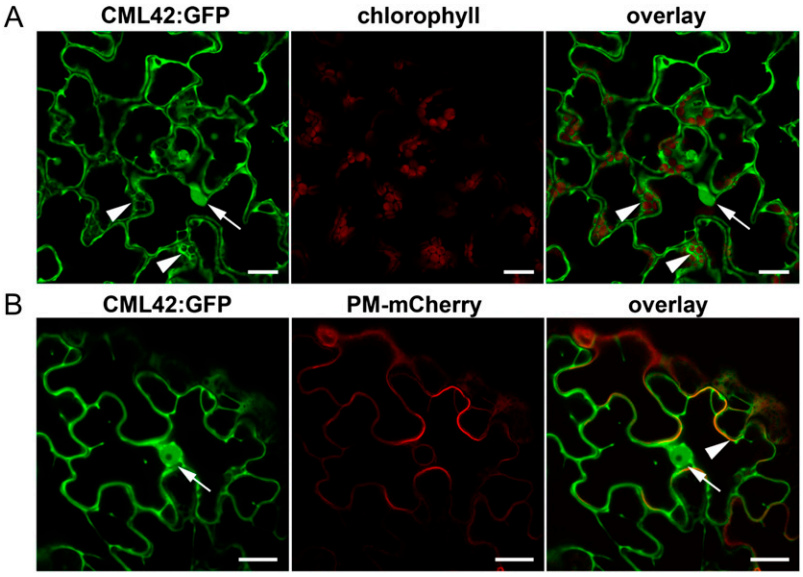

Figure 4. Subcellular CML42 localization. A, Visualization of CML42-GFP in leaves of transgenic Arabidopsis plants using confocal laser scanning microscopy. Green fluorescence of the fusion protein is visible in cytoplasm surrounding the chloroplasts (arrowheads) and the nucleus (arrows). B, Visualization of CML42-GFP in transiently transformed leaves of *N. benthamiana* using confocal laser scanning microscopy. Coinfiltration of a vector encoding mCherry directed to the plasma membrane (Nelson et al., 2007) was performed and visualizes the plasma membrane by red fluorescence. Note the location of CML42-GFP in the nucleus (arrows) and cytoplasm, which do not colocalize to the plasma membrane (arrowhead). Bars = 20 μm.

have higher expression of JA-responsive genes like the *PDF1.2*, *JAZ1*, and transcription factor *MYC2* when compared with wild-type plants without herbivory (Supplemental Fig. S5). JA perception occurs via the binding of bioactive jasmonate, JA-Ile, to the jasmonate receptor Coronatine Insensitive1 (COI1). It had been shown that 5 h after *S. littoralis* feeding, 119 genes were up-regulated, 71 of which showed no change in the JA receptor mutant *coi1-1*, hinting that JA perception is important for herbivory response (Bodenhausen and Reymond, 2007). To test if *CML42* is COI1 dependent, we analyzed the transcript accumulation of *CML42* upon *S. littoralis* OS treatment in the *coi1-1* mutant and wild-type plants. *CML42* transcript expression was higher and prolonged in the *coi1-1* mutant, with significant up-regulation at 60 and 90 min, as opposed to the wild-type plant, where at 90 min, *CML42* transcript was at basal levels (Fig. 5B). Hence, the expression of *CML42* is COI1 dependent and functional COI1 is required for maintaining controlled expression of *CML42* during plant defense. It has been shown that cytosolic Ca^{2+} elevations are induced by 300 μM to 2 mM JA in tobacco cell cultures and might be a part of its perception mechanism (Walter et al., 2007). In Arabidopsis, 500 μM JA induces a prolonged cytosolic Ca^{2+} elevation that does not reach basal levels after 10 min (Fig. 5C). To test if loss of function of *CML42* has an impact on JA-induced Ca^{2+} elevations, the *cml42* mutant was crossed to a Ca^{2+} reporter, aequorin, and F2 homozygous plants were selected. The *cml42* × aequorin mutant, when treated with 500 μM JA, showed a higher early cytosolic Ca^{2+} elevation than the wild-type aequorin plant (Fig. 5C). This indicates that *CML42* loss of function hyperactivates JA-induced Ca^{2+} elevation. However, treatment with *S. littoralis* OS on *cml42* × aequorin gave no differences, indicating that *CML42* is downstream of *S. littoralis* OS-induced Ca^{2+} elevation (Supplemental Fig. S6). One of the developmental phenotypes due to JA perception is inhibition of primary root elongation, which is COI1 dependent (Staswick et al., 1992; Feys et al., 1994). So we investigated the response of wild-type, JA-insensitive *coi1-16* and *cml42* roots to an increasing concentration of methyl jasmonate (MeJA) in a range from 10 to 50 μM. The *coi1-16* mutant showed a strong JA-insensitive phenotype, as reported previously (Ellis and Turner, 2002). We found that a lower concentration of MeJA (20 μM) is sufficient to induce maximum root growth inhibition in *cml42*, as opposed to 40 μM in wild-type plants, indicating that the *cml42* mutant is more sensitive to JA (Fig. 5D). All the data thus indicate that jasmonate perception is more sensitive upon *CML42* loss of function, and hence, *S. littoralis* feed less on plants.

GS Levels Are Modulated in *cml42* Plants

The secondary metabolite arsenal in Arabidopsis comprises many compounds, including GSs, flavonoids, and terpenoids, which are necessary for plant survival in unfavorable environments (Kliebenstein, 2004). GSs are important secondary metabolites involved in resistance to generalist insects like *S. littoralis*, and both aliphatic and indole GSs have additive roles (Müller et al., 2010). We wanted to evaluate the influence of *CML42* on GS accumulation to see if this could explain its effect on *S. littoralis* herbivory. Therefore, we analyzed the GS content of *cml42* and wild-type plants and found that *cml42* plants have an increased accumulation of GSs (Fig. 6A). The increase in total GSs was due to an increase in aliphatic GS (Met derived) rather than indolic components (Trp derived). Upon *S. littoralis* feeding for 24 h on wild-type and *cml42* plants, both indolic and aliphatic GSs increase to similar levels in both the lines and *cml42* does not show a greater increase as expected (Fig. 6B). The

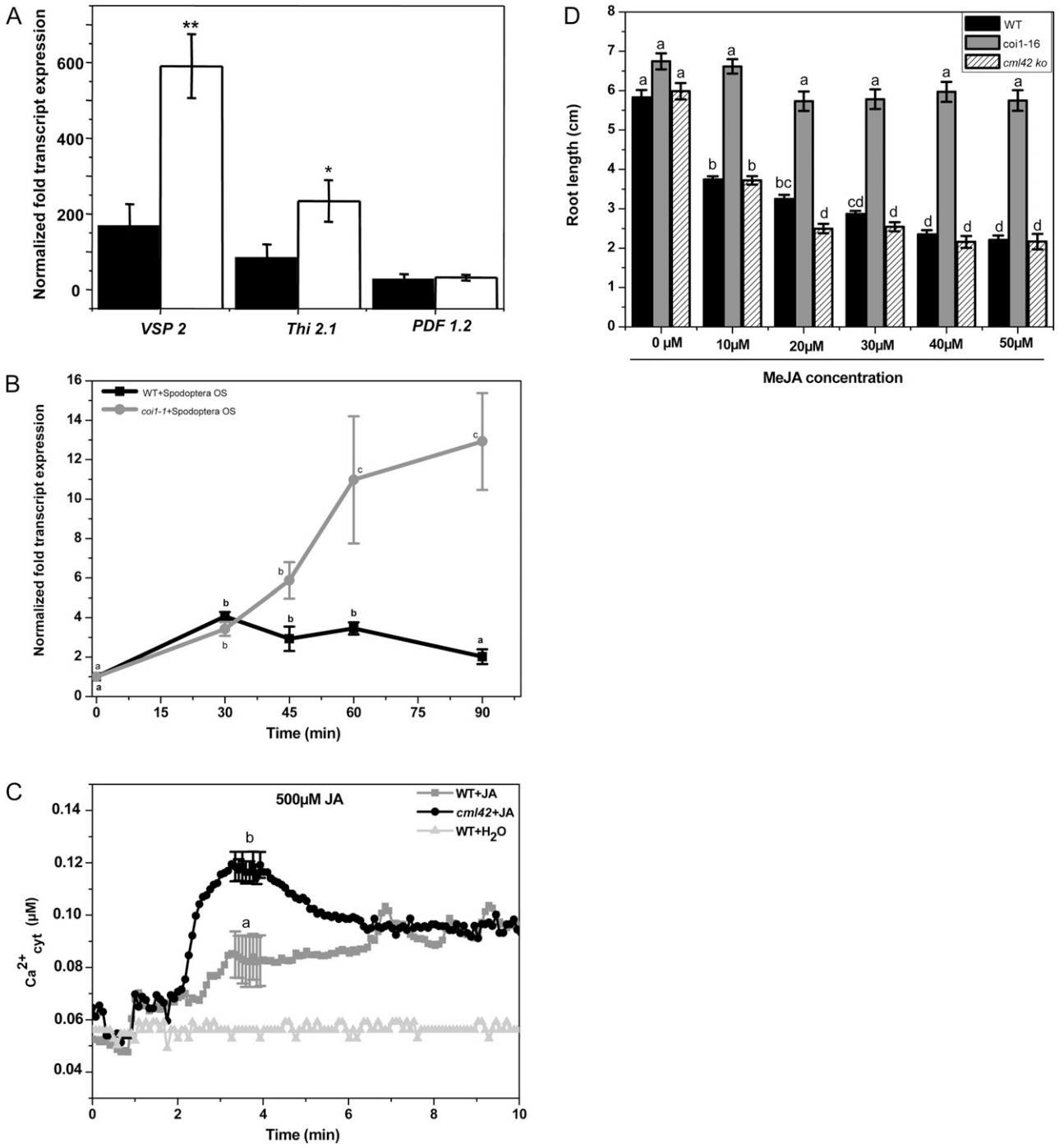

Figure 5. JA perception in *cml42* mutants. A, Relative mRNA levels of defense-related genes *VSP2*, *Thi2.1*, and *PDF1.2* in *S. littoralis*-infested leaves of wild-type (black bars) and *cml42* (white bars) plants. *S. littoralis* (fourth instar larvae) fed on both lines for 24 h. Undamaged wild-type and *cml42* plants served as controls. Means ± SE ($n = 4$) are shown. Statistically significant differences between Col-0 and *cml42* plants were analyzed by unpaired *t* test: ** $P < 0.001$, * $P < 0.05$. B, *CML42* transcript levels in *S. littoralis* OS-treated leaves of wild-type (WT; black squares) and the JA receptor mutant, *coi1-1* (gray circles), after 30, 45, 60, and 90 min of treatment. Leaves were elicited as described for Figure 2A. Different letters indicate significant differences among treatments (ANOVA; $P < 0.05$). Values are means ± SE ($n = 4$). C, Elevation in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) induced by JA in *cml42* plants. JA (500 μM) was applied to leaf discs of aequorin-expressing wild-type (dark gray squares) and *cml42* × aequorin (black circles) plants. Leaves were elicited as described for Figure 1A. Water was used as a control and gave background readings (light gray triangles). Values are means of two independent experiments ($n = 5$). Error bars represent SE, and different letters indicate significant differences among treatments (ANOVA; $P < 0.05$). D, Effects of increasing concentrations of MeJA on the inhibition of root growth in the wild type and *coi1-16* and *cml42* mutants. Seedlings were grown on MS plates with various MeJA concentrations (0–50 μM), and root length was measured after 14 d in continuous light. The experiment was repeated three independent times ($n = 20$ each line per experiment). Error bars represent SE, and different letters indicate significant differences among treatments (ANOVA, Student-Newman-Keuls post hoc test; $P < 0.05$).

plants maintain these high GS levels upon *S. littoralis* feeding even after 1 week and do not increase over this level (data not shown). This indicates that a certain threshold of GS levels might be sufficient for resistance, and these levels might be reached by the wild type and *cml42* at different time points. Also, a further increase from the constitutive high GS levels in *cml42* may be detrimental for plant fitness.

CML42 Loss of Function Affects Multiple Stress Response Pathways

To determine if metabolites other than GSs were altered upon *CML42* loss of function, we used the flow through after GS analysis (raw methanolic extracts minus the GS) and looked for metabolites that change in *cml42* over the wild type. It was found that the levels of the flavonol glycosides kaempferol-3-*O*-[6′′-*O*-(rhamnosyl)glucoside] 7-*O*-rhamnoside, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, and kaempferol 3,7-*O*-dirhamnoside (Matsuda et al., 2010) were significantly reduced in the *cml42* plants when compared with wild-type plants (Fig. 7A). Because flavonols serve in protection against UV-B radiation (D'Auria and Gershenzon, 2005), we tested if *CML42* loss of function had an effect on UV-B tolerance. It was revealed that 1 h of UV-B treatment resulted in 20% ± 5% survival in the wild type as opposed to only 4% ± 2% in *cml42* seedlings (Fig. 7B). Thus, loss of function of *CML42* is detrimental for plant resistance to UV-B stress. It is known that CMLs control many abiotic stress response pathways, and some act as regulators of ABA biosynthesis (Delk et al., 2005; Hubbard et al., 2008). Therefore, we looked for the impact of drought stress on the wild type and *cml42*. Water stress resulted in an increase of ABA content in all genotypes. But it was observed that ABA levels are higher in *cml42* as opposed to wild-type plants (Fig. 7C). This indicates that *CML42* also negatively affects ABA biosynthesis upon drought stress, as loss of function is more beneficial for increased ABA accumulation. The drought-resistant phenotype, however, was quite variable, and although the *cml42* plants showed a drought-resistant phenotype at 8 d after drought, both wild-type and *cml42* plants died upon prolonged drought stress.

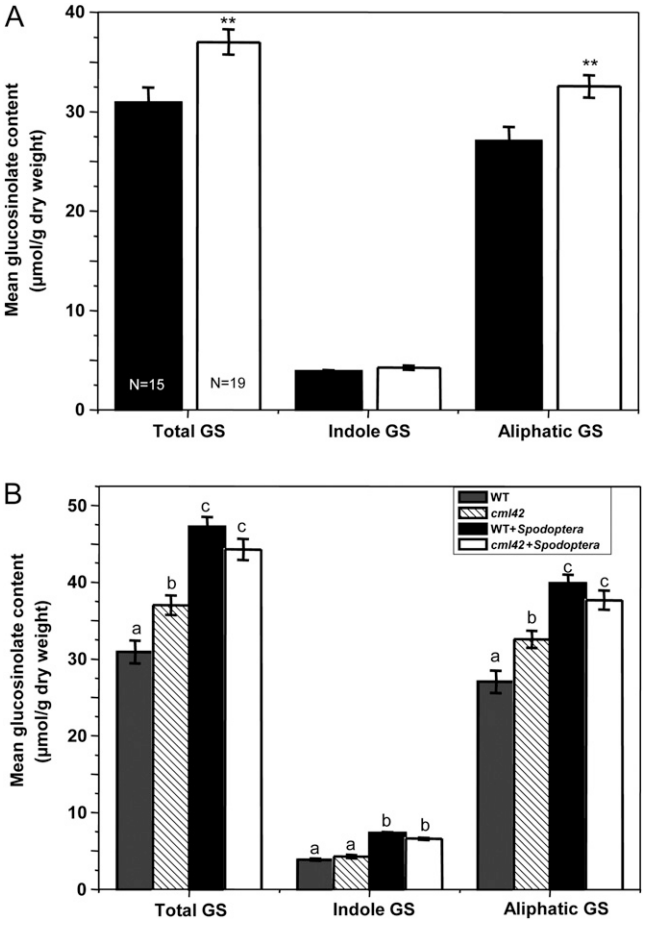

Figure 6. GS levels in *cml42* plants. A, Mean ± SE levels of total, indole, and aliphatic GSs in rosette leaves of 5-week-old untreated Arabidopsis wild-type (black bars) and *cml42* (white bars) plants. Statistically significant differences between Col-0 and *cml42* plants were analyzed by unpaired *t* test (** $P < 0.001$). Values are means of five independent experiments, and the total number of plants used is indicated on the bars. B, Mean ± SE ($n = 8$) levels of total, indole, and aliphatic GSs in rosette leaves of 5-week-old Arabidopsis wild-type (WT; gray and black bars) and *cml42* (white and hatched bars) plants fed on by *S. littoralis* for 1 d. Different letters indicate significant differences among treatments (ANOVA; $P < 0.05$). Values are means of two independent experiments.

DISCUSSION

S. littoralis Elicitor Induced Transcript Accumulation of *CML42*

The plant response to herbivory includes reaction to both mechanical wounding and elicitors in insect OS. Several studies have demonstrated that insect OS application to artificial wounds can mimic herbivory, suggesting that OS are primary sources of information for plant recognition of insects (Halitschke et al., 2003; Maffei et al., 2004; Consales et al., 2012). Our data here show that *S. littoralis* OS induces cytosolic Ca^{2+} and phytohormone elevation in Arabidopsis and thus activates defense. It is interesting that both JA and cis-OPDA show prolonged elevation upon OS treatment, indicating a sustained defense by plants, as reported for grasshopper OS in Arabidopsis (Schäfer et al., 2011).

Microarray analysis of early signaling genes induced upon the perception of *S. littoralis* OS led us to identify the *CML42* transcript, which was up-regulated in a transient manner. *S. littoralis* feeding, involving both mechanical wounding and OS perception, also up-regulated *CML42* transcripts. Wounding during insect herbivory is more complex than simple wounding; hence, we used MecWorm (a computer-controlled mechanical caterpillar that simulates mechanical wounding

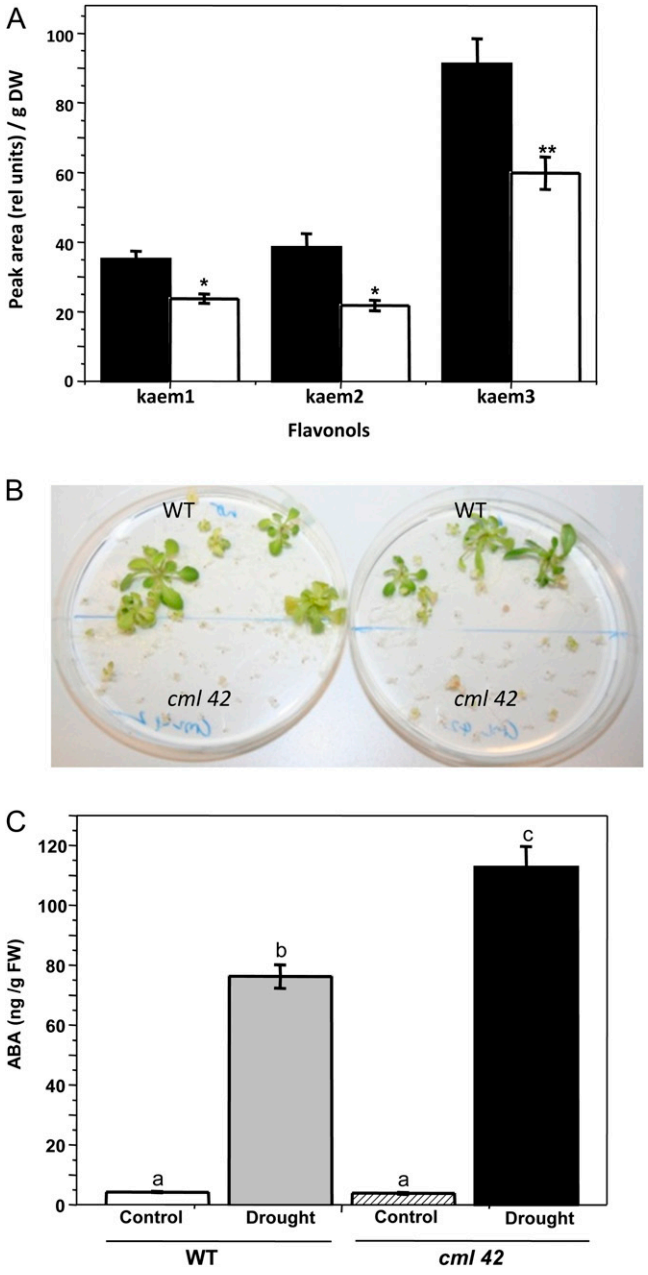

Figure 7. Multiple stress responses regulated by *CML42*. A, Relative concentrations of flavonol glycosides in rosette leaves of 5-week-old untreated Arabidopsis wild-type (black bars) and *cml42* (white bars) plants. Relative concentrations of flavonol glycosides are given in peak areas of the ion chromatogram in negative mode per gram dry weight. Flavonols were as follows: kaem1, kaempferol 3-*O*-[6′′*O*-(rhamnosyl) glucoside] 7-*O*-rhamnoside; kaem2, kaempferol 3-*O*-glucoside 7-*O*-rhamnoside; kaem3, kaempferol 3,7-*O*-dirhamnoside. Statistically significant differences between Col-0 and *CML42* plants were analyzed by unpaired *t* test (* $P < 0.05$, ** $P < 0.001$). Values are means ± SE ($n = 8$). DW, Dry weight. B, Arabidopsis wild-type (WT) and *cml42* seedlings were grown in standard conditions for 8 d and then treated for 60 min with UV-B stress. Plants of both lines were grown on either half of MS plates to avoid variations. Treated seedlings were further grown under standard conditions for 5 weeks. The photograph shows a representation of four independent experiments. C, Levels of ABA in wild-type (white and gray bars) and *cml42* (hatched and black bars) plants subjected to two cycles of drought stress of 8 d. ABA levels were measured from the whole plant rosette, which included both local and systemic leaves. Different letters indicate significant differences among treatments (ANOVA; $P < 0.05$). Values are means ± SE ($n = 10$). FW, Fresh weight.

in herbivory) to show that the *CML42* transcript was not MecWorm inducible. *CML42* is known to be expressed widely in all Arabidopsis tissues, constitutive expression was apparent in developing rosette and cauline leaves in the trichome support cells, and expression receded to veins and petioles as leaves matured (Dobney et al., 2009). We conclude that in mature leaves, *CML42* is an insect elicitor-activated gene induced upon plant perception of chemical signals in OS. However, a closely related member of the CML gene family of 50 members, *CML43*, was not up-regulated by OS, hinting at a specific role of *CML42* in herbivory elicitor perception. Arabidopsis *CML42* (AtACRE31) was also reported to be a flagellin (flg22) rapidly elicited gene and an ortholog of a similar gene rapidly activated upon treatment of Cf9 tobacco cell cultures with fungus-derived Avr9 peptide. Analysis of *CML42* and genes clustered with it identified it as a subset of genes potentially regulated by WRKY transcription factors (Navarro et al., 2004; Segonzac et al., 2011). *CML42* transcript was also identified to be rapidly induced by elicitors/effectors present in cell wall extract of a mutualistic fungus, *Piriformospora indica*, which promotes growth and not plant defense in Arabidopsis seedlings (Vadassery et al., 2009). This indicates that *CML42* transcript expression is highly inducible by many biotic stimuli and is a common element activated upon PAMP, HAMP, and specific elicitor perception in plants, indicating a conserved pathway at early signaling stages.

CML42 as a Negative Defense Regulator upon *S. littoralis* Herbivory

We found that loss of function of *CML42* results in reduced *S. littoralis* herbivory and that the larvae gain less weight on the mutants when compared with wild-type plants. Hence, it can be assumed that CML42 negatively regulates plant defense, as the loss of function of this gene is more beneficial to the plant in the presence of herbivores. The role of CML42 in plant development was identified based on the observation that CML42 interacts with KIC, a Ca^{2+}-binding protein that negatively regulates trichome branching (Dobney et al., 2009). CML42 controls the complex trichome-branching pathway and increases the number of trichome branches. Transgenic knockout plants lacking *CML42* expression had 50% of their trichomes with four branches, whereas the other 50% contained three branches, like wild-type plants. A small proportion of wild-type plants (10%) also show four branched

trichomes (Dobney et al., 2009). Arabidopsis trichomes affect the performance of small herbivores by interfering with the movement of insects over the plant surface and making it more difficult for the insects to access the leaf epidermis. However, trichomes not only affect herbivores but also their natural enemies and thus in the end are not always beneficial to the plant in multitrophic interactions (Styrsky et al., 2006; Dalin et al., 2008). In mature *cml42* mutant leaves, a 40% increase in trichome branching from three to four branches does not affect the mobility of bigger *S. littoralis* larvae, because in older leaves where the insects feed there are no changes in trichome density. Hence, it is unlikely that decreased feeding on *cml42* is due to trichome branching differences, and CML42 likely influences other factors.

It is surprising, however, that a negative regulator of plant defense is up-regulated during herbivory, as seen with *CML42* transcript accumulation. There are at least two possible explanations for this situation: (1) *CML42* can be part of a complex regulatory system for plant defenses or (2) can be an insect effector target, subject to manipulation during feeding. As part of a plant's readiness to respond to herbivory, positive regulators of the plant defense machinery are present that are subject to differential activation. This necessitates the involvement of negative regulators to prevent unnecessary prolonged activation of plant defense, which can be associated with significant costs for plant fitness and retarded plant growth (Heil and Baldwin, 2002; Du et al., 2009). Earlier reports in many systems have revealed that Ca^{2+} signaling negatively affects plant defenses, as in barley (*Hordeum vulgare*), where loss of calmodulin binding halves the ability of MLO proteins to negatively regulate defense against powdery mildew (Kim et al., 2002), and the Ca^{2+}-activated transcription factor CAMTA3 negatively regulates salicylic acid levels by binding to a salicylic acid regulator protein, EDS1 (Du et al., 2009). WRKY11 and -17 are also known negative regulators of basal resistance during bacterial infection (Journot-Catalino et al., 2006). In a biological context, the role of *CML42* could be to limit the expression of defense pathways upon herbivory, contributing to the balanced activation of defense-related functions.

A second hypothesis is that *CML42* might be a target of insect effectors that suppress plant defense. Recently, it was reported that *S. littoralis* OS contain effectors that suppress some wound-induced responses like the expression of ethylene response factor (ERF) transcription factor (Consales et al., 2012). However, the expression of *CML42* correlates with the phytohormone elevation pattern temporally and is up-regulated when plant defense is higher. Nevertheless, its role as an insect effector target cannot be ruled out, as *CML42* is one of the 77 flagellin-activated genes potentially targeted by *Pseudomonas syringae* pv *tomato* type III secretion effector proteins that suppress the flagellin-initiated defense response (Navarro et al., 2004).

JA Perception Affected in *cml42* Plants

The plant hormone JA and its derivatives are key players in the regulation of induced plant defense against insect herbivory, and 95% of protein repatterning near wound sites is JA dependent (Gfeller et al., 2011). The relatively broad effects of hormone signaling pathways on multiple plant physiological processes result in signaling pathways that are tightly regulated at multiple points, with both negative and positive feedback regulatory loops that regulate JA signaling (Kazan and Manners, 2008). We looked for alterations in phytohormone biosynthesis upon herbivory in wild-type and *cml42* plants and found no difference in the levels of JA and its derivatives between both lines. However, *cml42* showed higher up-regulation of the JA-responsive genes *VSP2* and *Thi2.1* upon *S. littoralis* herbivory, indicating higher resistance to herbivores. During insect herbivory, the JA pathway is rewired and the ERF and MYC2 pathways operate antagonistically, with activation of the MYC2 branch being beneficial for the plant and activation of the ERF branch performed by the insect for its advantage (Verhage et al., 2011). *VSP2* is a marker for the MYC2 branch of the pathway. *CML42* loss of function might affect the MYC2 branch directly, providing additional evidence for its role as a negative defense regulator. The *cml42* also resulted in a basal steady-state up-regulation of *MYC2*, *JAZ1*, and *PDF1.2* in older plants. None of the publicly available Arabidopsis microarrays identified *CML42* as a MeJA-induced gene, except one study that identified *CML42* to be induced by both MeJA and bestatin, which is an aminopeptidase inhibitor and activates JA signaling. Interestingly, bestatin, like *CML42*, exerts its effect by modulating the JA signaling pathway and not JA biosynthesis (Zheng et al., 2006). JA biosynthesis need not always precede JA signaling, and they could operate independently of each other, as in the JA-Ile biosynthetic mutant *jar1-1*, which cannot convert JA to bioactive JA-Ile, but reduction of JA-Ile levels in the *jar1-1* mutant has no effect on the wound-induced expression of *VSP2* (Suza and Staswick, 2008).

Most JA responses are mediated through the COI1 F-box protein, which is part of a JA-receptor complex. Upon insect attack, JA is rapidly synthesized and the bioactive JA-Ile conjugate binds to COI1, and this promotes the ubiquitination of transcriptional repressors, the JAZ proteins via the 26S proteasome, relieving the repression of AtMYC2 and facilitating the activation of JA-responsive genes (Chini et al., 2009). We found that *CML42* is highly up-regulated in *coi1-1* receptor mutants upon *S. littoralis* OS treatment, even for a prolonged time. Thus, the expression of *CML42* is COI1 dependent and the absence of JA perception ability in *coi1-1* mutants increases the expression of *CML42*. CML42 thus acts as a negative regulator of defense by decreasing COI1-mediated JA perception and subsequently the expression of JA-responsive genes. It might also be a target for negative feedback

regulation of JA signaling. Evidence for a role of Ca^{2+} in the regulation of JA biosynthesis comes from the fact that jasmonates induce Ca^{2+} elevation as well as the identification of a gain-of-function allele of the Arabidopsis *Two Pore Channel1* (*TPC1*) gene (Bonaventure et al., 2007; Walter et al., 2007). Down-regulation of TPC1, a tonoplast membrane-localized cation channel, amplifies the accumulation of JA after wounding (Bonaventure et al., 2007). How Ca^{2+}-mediated signals affect JA perception and signaling is unknown. In *cml42* lines, JA-induced Ca^{2+} elevation is higher than in the wild type. On the other hand, *S. littoralis* OS-induced Ca^{2+} elevation remains unchanged in *cml42* and wild-type plants. This means that (1) *CML42* is upstream of JA-induced Ca^{2+} elevation and negatively regulates it, and (2) *CML42* acts downstream of OS-induced Ca^{2+} elevation, as loss of function has no effect. This further strengthens the role of *CML42* in multiple JA perception mechanisms. Further evidence for increased JA perception in *cml42* mutants comes from the MeJA-induced root growth inhibition assays, which prove that a lower concentration of MeJA is sufficient for *cml42* to reach maximum root growth inhibition compared with wild-type plants. *cml42* showed an enhanced root growth inhibition phenotype only at 20 and 30 μM, implying that it is an upstream signaling component, and many parallel and downstream pathways coregulate JA-mediated root growth inhibition, thus reducing the effect of *cml42* loss of function. Taken together, this leads to the conclusion that *cml42* mutation results in hyperperception of JA and activation of JA-responsive genes, which might contribute to increased resistance. A model for its possible functions is presented in Figure 8.

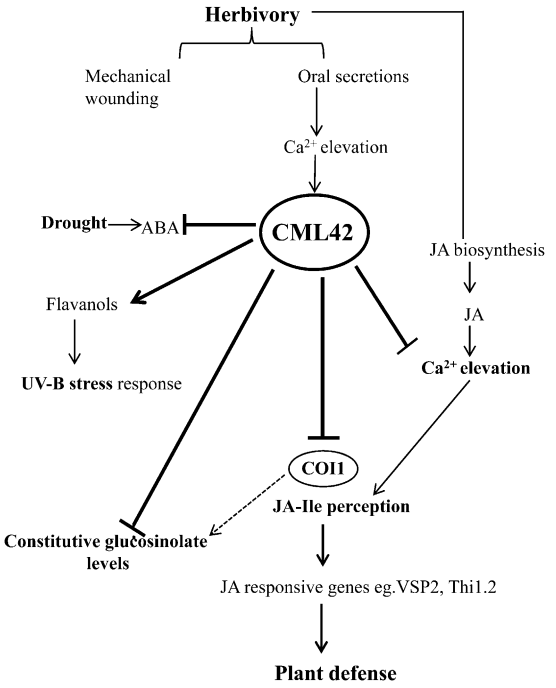

Figure 8. Model for multiple and varied roles of CML42 in insect herbivory and responses to abiotic stress. CML42 acts as a negative regulator of plant defense against *S. littoralis* by COI1-mediated JA perception, JA-induced Ca^{2+} elevation, ABA accumulation upon drought stress, and constitutive GS accumulation. CML42 also acts as a positive regulator of flavonol (kaempferol) accumulation and UV-B-mediated stress response. In insect herbivory, cytosolic Ca^{2+} elevation and *CML42* up-regulation are early plant responses upon the perception of *S. littoralis* OS. CML42 might act as a component for modulating excess defense or as an insect effector target, because loss of function of *CML42* results in increased plant defense. *CML42* up-regulation is negatively regulated by COI1, and loss of functional COI1 results in prolonged *CML42* activation. CML42 acts as a negative regulator of plant defense by decreasing COI1-mediated JA sensitivity and the expression of JA-responsive genes and is independent of herbivory-induced JA biosynthesis. CML42 acts upstream of JA-induced Ca^{2+} elevation and negatively regulates it. CML42 also negatively regulates constitutive aliphatic GSs, which are COI1 dependent (dotted arrow; Mewis et al., 2005). Thus, CML42 negatively regulates plant defense via its action on COI1-mediated JA perception.

Constitutive GS Levels Are Affected in *cml42* Plants

GSs represent a diverse group of defense secondary metabolites specific to the order Brassicales. GSs are stored separately from the activating enzyme myrosinase and only come in contact upon tissue disruption. Myrosinase action finally results in the production of biologically active breakdown products such as isothiocyanates, nitriles, and epithionitriles, all of which are toxic to generalist insects (Tohge et al., 2005; Textor and Gershenzon, 2009). Increased production of both indole and aliphatic GSs is detrimental for the survival of *S. littoralis* on Arabidopsis (Müller et al., 2010). We found that *cml42* has an increased accumulation of aliphatic GSs in the whole rosette. In Arabidopsis, overexpression of the calmodulin-binding protein IQD1, localized to nucleus, also increased GS biosynthesis (Levy et al., 2005). Upon *S. littoralis* feeding, the GS increases in both wild-type and *cml42* plants are similar. The fact that GSs are not induced more strongly in *cml42* might be attributed to a certain threshold of GSs already being sufficient for insect resistance or to a drastic increase from the already constitutive high GS levels in *cml42* being detrimental for plant fitness. It is known that normal constitutive GS accumulation requires the functional JA receptor COI1, and *coi1-1* mutants have reduced constitutive GS levels. However, GS accumulation upon insect feeding requires functional salicylic acid signaling mediator protein, NPR1, and ethylene signaling protein, ETR1, but not COI1, as in the *coi1-1* mutant GSs increase significantly upon insect feeding (Mewis et al., 2005). Furthermore, it is known that MYC2 transcription factors, which regulate JA-responsive genes, negatively regulate JA-dependent indole GS biosynthesis (Dombrecht et al., 2007). Hence, an increased sensitivity to JA in *cml42* resulting in increased expression of *VSP2* and *Thi1.2* need not necessarily also result in increased GSs upon herbivory. A combined action of both increased JA

sensitivity and GSs might also contribute to *cml42* resistance.

CML42 Plays Various Roles in Abiotic Stress Perception

Kaempferol glycosides are the major flavonols in leaves, stems, and flowers of Arabidopsis and serve to protect against UV-B radiation (D'Auria and Gershenzon, 2005). We found that *CML42* loss of function resulted in a constitutively reduced accumulation of flavonol glycosides and thus acts as a positive regulator of flavonoid induction. *cml42* seedlings were also less resistant to UV-B stress, supporting the role of flavonol glycosides in this regard and the varied roles that Ca^{2+} sensors such as CML42 play in stress perception. Among other abiotic stresses, drought triggers the production of the phytohormone ABA, which in turn causes stomatal closure and induces the expression of stress-related genes (Shinozaki and Yamaguchi-Shinozaki, 2007). *cml42* also displayed an increased ABA accumulation upon multiple drought stress cycles, although it does not show a clear drought-resistant phenotype. Thus, *CML42* negatively regulates ABA levels upon drought stress. Consistent with this finding, an Arabidopsis genome-wide ABA-responsive gene expression study found *CML42* transcript to be repressed by ABA treatment in wild-type plants (Hoth et al., 2002). CML9, another CML family member, is known to be a negative regulator in the ABA signaling pathway as well (Magnan et al., 2008). A calcium sensor that belongs to the calcineurin B-like (CBL) protein family, CBL9 was also shown to participate as a negative regulator in ABA pathways and acts through a mechanism that involves ABA biosynthesis control (Pandey et al., 2004). Interestingly, when we conducted a bioinformatics-based prediction of CML42 targets in Arabidopsis by predicted Arabidopsis interactome resource (Lin et al., 2011), it resulted in the identification of AKIN10/SnRK1.1 and AKIN11/SnRK1.2 as possible targets. AKIN10 and -11 have been shown to affect ABA signaling and are central integrators of transcription networks in plant stress and energy signaling (Baena-González et al., 2007; Jossier et al., 2009). Thus, apart from its role in herbivory, CML42 play distinct roles in responses to varied abiotic stresses through interaction with key players in hormone signaling (Fig. 8).

CML42 Is Targeted to Cytosol and Nucleus

In eukaryotic cells, various stimuli mobilize different pools of Ca^{2+} to trigger characteristic changes in Ca^{2+}, and these changes can also occur in different cell compartments (Mazars et al., 2009). The majority of CMLs are predicted to be cytosolic or nuclear (McCormack and Braam, 2003).We have been able to show that *S. littoralis* OS induce cytosolic Ca^{2+} elevation and also the expression of *CML42*. To prove the hypothesis that the localization of Ca^{2+} sensor proteins might corelate with the spatial pattern of Ca^{2+} elevation, we looked at the localization of CML42. We found that stable Arabidopsis CML42-GFP fusion lines show fluorescence localized to both cytosol and nucleus. So it can be assumed that these proteins are physically located in the cytosol, where the Ca^{2+} elevation occurs, and this also hints at the possibility of nuclear Ca^{2+} elevation by *S. littoralis*. Nuclear localization of CML42 also hints at possible roles in regulating transcription factors like WRKY, which is a predicted CML42 target (Navarro et al., 2004). It is not known, however, how important the cellular localization is for the negative regulation of herbivory, and this will be an important area for further studies.

CML family proteins are sensor-relay proteins, and their only known function is to bind Ca^{2+} and undergo conformational changes, which results in binding to targets. Thus, the identification of targets of CML42 with only three EF hand domains will throw light on the early signaling pathways activated by this protein upon the perception of various stresses. The role of CML42 as a negative regulator in defense against herbivory and ABA biosynthesis and its positive regulation of the response to UV-B stress would all depend on the varied targets the proteins interact with under different conditions. Based on the data provided, it seems that defense signaling and abiotic stress responses can cross talk through CML42. There is a good rationale for this scenario if some abiotic stresses can be so severe that defense would cost too much. When such stresses are low, there may be more resources for defenses, and this could also be signaled through CML42. It is also conceivable that under certain abiotic stresses such as UV or drought, there is less risk of herbivory. This study also proves that the JA signaling pathway is modulated negatively by the Ca^{2+} sensor protein CML42, thus highlighting that Ca^{2+} signaling in insect herbivory is an intricate and complex network with multiple components involving positive and negative regulators.

MATERIALS AND METHODS

Plant and Insect Materials

Arabidopsis (*Arabidopsis thaliana*) seeds (ecotype Columbia [Col-0]) and mutant lines with a T-DNA insertion in the exon of *AtCML42* (At4g20780), SALK_041400C (Dobney et al., 2009), and SALK_ 040227 with T-DNA in the promoter, provided by the SALK Institute (Alonso et al., 2003), were used for the insect feeding assay. The absence of *CML42* mRNA in the homozygous SALK_ 040227 was checked by reverse transcription (RT)-PCR using *CML42* gene-specific primers. A reduced transcript level (knockdown) was detected in SALK_ 040227 (Supplemental Fig. S7). SALK_041400C (Dobney et al., 2009), a complete knockout line, was used for all further experiments. *coi1-1* homozygous plants were selected by a cleaved-amplified polymorphic sequence marker (Xie et al., 1998).

Seeds were sown in 10-cm round pots and stratified for 2 d at 4°C. Afterward, plants were moved to ventilated growth rooms with constant air flow and 40% humidity at 23°C. Plants were grown at a distance of 30 cm from fluorescent light banks with six bulbs of cool-white light and two bulbs of wide-spectrum lights at a 10-h-light/14-h-dark photoperiod and a light intensity of 150 μmol m^{-2} s^{-1}. The plants were shifted to a wide-spectrum light source 3 weeks after growth, and all the experiments were done on 5-week-old

plants. Larvae of *Spodoptera littoralis* were hatched from eggs and reared on an agar-based optimal diet (Bergomaz and Boppre, 1986). Temperature was kept at 23°C to 25°C with an 8-h-light/16-h-dark cycle. The insect biomass assay was done using first instar larvae (freshly hatched larvae grown for 3 d in light), which were preweighed before the experiment and selected to have equal weights. Three larvae were placed on a single plant and covered with perforated plastic covers. After 8 d of feeding, single larvae were removed and weighed. Each experiment had 10 plants, and the experiments were repeated six times independently. Herbivory screens for 1- and 2-d feeding were performed with fourth instar *S. littoralis* larvae that were starved 12 h prior to plant feeding.

Plant Treatments

All induction experiments were performed 5 weeks post germination at a vegetative (prebolting) growth stage. Insect herbivory screens were carried out with three larvae per plant. For experiments with insect OS, wounding was done with a pattern wheel (six vertical motions) on either side of the leaf. Fourth instar *S. littoralis* larvae reared on artificial diet were fed on Arabidopsis leaves for 24 h prior to collecting OS on ice. The OS was centrifuged at 13,000 rpm for 2 min and diluted 1:1 with water. A total of 20 μL of fresh diluted OS was spread across all the holes on a single leaf. In control plants, water was added. The samples were harvested and stored in liquid nitrogen. Mechanical wounding was performed with MecWorm (Mithöfer et al., 2005). MecWorm operation was programmed to generate two, four, or six circles of damaged leaf area in the primary leaf (r = 1.5 mm) using six punches per minute at time points of 1, 2, and 3 h, respectively. All the experiments were repeated three times independently.

The root growth inhibition assay was done by growing Arabidopsis seedlings on control, 10, 20, 30, 40, and 50 μM MeJA (Sigma). Plants were grown vertically on Murashige and Skoog (MS) agar medium under continuous light. Wild-type, JA-insensitive mutant *coi1-16*, and *cml42* knockout lines were grown on each plate. All the seeds germinated at the same time, and 14 d later the root length was measured.

For drought assays, watering was withheld from 3-week-old plants for 8 d (normally they were watered daily). These were rewatered and again subjected to drought stress for 8 d before collecting samples for ABA levels at the 5-week stage. In a separate experiment, the plants were left unwatered for 16 d to assess plant survival rate. To minimize experimental variation, wild-type and mutant plants were placed in the same tray. For UV-B experiments, wild-type and *cml42* knockout seeds were surface sterilized and grown on MS plates for 8 d. To minimize variation, each plate was divided and sown one half with wild-type plants and the other half with *cml42* knockout plants. In 8-d-old seedlings, the UV-B stress was applied by 1 h of UV-B radiation at an intensity of 100 μW cm^{-2} (measured with a digital ultraviolet radiometer; Solartech Solarimeter). The plants were then allowed to grow for 5 weeks under standard conditions until they were photographed. Survival was measured by comparing the number of living plants on the UV-treated plates versus the bleached dead plants.

Ca^{2+} Measurements

Transgenic Arabidopsis (Col-0) plants expressing cytosolic apoaequorin were used for Ca^{2+} measurements (Knight et al., 1997). Plants were grown in 10-cm pots for 4 weeks. For Ca^{2+} measurements, a leaf disc was taken and reconstituted in 5 μM coelenterazine (PJK; http://www.pjk-gmbh.de/) in the dark overnight at 21°C. Bioluminescence counts in leaf discs were recorded at 5-s intervals for 10 min, recorded as relative light units with a microplate luminometer (Luminoscan Ascent version 2.4; Thermo Fischer Scientific). After a 1-min background reading, *S. littoralis* OS were added manually to the well and readings in relative light units were taken for 10 min. Calibrations were performed by estimating the amount of aequorin remaining at the end of the experiment by discharging all remaining aequorin in 1 M $CaCl_2$ and 10% ethanol, and the counts were recorded for 5 min. The luminescence counts obtained were calibrated using the equation of Rentel and Knight (2004).

Aequorin-expressing *cml42* mutants (SALK_041400C) were generated by crossing the *cml42* line with aequorin plants. The F2 progeny from the aequorin × *cml42* crosses that were homozygous for the *cml42* mutation were used. F2 plants were screened by PCR using primers spanning the T-DNA insertion (CML42-1-RP and LBb1.3) and the wild-type gene (CML42-1-LP and CML42-1-RP). The aequorin positives were identified by the presence or absence of measurable Ca^{2+} discharge using 1 M $CaCl_2$ and 10% ethanol. Three-week-old plants were used for the experiments. The primer used are CML42-1-LP (5′-CAACCCCATGACACCTCATAC-3′), CML42-1-RP (5′-CTTGGTCTCAACGCTGATCTC-3′), and LBb1.3 (5′-ATTTTGCCGATTTCGGAAC-3′).

Expression Analysis by Real-Time PCR

Leaf material was ground to a fine powder in liquid N_2, and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. An additional DNase (Turbo DNase; Ambion) treatment was included to eliminate any contaminating DNA. RNA quantity was determined photospectrometrically. DNA-free total RNA (1 μg) was converted into single-stranded cDNA using a mix of oligo$(dT)_{20}$ primers and the Omniscript cDNA synthesis kit (Qiagen).Gene-specific primers (placed at the exon-exon junction for specific amplification of cDNA, whenever possible) were designed using the National Center for Biotechnology Information primer-design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast). For real-time PCR, primers producing 150- to 170-bp amplicons were used. Quantitative RT-PCR was done on optical 96-well plates on an MX3000P Real-Time PCR Detection System (Stratagene) using the Brilliant QPCR SYBR Green Mix (Agilent) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix. Dissociation curve analysis was performed for all primer pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. Furthermore, we tested four genes as invariant endogenous controls in the assay to correct for sample-to-sample variation in RT-PCR efficiency and errors in sample quantification, and we found that *RPS18B* performed best as an endogenous control ("normalizer") upon herbivory. Thus, the mRNA levels for each cDNA probe were normalized with respect to the *RPS18B* mRNA level. Fold induction values of target genes were calculated with the ΔΔCP equation (Pfaffl, 2001) and related to the mRNA level of target genes in control leaf, which were defined as 1.0. All of the assays were run in triplicate (biological replication) to control for overall variability. The primer pairs used are as follows: for *RPS18B* (*At1g34030*), 5′-GTCTCCAATGCCCTTGACAT-3′ and 5′-TCTTTCCTCTGCGACCAGTT-3′; for *CML42* (*At4g20780*), 5′-TCGGATCTCGCCGAGGCGTT-3′ and 5′-ACGCGACCATCTTGATTCCGGT-3′; for *CML43* (*At5g44460*), 5′-ACGCTCTTCGTCTCCACCGAGT-3′ and 5′-CGGAGTCCGGTTTTGTCCGGT-3′; for *MYC2* (*At1g32640*), 5′-CGGAGATCGAGTTCGCCGCC-3′ and 5′-AATCCCGCACCGCAAGCGAA-3′; for *JAZ1* (*At1g19180*), 5′-GGCGAGCAAAGGCACCGCTA-3′ and 5′-TCCAAGAACCGGTGAAGTGAAGC-3′; for *PDF1.2* (*At5g44420*), 5′-CTGCTTTCGACGCACCGGCA-3′ and 5′-GTTGCATGATCCATGTTTGGCTCCT-3′; for *Thi2.1* (*At1g72260*), 5′-CGCCATTCTCGAAAACTCAGCTGA-3′ and 5′-GTTTAGGCGGCCCAGGTGGG-3′; and for *VSP2* (*At5g24770*), 5′-ACGACTCCAAAACCGTGTGCAA-3′ and 5′-CGGGTCGGTCTTCTCTGTTCCGT-3′.

Vector Construction and Expression of a GFP Fusion Protein

Gateway technology (Invitrogen) was used for the generation of pB7FWG2 transformation constructs, which consisted of a target gene (CML42 open reading frame cDNA) bearing a C-terminal fusion to e-GFP under the control of the dual cauliflower mosaic virus 35S promoter for plant transformations (Karimi et al., 2007). The attB adaptor-bearing PCR primers (see below) were designed for the generation of attB PCR products for recombination with the donor vector pDONR207 via BP Clonase reactions (Invitrogen). Fully sequenced entry clones were recombined in LR Clonase reactions with the pB7FWG2 destination vector. The binary constructs were introduced into *Agrobacterium tumefaciens* (GV3101) and used to transform Arabidopsis ecotype Col-0 by the standard flower-dip method (Clough and Bent, 1998; Zhang et al., 2006) and selected with phosphinotricin. The T1 positive lines were used for GFP localization. For transient transformation of *Nicotiana benthamiana* leaves, mixtures (1:1:1) of *A. tumefaciens* harboring the *CML42* construct, organelle markers for either plasma membrane or plastids (Nelson et al., 2007), and the silencing inhibitor p19 (Voinnet et al., 2003) were infiltrated into fully developed leaves. Plants were cultivated for 3 d under greenhouse conditions, and infiltrated leaf area was analyzed by confocal laser scanning microscopy using a LSM710 (Carl Zeiss; http://www.zeiss.com) equipped with appropriate lasers (488 and 594 nm for GFP and mCherry, respectively). Primers used are as follows: CML42F-attB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGAGTAACAACAACGAGA-3′) and CML42R-attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTAGAAGAAGGGATGACAACAGTA-3′).

Protein Extraction and Western Blot

Proteins were extracted by homogenizing single leaves in lysis buffer, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton, 1 mM dithiothreitol, and protease inhibitor cocktail (Roche). Total crude proteins were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was incubated first with mouse anti-GFP antibody (Roche) and then with rabbit anti-mouse IgG-horseradish peroxidase conjugates (Sigma-Aldrich). Proteins were detected using the enhanced chemiluminescence western-blotting detection reagent kit (Thermo Scientific).

Quantification of JA, JA-Ile, cis-OPDA, and Salicylic Acid in Arabidopsis Leaves

Plant material was weighed (250 mg) and frozen with liquid nitrogen, and samples were kept at −80°C until used. For phytohormone analysis, finely ground leaf material was extracted with 1.5 mL of methanol containing 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid, 60 ng of D_4-salicylic acid, 60 ng of D_6-ABA (Santa Cruz Biotechnology), and 15 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. JA-[$^{13}C_6$]Ile conjugate was synthesized as described by Kramell et al. (1988) using [$^{13}C_6$]Ile (Sigma). The homogenate was mixed for 30 min and centrifuged at 14,000 rpm for 20 min at 4°C. After the supernatant was collected, the homogenate was reextracted with 500 μL of methanol, mixed, and centrifuged, and supernatants were pooled. The combined extracts was evaporated in a SpeedVac at 30°C and redissolved in 500 μL of methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm; Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was as follows: 0 to 0.5 min, 5% B; 0.5 to 9.5 min, 5% to 42% B; 9.5 to 9.51 min, 42% to 100% B; 9.51 to 12 min, 100% B; and 12.1 to 15 min, 5% B. The mobile phase flow rate was 1.1 mL min^{-1}. The column temperature was maintained at 25°C. An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in the negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at −4,500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 ψ, curtain gas at 25 ψ, heating gas at 60 ψ, and collision gas at 7 ψ. Multiple reaction monitoring was used to monitor analyte parent ion → product ion: mass-to-charge ratio [m/z] 136.9 → 93.0 (collision energy [CE], −22 V; declustering potential [DP], −35 V) for salicylic acid; m/z 140.9 → 97.0 (CE, −22 V; DP, −35 V) for D_4-salicylic acid; m/z 209.1 → 59.0 (CE, −24 V; DP, −35 V) for JA; m/z 213.1 → 56.0 (CE, −24 V; DP, −35 V) for 9,10-D_2-9,10-dihydrojasmonic acid; m/z 263.0 → 153.2 (CE, −22 V; DP, −35 V) for ABA; m/z 269.0 → 159.2 (CE, −22 V; DP, −35 V) for D_6-ABA; m/z 322.2 → 130.1 (CE, −30 V; DP, −50 V) for the JA-Ile conjugate; and m/z 328.2 → 136.1 (CE, −30 V; DP, −50 V) for the JA-[$^{13}C_6$]Ile conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. The peak of the endogenous bioactive form of JA-Ile, (+)-7-iso-jasmonoyl-L-Ile (Fonseca et al., 2009), was used for JA-Ile quantification. For quantification of cis-OPDA, 9,10-D_2-9,10-dihydrojasmonic acid was used as the internal standard, applying an experimentally determined response factor of 2.24.

GS Analysis

Whole plants were used for GS analysis as opposed to single leaves due to vast leaf-to-leaf variation (Brown et al., 2003). Samples were freeze dried to a constant weight and ground to a fine powder. Twenty-five milligrams of freeze-dried and pulverized material per plant was used for GS analysis. GSs were extracted with 1 mL of 80% methanol solution containing 0.05 mM intact 4-hydroxybenzylglucosinolate as an internal standard. After centrifugation, extracts were loaded onto DEAE-Sephadex A25 columns (flow through was collected for further metabolite analysis; see below) and treated with arylsulfatase for desulfation (Sigma-Aldrich). The eluted desulfoglucosinolates were separated using HPLC (Agilent 1100 HPLC system; Agilent Technologies) on a reverse-phase C-18 column (Chromolith Performance RP18e, 100 × 4.6 mm; Merck) with a water-acetonitrile gradient (0%–3% acetonitrile from 0 to 3 min, 3%–20.5% acetonitrile from 3 to 10 min, 20.5%–50% acetonitrile from 10 to 13 min, followed by a washing cycle; flow, 1.5 mL min^{-1}). Detection was performed with a photodiode array detector, and peaks were integrated at 229 nm. We used the following response factors, aliphatic GS 2.0, indole GS 0.5, and 2-phenylethyl GS 2.0 (Burow et al., 2006), for quantification of individual GSs.

Flavonoid Analysis by HPLC-Ion Trap Mass Spectrometry

Flavonoids were analyzed in the flow through from the DEAE-Sephadex A25 columns used in GS analysis (see above). These flow-through extracts are basically methanolic raw plant extracts minus GSs. Chromatographic analyses were carried out on an 1100 series HPLC apparatus (Agilent Technologies) coupled to an Esquire 6000 electrospray ionization-ion trap mass spectrometer (Bruker Daltonics) operated in alternating ionization mode in the range m/z 60 to 1,400. Capillary voltage was −4,000 V/4,000 V; nebulizer pressure was 35 ψ; drying gas was 11 L min^{-1}; gas temperature was 330°C. Elution was accomplished using an EC 250/4.6 Nucleodur Sphinx RP column (25 cm × 4.6 mm, 5 μm; Macherey-Nagel) with a gradient of 0.2% (v/v) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL min^{-1} at 25°C as follows: 0% to 100% (v/v) B (15 min), 100% B (3 min), 100% to 0% (v/v) B (6 s), and 0% B (8 min, 54 s). Flow coming from the column was diverted in a 4:1 ratio before reaching the electrospray ionization unit. Flavonols were identified based on data in the literature (Tohge et al., 2005). Relative peak intensities were derived from the peak areas of the extracted ion chromatogram ([M-H]$^-$) in negative mode (i.e. m/z 739 for kaempferol 3-*O*-[6′′*O*-(rhamnosyl)glucoside] 7-*O*-rhamnoside, m/z 593 for kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, and m/z 577 for kaempferol 3,7-*O*-dirhamnoside).

Statistical Analysis

Statistical differences between different groups were detected by one-way ANOVA and post hoc Student-Newman-Keuls test in SigmaStat 2.03. Different letters indicate significant difference between treatments.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *CML43* regulation by *S. littoralis* OS.

Supplemental Figure S2. Trichome morphology of Arabidopsis wild-type and *cml42* mutant 5-week-old plants.

Supplemental Figure S3. Phytohormone changes upon *S. littoralis* OS application in *cml42* mutant plants.

Supplemental Figure S4. CML42:GFP full-length protein expression in transformed Arabidopsis.

Supplemental Figure S5. Relative mRNA levels of defense-related genes *MYC2*, *JAZ1*, and *PDF1.2* in undamaged *cml42* mutant and control wild-type plants.

Supplemental Figure S6. Elevation in cytosolic calcium concentration induced by *S. littoralis* OS is not altered in *cml42* plants.

Supplemental Figure S7. Verification of SALK_ 040227 T-DNA lines.

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Figure S1

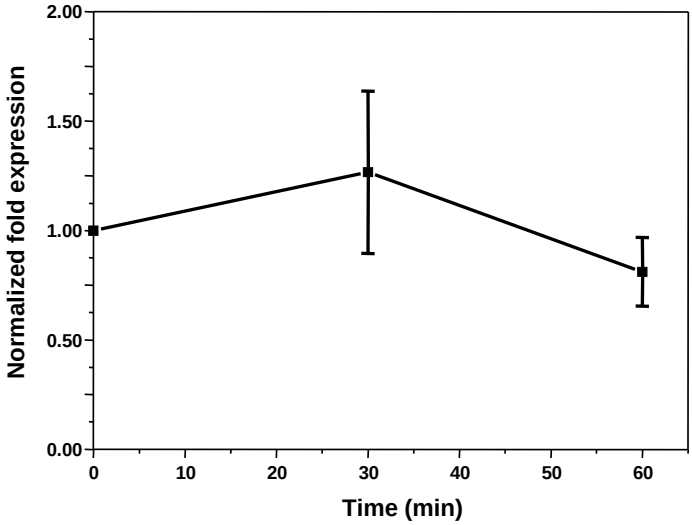

Figure S1: *CML43* regualtion by *S. littoralis* oral secretions.
Mean (± SE) *CML43* transcript levels in OS treated leaves of Arabidopsis after 30, and 60 min of treatment; elicitation and calculation as in Fig. 2A.

Figure S2

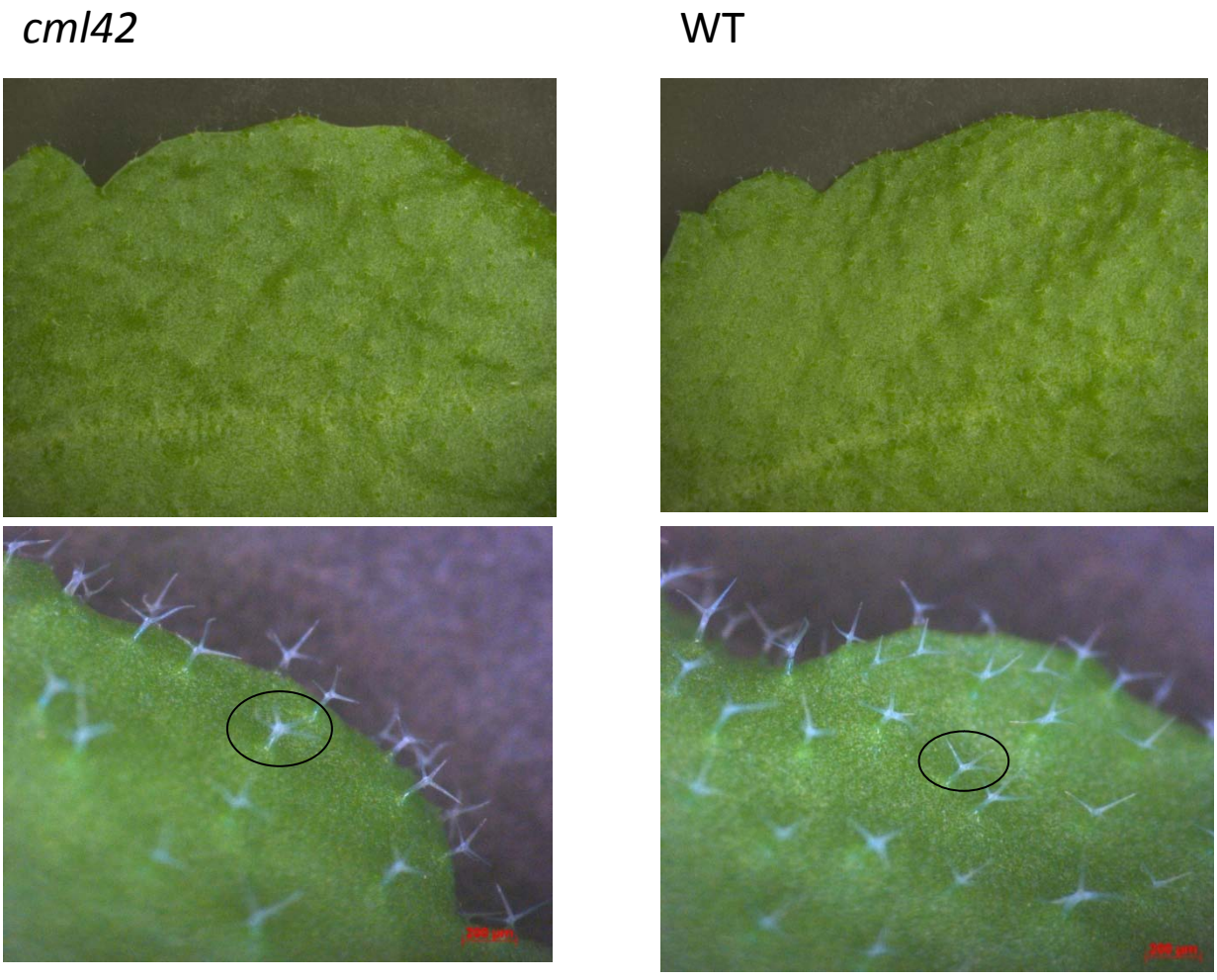

Figure S2: Trichome morphology of Arabidopsis WT and *cml42* mutant (5-week-old plants).

Figure S3

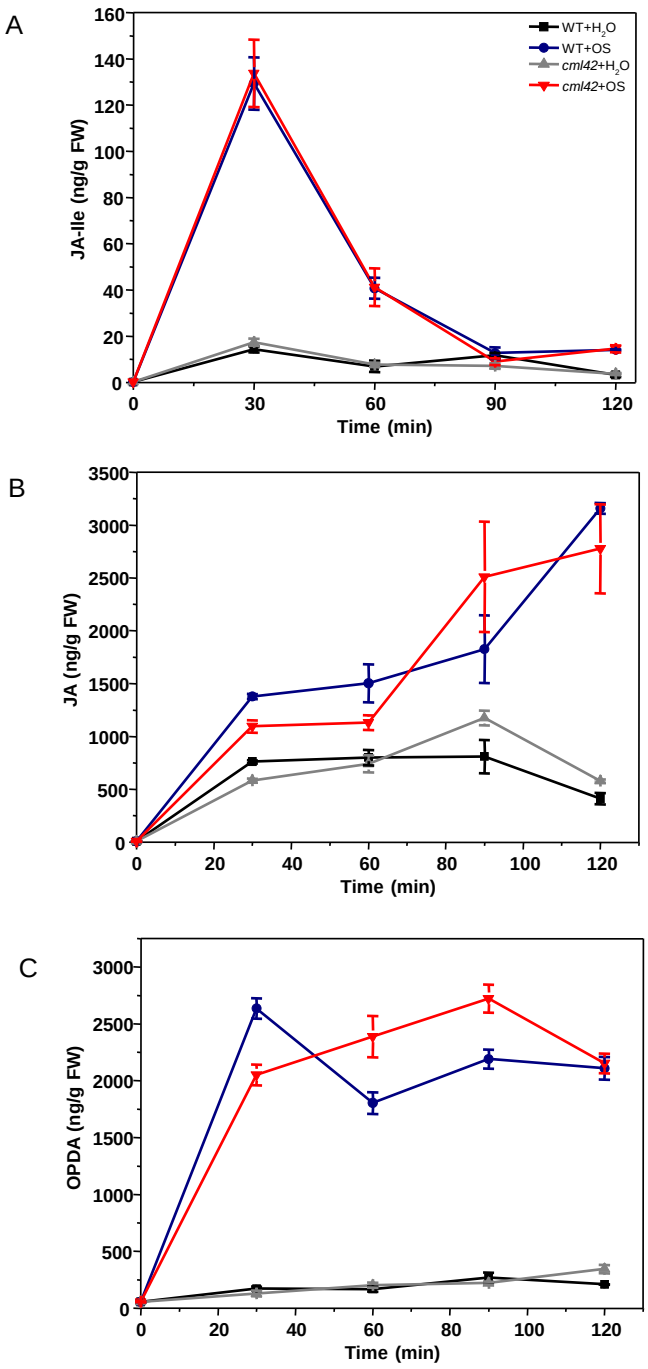

Figure S3: Phytohormone changes upon *S. littoralis*-OS application in *cml42* mutant plants. Levels of (+) JA-Ile (**A**), JA (**B**) and *cis*-OPDA (**C**) in Arabidopsis WT and *cml42* mutant line treated with *S. littoralis* OS (1:1 diluted) for 30, 60, 90 and 120 min. The phytohormone levels were measured from the local leaves.

Figure S4

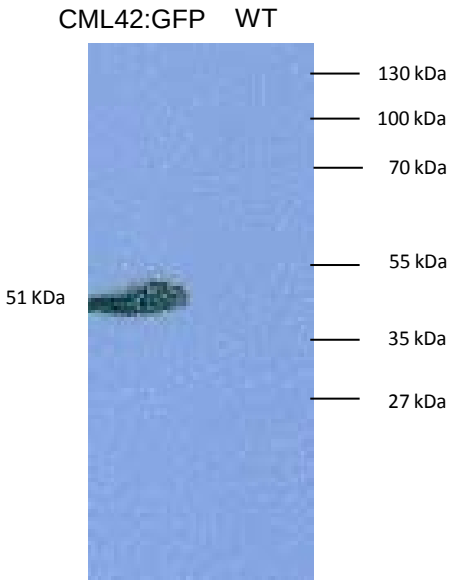

Figure S4: CML42:GFP full length protein expression in transformed Arabidopsis. Protein was extracted from GFP:CML42-transformed stable Arabidopsis lines. Supernatant of crude lysate was analyzed by western blotting using anti-GFP antibodies.

Figure S5

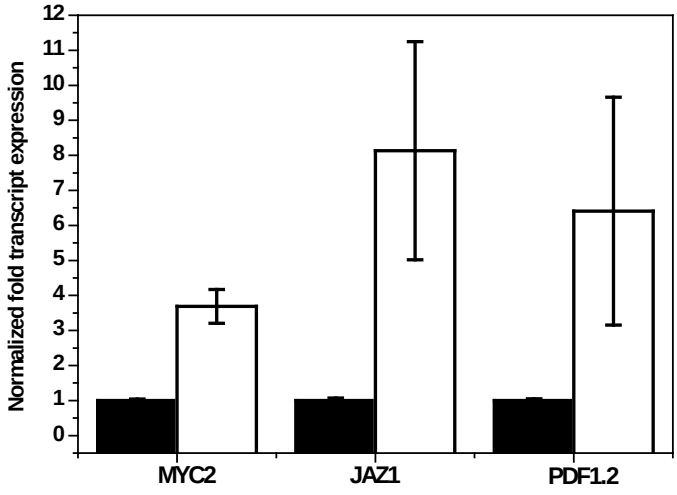

Figure S5: Relative mRNA levels (n=5) of defense-related genes *MYC2*, *JAZ1* and *PDF1.2* in undamaged *cml42* mutants (white) and control WT (black).

Transcript abundance in leaves were determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. Fold change in leaf tissue was calculated by comparative Ct method using an independent WT plant as control. Differences between Col-0 and *cml42* plants were analyzed by unpaired t-test and are statistically significant *P = <0.05.

Figure S6

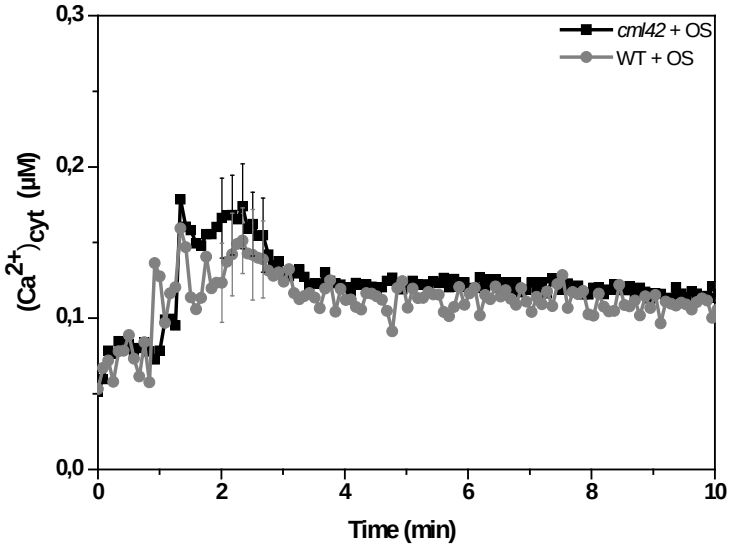

Figure S6: Elevation in cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ induced by *S. littoralis* oral secretions is not altered in *cml42* plants.
Application of 40 µL *S. littoralis* oral secretions (1:1 diluted) to 4-week-old Arabidopsis leaf disc of aequorin WT (dark grey), *cml42* x aequorin (black).

Figure S7

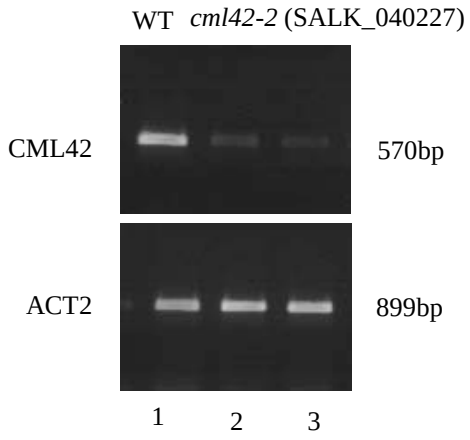

Figure S7: Verification of SALK_ 040227 T-DNA lines

Semi-quantitative RT-PCR analysis of *CML42* transcript expression in wild type control (WT,1) and *cml42-2* (SALK_040227) mutant line (2, 3) using total RNA isolated from leaves. Expression of the house-keeping gene *ACT2* (*Actin 2*) was used as quantitative control .

SALK_040227 plants homozygous for T-DNA were identified by PCR, using primer pairs *CML42-2-LP* and *CML42-2-RP* for verification of wild type gene and *LBa1.3* and *CML42-2-RP* for T-DNA insertion. The absence of *CML42* mRNA in the homozygous SALK_ 040227 was checked by RT-PCR using *CML42* gene specific primers and reduced transcript (knock-down) was detected.

CML42-2-LP	5’- CGAAGAAAGAATCGTCGAGTG -3’
CML42-2-RP	5’- CCATTAAAGCAACCAAGCTTG -3’
LBb1.3	5’- ATTTTGCCGATTTCGGAAC -3’
ACT2-F	5’- GTTGGGATGAACCAGAAGGA-3’
ACT2-R	5’- GAACCACCGATCCAGACACT -3’
CML42-F	5’- ATGGAGAGTAACAACAACGAGA-3’
CML42-R	5’- AGAAGAAGGGATGACAACAGTA-3’