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CML42-mediated calcium signaling co-ordinates responses to *Spodoptera* herbivory and abiotic stresses in Arabidopsis¹

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Abstract

In the interaction between Arabidopsis 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 (OS) induced both Ca\(^{2+}\) and phytohormone elevation in Arabidopsis. Plant gene expression induced by OS revealed up-regulation of a gene encoding calmodulin like protein, CML42. Functional analysis of cml42 plants revealed more resistance to herbivory than in 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 hyper-activated transcript accumulation of JA-responsive genes, VSP2 and Thi2.1, upon herbivory which might contribute to increased resistance. CML42 up-regulation is negatively regulated by jasmonate receptor, COI1, as loss of functional COI1 resulted in prolonged CML42 activation. CML42 thus acts as negative regulator of plant defense by decreasing COI1-mediated JA sensitivity and expression of JA responsive genes and is independent on 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 response as kaempferol glycosides were down-regulated in cml42, and impaired in UV-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.
Introduction

Plants respond actively to insect herbivory by production of phytohormones and anti-herbivore secondary metabolites and proteins (Mithöfer et al., 2009). Many of these defenses are co-ordinated by a jasmonic acid (JA) dependent signaling cascade which 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 perspective of plant-pathogen interaction, plant defense involves two components. One is immunity due to recognition of general elicitors, like microbe or pathogen associated molecular patterns (MAMP/PAMP), by specific pattern recognition receptors (PRRs) which trigger basal resistance or PAMP-triggered immunity (PTI). However, during co-evolution of pathogens with host plants, pathogens acquired effector molecules to suppress PTI. The plants then counter-acted this with a second component of immunity, recognition of effectors by specific resistance proteins (R) resulting in effector triggered immunity (ETI) 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 MAPK 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 and are of two kinds: (i) chemical elicitors derived from insect oral secretions (OS) and oviposition fluids; and (ii) plant-derived self-recognition factors occurring due to a specific pattern of wounding (Mithöfer and Boland, 2008; Heil, 2009). Insect OS contain elicitors which 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 formed in caterpillar midgut, and fatty acid-amino acid conjugates (FACs) such as volicitin in maize. However in Arabidopsis 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 oral secretions which result in specific ligand-receptor interactions mediating recognition (Schmelz et al., 2009). The identity of many HAMPs and knowledge of their perception mechanisms is 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 sub- cellular 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 points 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 activity, eg., CDPK) and sensor relay proteins (only bind Ca\(^{2+}\) and undergo conformational changes). Calmodulin like proteins (CMLs) are sensor relay proteins which are unique to plants with 50 members in Arabidopsis. CMLs are defined by the presence of 2-6 predicted EF hand motifs, by the absence of any other identifiable functional domains and at least 15% amino acid identity with calmodulins, CaMs (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, hormone and chemical treatment (Vanderbeld and Snedden, 2007). CML24 is known to cause alterations in flowering time, abscisic acid and ion stress (Delk et al., 2005; Hubbard et al., 2008). CML9 alters plant responses to abscisic acid 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 [Ca\(^{2+}\)]\(_{cyt}\) in cells adjacent to the insect bite (Maffei et al., 2004). An FAC fraction from larval oral secretions results in an immediate Ca\(^{2+}\) influx in soybean 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 oral secretion 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 spider mite attack *Tetranychus urticae* shows activated defense gene expression
requiring Ca\(^{2+}\) influx and protein phosphorylation (Arimura et al., 2000). In Arabidopsis, a 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 biosynthesis (Levy et al., 2005). In 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 oral secretions (GS) to puncture wounds elicited increased cytosolic Ca\(^{2+}\) and phytohormone levels (Schafer et al., 2011). Ca\(^{2+}\) has also been implicated in downstream parts of herbivore-triggered signaling cascades since jasmonates and synthetic analogues induce Ca\(^{2+}\) elevations in tobacco BY-2 cells culture (Walter et al., 2007).

In the current 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 perception of elicitors in *S. littoralis* oral secretions. Loss of CML42 negatively regulates defense and results in reduced herbivory due to increased JA sensitivity. Apart from its role in herbivory, CML42 also co-ordinates the perception of abiotic stress responses.

**Results**

*S. littoralis* oral secretions induce cytosolic Ca\(^{2+}\) elevation and a phytohormone burst in Arabidopsis

As they feed, caterpillars use oral secretions (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 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 seconds the Ca\(^{2+}\) levels increased and reached a maximum at 2 minutes followed by a slow decline to resting levels in 10 minutes (Fig. 1A). To analyze whether OS induces downstream defense responses in the leaf, we measured phytohormone changes upon wounding + H\(_2\)O 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 minutes and declining gradually to resting levels by 90 minutes (Fig. 1B). JA levels upon OS application increased gradually reaching a peak at 60 minutes and then decreasing gradually, but did not reach resting levels after 120 minutes (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 oral secretion application and their levels increased equally upon both treatments (Fig. 1E).

**Elicitors in *S. littoralis* oral secretion 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 minutes each (unpublished data). Microarray analysis revealed that gene encoding calmodulin like protein (CML), *CML42* (At4g20780), are 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 minutes and sustaining for 60 minutes (Fig. 2A). Plants may respond to two aspects of herbivory: mechanical wounding and application of oral secretions. 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 minutes and the transcript is down-regulated at 180 minutes indicating that the expression of *CML42* is induced by oral secretions 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 hour and returning to baseline expression in 2 hours (Fig. 2C). Since the kinetics of CML42 expression (30 – 60 minutes) 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, though the marker gene VSP2 was up-regulated (Fig. 2D). Since 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 CaM 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 oral secretion application (Supplementary Fig.1).

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. Since 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 to the wild type after they feed for 8 days on either plants (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 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 (Supplementary 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 WT and cml42 plants were challenged with S. littoralis larvae and oral secretions 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 after 24 hours and 1 week after feeding (Figs. 3A, B, C and D). SA levels remain unchanged upon insect herbivory. Similar results were obtained by wounding and OS treatment (Supplementary Fig. S3) indicating that increased resistance in cml42 is not due to an increase in phytohormone production.

**CML42 is targeted to cytosol and nucleus**

Specificity of a common signal like Ca\(^{2+}\) is due to both the spatial and temporal pattern of Ca\(^{2+}\) elevation and due to spatially distinct Ca\(^{2+}\) decoding protein complexes. Hence it was important to study the sub-cellular localization of CML42, and so we generated transgenic Arabidopsis lines expressing CML42-GFP fusion protein under the control of the constitutive 35S CaMV promoter. The CML42-GFP fusion 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 co-localization experiment using *Nicotiana benthamiana* transiently co-transformed with plasma membrane markers (Fig. 4B). In both the cases CML42 was localized to the cytosol and nucleus. The CML42-GFP fusion protein was of the expected size, 51 kDa, and the full length protein was expressed in Arabidopsis (Supplementary 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 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 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 cml42 mutant the mRNA level was up-regulated 200-fold, in contrast to a 100-fold higher level in wild type (Fig. 5A). *PDF1.2* showed no differences and was up-regulated 25-fold in wild type and 30-fold in cml42 upon *S. littoralis* herbivory. Further it was observed that cml42 plants *per se* have higher expression of JA responsive genes like the *PDF1.2, JAZ1* and transcription factor *MYC2* when compared to wild type plants without herbivory (Supplementary Fig. S5). JA perception occurs via binding of bioactive jasmonate, JA-Ile to jasmonate receptor, COI1. It had been shown that 5 h after *S. littoralis* feeding 119
genes were up-regulated 71 of which showed no change in 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 *coi1-1* mutant, with significant up-regulation at 60 minutes and 90 minutes as opposed to the wild type plant where at 90 minutes *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 minutes (Fig. 5C). To test if loss-of-function of *CML42* has an impact on JA-induced Ca$^{2+}$ elevations, *cml42* mutant was crossed to Ca$^{2+}$ reporter, aequorin, and F$_2$ homozygous plants were selected. *cml42* x 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 hyper-activates JA-induced Ca$^{2+}$ elevation. However, treatment with *S. littoralis* OS on *cml42* x aequorin gave no differences indicating that *CML42* is downstream of *S. littoralis* OS-induced Ca$^{2+}$ elevation (Supplementary 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 µM to 50 µM. The *coi1-16* mutant showed strong JA insensitive phenotype as reported previously (Ellis and Turner, 2002). We found that 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 *cml42* mutant is more sensitive to JA (Fig. 5D). All the data thus indicates that jasmonate perception is more sensitive upon *CML42* loss-of-function and hence *S. littoralis* feed less on plants.

**Glucosinolates level are modulated in *cml42* plants**

The secondary metabolite arsenal in Arabidopsis comprises many compounds including glucosinolates, flavonoids, and terpenoids which are necessary for plant survival in unfavorable environments (Kliebenstein, 2004). Glucosinolates (GS), are important secondary metabolites involved in resistance to generalist insects like *S. littoralis* and both aliphatic and
indole glucosinolates have additive roles (Müller et al., 2010). We wanted to evaluate the influence of CML42 on glucosinolate 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 increased accumulation of glucosinolates (Fig. 6A). The increase in total glucosinolates was due to an increase in aliphatic GS (methionine-derived) rather than indolic components (tryptophan derived). Upon S. littoralis feeding for 24 hours on WT and cml42, both indolic and aliphatic glucosinolates increase to similar levels in both the lines and cml42 does not show a greater increase as expected (Fig. 6B). The 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 WT 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 glucosinolates 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 which change in cml42 over the wild type. It was found that the level of the flavonol glycosides, kaempferol-3-O-[6”-O-(rhamnosyl)glucoside] 7-O-rhamnoside, kaempferol 3-O-glycoside 7-O-rhamnoside and kaempferol 3,7-O-dirhamnoside (Matsuda et al., 2010) were significantly reduced in the cml42 plants when compared to wild type plants (Fig. 7A). Since 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 hour UV-B treatment resulted in 20 ± 5% survival in WT 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 abscisic acid (ABA) biosynthesis (Delk et al., 2005; Hubbard et al., 2008). Therefore, we looked for the impact of drought stress, on WT and cml42. Water stress resulted in increase of ABA content in all genotypes. But it was observed that ABA levels are higher in cml42 as opposed to WT 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 very variable and though the cml42 plants showed a drought resistance phenotype at 8 days after drought, both WT and cml42 plants died upon prolonged drought stress.
Discussion

*S. littoralis* elicitor induced transcript accumulation of *CML42*

Plant response to herbivory includes reaction to both mechanical wounding and elicitors in insect oral secretions (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., 2011). Our current data show that *S. littoralis* OS induces cytosolic Ca\(^{2+}\) and phytohormone elevation in Arabidopsis and thus activates defense. It is interesting to note 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 (Schafer et al., 2011).

Microarray analysis of early signaling genes induced upon 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 and hence we used MecWorm (a computer controlled mechanical caterpillar that simulates mechanical wounding in herbivory) to show that *CML42* transcript was not MecWorm inducible. *CML42* is known to be expressed widely in all Arabidopsis tissues and in leaves 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 oral secretions 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 (FLARE) and an ortholog of a similar gene rapidly activated upon treatment of Cf9-tobacco cell cultures with fungal derived Avr9 peptide. Analysis of *CML42* and genes clustered with it identified it as a sub-set 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 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 the larvae gain less weight on the mutants when compared to 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 observation that CML42 interacts with KIC, a Ca²⁺-binding protein which negatively regulates trichome branching (Dobney et al., 2009). CML42 controls the complex trichome branching pathway and it increases the number of trichome branches. Transgenic knock out 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 performance of small herbivores by interfering with the movement of insects over 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 multi-trophic 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, since 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 however surprising 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 of 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 plant defense machinery are present which 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 Ca²⁺ signaling negatively affects plant defenses, as in barley 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\textsuperscript{2+} activated transcription factor CAMTA3 negatively regulates salicylic acid (SA) levels by binding to an SA 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 \textit{CML42} could be to limit the expression of defense pathways upon herbivory contributing to balanced activation of defense-related functions.

A second hypothesis is that \textit{CML42} might be a target of insect effectors which suppress plant defense. Recently it was reported that \textit{S. littoralis} OS contain effectors that suppress some wound induced responses like expression of ERF transcription factor (Consales et al., 2011). However, expression of \textit{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 \textit{CML42} is one of the 77 flagellin activated genes potentially targeted by \textit{Pseudomonas syringae} pv. \textit{tomato} type III secretion effector proteins that suppress the flagellin-initiated defense response (Navarro et al., 2004).

\textbf{JA perception affected in \textit{cml42} plants}

The plant hormone jasmonic acid and its derivatives are key players in regulation of induced plant defense against insect herbivory and 95\% of protein re-patterning 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 \textit{cml42} plants and found no difference in the levels of JA and its derivatives between both lines. However, \textit{cml42} showed higher up-regulation of the JA responsive genes, \textit{VSP2} and \textit{Thi2.1} upon \textit{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). \textit{VSP2} is a marker for the MYC2 branch of the pathway. \textit{CML42} loss-of-function might affect MYC2 branch directly providing additional evidence for its role as negative defense regulator. The \textit{cml42} also resulted in a basal steady state up-regulation of \textit{MYC2}, \textit{JAZ1} and \textit{PDF1.2} in older plants. None of the publicly available Arabidopsis microarrays identified \textit{CML42} as MeJA-induced gene, except one study that identified \textit{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 JA signaling pathway, and not JA biosynthesis (Zheng et al., 2006). JA biosynthesis needs not always precede JA signaling and could operate independently of each other as in case of JA-Ile biosynthetic mutant jar1-1 that cannot convert JA to bioactive JA-Ile, but reduction in JA-Ile levels in jar1-1 mutant has no effect on the wound-induced expression of VSP2 (Suza and Staswick, 2008).

Most JA responses are mediated through the coronatine insensitive 1 (COI1) F-box protein which is part of a JA-receptor complex. Upon insect attack, JA is rapidly synthesized and the bio-active JA-Ile conjugate binds to COI1 and this promotes ubiquitination of transcriptional repressors, the JAZ proteins via the 26S proteasome, relieving repression on AtMYC2 and facilitating 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 Channel 1 (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 wild type. On the other hand, S. littoralis OS-induced Ca^{2+} elevation remains unchanged in cml42 and WT. This means that a) CML42 is upstream of JA-induced Ca^{2+} elevation and negatively regulates it; and b) 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 come from the MeJA-induced root growth inhibition assays, which prove that lower concentration of MeJA is sufficient for cml42 to reach maximum root growth inhibition compared to wild type plants. cml42 showed enhanced root growth inhibition phenotype only at 20 and 30µM implying that it is an upstream signaling component and many parallel and downstream pathways co-regulate 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 hyper-perception of JA and
activation of JA responsive genes which might contribute to increased resistance. A model for its possible functions is presented in Fig. 8.

**Constitutive glucosinolates levels are affected in cml42 plants**

Glucosinolates (GS) represent a diverse group of defense secondary metabolites specific to the order Brassicales. Glucosinolates 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 glucosinolates are detrimental for survival of *S. littoralis* on Arabidopsis (Müller et al., 2010).

We found that *cml42* has increased accumulation of aliphatic glucosinolates in the whole rosette. In Arabidopsis, over-expression of a calmodulin binding protein IQD1, localized to nucleus also increased glucosinolate biosynthesis (Levy et al., 2005). Upon *S. littoralis* feeding the glucosinolate increases in both WT and *cml42* are similar. The fact that glucosinolates are not induced more strongly in *cml42* might be attributed to a certain threshold of glucosinolates 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 glucosinolate 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 *coi1-1* mutant GS increase significantly upon insects feeding (Mewis et al., 2005). Furthermore, it is known that MYC2 transcription factors, which regulate JA responsive genes negatively regulate JA dependent-indole glucosinolate 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 glucosinolates upon herbivory. A combined action of both increased JA sensitivity and glucosinolates 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 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 phytohormone abscisic acid (ABA), which in turn causes stomatal closure and induces expression of stress-related genes (Shinozaki and Yamaguchi-Shinozaki, 2007). *cml42* also displayed an increased ABA accumulation upon multiple drought stress cycles, though 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 protein, (CBL) 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 PAIR (Lin et al., 2010) it resulted in 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-Gonzalez 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* oral secretions induce cytosolic Ca\(^{2+}\) elevation and also expression of *CML42*. To prove the hypothesis that localization of Ca\(^{2+}\) sensor proteins might co-relate 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 also hints at 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 however not known 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, identification of targets of CML42 with only 3 EF hand domains will throw light on the early signaling pathways activated by this protein upon perception of various stresses. The role of CML42 as a negative regulator in defense against herbivory and ABA biosynthesis and its positive regulation of 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 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**

*A. thaliana* seeds (ecotype Columbia) 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 promoter, provided by the SALK institute (Alonso et al., 2003) were used for insect feeding assay. The absence of CML42 mRNA in the homozygous SALK_040227 was checked by RT-PCR using CML42 gene specific primers. Reduced transcript level (knock-down) was detected in SALK_040227 (Supplemental Figure S7). SALK_041400C (Dobney et al., 2009), a complete knock-out line, was used for all further experiments. *coi1-1* homozygous plants were selected by CAPS marker (Xie et al., 1998).
Seeds were sown in 10 cm round pots and stratified for 2 d at 4°C. Afterwards, 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 6 bulbs of cool white and 2 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 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 23–25°C with 8 h light/16 h dark cycles. The insect biomass assay was done using 1st instar larvae (freshly hatched larvae grown for 3 d in light), which were pre-weighed 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 6 times independently. Herbivory screens for 1 d and 2 d feeding were performed with 4th instar *S. littoralis* larvae which were starved 12 h prior to plant feeding.

**Plant treatments**

All induction experiments were performed 5 weeks post germination at a vegetative (pre-bolting) growth stage. Insect herbivory screens were carried out with 3 larvae per plant. For experiments with insect oral secretions, wounding was done with a pattern wheel (6 vertical motions) on either side of the leaf. 4th 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 2, 4, or 6 circles of damaged leaf area in the primary leaf (r = 1.5 mm) using 6 punches min⁻¹ at time points at 1, 2 and 3 h, respectively. All the experiments were repeated three times independently.

Root growth inhibition assay was done by growing Arabidopsis seedlings on control, 10 µM, 20 µM, 30 µM, 40 µM and 50 µM MeJA (Sigma). Plants were grown vertically on MS agar medium under continuous light. WT, JA insensitive mutant *coi1-16*, and *cml42* knock-out
lines were grown in each plate. All the seeds germinated at same time and 14 days 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 re-watered and again subjected to drought stress for 8 d before collecting samples for ABA levels at 5 week stage. In a separate experiment, the plants were left unwatered for 16 d to assess plant survival rate. To minimize experimental variation, WT and mutants were placed in the same tray. For UV-B experiments WT and cml42 ko seeds were surface sterilized and grown on MS plates for 8 d. To minimize variation, each plate was divided and sown one half with WT and other half with cml42 ko plants. In 8-d-old seedlings the UV-B stress was applied by 1 h of UV-B radiation at the 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.

**Ca²⁺ measurements**

Transgenic *A. thaliana* (Col) expressing cytosolic apoaequorin were used for Ca²⁺ measurements (Knight et al., 1997). Plants were grown in 10 cm pots for 4 weeks. For Ca²⁺ measurements a leaf disc was taken and reconstituted in 5 µm coelenterazine (P.J.K. GmbH, 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 (RLU/sec) 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 RLU were taken for 10 min. Calibrations were performed by estimating the amount of aequorin remaining at the end of experiment by discharging all remaining aequorin in 1 M CaCl₂ and 10% ethanol, and the counts were recorded for 5 min. The luminescence counts obtained were calibrated using the equation by (Rentel and Knight, 2004).

Aequorin-expressing cml42 mutants (SALK_041400C) were generated by crossing cml42 line with aequorin plants. The F2 progeny from the aequorin x cml42 crosses which 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 (CML42-1-LP and CML42-1-RP) for wild type gene. The aequorin positives were identified by presence or absence of measurable Ca²⁺ discharge using 1 M CaCl₂ and 10% ethanol. Three week old plants were used for experiments. The primer pairs are listed:
Expression analysis by Real Time PCR

Leaf material was ground to a fine powder in liquid N₂, and total RNA was isolated using the TRIzol Reagent (Invitrogen) according to the manufacturers’ 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-dT₂₀ primers using 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 NCBI 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. Q-RT-PCR was done in optical 96-well plates on a 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. A 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 4 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 found 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 ΔΔCT 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 listed below

Primers

RPS18B (At1g 34030)  5’- GTCTCCAATGCCTTGACAT -3’
5’- TCTTTCTCCTGCGACCAGTT -3’

CML 42 (At 4g20780)  5’- TCGGATCTCGCCGAGGCGT -3’
5’- ACGCGACCATCTTGTACCGGT -3’
Vector construction and expression of GFP fusion protein

Gateway Technology (Invitrogen) was used for the generation of pB7FWG2 transformation constructs, which consisted of a target gene (CML42 ORF 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 Columbia-0 by the standard flower dip method (Clough and Bent, 1998; Zhang et al., 2006) and selected with BASTA. The T1 positive lines were used for GFP localization. For transient transformation of N. 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:

CML42F-attB1 5’-GGGGACAAAGTTTGTACAAAAAGCAGGCTTC
  ATGGAGAGTAACCAAACACGAGA-3’

CML42R-attB2 5’-GGGGACCACCTTTGTACAAGAAAGCTGGGA
  AGAAGAAAGGATGACAACAGTA -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 DTT and protease inhibitor cocktail (Roche). Total crude proteins were separated by SDS-polyacrylamide gel electrophoresis (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-HRP conjugates (Sigma-Aldrich). Proteins were detected using ECL western blotting detection reagent kit (Thermo Scientific).

Quantification of jasmonic acid, JA-Ile, OPDA, and salicylic acid in Arabidopsis thaliana 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-D2-9,10-dihydrojasmonic acid, 60 ng D4-salicylic acid, 60 ng D6-abscisic acid (Santa Cruz Biotechnology), and 15 ng of jasmonic acid-[13C6]isoleucine conjugate as internal standards. Jasmonic acid-[13C6]isoleucine conjugate was synthesized as described by (Kramell et al., 1988) using [13C6]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 re-extracted with 500 µL methanol, mixed and centrifuged and supernatants were pooled. The combined extracts was evaporated in a speed-vac at 30°C and re-dissolved in 500 µL methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 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: 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 -4500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: 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 D₄-salicylic acid; m/z 209.1 → 59.0 (CE -24 V; DP -35 V) for jasmonic acid; m/z 213.1 → 56.0 (CE -24 V; DP -35 V) for 9,10-D₂-9,10-dihydrojasmonic acid; m/z 263.0 → 153.2 (CE -22 V; DP -35 V) for abscisic acid; m/z 269.0 → 159.2 (CE -22 V; DP -35 V) for D₆-abscisic acid; m/z 322.2 → 130.1 (CE -30 V; DP -50 V) for the jasmonic acid-isoleucine conjugate; m/z 328.2 → 136.1 (CE -30 V; DP -50 V) for the jasmonic acid-[¹³C₆]isoleucine 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-isoleucine (Fonseca et al., 2009) was used for JA-Ile quantification. For quantification of 12-oxophytodienoic acid (cis-OPDA), 9,10-D₂-9,10-dihydrojasmonic acid was used as the internal standard applying an experimentally determined response factor of 2.24.

**Glucosinolate (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 until constant weight and ground to a fine powder. 25 mg of freeze-dried and pulverised material per plant was used for GS analysis. GS were extracted with 1 mL of 80% methanol solution containing 0.05 mM intact 4-hydroxybenzylglucosinolate as internal standard. After centrifugation, extracts were loaded onto DEAE Sephadex A 25 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 high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies) on a reversed phase C-18 column (Chromolith Performance RP18e, 100 x 4.6 mm, Merck, Darmstadt) with an water-acetonitrile gradient (0 to 3% acetonitrile from 0 to 3 min, 3 to 20.5% acetonitrile from 3 to 10 min, 20.5 to 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 glucosinolates 2.0, indole glucosinolates 0.5, 2-
phenylethyl glucosinolate 2.0 (Burow et al., 2006) for quantification of individual glucosinolates.

**Flavonoid analysis by HPLC-Ion trap mass spectrometry**

Flavonoids were analysed in the flow through from the DEAE Sephadex A 25 columns used in GS analysis (see above). These flow-through extracts are basically methanolic raw plant extracts minus glucosinolates. Chromatographic analyses were carried out on an 1100 series HPLC (Agilent Technologies) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics) operated in alternating ionization mode in the range \( m/z \) 60 to 1400. Capillary voltage, -4,000 V/4,000 V; nebulizer pressure, 35 psi; drying gas, 11 L min\(^{-1}\); gas temperature, 330ºC. Elution was accomplished using a EC 250/4.6 Nucleodur Sphinx RP column (25 cm x 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), 0% B (8 min 54 s). Flow coming from the column was diverted in a ratio 4:1 before reaching the ESI unit. Flavonols were identified based on data in the literature (Tohge et al., 2005). Relative peak intensities were derived from the peak area 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 posthoc SNK test in SigmaStat 2.03. Different letters indicate significant difference between treatments.

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Legends

**Figure 1.** *S. littoralis* oral secretions (OS)-induced changes in $[Ca^{2+}]_{cyt}$ and phytohormones in Arabidopsis.

**A** Application of 40 µL *S. littoralis* oral secretions (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 disc at 5 s integration time for 10 min. In all the experiments, water was used as control and gave background readings.

**B-E** Mean (± SE, n=4) content of jasmonic acid-isoleucine, (+)-JA-Ile (B), jasmonic acid, JA (C), 12-oxophytodienoic acid, cis-OPDA (D), and salicylic acid, SA (E) in mechanical wounding + *S. littoralis* oral secretion-elicited leaves and control with mechanical wounding + H$_2$O treatment. Phytohormones were measured in local treated leaves.

**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 water or 1:1 diluted OS per leaf. Transcript abundance in leaves was determined by real-time PCR analysis and normalised to the plant RPS18B mRNA level. The fold change was calculated relative to control which was mechanical wounding + H$_2$O. The graph shows x-fold induction of the mRNA levels by the *S. littoralis* OS relative to the levels in the H$_2$O treated control leaves. Mean (± 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. Mean (± SE, n=3).

**C** *CML42* transcript levels in Arabidopsis leaves after 30 min, 1, 2 and 24 h of *S. littoralis* feeding. Undamaged leaves were used as controls for quantification. Mean (± SE, n=5).

**D** *CML42* and *VSP2* transcript levels in JA-treated (50µM) Arabidopsis leaves after 0.5, 3 and 6 hours of treatment. Elicitation and calculation as in Fig. 2A. *VSP2* was used as a marker for JA responsiveness in plants. The fold change was calculated relative to control, which were leaves with 0.015% methanol spray. Mean (± SE, n=6).

**E,F** Larval weight after feeding on Col-0 and two different *cml42* lines, (D) SALK_041400; (E) SALK_040227. *S. littoralis* 1st instar larvae growing in light for 3 d after hatching were pre-weighed 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 (N) is indicated in the bars. Values are mean of 4 and 3 independent experiments for E and F respectively. Statistically significant differences between Col-0 and *cml42* plants after feeding of the larvae were analyzed by Mann-Whitney Rank Sum Test, **P = <0.001.

**Figure 3.** Phytohormone elevation upon *S. littoralis* herbivory in *cml42* plants.

Mean (± SE, n=5) levels of JA (A), (+) JA-Ile (B), cis-OPDA (C) and SA (D) in Arabidopsis wild type, WT (white) and *cml42* (black) fed by *S. littoralis* for one and eight days. The
phytohormone levels were measured from the whole plant rosette which includes both local and systemic leaves. Statistical differences in phytohormone levels among WT and cml42 (ANOVA; P<0.05) were calculated and no significant changes determined.

**Figure 4.** Subcellular CML42 localization.

**A** Visualization of CML42:GFP in leaves of transgenic Arabidopsis plants using CLSM. Green fluorescence of the fusion protein is visible in cytoplasm surrounding chloroplasts (arrow heads) and in the nucleus (arrows).

**B** Visualization of CML42:GFP in transiently transformed leaves of *Nicotiana benthamiana* using CLSM. Co-infiltration 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 co-localize to the plasma membrane (arrow head).

Bars represent 20 µm in all micrographs.

**Figure 5.** Jasmonic acid perception in cml42 mutants.

**A** Relative mRNA levels of defense-related genes *VSP2*, *Thi2.1*, and *PDF1.2* in *S. littoralis* infested leaves of WT (black) and cml42 (white). *S. littoralis* (4th instar larvae) fed on both lines for 24 h. Undamaged WT 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 and *P = <0, 05.

**B** CML42 transcript levels in *S. littoralis* OS-treated leaves of wild type (black) and jasmonic acid receptor mutant, *coi1-1* (grey) after 30, 45, 60 and 90 min of treatment. Leaves were elicited as described for Fig. 2A. Different letters indicate significant differences among treatments (ANOVA; P<0.05). Mean (± SE, n=4).

**C** Elevation in cytosolic calcium concentration [Ca$^{2+}$]$_{cyt}$ induced by jasmonic acid in cml42 plants. Jasmonic acid (500 µM) was applied to leaf disc of aequorin-expressing WT (dark grey) and cml42 x aequorin (black) plants. Leaves were elicited as described for Fig. 1A. Water was used as control and gave background readings (light grey). N=5 and mean of 2 independent experiments. Error bars represent SE, different letters indicate significant differences among treatments (ANOVA; P<0.05).

**D** Effects of increasing concentrations of MeJA on inhibition of root growth in wild type, *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 days in continuous light. The experiment was repeated 3 independent times (N=20 each line per experiment). Error bars represent SE, different letters indicate significant differences among treatments (ANOVA, SNK post hoc test: P<0.05).
Figure 6. Glucosinolate levels in cml42 plants.

A Mean (± SE) levels of total, indole and aliphatic glucosinolates in rosette leaves of 5-week-old, untreated Arabidopsis WT (black) and cml42 (white) plants. Statistically significant differences between Col-0 and cml42 plants were analyzed by unpaired t-test, **P = <0.001. The values were mean of 5 independent experiments, total number of plants used (N) is indicated in the bars.

B Mean (± SE, n=8) levels of total, indole and aliphatic glucosinolates in rosette leaves of 5-week-old Arabidopsis WT (black) and cml42 (white) plants fed by S. littoralis for one day. Different letters indicate significant differences among treatments (ANOVA; P<0.05). The values were mean of 2 independent experiments.

Figure 7. Multiple stress responses regulated by CML42.

A Relative concentrations of flavonol glycosides in rosette leaves of 5-week-old, untreated Arabidopsis WT (black) and cml42 (white) plants. Relative concentration of flavonol glycosides is given in peak area of the ion chromatogram in negative mode per gram dry weight. Flavonols: kaempferol 3-O-[6”-O-(rhamnosyl)glucoside] 7-O-rhamnoside, kaem1; kaempferol 3-O-glucoside 7-O-rhamnoside, kaem2; kaempferol 3,7-O-dirhamnoside, kaem3. Statistically significant differences between Col-0 and CML42 plants were analyzed by unpaired t-test, *P = <0.05 and **P = <0.001. Mean (± SE, n=8).

B Arabidopsis 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. Picture is a representation of 4 independent experiments

C Levels of abscisic acid (ABA) in WT (white) and cml42 (black) plants subjected to 2 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). Mean (± SE, n=10).

Figure 8. Model for multiple and varied roles of CML42 in insect herbivory and response to abiotic stress

CML42 acts as a negative regulator of: Plant defense against Spodoptera littoralis by COI1-mediated JA perception, JA-induced Ca$^{2+}$ elevation, ABA accumulation upon drought stress, and constitutive glucosinolate 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 perception of S. littoralis oral secretion. CML42 might act as a component for modulating excess defense or as an insect effector target, since 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 negative
regulator of plant defense by decreasing COI1-mediated JA sensitivity and expression of JA responsive genes and is independent on herbivory- induced JA biosynthesis. CML42 acts upstream of JA-induced Ca^{2+} elevation and negatively regulates it. CML42 also negatively regulates constitutive aliphatic glucosinolates, which are COI1 dependent (dotted arrows) (Mewis et al., 2005). Thus, CML42 negatively regulates plant defense via its action on COI1-mediated JA perception.

Supplemental Data

Figure S1: CML43 regulation 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: Trichome morphology of Arabidopsis WT and cml42 mutant (5-week-old plants).

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: 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: 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 (Schmittgen and Livak, 2008) 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: 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:** 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 Lb1.3 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’
- **Lb1.3**: 5’- ATTTTGCCGATTTCGGAAC -3’
- **ACT2-F**: 5’- GTTGGGATGAACCAGAAGGA-3’
- **ACT2-R**: 5’- GAACCACCGATCCAGACACT -3’
- **CML42-F**: 5’- ATGGAGAGTAACAAACAACGAGA-3’
- **CML42-R**: 5’- AGAAGAAGGGATGACACAGTA-3’
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**Figure 1.** *S. littoralis* oral secretions (OS)-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ and phytohormones in Arabidopsis.

**A** Application of 40 µL *S. littoralis* oral secretions (1:1 diluted) to 5-week-old Arabidopsis leaf discs expressing aequorin. Mean (± SE, n=4) $[\text{Ca}^{2+}]_{\text{cyt}}$ was calculated from the relative light units measured in leaf disc at 5 s integration time for 10 min. In all the experiments, water was used as control and gave background readings.

**B-E** Mean (± SE, n=4) content of jasmonic acid-isoleucine, (+)-JA-Ile (**B**), jasmonic acid, JA (**C**), 12-oxophytodienoic acid, cis-OPDA (**D**), and salicylic acid, SA (**E**) in mechanical wounding + *S. littoralis* oral secretion-elicited leaves and control with mechanical wounding + H$_2$O treatment. Phytohormones were measured in local treated leaves.
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 water or 1:1 diluted OS per leaf. Transcript abundance in leaves was determined by real-time PCR analysis and normalised to the plant *RPS18B* mRNA level. The fold change was calculated relative to control which was mechanical wounding + H2O. The graph shows x-fold induction of the mRNA levels by the *S. littoralis* OS relative to the levels in the H2O treated control leaves. Mean (± 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. Mean (± SE, n=3).

**C** CML42 transcript levels in Arabidopsis leaves after 30 min, 1, 2 and 24 h of *S. littoralis* feeding. Undamaged leaves were used as controls for quantification. Mean (± SE, n=5).

**D** CML42 and VSP2 transcript levels in JA-treated (50µM) Arabidopsis leaves after 0.5, 3 and 6 hours of treatment. Elicitation and calculation as in Fig. 2A. VSP2 was used as a marker for JA responsiveness in plants. The fold change was calculated relative to control, which were leaves with 0.015% methanol spray. Mean (± SE, n=6).

**E, F** Larval weight after feeding on Col-0 and two different cml42 lines, (D) SALK_041400; (E) SALK_040227. *S. littoralis* 1st instar larvae growing in light for 3 d after hatching were pre-weighed 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 (N) is indicated in the bars. Values are mean of 4 and 3 independent experiments for E and F respectively. Statistically significant differences between Col-0 and cml42 plants after feeding of the larvae were analyzed by Mann-Whitney Rank Sum Test, **P = <0.001.**
Figure 3. Phytohormone elevation upon *S. littoralis* herbivory in *cml42* plants. Mean (± SE, n=5) levels of JA (A), (+) JA-Ile (B), cis-OPDA (C) and SA (D) in Arabidopsis wild type, WT (white) and *cml42* (black) fed by *S. littoralis* for one and eight days. The phytohormone levels were measured from the whole plant rosette which includes both local and systemic leaves. Statistical differences in phytohormone levels among WT and *cml42* (ANOVA; P<0.05) were calculated and no significant changes determined.
Figure 5. Jasmonic acid perception in cml42 mutants.

A Relative mRNA levels of defense-related genes VSP2, Thi2.1, and PDF1.2 in S. littoralis infested leaves of WT (black) and cml42 (white). S. littoralis (4th instar larvae) fed on both lines for 24 h. Undamaged WT 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 and *P = <0.05.

B CML42 transcript levels in S. littoralisOS-treated leaves of wild type (black) and jasmonic acid receptor mutant, coi1-1 (grey) after 30, 45, 60 and 90 min of treatment. Leaves were elicited as described for Fig. 2A. Different letters indicate significant differences among treatments (ANOVA; P<0.05). Mean (± SE, n=4).

C Elevation in cytosolic calcium concentration [Ca^{2+}]_{cyt} induced by jasmonic acid in cml42 plants. Jasmonic acid (500 µM) was applied to leaf disc of aequorin-expressing WT (dark grey) and cml42 x aequorin (black) plants. Leaves were elicited as described for Fig. 1A. Water was used as control and gave background readings (light grey). N=5 and mean of 2 independent experiments. Error bars represent SE, different letters indicate significant differences among treatments (ANOVA; P<0.05).

D Effects of increasing concentrations of MeJA on inhibition of root growth in wild type, 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 days in continuous light. The experiment was repeated 3 independent times (N=20 each line per experiment). Error bars represent SE, different letters indicate significant differences among treatments (ANOVA, SNK post hoc test; P<0.05).
Figure 6. Glucosinolate levels in *cml42* plants.

**A** Mean (± SE) levels of total, indole and aliphatic glucosinolates in rosette leaves of 5-week-old, untreated Arabidopsis WT (black) and *cml42* (white) plants. Statistically significant differences between Col-0 and *cml42* plants were analyzed by unpaired t-test, **P = <0.001.** The values were mean of 5 independent experiments, total number of plants used (N) is indicated in the bars.

**B** Mean (± SE, n=8) levels of total, indole and aliphatic glucosinolates in rosette leaves of 5-week-old Arabidopsis WT (black) and *cml42* (white) plants fed by *S. littoralis* for one day. Different letters indicate significant differences among treatments (ANOVA; P<0.05). The values were mean of 2 independent experiments.
Figure 7. Multiple stress responses regulated by CML42.

A. Relative concentrations of flavonol glycosides in rosette leaves of 5-week-old, untreated Arabidopsis WT (black) and cml42 (white) plants. Relative concentration of flavonol glycosides is given in peak area of the ion chromatogram in negative mode per gram dry weight. Flavonols: kaempferol 3-O-[6"-O-(rhamnosyl)glucoside] 7-O-rhamnoside, kaem1; kaempferol 3-O-glucoside 7-O-rhamnoside, kaem2; kaempferol 3,7-O-dirhamnoside, kaem3. Statistically significant differences between Col-0 and CML42 plants were analyzed by unpaired t-test, *P = <0.05 and **P = <0.001. Mean (± SE, n=8).

B. Arabidopsis 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. Picture is a representation of 4 independent experiments.

C. Levels of abscisic acid (ABA) in WT (white) and cml42 (black) plants subjected to 2 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). Mean (± SE, n=10).

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Figure 8

CML42 acts as a negative regulator of: Plant defense against *Spodoptera littoralis* by COI1-mediated JA perception, JA-induced Ca²⁺ elevation, ABA accumulation upon drought stress, and constitutive glucosinolate accumulation. CML42 also acts as a positive regulator of flavonol (kaempferol) accumulation and UV-B-mediated stress response. In insect herbivory, cytosolic Ca²⁺ elevation and CML42 up-regulation are early plant responses upon perception of *S. littoralis* oral secretion. CML42 might act as a component for modulating excess defense or as an insect effector target, since 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 negative regulator of plant defense by decreasing COI1-mediated JA sensitivity and expression of JA responsive genes and is independent on herbivory-induced JA biosynthesis. CML42 acts upstream of JA-induced Ca²⁺ elevation and negatively regulates it. CML42 also negatively regulates constitutive aliphatic glucosinolates, which are COI1 dependent (dotted arrows) (Mewis et al., 2005). Thus, CML42 negatively regulates plant defense via its action on COI1-mediated JA perception.
**Figure 4.** Subcellular CML42 localization.

A Visualization of CML42:GFP in leaves of transgenic Arabidopsis plants using CLSM. Green fluorescence of the fusion protein is visible in cytoplasm surrounding chloroplasts (arrow heads) and in the nucleus (arrows).

B Visualization of CML42:GFP in transiently transformed leaves of *Nicotiana benthamiana* using CLSM. Co-infiltration 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 co-localize to the plasma membrane (arrow head).

Bars represent 20 µm in all micrographs.