CML24, Regulated in Expression by Diverse Stimuli, Encodes a Potential Ca^{2+} Sensor That Functions in Responses to Abscisic Acid, Daylength, and Ion Stress[1]

Nikkí A. Delk, Keith A. Johnson, Naweed I. Chowdhury, and Janet Braam*

Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005–1892 (N.A.D., N.I.C., J.B.); and Department of Biology, Bradley University, Peoria, Illinois 61625 (K.A.J.)

Changes in intracellular calcium (Ca^{2+}) levels serve to signal responses to diverse stimuli. Ca^{2+} signals are likely perceived through proteins that bind Ca^{2+}, undergo conformation changes following Ca^{2+} binding, and interact with target proteins. The 50-member *calmodulin-like* (*CML*) Arabidopsis (*Arabidopsis thaliana*) family encodes proteins containing the predicted Ca^{2+}-binding EF-hand motif. The functions of virtually all these proteins are unknown. CML24, also known as TCH2, shares over 40% amino acid sequence identity with calmodulin, has four EF hands, and undergoes Ca^{2+}-dependent changes in hydrophobic interaction chromatography and migration rate through denaturing gel electrophoresis, indicating that CML24 binds Ca^{2+} and, as a consequence, undergoes conformational changes. *CML24* expression occurs in all major organs, and transcript levels are increased from 2- to 15-fold in plants subjected to touch, darkness, heat, cold, hydrogen peroxide, abscisic acid (ABA), and indole-3-acetic acid. However, CML24 protein accumulation changes were not detectable. The putative *CML24* regulatory region confers reporter expression at sites of predicted mechanical stress; in regions undergoing growth; in vascular tissues and various floral organs; and in stomata, trichomes, and hydathodes. *CML24*-underexpressing transgenics are resistant to ABA inhibition of germination and seedling growth, are defective in long-day induction of flowering, and have enhanced tolerance to $CoCl_2$, molybdic acid, $ZnSO_4$, and $MgCl_2$. $MgCl_2$ tolerance is not due to reduced uptake or to elevated Ca^{2+} accumulation. Together, these data present evidence that *CML24*, a gene expressed in diverse organs and responsive to diverse stimuli, encodes a potential Ca^{2+} sensor that may function to enable responses to ABA, daylength, and presence of various salts.

Calcium (Ca^{2+}) signaling is implicated in plant responses to diverse stimuli, such as touch, light, pathogens, temperature, and hormones (Reddy, 2001). Proteins that bind Ca^{2+} are potential sensors that detect changes in cytosolic Ca^{2+} levels and mediate appropriate cellular responses through interaction with target proteins.

The Arabidopsis (*Arabidopsis thaliana*) *calmodulin-like* (*CML*) gene family encodes potential Ca^{2+} sensors that contain conserved Ca^{2+}-binding domains, called EF hands, and share sequence similarity with the essential, ubiquitous, and highly conserved Ca^{2+} receptor, calmodulin (CaM; McCormack and Braam, 2003). There are 50 *CML*s in Arabidopsis (McCormack and Braam, 2003); the large size of this family may reflect the importance of Ca^{2+} signaling and the diversity of physiological responses that may be regulated through Ca^{2+} signaling. Despite the implication of the importance of Ca^{2+} signaling in many fundamental cellular processes, our understanding of the regulation and function(s) of this potentially critical family of proteins is very limited.

CML24 is a CaM-related protein that shares approximately 40% overall sequence identity with CaM (Khan et al., 1997; McCormack and Braam, 2003). Primary amino acid sequence analyses and modeling studies predict that CML24 has four functional EF hands for Ca^{2+} binding and that upon Ca^{2+} binding conformational changes likely result in the exposure of hydrophobic patches, enabling target interaction (Khan et al., 1997). Sequence diversity from CaM suggests that CML24 target proteins would be distinct from those of CaM.

CML24, also known as *TCH2*, was first identified as a gene dramatically up-regulated in expression by touch (Braam and Davis, 1990). *CML24* expression is also induced by darkness, heat, and cold (Braam and Davis, 1990; Braam, 1992; Polisensky and Braam, 1996; Lee et al., 2005). Therefore, *CML24* is highly responsive to diverse stimuli and encodes a protein that likely functions in a Ca^{2+}-influenced manner. Here we present evidence implicating CML24 function in seed germination, seedling growth, transition to flowering, and ion homeostasis, physiological processes that may involve Ca^{2+} signaling.

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* Corresponding author; e-mail braam@bioc.rice.edu; fax 713–348–5154.

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Seed germination is regulated through the antagonistic actions of the plant hormones gibberellin (GA) and abscisic acid (ABA), which promote and inhibit seed germination, respectively (Finkelstein et al., 2002; Olszewski et al., 2002). In plants such as barley (*Hordeum vulgare*), increases in cytosolic Ca^{2+} levels mediate GA-induced endosperm hydrolysis, mobilizing storage reserve energy for the germinating embryo, while ABA negatively regulates this process (Gilroy, 1996; Gomez-Cadenas et al., 1999). Arabidopsis plants with mutations in the SCaBP5 and CBL9 Ca^{2+} sensors are hypersensitive to ABA inhibition of germination (Guo et al., 2002; Pandey et al., 2004). These data implicate a role for Ca^{2+} in the regulation of seed germination.

The transition from the vegetative phase (leaf production) to the reproductive phase (flower production) may also be influenced by Ca^{2+} signaling. Long-day photoperiods induce flowering in Arabidopsis through the circadian clock (Mouradov et al., 2002). Circadian rhythmic oscillations in cytosolic Ca^{2+} are photoperiod sensitive and may encode information about daylength (Love et al., 2004). Vernalization, an extended exposure to low temperatures, also induces flowering (Mouradov et al., 2002). Induction of expression by cold of *EARLI1*, a vernalization- and photoperiod-responsive Arabidopsis gene encoding a putative lipid transfer protein, is Ca^{2+} dependent (Wilkosz and Schlappi, 2000; Bubier and Schlappi, 2004). Furthermore, antisense Arabidopsis transgenics for a putative Ca^{2+} receptor and Arabidopsis transgenics overexpressing *PPF1*, which encodes a putative Ca^{2+} transporter in pea (*Pisum sativum*), are delayed in flowering (Han et al., 2003; Wang et al., 2003).

Ca^{2+} may also influence ion homeostasis. For example, the Arabidopsis Ca^{2+} sensor SOS3 interacts with the SOS2 kinase in response to salt stress-induced increases in cytosolic Ca^{2+} (Zhu, 2002). The SOS2/SOS3 interaction leads to gene induction and functional activation of the Na^+/H^+ antiporter, SOS1, which removes Na^+ from the cytosol (Zhu, 2002). The Arabidopsis Ca^{2+}/CaM-activated Ca^{2+}-ATPase, ACA4, may also affect Na^+ homeostasis, as yeast expressing *ACA4* are more salt tolerant (Geisler et al., 2000). Ca^{2+} signaling induces stomatal closure through the regulation of K^+ channels and H^+ pumps (Schroeder et al., 2001). Finally, Arabidopsis plants suffering from insertions in the *CAX1* gene encoding a Ca^{2+}/H^+ antiporter are Mg^{2+}, Mn^{2+}, K^+, and Na^+ tolerant (Cheng et al., 2003).

Here, we demonstrate that regulation of *CML24* expression is influenced by diverse environmental and hormonal stimuli, and *CML24* is expressed throughout plant development. CML24 binds Ca^{2+} and undergoes Ca^{2+}-dependent conformational changes. Through the isolation and characterization of transgenic plants with reduced *CML24* expression levels, we demonstrate that CML24 has roles in ABA inhibition of germination and seedling growth, photoperiod-induced transition to flowering, and ion homeostasis.

RESULTS

CML24 Encodes a CaM-Like Ca^{2+}-Binding Protein with Four EF-Hand Motifs

CML24 encodes a 161-amino acid, 16-kD protein that shares 66% similarity and 40% to 41% identity to the Arabidopsis CaMs 1, 2, 6, and 7 (Fig. 1A). The sequence divergence of CML24 from CaM argues that CML24 is not a typical CaM. However, based on the high conservation of the EF-hand motifs (Fig. 1A, underlined), one would predict that CML24 can bind Ca^{2+}. Ca^{2+} binding by CaM and the resulting conformational change can be detected as a mobility shift in SDS-PAGE (Burgess et al., 1980). To determine whether CML24 binds Ca^{2+} and undergoes an induced conformational change, total protein from wild-type plants was subjected to SDS-PAGE in the presence of either Ca^{2+} (Fig. 1B, Ca^{2+}) or the Ca^{2+} chelator EGTA (Fig. 1B, EGTA); CML24 was detected with anti-CML24 antibody. The increased CML24 mobility in the presence of Ca^{2+} relative to EGTA (Fig. 1B) strongly suggests that CML24 can bind Ca^{2+} and undergo a Ca^{2+}-dependent conformational change. Furthermore, recombinant CML24 binds phenyl-Sepharose in a Ca^{2+}-dependent manner (data not shown), indicating that CML24 can undergo Ca^{2+}-dependent changes that reveal hydrophobic surfaces for interaction. These results, along with presence of CaM-related EF hands in CML24 (Fig. 1A) and modeled tertiary structure (Khan et al., 1997), are consistent with the idea that *CML24* may function as a Ca^{2+} sensor.

CML24 Expression Is Highly Responsive to Diverse Environmental and Hormonal Stimuli

CML24 was first identified as a gene strongly induced by touch stimulation (Braam and Davis, 1990), and subsequent studies indicated *CML24* expression induction by a variety of stimuli (Braam, 1992; Polisensky and Braam, 1996). However, quantitative assessment of expression levels has not been previously conducted. To quantify and more fully ascertain *CML24* expression behavior, we performed quantitative real-time reverse transcription (RT)-PCR (QRT-PCR) on RNA from plants subjected to various stimuli (Fig. 2A). *CML24* transcript levels are increased approximately 9-fold in plants 30 min after a touch stimulus. Darkness results in a 2-fold increase in *CML24* RNA levels. One-hour exposure to 37°C and 4-h exposure to 4°C cause approximately 5- and 15-fold increases in *CML24* transcript levels, respectively. A 4-fold increase in *CML24* transcript levels occurs in plants 30 min after treatment with 20 mM hydrogen peroxide (H_2O_2). The hormones ABA and auxin (indole-3-acetic acid [IAA]) also rapidly increase *CML24* RNA levels 5- and 3-fold, respectively. Treatment with the ethylene precursor aminocyclopropanecarboxylic acid (ACC) or GA does not result in an increase in *CML24* transcript levels after 45 min of plant exposure. No detectable changes in CML24 protein levels are

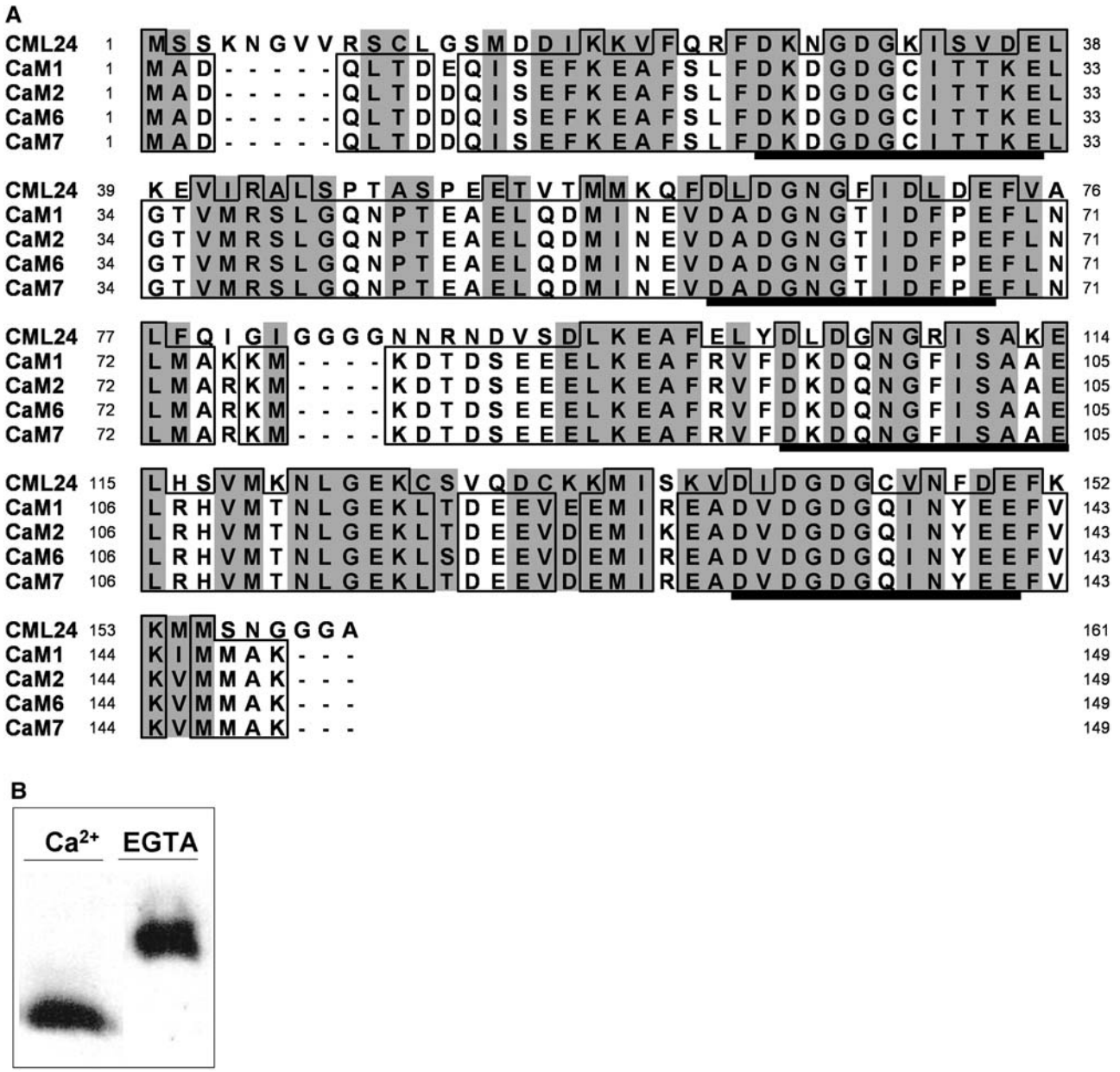

Figure 1. *CML24* encodes a CaM-like, Ca^{2+}-binding protein. A, Amino acid sequence of CML24 and the four Arabidopsis CaM isoforms represented by CaM1, CaM2, CaM6, and CaM7. Shaded and boxed residues indicate similarity and identity, respectively. The Ca^{2+}-binding domains of the four EF hands are underlined. B. Western blot analyzed with anti-CML24 antibody. The faster migration of CML24 through the SDS-polyacrylamide gel after incubation with Ca^{2+} is an indication that CML24 can bind Ca^{2+} and change conformation upon interaction with Ca^{2+}.

detected by western-blot analysis of total proteins from the touch-, darkness-, cold-, heat-, H_20_2-, ABA-, or IAA-treated plants (data not shown). A possible explanation for this lack of correlation between RNA and protein is that the nascent *CML24* transcripts are not translated. Another possibility is that western analyses are not sensitive enough to detect modest increases in CML24 protein over the basal levels. Alternatively, the stimuli that induce *CML24* expression may also lead to CML24 degradation; in this way, the new transcripts may be generated to replenish the steady-state CML24 protein level as a homeostatic feedback mechanism.

CML24 and *CML24*::*GUS* Are Expressed in Diverse Organs and Tissues throughout Development

RT-PCR analyses indicate that *CML24* transcripts are detectable in all major organs of adult plants, including roots, rosette leaves, inflorescence stems, cauline leaves, flowers, and immature siliques (Fig. 2B). To visualize spatial patterns of expression within

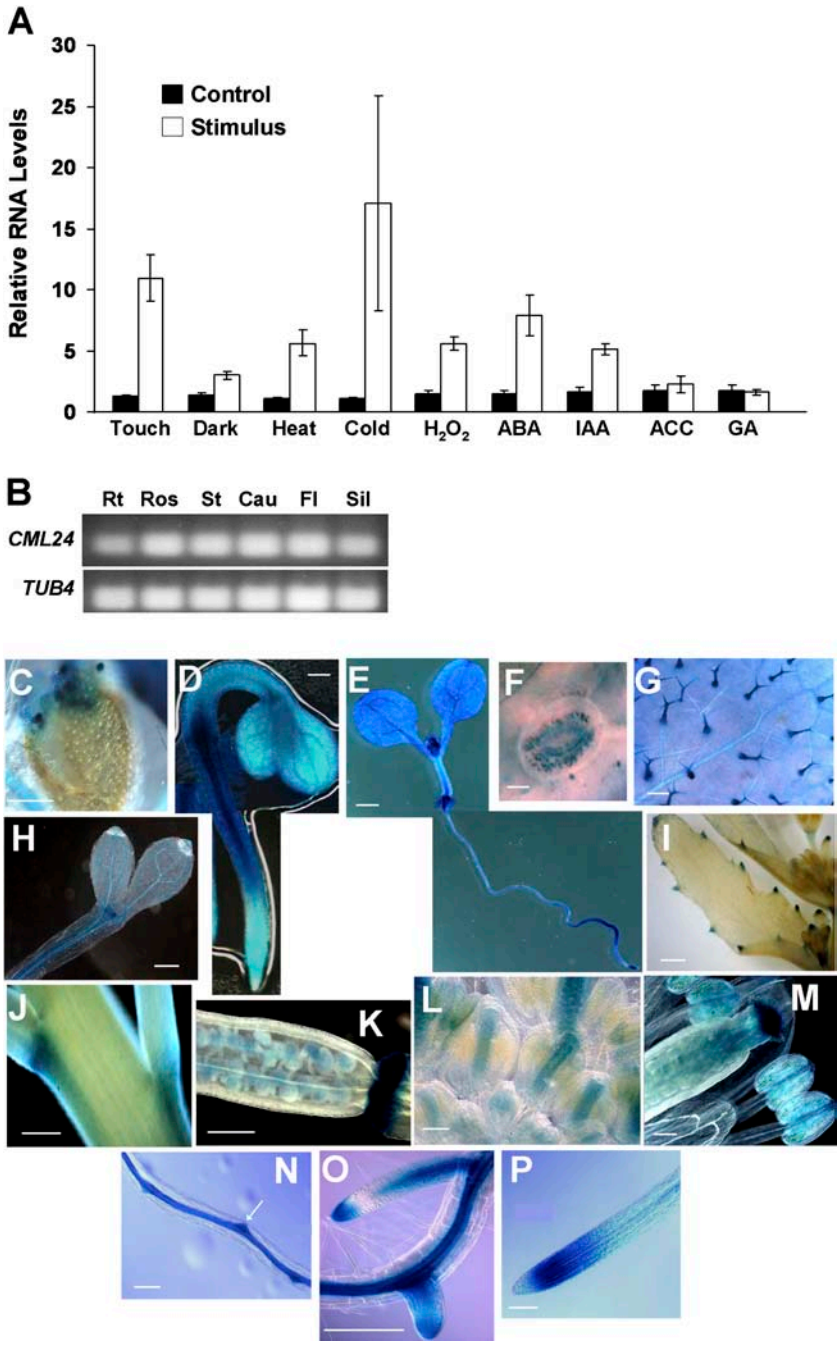

Figure 2. *CML24* and *CML24*::*GUS* expression. A, *CML24* expression is induced by a variety of stimuli. Plants were left alone (controls; black bars) or treated (white bars) with the following stimuli and harvested at the time points indicated in parentheses: touch (30 min), dark (30 min), 37°C heat (1 h), 4°C cold (4 h), 20 mM H_2O_2 (30 min), 100 μM ABA (30 min), 100 μM IAA (30 min), 100 μM ACC (45 min), and 100 μM GA (45 min). QRT-PCR was conducted as described in "Materials and Methods"; three independent biological replicates were analyzed. Error bars represent SE. B, *CML24* is expressed in many plant organs. Total RNA purified from roots of 4-week-old plants (Rt), and rosette leaves (Ros), inflorescence stems (St), cauline leaves (Cau), flowers (Fl), and siliques (Sil) of 7-week-old plants was subjected to RT-PCR with primers specific for *CML24* and *TUB4* (encoding tubulin). *TUB4* serves as a control for RNA integrity and as a semiquantitative standard. C to P, *CML24*::*GUS* is expressed in many plant organs and throughout plant development. *CML24*::*GUS* is expressed in (C) the shed seed coat, (D) 2-d-old seedlings, (E) 7-d-old seedlings, (F) guard cells, (G) leaf vasculature and trichomes of 14-d-old seedlings, (H) 6-d-old dark-grown seedlings, (I) hydathodes, (J) branch points, (K) developing seed and abscission zone, (L) styles of young flowers, (M) mature anthers and stigmatic papillae, (N) root vasculature and lateral root initiation sites (arrow) of 7-d-old seedlings, and (O) lateral root tip and (P) primary root tip of 14-d-old seedlings. Bars = 100 μm in C, D, G, H, and K to P; bars = 1 mm in E and I; bars = 5 μm in F; bars = 500 μm in J.

these organs, we generated and analyzed transgenic plants harboring *CML24*::*β-glucuronidase* (*GUS*) gene fusions composed of a 1-kb region upstream of the *CML24* transcribed region fused to the *GUS* reporter gene. GUS activity, detected by the formation of a blue precipitate in the presence of 5-bromo-4-chloro-3-indolyl-β-D-GlcUA, reveals sites where the putative *CML24* regulatory region confers expression (Fig. 2, C–P). *CML24*-driven *GUS* activity is in the seed coat, particularly where the coat is broken open to enable the emergence of the seedling radical (Fig. 2C). Newly emerged *CML24*::*GUS* seedlings stain throughout but have highest GUS activity in the proximal radical (Fig. 2D). Seven-day-old seedlings have GUS activity throughout the cotyledons, hypocotyl, and elongating root (Fig. 2E). Higher levels of GUS activity are detected in the shoot apex, the root-shoot junction, and distal regions of the root (Fig. 2E). *GUS* expression is also seen throughout 6-d-old *CML24*::*GUS* transgenics grown in the dark (Fig. 2H); however, activity is highest in the vascular tissues. *CML24*-driven *GUS* expression is also in the vasculature of cotyledons (Fig. 2E), leaves (Fig. 2G), and roots (Fig. 2, N and O) of light-grown plants. Specialized leaf structures such as

guard cells (Fig. 2F), trichomes (Fig. 2G), and hydathodes (Fig. 2I) have high *GUS* expression in the *CML24*::*GUS* transgenics. *CML24*::*GUS* is also expressed at inflorescence stem branch points (Fig. 2J), the silique abscission zone (Fig. 2K), in young (Fig. 2L) and mature (Fig. 2M) styles and stigmatic papillae (Fig. 2M), mature anthers (Fig. 2M), and developing seed (Fig. 2K). Finally, *CML24*::*GUS* is expressed at the lateral root initiation sites, localized regions of lateral root tips, and primary root tips (Fig. 2, N–P, respectively). *CML24* expression behavior, as monitored by RT-PCR and reporter activity analyses, indicates that *CML24* has diverse developmental expression regulation and thus may function in many organs and tissues throughout development and morphogenesis.

CML24-Underexpressing Transgenics Are Specific for *CML24* Cosuppression

To gain insight into the physiological functions of CML24, we sought to assess the consequences of loss of *CML24*. We generated transgenic plants that have reduced expression of *CML24* as a consequence of cosuppression of *CML24*::*GUS* transgenes and the endogenous *CML24*. Two independent lines of *CML24*-underexpressing plants, called *U1* and *U2*, were identified as plants with undetectable GUS activity and the absence of *CML24* transcripts among several *CML24*::*GUS* transformants (data not shown). The loss of *CML24* transcripts leads to a strong reduction in CML24 protein levels as detected by western-blot analyses (Fig. 3A). Levels of CML24 protein are compared among wild type (Fig. 3A, WT, ecotype RLD), transgenics harboring a cauliflower mosaic virus *35S*-driven *CML24* gene that over produce CML24 (Fig. 3A, *O1* and *O2*), and the two independent

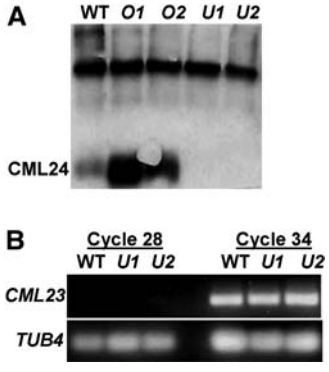

Figure 3. *CML24*-underexpressing transgenics (*U1*, *U2*) have reduced *CML24* levels, and this effect is likely gene specific. A, Western blot of total protein from wild-type (WT) and transgenic plants interacted with anti-CML24 antibodies. The antibody cross reacts with an abundant, larger protein that serves as a loading control. *U1* and *U2* have undetectable levels of CML24. Lanes *O1* and *O2* serve to verify the migration position of CML24 as these samples are from transgenics engineered to overexpress *CML24*. B, Semiquantitative RT-PCR of *CML23* and *TUB4* (control) from wild type, *U1*, and *U2*. PCR products after cycles 28 and 34 were analyzed by gel electrophoresis. No effect on *CML23* expression is detected in the *CML24*-underexpressing transgenics.

CML24-underexpressing transgenics (Fig. 3A, *U1* and *U2*). The *CML24*-overexpressing plants were used to verify the gel migration position of CML24. Reproducible phenotypic consequences of *CML24* overexpression have not been detected and therefore are not further discussed here. To check whether the gene silencing that led to the reduction in *CML24* expression is specific for *CML24*, we sought to assess the expression levels of a related gene that we would predict, based on sequence relatedness, to be most likely affected if the silencing were not gene specific. *CML23* and *CML24* share 33% nucleotide identity and encode proteins that are 78% identical at the amino acid level (McCormack and Braam, 2003). Semiquantitative RT-PCR detection of *CML23* RNA reveals transcript levels in *U1* and *U2* that are indistinguishable from the wild-type control (Fig. 3B). Thus, *CML23* expression is not significantly affected in the *CML24*-underexpressing transgenics. Because *CML23* is the closest relative to *CML24* and is not significantly affected by the *CML24* silencing, it is probable that other genes with less sequence similarity to *CML24* are also not significantly affected. These analyses also indicate that the anti-CML24 antibody used in Figures 1B and 3A does not recognize CML23 or CaM (described in ''Materials and Methods''), and therefore is likely specific for CML24 among the CaM and CML proteins. Most importantly, these data indicate that phenotypes observed in *CML24 U1* and *U2* are most likely consequences of specific reduction in CML24 levels and, hence, CML24 function.

CML24 U1 and *U2* Have Increased Germination and Seedling Growth on ABA

ABA promotes seed dormancy, inhibits seed germination and seedling development, and promotes drought tolerance in plants through stomatal regulation (Finkelstein et al., 2002). Furthermore, it is well established that changes in cytosolic Ca^{2+} levels mediate ABA-induced stomatal closure (McAinsh et al., 1990, 1991; Irving et al., 1992; Allen et al., 1999; Pei et al., 2000; Schroeder et al., 2001; Webb et al., 2001). To test whether *CML24* may have a role in ABA responses, we examined the ability of the *CML24*-underexpressing transgenics to respond to exogenous ABA. Wild-type and the *CML24*-underexpressor seeds were plated on growth media supplemented with different concentrations of ABA. After 8 d, the percentage of seed germination was determined. The *CML24* underexpressors show an enhanced ability to germinate in the presence of 1 μM and 2 μM ABA compared to wild type (Fig. 4, left). Shoot tissue greening in the presence of ABA is another indication of reduced ABA sensitivity (Lopez-Molina and Chua, 2000). *CML24 U1* and *U2* develop green, leaf-like structures when grown on media supplemented with 5 μM ABA and 0.5% (w/v) Suc; wild-type development is strongly arrested when grown under these conditions (Fig. 4, right). Some ABA-insensitive and -deficient mutants have less

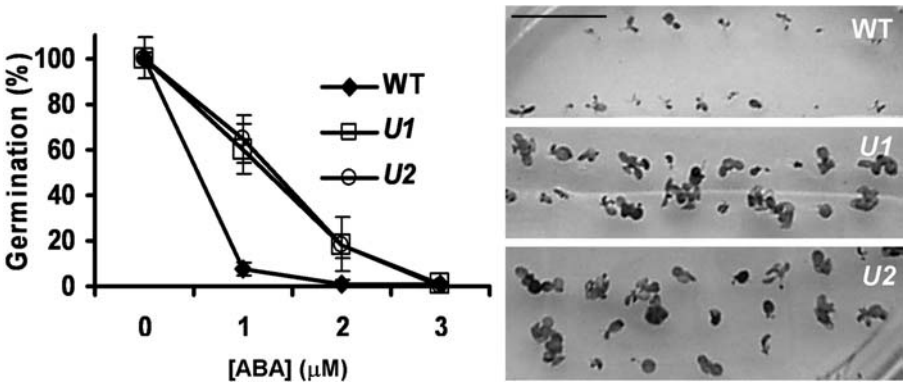

Figure 4. *CML24*-underexpressing transgenics (*U1*, *U2*) show altered responses to ABA. *U1* and *U2* are less sensitive to ABA-induced dormancy. The graph to the left shows a greater percentage of *U1* and *U2* seedlings germinate on 1 μM and 2 μM ABA 6 d post sowing than wild type (WT). $n \geq 80$ on PN and $n \geq 140$ on ABA. The photograph on the right shows *U1* and *U2* seedlings have more enhanced development and are greener than wild type when grown on media supplemented with 5 μM ABA and 0.5% Suc. Scale bar = 15 mm.

robust dormancy than wild type (Koornneef et al., 1984; Leon-Kloosterziel et al., 1996; Finkelstein et al., 2002). When sown on growth media without stratification to determine dormancy, the *CML24*-underexpressing transgenics germinate at the same rate as wild type (data not shown). Reduced sensitivity to ABA can also result in reduced sensitivity to the osmoticum mannitol (Carles et al., 2002). The *CML24*-underexpressing transgenics show wild-type germination rates in the presence of mannitol (data not shown).

Some ABA-deficient and -insensitive mutants have a wilty phenotype due to increased transpiration through aberrantly functioning stomata (Leon-Kloosterziel et al., 1996; Leung et al., 1997; Xiong et al., 2002). Detached leaves and shoots from the *CML24*-underexpressing transgenics lose fresh weight at a similar rate as wild type (data not shown). Furthermore, the *CML24*-underexpressing transgenics show wild-type response to ACC, IAA, GA, and the GA biosynthesis inhibitor paclobutrazol (data not shown). Therefore, the *CML24*-underexpressing transgenics appear to be specifically affected in germination responses to exogenous ABA.

CML24-Underexpressing Transgenics Flower Late in Long Days

Multiple environmental and endogenous signals influence Arabidopsis flowering such as photoperiod, vernalization, GA, and autonomous signals (Mouradov et al., 2002). Arabidopsis is a facultative long-day flowering plant (Mouradov et al., 2002). Long-day photoperiods (e.g. 16 h of light) induce Arabidopsis flowering, while short days (e.g. 8 h of light) significantly delay flowering. *CML24*-underexpressing transgenics flower later than wild type when grown in 24-h (Fig. 5A) and 16-h photoperiods (Fig. 5, B and C). Whereas all of the wild-type plants flowered by 32 d after sowing, none of the *CML24*-underexpressing plants flowered before 40 d of age when grown under 16-h photoperiods (Fig. 5C). Over 60 d of growth were required for 100% of the *CML24*-underexpressing transgenics to flower (Fig. 5C). Furthermore, while wild-type plants produced an average of nine leaves before flowering, the *CML24*-underexpressing transgenics produce an average of 25 rosette leaves before flowering (Fig. 5B); increased leaf number at the transition to flowering is typical of late-flowering mutants (Koornneef et al., 1991). The flowering time delay of the *CML24*-underexpressing transgenics is specific for long-day conditions; under an 8-h photoperiod, the *CML24*-underexpressing transgenics flower simultaneously with the wild-type plants, and wild type and the *CML24*-underexpressing transgenics generate comparable numbers of rosette leaves prior to flowering (Fig. 5, B and D). Furthermore, the *CML24*-underexpressing transgenics are responsive to vernalization. Vernalization treatments of 30 or 40 d accelerate short-day flowering by 35 and 50 d, respectively, in both the wild type and the *CML24*-underexpressing transgenics (Fig. 5E; data not shown); under these conditions wild type and the *CML24*-underexpressing transgenics make an average of 15 leaves before flowering (Fig. 5B; data not shown). Finally, the transition to flowering of the *CML24*-underexpressing transgenics is responsive to GA (Fig. 5, B and F). Plants grown in an 8-h photoperiod were sprayed once or twice a week for 8 weeks with 100 μM GA_3. Forty days after the first GA treatment, wild type and the *CML24*-underexpressing transgenics began to flower, accelerating short-day flowering by 60 d (Fig. 5F). Taken together, these data demonstrate the *CML24*-underexpressing transgenics are specifically defective in long-day induction of transition to flowering.

CML24-Underexpressing Transgenics Are Less Sensitive to Various Salts

The *CML24*-underexpressing transgenics were assayed for tolerance to a variety of salts. The *CML24*-underexpressing transgenics show wild-type responses to KCl, LiCl, NaCl, $CdCl_2$, $NiCl_2$, $MnCl_2$, and $CuSO_4$ (data not shown). However, the *CML24*-underexpressing transgenics have enhanced resistance to $CoCl_2$, molybdic acid (Na_2MoO_4), $ZnSO_4$, and $MgCl_2$ (Figs. 6 and 7).

The *CML24*-underexpressing transgenics and wild type have comparable growth on unsupplemented plant nutrient (PN; Haughn and Somerville, 1986) growth media (Figs. 6, C and H, and 7B). However, when germinated on PN supplemented with 90 to 120 μM $CoCl_2$, most of the wild-type seedlings display

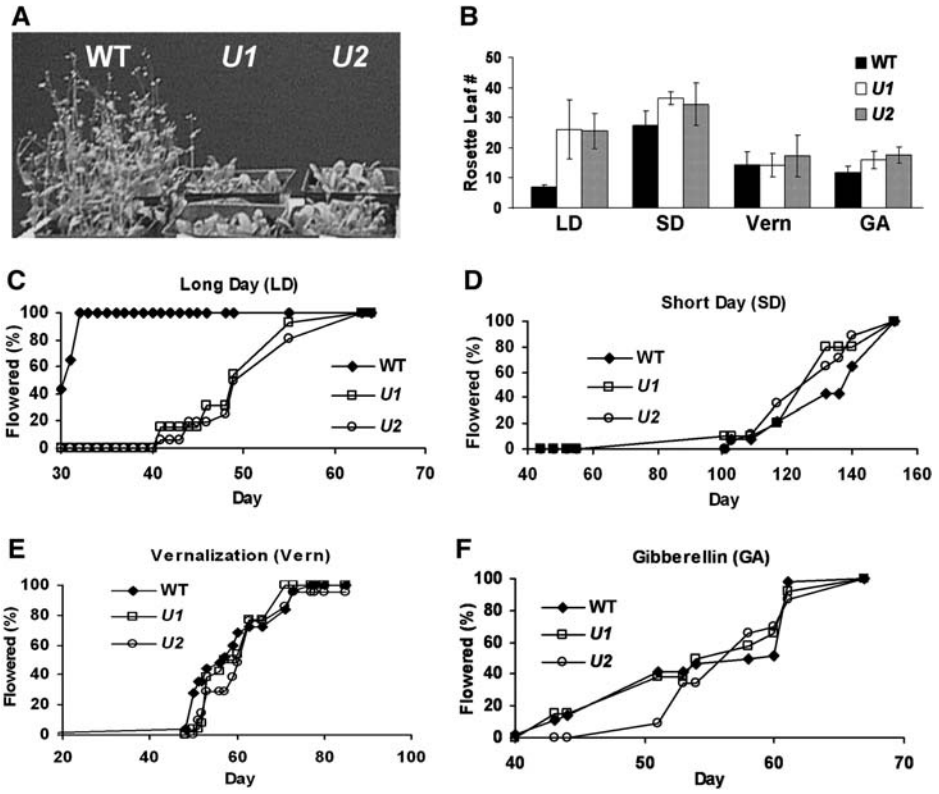

Figure 5. *CML24*-underexpressing transgenics (*U1*, *U2*) flower late in long days. A, Seven-week-old wild-type plants flower earlier than *U1* and *U2* when grown in a 24-h photoperiod. B, Rosette leaf numbers for plants grown in a 16-h photoperiod (LD, $n \geq 13$), an 8-h photoperiod (SD, $n \geq 10$), after a 40-d vernalization treatment (Vern, $n \geq 14$), or in response to a 100 μM GA treatment (GA, $n \geq 33$). Error bars represent SDs. Flowering time was recorded for plants grown in (C) a 16-h photoperiod, $n \geq 13$; (D) an 8-h photoperiod, $n \geq 10$; (E) after a 40-d vernalization treatment, $n \geq 14$; and (F) in response to a 100-μM GA treatment, $n \geq 33$.

arrested development after cotyledon expansion and become chlorotic (Fig. 6A, left; data not shown). Although the growth of the *CML24*-underexpressor transgenics is also affected by the presence of $CoCl_2$, most of the seedlings remain green and develop their first pair of true leaves (Fig. 6A, right). The overall growth and development of the seedlings were assessed by measuring the amount of chlorophyll accumulated per seedling (Fig. 6, B and C). The *CML24*-underexpressing transgenics are more tolerant of $CoCl_2$ than wild type as demonstrated by their 3-fold higher level of chlorophyll accumulation per seedling than wild type when grown in the presence of 100 μM $CoCl_2$ (Fig. 6B).

The *CML24*-underexpressing transgenics show faster development than wild type on growth media supplemented with 1 to 2 mM molybdic acid (Fig. 6, D and E; data not shown). Within 4 d of growth on 2 mM molybdic acid, approximately 41% to 60% of the *CML24*-underexpressing transgenics have expanded cotyledons, while less than 2% of wild-type plants have reached this developmental stage (Fig. 6E). This demonstrates the *CML24*-underexpressing transgenic growth is more tolerant of excess molybdic acid in the growth media than wild type.

The growth of the *CML24*-underexpressing transgenics is also more tolerant of the presence of $ZnSO_4$. When grown on media supplemented with 200 to 350 μM $ZnSO_4$, the *CML24*-underexpressing transgenics have longer roots than wild type (Fig. 6, F, top, and G; data not shown). After 10 d, wild-type roots are approximately one-half the length of the roots of the *CML24*-underexpressing transgenics when grown on 250 μM $ZnSO_4$ (Fig. 6G). On unsupplemented growth media, wild type and the *CML24*-underexpressing transgenics have comparable root lengths (Fig. 6, F, bottom, and H).

Finally, CML24-underexpressing transgenic growth is less inhibited by the presence of $MgCl_2$ than wild type. Figure 7A shows wild type and the CML24-underexpressing transgenics on growth media supplemented with 25 mM $MgCl_2$ (left) and 30 mM $MgCl_2$ (right) for 20 d. Chlorophyll levels were measured to quantitate the difference in vegetative development among wild type and the two CML24-underexpressing transgenics (Fig. 7B). On $MgCl_2$ concentrations $\geq$20 mM, the underexpressing transgenics have, on average, twice the chlorophyll level of wild type. These data indicate the CML24-underexpressing transgenics are more tolerant of high levels of $MgCl_2$ than wild type.

CML24-Underexpressing Transgenics Show Normal Ion Accumulation

To test whether the metal tolerance of the *CML24*-underexpressing transgenics is a consequence of reduced uptake, we examined metal accumulation in whole plants grown on increasing concentrations of Mg^{2+}. This condition was chosen for more in-depth analysis because overall plant growth was gradually inhibited with increasing concentrations, and sufficient tissue could be obtained for analysis. We used inductively coupled plasma mass spectroscopy (ICP-MS) to measure Mg^{2+} accumulation in plants grown on

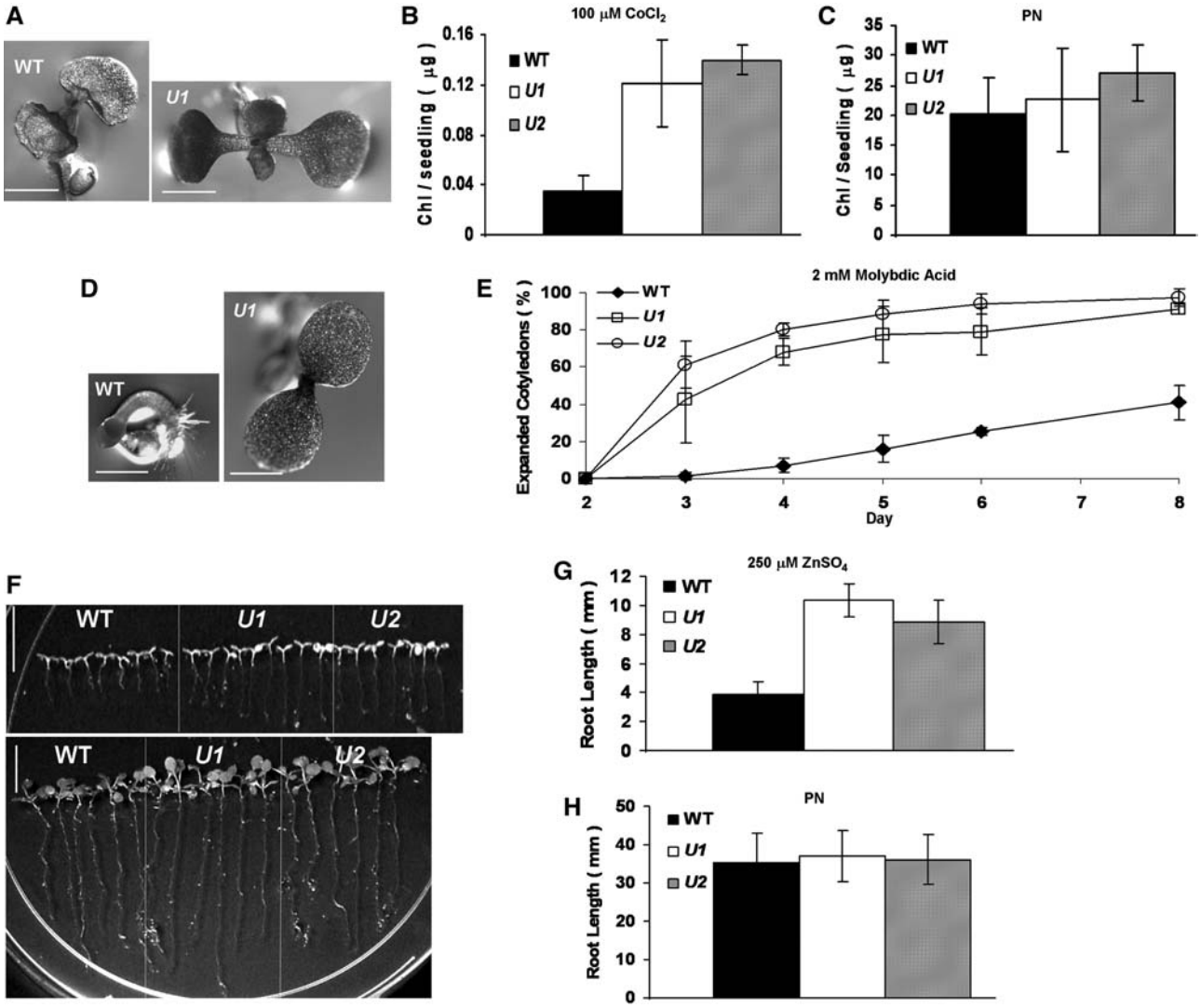

Figure 6. The growth of the *CML24*-underexpressing transgenics (*U1*, *U2*) is more tolerant of $CoCl_2$, molybdic acid, and $ZnSO_4$ than that of wild type. A, Representative wild type (WT, left) and *CML24*-underexpressing transgenic (*U1*, right) 20 d after growth on PN media supplemented with 100 μM $CoCl_2$. Wild type is chlorotic and does not develop a first set of true leaves. The *CML24*-underexpressing transgenics maintain some chlorophyll in the cotyledons and have expanded true leaves. B, *U1* and *U2* produce more chlorophyll (Chl) per seedling than wild type when grown on 100 μM $CoCl_2$. The average chlorophyll per seedling was determined by dividing the total chlorophyll absorbance (A_{652}) by the total number of seedlings in a pool. The number of seedling per pool was $\geq$10; three pools were analyzed. C, Wild type, *U1*, and *U2* have comparable chlorophyll levels when grown on unsupplemented PN growth media; $n \geq 5$. D, Representative 4-d-old wild type (left) and *CML24*-underexpressing transgenic (right) plant grown on PNS PN supplemented with of 2 mM molybdic acid (Na_2MoO_4). E, *U1* and *U2* develop expanded cotyledons at a faster rate than wild type; $n \geq 10$. F, Seedlings grown vertically on PN supplemented with 250 μM $ZnSO_4$ (top) or unsupplemented PN (bottom) for 10 d. G, *U1* and *U2* develop longer roots than wild type after 10-d growth on 250 μM ZnSO4; $n \geq 39$. H, Wild type, *U1*, and *U2* have comparable roots lengths on unsupplemented PN; $n \geq 29$. Error bars represent SDS. Scale bars = 1 mm in A and D, and 10 mm in F.

unsupplemented media and on media supplemented with increasing concentrations of $MgCl_2$. The *CML24*-underexpressing transgenics accumulate similar levels of Mg^{2+} as wild type (Fig. 7C). As the $MgCl_2$ concentration is elevated, the levels of Mg^{2+} in both wild-type and *CML24*-underexpressing plants increase. These data indicate that reduced Mg^{2+} uptake is not the basis for the *CML24*-underexpressing transgenics' increased growth on Mg^{2+}-supplemented media.

An alternative explanation for the enhanced tolerance of the *CML24*-underexpressing transgenics could be that excess Ca^{2+} accumulation relieves toxic effects of elevated Mg^{2+} (Cheng et al., 2003). To assess whether the *CML24*-underexpressing transgenics accumulate higher Ca^{2+} levels than wild type, we monitored total Ca^{2+} levels in plants. The *CML24*-underexpressing transgenics accumulate similar levels of Ca^{2+} as wild type under normal growth conditions

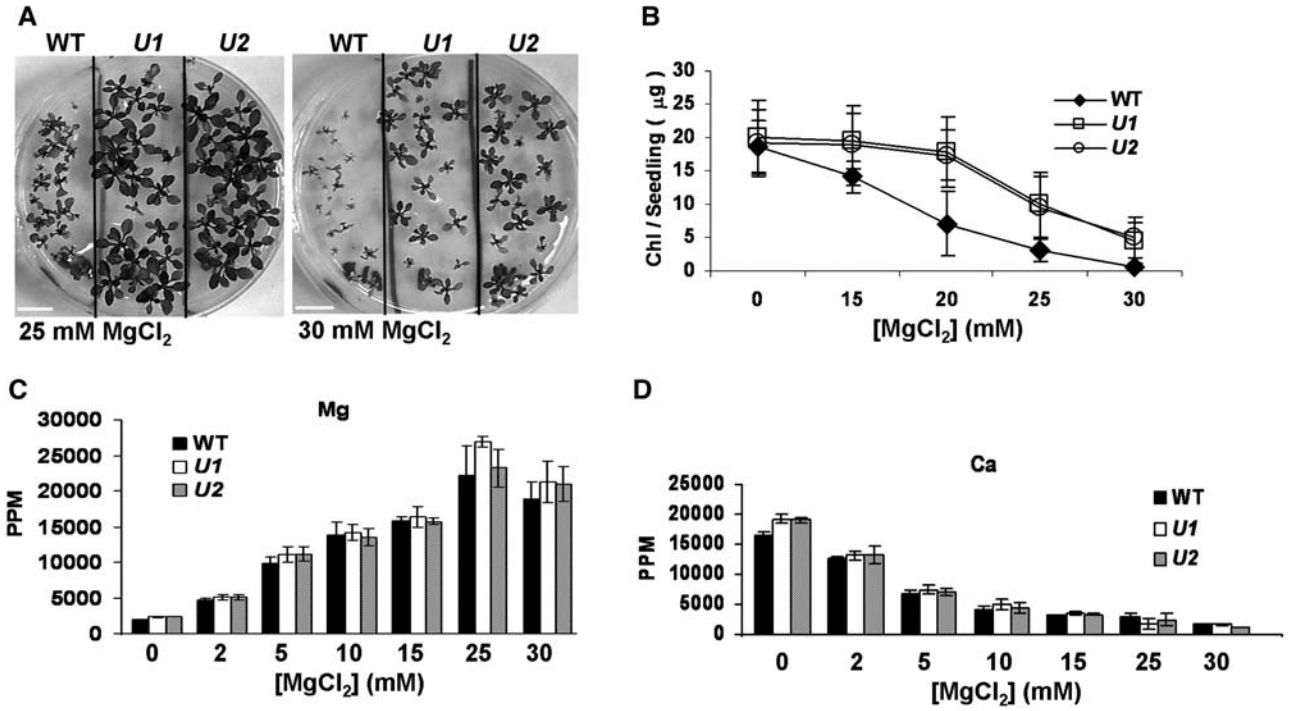

Figure 7. The *CML24*-underexpressing transgenics (*U1*, *U2*) are more tolerant of $MgCl_2$ than wild type (WT) and accumulate comparable levels of Mg^{2+} and Ca^{2+}. A, *U1* and *U2* show further development than wild type on growth media supplemented 25 mM $MgCl_2$ (left) or 30 mM $MgCl_2$ (right) for 20 d. Scale bar = 15 μm. B, Chlorophyll (Chl) levels were determined on increasing concentrations of $MgCl_2$ to represent differences in vegetative development. Error bars represent SDS, $n \geq 10$. C and D, ICP-MS was performed on approximately 3-week-old plants grown on increasing concentrations of $MgCl_2$. C, Wild type, *U1*, and *U2* accumulate comparably increased levels of Mg^{2+} when grown on PNS supplemented with increasing concentration of $MgCl_2$. D, All plants accumulate reduced levels of Ca^{2+} when grown on increasing concentrations of $MgCl_2$. Error bars represent SDS of four pools of four seedlings per pool for 0 to 15 mM $MgCl_2$ or three pools of two to five seedlings per pool for 25 and 35 mM $MgCl_2$. PPM, Parts per million.

and when grown with elevated exogenous $MgCl_2$ (Fig. 7D). Wild type and the *CML24*-underexpressing transgenics exposed to increasing levels of exogenous $MgCl_2$ show lower overall Ca^{2+} accumulation. Therefore, enhanced $MgCl_2$ tolerance in the *CML24*-underexpressing transgenics is neither due to increased Ca^{2+} accumulation nor decreased Mg^{2+} uptake.

DISCUSSION

CML24 Encodes a Ca^{2+}-Binding Protein Distinct from CaM

CML24 belongs to the Arabidopsis *CML* gene family (McCormack and Braam, 2003). This family of 50 genes encodes proteins that share at least 16% amino acid identity with CaM, contain one or more EF hands, and have no other predicted functional domains (McCormack and Braam, 2003). Comparisons of primary and tertiary protein structure (Khan et al., 1997) have revealed several features that distinguish CML24 from CaMs and other CMLs; these features may be important for differences in target interaction, localization, and/or stability.

For example, although modeling reveals CML24 may form a structure similar to CaM, CML24 has five Glys within the first six residues of the linker region (Fig. 1A), which are predicted to provide increased flexibility (Khan et al., 1997). CML24 is predicted to have a higher pI (4.78) than CaM (approximately 4.6). CML24 contains more positively charged residues than CaM, some of which replace three Glus (E) found in CaM (Fig. 1A). These primary sequence differences suggest that CML24 may have different target preferences from CaM.

CML24 is able to bind Ca^{2+} and undergo conformational changes that are detectable as an enhanced rate of SDS-PAGE migration in the presence of Ca^{2+} (Fig. 1B). The ability of EF-hand proteins such as CML24 to show Ca^{2+}-dependent migration rate changes, even under the denaturing conditions of SDS-PAGE, suggests profound Ca^{2+}-induced conformational changes and high Ca^{2+} affinity. In addition, purified CML24 binds to phenyl sepharose in a Ca^{2+}-dependent manner (data not shown), indicating that hydrophobic surfaces become available for binding in the presence of Ca^{2+}. This behavior predicts the potential for CML24 to function as a Ca^{2+} sensor in plant cells.

CML24 Expression Is Increased by Diverse Stimuli and Occurs in Many Major Organs

CML24 is a highly regulated gene; expression increases in plants subjected to the diverse stimuli of touch, darkness, heat, cold, H_2O_2, ABA, and IAA (Fig. 2A). Many of the inducing stimuli cause increases in cytosolic Ca^{2+}. When heat shock- or cold shock-induced Ca^{2+} increases are perturbed by Ca^{2+} channel blockers or chelators, induction of *CML24* expression is strongly inhibited (Braam, 1992; Polisensky and Braam, 1996), suggesting that *CML24* expression induction by heat and cold is Ca^{2+} dependent. However, at least by western analyses, there is a lack of detectable changes in protein level (data not shown). Although this may indicate that the nascent *CML24* transcripts are not translated, it may be more plausible that new protein synthesis is not detected by our methods or that new synthesis replaces degraded protein. Taken together, these data suggest that *CML24* expression may be induced by stimuli that use Ca^{2+} as a second messenger and may represent a feedback mechanism to produce or at least be poised to produce the Ca^{2+}-binding CML24 protein under conditions of stress-induced Ca^{2+}-signaling events.

Although RT-PCR indicates that *CML24* transcripts are present in all major organs (Fig. 2B), *CML24*::*GUS* reporter gene activities are found in more restricted sites. *CML24*::*GUS* is expressed at sites that are predicted to have been under mechanical stress, including the ruptured seed coat (Fig. 2C), the root-shoot junction (Fig. 2E), inflorescence branch points (Fig. 2J), and the developing abscission zone of the silique (Fig. 2K). Thus, the *CML24* regulatory region may be activated not only by externally applied mechanical force such as touch, but also by mechanical stresses that become manifest during development. In addition, *CML24*::*GUS* expression in guard cells (Fig. 2F), hydathodes (Fig. 2I), and vascular tissue (e.g. Fig. 2, N and O) may be a consequence of possible turgor pressure changes that these cells may experience.

CML24 May Positively Regulate ABA Inhibition of Germination and Seedling Development

ABA regulates many stress responses and developmental processes. ABA signaling is involved in plant response to cold, drought, and osmotic stress (Finkelstein et al., 2002). ABA triggers stomatal closure via H_2O_2 and cytosolic Ca^{2+} signals (Schroeder et al., 2001; Finkelstein et al., 2002). Darkness also induces stomatal closure (for review, see Schroeder et al., 2001). *CML24* expression is induced by ABA, H_2O_2, external Ca^{2+}, and darkness (Fig. 2A; Braam, 1992), and *CML24*::*GUS* expression is found in guard cells (Fig. 2F). However, under the conditions tested, the *CML24*-underexpressing transgenics show wilting behaviors indistinguishable from wild type (data not shown); therefore, reduced CML24 levels do not significantly affect guard cell function.

ABA also maintains seed dormancy, prevents germination, and inhibits seedling growth (Finkelstein et al., 2002). Increases in cytosolic Ca^{2+} mediate ABA-induced gene expression (Van der Meulen et al., 1996; Wu et al., 1997; Webb et al., 2001), and a role for Ca^{2+} in ABA regulation of stomatal aperture is well established (McAinsh et al., 1990, 1991; Irving et al., 1992; Allen et al., 1999; Pei et al., 2000; Schroeder et al., 2001; Webb et al., 2001). However, it is unclear whether Ca^{2+} plays a role in ABA regulation of germination. Mutants in the Ca^{2+} sensors, SCaBP5 and CBL9, and their respective target kinases, PKS3 and CIPK3, are hypersensitive to ABA inhibition of germination and seedling growth (Guo et al., 2002; Kim et al., 2003; Pandey et al., 2004), suggesting a role for Ca^{2+} sensing in these ABA-regulated processes. However, while SCaBP and CBL9 may negatively regulate the ABA-signaling pathway (Guo et al., 2002; Pandey et al., 2004), the reduced sensitivity of the *CML24*-underexpressing transgenics to exogenous ABA (Fig. 4) and the *CML24*::*GUS* expression in seed coats (Fig. 2C), developing embryos (Fig. 2K), and 2-d-old seedlings (Fig. 2D) suggest that CML24 may act downstream of ABA perception, perhaps mediating cellular responses to ABA-induced Ca^{2+} fluctuations to delay germination and seedling growth.

CML24 May Be Required for Photoperiod-Induced Flowering

The *CML24*-underexpressing transgenics are delayed in flowering compared to wild type when grown under 16- or 24-h photoperiods (Fig. 5, A–C). However, the reduction in CML24 levels has no effect on the timing of transition to flowering when plants are grown in 8-h photoperiods or subjected to vernalization or GA-induced flowering (Fig. 5, B and D–F). This phenotype is characteristic of flowering-time mutants disrupted in the long-day flower induction pathway (Koornneef et al., 1991, 1998; Bagnall, 1993; Putterill et al., 1995).

The long-day flower induction pathway is influenced by circadian rhythms (Mouradov et al., 2002). Cytosolic Ca^{2+} oscillates in a photoperiod-sensitive rhythm (Love et al., 2004). These Ca^{2+} fluctuations may reflect a role for Ca^{2+} in photoperiod-induced flowering. One possibility is that CML24 may play a role in mediating flowering transition responses to potential Ca^{2+} signals.

CML24 May Function in Ion Homeostasis

The *CML24*-underexpressing transgenics undergo further development than wild type in the presence of excess $CoCl_2$ (Fig. 6, A and B), molybdic acid (Fig. 6, D and E), $ZnSO_4$ (Fig. 6, F and G), and $MgCl_2$ (Fig. 7, A and B). Thus, CML24 is required for normal seedling growth inhibition that occurs in plants grown under these conditions. Based on ICP-MS analysis, we have ruled out the possibility that CML24 may normally

function to promote Mg^{2+} uptake and that the loss of CML24 prevents the accumulation of the toxic ions. The transgenics underexpressing *CML24* accumulate similar Mg^{2+} levels as wild type when grown on Mg^{2+}-supplemented media (Fig. 7C). Apparently, the *CML24*-underexpressing transgenics are better able to tolerate the accumulated ions. One possibility is that CML24 functions as a negative regulator of ion sequestration, and the tolerance of the *CML24*-underexpressing transgenics may be due to more efficient sequestering of ions into internal stores. *CML24*::*GUS* expression is found in lateral roots and root tips, as well as the vasculature (Fig. 2, N–P). These patterns of expression are consistent with the possibility that CML24 may support ion uptake and transport. Metals such as Co^{2+}, Mo^{2+}, Zn^{2+}, and Mg^{2+} serve as cofactors for diverse enzymes (Clarkson and Hanson, 1980); therefore, the *CML24*-underexpressing transgenic response to these ions could reflect a role for CML24 in regulating various physiological processes that involve Co^{2+}, Mo^{2+}, Zn^{2+}, or Mg^{2+}.

Diverse Roles of *CML24*

The diverse phenotypes of the CML24-underexpressing transgenics could be a consequence of the loss or reduction of CML24 interactions with distinct targets and thus having impact on distinct physiological processes. Alternatively, the phenotypes may be related in some way. There is evidence that Ca^{2+} signaling may be involved in ABA responses, timing of transition to flowering, and salt stress responses. Alternatively, the reduced sensitivity of the *CML24*-underexpressing transgenics to ions and ABA and their delayed flowering could be due to reduced sensitivity to and/or reduced production of reactive oxygen species (ROS). High ion levels induce ROS production (Arora et al., 2002; Mittler, 2002), H_2O_2 mediates ABA signal transduction in guard cells (Pei et al., 2000), and the timing of transition to flowering may involve ROS (Kurepa et al., 1998). Some mechanical perturbations can also cause ROS production (Braam, 2005). Therefore, the *CML24*-underexpressing transgenics' growth response to high levels of ions and exogenous ABA, the delay in photoperiod-induced flowering, as well as the induction of *CML24* expression by touch, ABA, and H_2O_2 suggest that CML24 may have a role in redox signaling and stress responses. These possibilities are under current investigation.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stimuli Treatments

Arabidopsis (*Arabidopsis thaliana* ecotypes Columbia [Col-0] and RLD) were cultivated in Baccto soil (Southwest Fertilizer), on agar plates, or in liquid growth media. Seeds sown on plates or in liquid media were surface sterilized with 100% bleach (v/v; 6% sodium hypochlorite) for 10 min followed by three washes with sterile water. Seeds were sown in soil and plants were grown under continuous light (28 μmol m^{-2} s^{-1}) at 25°C. Seeds sown on agar plates were placed in a 22°C growth chamber under continuous light (37 μmol m^{-2} s^{-1}). Seeds sown in liquid media grew at 25°C with continuous shaking at 96 rpm under continuous light (37 μmol m^{-2} s^{-1}).

Growth Conditions and Stimuli Treatments for Expression Analysis

Touch, dark, heat, and cold treatments were administered to 5-week-old soil-grown plants (ecotype Col-0). The touch stimulus was administered by bending the petioles and leaves back and forth for 2 min. The darkness stimulus was administered by covering plants with a black box for 30 min. Heat and cold treatments were administered by placing potted plants at 37°C for 1 h or at 4°C for 4 h, respectively. Control plants for the touch, dark, heat, and cold experiments were left unstimulated. Hormone and hydrogen peroxide (H_2O_2) treatments were administered to plants grown for 3 weeks in 25 mL of PN (Haughn and Somerville, 1986) liquid growth media supplemented with 0.5% (w/v) Suc (PNS). The growth media was replaced with 25 mL of fresh PNS 24 h prior to treatment with 100 μM ABA (Sigma-Aldrich), 100 μM IAA (Sigma-Aldrich), 100 μM GA_3 (Sigma-Aldrich), 100 μM ACC (Sigma-Aldrich), 20 mM H_2O_2 (3% hydrogen peroxide solution; Walgreens), 0.32% (v/v) ethanol (hormone solvent control), or sterile water (H_2O_2 solvent control). After treatments, whole-plant tissue was collected at time points indicated in the text. For organ-specific expression analysis, rosette leaves, inflorescences, cauline leaves, flowers, and nonsenescent siliques were collected from 7-week-old soil-grown plants (ecotype Col-0). Roots were isolated from plants (ecotype Col-0) grown in 25 mL of PNS for 4 weeks. For *CML23* expression analysis in the *CML24*-underexpressing transgenics, plants (ecotype RLD) were grown in 25 mL of PNS for 6 weeks and tissue was collected from unstimulated whole plants. After collection, tissue was immediately frozen in liquid nitrogen and stored at −80°C prior to RNA isolation.

Growth Conditions and Stimuli Treatments for Phenotype Analysis

Seeds were stratified at 4°C for at least 3 d before sowing on 0.8% to 1.6% (w/v) agar plates containing PN growth media or PN supplemented with ABA, or additional molybdic acid (Na_2MoO_4; Sigma-Aldrich), $ZnSO_4$ (Sigma-Aldrich), $CoCl_2$ (Sigma-Aldrich), or $MgCl_2$ (Sigma-Aldrich). Control plates for ABA germination experiments were supplemented with 0.005% ethanol (v/v; ABA solvent). For ICP-MS analysis, seeds were sown on PN agar plates with or without 2 mM $MgSO_4$ (component of PN media) or PN agar plates supplemented with 5 to 30 mM $MgCl_2$. For seedling greening on ABA experiments, plants were grown on 0.8% (w/v) agar plates containing 0.5× Murashige and Skoog basal media with Gamborg's vitamins (Sigma-Aldrich) supplemented with 0.5% (w/v) Suc, with or without 5 μM ABA.

ABA Germination Analysis

The number of germinated seeds (radical emerged from seed coat) was counted at least every 2 d for approximately 3 weeks. Percent germination was determined by calculating the total number of seeds germinated divided by the total number of seeds sown.

Transition to Flowering Analysis

To assess flowering-time response to photoperiod, seeds were stratified at 4°C for at least 3 d and germinated in soil. Plant were grown at 25°C in a long day of 16- or 24-h light (37 μmol m^{-2} s^{-1} or 28 μmol m^{-2} s^{-1} light intensity, respectively) or a short day of 8-h light (37 μmol m^{-2} s^{-1} light intensity). To assess flowering-time response to GA, stratified seed were germinated and grown in 8-h light at 22°C. After 4 weeks growth, plants were sprayed with 100 μM GA_3 and 0.2% (v/v) Triton X-100 1 to 2 times a week for 8 weeks. Control plants were sprayed with 0.16% (v/v) ethanol (GA_3 solvent) and 0.2% (v/v) Triton X-100. Vernalization treatment was administered by germinating seeds in 24-h light at 22°C for 2 d to break dormancy and then placing seed at 4°C under continuous light (11 μmol m^{-2} s^{-1} light intensity) for 30 or 40 d. Vernalized seedlings were transplanted to soil and grown in 8-h light at 22°C for flowering-time analysis. For all treatments, day of flowering, defined as the emergence of the primary inflorescence, and rosette leaf number were recorded.

Chlorophyll Measurement

Shoot tissue was frozen and ground in liquid nitrogen. Chlorophyll was extracted in 80% (v/v) acetone on ice or at −20°C for 10 min to overnight with occasional vortexing. During extraction, sample tubes were covered with aluminum foil to prevent light degradation of the chlorophyll. Supernatants were isolated by 12,000*g* centrifugation. Absorbance was measured at 652 nm using the Beckman DU-64 spectrophotometer (Beckman Instruments). Chlorophyll content was measured using the following equation: Chl (mg/mL) = A_{652}/34.5 (Danilov and Ekelund, 2001).

ICP-MS Analysis

Three-week-old plants were harvested and dried at 65°C overnight. Approximately 4 mg of dried plant material was weighed and placed into Pyrex digestion tubes. Digestions were carried out using 1 mL HNO_3 at 114°C for 4 h. Each sample was then diluted to 10 mL with sterile water and analyzed on a Perkin-Elmer Elan DRC-e ICP-MS using a glass Conikal nebulizer drawing 1 mL per min. Methane was used as the collision cell gas for Fe.

Protein Sequence Alignment and Comparisons

Protein sequences (CML24/TCH2, At5g37770; CaM1, At5g37780; CaM2, At2g41110; CaM6, At5g21274; CaM7, At3g43810) were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Protein sequences were aligned and percent identities and similarities were determined as described in McCormack and Braam (2003).

Generation of CML24 Antibodies

The *CML24* coding region was amplified by PCR using foward primer 5′-AAG CTT CAT ATG TCA TCG AAG AAC GGA G-3′ and reverse primer 5′-CGG GAT CCT CAA GCA CCA CCA CCA TTA CT-3′. The underlined bases introduce restriction enzyme sites into the amplicon. The PCR products were ligated into *Nde*I/*Bam*HI site of pET21a (Novagen) to create plasmid pKAJ263. The recombinant CML24 protein was expressed in BL21 (DE3)-RIL cells by growing cells at 37°C overnight in Luria-Bertani containing 100 μg/mL ampicillin. Twenty-five milliliters of the overnight culture was used to inoculate a 500-mL culture of Luria-Bertani/ampicillin that was then grown to mid-log phase (OD_{600} of approximately 0.6). At mid-log phase, isopropylthio-β-galactoside was added to a final concentration of 1 mM. After an additional 2 h of growth, the cells were pelleted at 8,000*g* for 10 min at 4°C. Protein was purified from the pellet using a modified CaM purification protocol (Fromm and Chua, 1992). The cell pellet was resuspended in 20 mL of buffer A (20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA) and then lysed by three passages through a French pressure cell. The solution was centrifuged at 30,000*g* for 30 min at 4°C. The supernatant was transferred to a clean tube and 1 M $CaCl_2$ was added to a final concentration of 3 mM. The supernatant was loaded onto a 10-mL phenyl-Sepharose column previously equilibrated with buffer B (20 mM Tris, pH 7.5, 200 mM NaCl, 1 mM $CaCl_2$). The column was then washed with 75 mL of buffer B. Recombinant CML24 protein was eluted with 50 mL of buffer A. Fractions containing protein (as determined using Pierce BCA kit; Pierce Chemical) were pooled together and adjusted to 3 mM $CaCl_2$. Pooled fractions were purified a second time by phenyl-Sepharose chromatography. Eluted protein was concentrated using an Amicon Centricon centrifugal filtration device with a 3,000 molecular weight cutoff (Millipore). Purified protein was excised from a 15% (w/v) polyacrylamide gel after staining with Coomassie Brilliant Blue G250. Antibody was produced in rabbits by Cocalico Biologicals. The antibody was IgG purified using the Bio-Rad Econo-Pac Serum IgG Purification kit. The anti-CML24 antibodies react with 60 ng of purified CML24 on a western blot (data not shown); however, the antibodies fail to detect up to 20 μg of purified mammalian CaM (a gift from Kate Beckingham, Rice University; data not shown). Because mammalian CaM shares 91% amino acid identity with Arabidopsis CaM, this result suggests that the anti-CML24 antibody probably does not have significant cross reactivity with Arabidopsis CaMs.

Protein Analyses

Total plant protein was extracted from whole-plant tissue using a lysis buffer of 4% (w/v) SDS, 20% (v/v) glycerol, and 120 mM Tris, pH 6.8 as described in Sistrunk et al. (1994). Protein concentration was determined using the Pierce BCA kit. For western-blot analysis, 20 to 60 μg of protein were separated in a 15% (w/v) SDS-polyacrylamide gel. The protein was then transferred to nitrocellulose membrane using Towbain's buffer (25 mM Tris, 192 mM Gly, and 20% [v/v] methanol, pH 8.3; Harlow and Lane, 1988) supplemented with 1 to 2 mM $CaCl_2$ to assist in transfer of low M_r proteins (McKeon and Lyman, 1991). To enhance retention of CML24 protein, blots were baked overnight at 65°C in a vacuum oven (Sistrunk et al., 1994). Blots were incubated with 2.4 to 3 μg/mL of primary antibody followed by Pierce horseradish peroxidase-conjugate goat anti-rabbit secondary antibody. Both antibody solutions were diluted in 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% (v/v) Tween 20, and 1% (w/v) nonfat milk. Detection of the interaction was performed using the Pierce SuperSignal West Pico Chemluminescent kit. To assay for Ca^{2+}-dependent mobility shift, total plant protein was run on SDS-PAGE in the presence of 5 mM EGTA or 5 mM $CaCl_2$ before transfer to nitrocellulose membrane and interaction with antibody as described above.

Generation and Identification of *GUS* Reporter Transgenics

Following amplification of the *CML24* upstream sequence with forward primer 5′-CCC AAG CTT ACA TAA ACG GAC AAG TTC G-3′ and reverse primer 5′-TGC TCT AGA TTG AGA TTT GAG AGA AG- 3′, a transcriptional fusion of 1,061 bp of upstream genomic sequence was directionally ligated into the *Hin*dIII/*Xba*I sites in the *GUS* binary vector pBI101 (CLONTECH Laboratories) to generate the plasmid pBI-1114b. The underlined bases introduce restriction enzyme sites into the amplicon.

Transformation of Arabidopsis ecotypes RLD or Col-0 were performed using vacuum infiltration (Bechtold et al., 1993) or floral dip (Clough and Bent, 1998), respectively, in an Agrobacteria infiltration media. Seeds from the transformed plants were collected and screened for resistance to 50 μg/mL kanamycin on growth media supplemented with 0.8% (w/v) agar. T_1 plants were transplanted into soil and grown to maturity. Approximately 30 T_2 plants were screened for GUS accumulation. Three independent transgenics showing similar *GUS* expression patterns (lines 1114b-3 [RLD], *CML24*::*GUS* no. 4 [Col-0], and *CML24*::*GUS* no. 10 [Col-0]) were analyzed for *CML24*::*GUS* expression.

Identification of *CML24*-Underexpressing Transgenics

Transgenes can induce silencing of endogenous loci containing sequences homologous to the transgene through the phenomenon of cosuppression (Matzke and Matzke, 1995). Cosuppressed *CML24*::*GUS* transgenics (harboring 1.5 kb of *CML24* upstream sequence plus 210 bp of *CML24* coding sequence directionally cloned into the *Xba*I/*Bam*HI sites of the pBI101 *GUS* vector; plasmid pKAJ259) were identified from transformants showing kanamycin resistance but lacking GUS accumulation. Three independent *CML24*::*GUS* transgenics (ecotype RLD) lacking GUS accumulation were checked for *CML24* RNA and protein accumulation; two of these lines had undetectable levels of *CML24* RNA (data not shown) and CML24 protein (Fig. 3A). These lines were named *CML24 U1* and *CML24 U2*.

GUS Staining

CML24::*GUS* transgenics were submerged in a Na-P buffer (0.1 M Na_2HPO_4, 0.1 M $NaHPO_4$, pH 7.0, 1 mM EDTA) staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcUA with or without paraformaldehyde fixative and with or without cyanide, as described in Jefferson et al. (1987), Martin et al. (1992), and Bartel and Fink (1994). Staining was visualized and photographed using the Zeiss axioscope (Carl Zeiss GmbH) and Leica MZFL III stereoscope.

RNA Isolation

Total RNA was extracted from plants according to Verwoerd et al. (1989) or by using TRIzol reagent (Invitrogen) according to manufacturer instructions. The first method, in brief, utilizes equal volumes of 80°C extraction buffer (0.1 M LiCl, 0.1 M Tris, pH 8.0, 0.01 M EDTA, 1% [w/v] SDS) to break open the cells, and 80°C phenol (equilibrated at pH 8.0) and chloroform to extract protein and tissue. Samples were centrifuged at 12,000*g* for 10 min and a second phenol/chloroform extraction was performed. RNA was precipitated from the supernatant using an equal volume of 4 M LiCl (RNase free) followed

by an ethanol precipitation. RNA was resuspended in RNase-free water. Alternatively, an equal volume of TRIzol reagent and 0.2× volume chloroform (RNase free) were added to tissue and centrifuged at 12,000*g* at 4°C for 15 min. RNA was precipitated with 100% (v/v) isopropanol (RNase free) and washed with 75% (v/v) ethanol (RNase free). RNA was resuspended in RNase-free water. RNA concentrations were determined at absorbance A_{260}, and integrity was visualized by separation on a 1% (w/v) formaldehyde agarose gel stained with ethidium bromide.

RT and RT-PCR

One microgram of purified total RNA was DNase treated (Roche Diagnostics) for 30 min at 37°C and heat inactivated at 65°C for 10 min. DNase-treated RNA was then reverse transcribed using a reaction mix of 8 μM oligo(dT) (Integrated DNA Technologies), 1.6 mM dNTPs (Bioline), 1× NEB buffer (New England Biolabs), and NEB M-MuLV reverse transcriptase (New England Biolabs), which was incubated at 37°C for 1 h. Alternatively, DNase-treated RNA was reverse transcribed using a reaction mix of 4 μM oligo(dT), 0.2 μM dNTPs, 4 mM diothiothreitol (Invitrogen), 1× First Strand buffer (Invitrogen), and SuperScript III RNase H− reverse transcriptase (Invitrogen). RNA, oligo(dT), and dNTPs were denatured at 65°C for 5 min and snap cooled on ice for 5 min, after which buffer, diothiothreitol, and reverse transcriptase were added to the mixture and the reaction carried out at 50°C for 1 h.

For RT-PCR, the volume of cDNA mixture equivalent to 40 ng of RNA was amplified using primers for *CML23* (forward primer, 5′GGACATGTCGAAGAACGTTTCGAGAAACTG3′; reverse primer, 5′CTGGCGCGCCAGAGAGCCATTAAAGAAGCAAC3′), *CML24* (forward primer, 5′-GAG TAA TGG TGG TGG TGC TTG A-3′; reverse primer, 5′-ACG AAT CAT CAC CGT CGA CTA A-3′), or *TUB4* (forward primer, 5′-CTG TTT CCG TAC CCT CAA GC-3′; reverse primer, 5′-AGG GAA ACG AAG ACA GCA AG). For semi-quantitative RT-PCR, reactions were amplified for 28 and 34 cycles. To verify the absence of DNA contamination of the RNA samples, the RNA samples were subjected to PCR using *CML23* or *CML24* primers in the absence of RT. No products were detectable.

QRT-PCR

The volume of cDNA mixture equivalent to 100 ng of RNA, 0.5 μM primers, and 1 × SYBR Green I mix (EpiCentre) were used for QRT-PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The ΔΔCT method was used to determine relative cDNA levels. Briefly, each sample was amplified using primers for *TUB4* (normalizer) and *CML24* (gene of interest). The difference in cycle number where product amplification resulted in a fixed threshold amount of fluorescence was determined by the following equation: $\Delta CT_{sample} = \Delta CT_{TUB4} - \Delta CT_{CML24}$. One sample was chosen as a calibrator and ΔΔCT was determined for each sample according to the following equation: $\Delta\Delta CT_{sample} = \Delta CT_{sample} - \Delta CT_{calibrator}$. Relative RNA levels were calculated using inverse $\log_2$, $2^{\wedge(\Delta\Delta CT_{sample})}$. The average and SE of the relative RNA levels for three biological replicates was calculated and graphed for each sample. Finally, the relative fold induction was determined by dividing the average relative RNA levels of the stimulated plants by the average relative RNA levels of the control plants.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all parts of the material. Obtaining permission will be the responsibility of the requestor.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers CML24 (TCH2) P25070, CaM1 P25854, CaM2 P25069, CaM6 Q03509, and CaM7 P59220.

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