PROMOTER ANALYSIS OF A SUBFAMILY OF CALMODULIN-LIKE GENES IN *ARABIDOPSIS*

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ABSTRACT

Ca^{2+} ions participate as second messengers in many stress-response and developmental pathways. Among eukaryotes, plants possess a remarkable diversity of Ca^{2+} binding proteins (Ca^{2+} sensors) such as calmodulin (CaM) and CaM-related proteins (CMLs) that regulate downstream targets and coordinate signal transduction events in response to stimuli. Previous studies have shown that a small subfamily of *CMLs* (*CML37*, *CML38*, *CML39*) in *Arabidopsis* show differential tissue expression as well as a dramatic induction of expression in response to environmental stress. For example, *CML37* and *CML38* respond very strongly to wounding, while *CML39* is induced significantly by jasmonate. In order to understand the underlying regulatory mechanisms of the genes, promoter analysis experiments using the 5′ upstream regions of these *CMLs* driving β-glucuronidase (GUS) reporter expression were conducted. This empirical approach is a critical complement to algorithm-based prediction methods. It was found that the gateway vector pMDC163 was unsuitable for 5′ deletion analyses. Three regions within the *CML37* promoter were identified as having wound-responsiveness. Several known wound-responsive *cis*-elements were identified in these regions. A putative *cis*-element that is overrepresented in genes coexpressed with *CML37* was also identified. Together, these data should lay the groundwork to identify the transcriptional regulators that direct stress-responsive *CML* gene expression.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
AGRIS	*Arabidopsis* gene regulatory information server
AP2/EREBP	APETALA2/ethylene-responsive-element-binding protein TF family
Athena	*Arabidopsis thaliana* expression network analysis database
ATTED II	*Arabidopsis thaliana* trans-factor and *cis*-element prediction database
BAR	botany array resource
bp	base pair(s)
bZIP	basic-domain leucine-zipper TF family
$[Ca^{2+}]_{cyt}$	cytosolic free Ca^{2+} concentration
CaBP	Ca^{2+}-binding protein(s)
CaM	calmodulin
CaMBP	CaM binding protein(s)
CBL	calcineurin B-like
CDPK	Ca^{2+}-dependent protein kinase
CML	calmodulin-like protein
DACC	depolarization-activated Ca^{2+} channel
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
ERF	ethylene response factor(s)
GA	gibberellic acid

GFP	green fluorescent protein
GUS	β-glucuronidase
HACC	hyperpolarization-activated Ca^{2+} channel
JA	jasmonic acid
JAZ	JASMONATE ZIM-domain proteins
KIC	kinesin-like CaM-binding protein-interacting Ca^{2+}-binding protein
LUC	firefly luciferase
MCC	mechanosensitive Ca^{2+} channel
MeJA	methyl jasmonate
MEME	multiple em for motif elicitation database
MS	Murashige and Skoog
MUG	4-methylumbelliferyl glucuronide
PCR	polymerase chain reaction
PLACE	plant *cis*-acting regulatory DNA elements database
PlantCARE	plant *cis*-acting regulatory elements database
ROS	reactive oxygen species
SA	salicylic acid
TCH	touch-induced
TF	transcription factor(s)
TFII	general transcription factor for RNA polymerase II
VICC	voltage-independent Ca^{2+} channel
X-Gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronide

CHAPTER 1: Introduction and literature review

Plant defence responses

Plants are sessile organisms and therefore must find a way to respond to changing environments. One of the major factors with which plants must cope is wounding of tissues, which can be caused by abiotic factors such as wind, rain, or hail, as well as biotic factors, such as insect herbivory. Not only do these factors destroy plant tissues, they provide a pathway for opportunistic pathogen invasion. It appears that plants are able to distinguish between mechanical wounding and insect herbivory and induce different, yet similar, genes to respond to each stress type (Korth and Dixon, 1997; Reymond, et al., 2000). This differential response is likely due to the fact that herbivores release elicitors such as fatty-acid amino conjugates (Alborn, et al., 1997), ATP synthase γ-subunit-derived peptides (Schmelz, et al., 2006), and disulfooxy fatty acids (Alborn, et al., 2007) into the wound site. Alone, these elicitors are capable of initiating plant defence responses (Schmelz, et al., 2009). Pathogen and herbivore response mechanisms are also quite different. Although many of the same genes are activated in both pathways (Cheong, et al., 2002), the ultimate physiological response to each pathway is unique. Following pathogen attack, plants activate salicylic acid (SA) pathways that instigate the hypersensitive response. This response causes the cells immediately surrounding the site of infection to undergo programmed cell death and fill with antimicrobial compounds (Kessler and Baldwin, 2002). Understandably, this response is only effective against pathogens, as herbivores live independently of plants and can simply move to another feeding site. Additionally, in *Arabidopsis*, gene regulation following herbivory and pathogen attack is specific to the type of insect or pathogen that is attacking the plant (De

Vos, et al., 2005). Wounding response has also been found to overlap with other abiotic stress responses; many of the same genes are induced following drought, cold, salinity, heat, and anaerobic stresses (Reymond, et al., 2000; Cheong, et al., 2002; Delessert, et al., 2004).

Defence mechanisms have a high metabolic cost and so must be induced only following damage (Agrawal, 2005). Plants have many natural physical defences, such as cuticles, lignification of cell walls, thorns, trichomes, and hardened woody bark (Delessert, et al., 2004; León, et al., 2001). Wounding generates many signals that can be recognized by plants. Cell contents are released, as are oligogalacturonides from damaged cell walls. Additionally, undamaged cells surrounding the wound site may experience pressure and water differentials (Van Poecke, 2007). Following wounding, plants display both local (in the immediate vicinity of the wound site) and systemic responses. Locally, plants transiently produce both reactive oxygen species (ROS) and Ca^{2+} (León, et al., 2001; Howe and Jander, 2008). Genes involved in Ca^{2+} signalling, as well as in ROS production and scavenging are both found to be upregulated following wounding (Cheong, et al., 2002; Kuśnierczyk, et al., 2008), indicating that both these signalling molecules have roles in wound response. Genes induced in the local response mainly have functions in repairing damaged plant tissues, adjusting plant metabolism, or producing substances that inhibit further predation (León, et al., 2001). Lignin biosynthetic genes are induced locally to repair the wound site, while photosynthetic genes are downregulated to adjust plant metabolism (Delessert, et al., 2004). Many substances are produced to deter further predation. For instance, protease inhibitor genes, which decrease insect growth and performance, are upregulated. These genes decrease nutrient uptake by herbivores and increase digestive proteolytic enzyme activity, which

results in the depletion of essential amino acids (Van Poecke, 2007). A host of secondary metabolites are also produced that function as poisons, digestibility reducers, and insect repellents (Howe and Jander, 2008). Phytoalexins, flavonoids, and alkaloids function as anti-herbivore compounds, and their biosynthetic pathways are all upregulated following attack (Cheong, et al., 2002; Delessert, et al., 2004). Upon wounding, *Arabidopsis* myrosinases hydrolyze sulphur-containing glucosinolates into toxic proteins such as isothiocyanates and epithionitriles (Kessler and Baldwin, 2002; Kuśnierczyk, et al., 2008). This mechanism of defence is effective because prior to wounding, the myrosinases are spatially separated from the glucosinolates to prevent premature formation of these toxic molecules. Isothiocyanates have been found to reduce the survival and growth of the specialist herbivore *Pieris rapae* on *Arabidopsis* (Agrawal and Kurashige, 2003).

The systemic response is actually established earlier than the local response and it involves mainly signalling and regulatory factors (Delessert, et al., 2004), as it requires mobile signals to be transported around the plant to prepare unwounded areas of the plant for possible attack. In solanaceous plants, an 18 amino acid oligopeptide called systemin is thought to be the primary wound signal. This oligopeptide does not have to be synthesized following wounding, but is cleaved from a 200 amino acid precursor called prosystemin (Kessler and Baldwin, 2002; León, et al., 2001). In tomatoes, systemin binds a receptor kinase called SR160 (Meindl, et al., 1998) in plasma membranes that initiates a signalling cascade that results in the activation of phospholipase A2, which in turn releases linolenic acid from the plasma membrane (Kessler and Baldwin, 2002). Linolenic acid is a member of the octadecanoid pathway that controls jasmonic acid (JA) synthesis. JA and related compounds (jasmonates) are known defence signalling

phytohormones. Members of the octadecanoid pathway are upregulated following wounding as well as JA treatment, indicating a positive feedback mechanism (Delessert, et al., 2004). JA signalling is dependent on the gene *COI1*. This gene codes for a leucine-rich-repeat/F-box protein that determines substrate specificity of the SCF-type E_3 ubiquitin ligase SCF^{COI1}. Following JA application, SCF^{COI1} targets JASMONATE ZIM-domain (JAZ) proteins for degradation (Chini, et al., 2007; Thines, et al., 2007). These JAZ proteins are known repressors of key transcriptional activators of JA responses, such as MYC2 and other transcription factors (Boter, et al., 2004; Wang, et al., 2008). Strangely, JAZ proteins are upregulated by wounding and herbivory (Chung, et al., 2008), perhaps to limit JA signalling.

Other members of the octadecanoid pathway also have roles in defence response. Plants release volatile organic compounds following herbivore attack. These compounds serve two purposes: to signal distal leaves in order to overcome the restrictions of the vascular system, and to attract natural predators of herbivores (Heil and Ton, 2008). These volatile organic compounds are synthesized mainly through three biosynthetic pathways. Firstly, the so-called "green-leaf volatiles" are C6 compounds that are produced early in the wound response from precursors of JA (Kessler and Baldwin, 2002). Additionally, methylated JA (MeJA) is volatile and mobile in the gas phase and likely to function as long-distance defence signalling molecule (Heil and Ton, 2008). Secondly, terpenes derived from the isoprenoid pathway are volatile compounds that attract predators and parasitoids and may function as phytoalexins (Kessler and Baldwin, 2002). Thirdly, volatiles are produced through the shikimate pathway, which links metabolism of carbohydrates to the biosynthesis of aromatic compounds (Kessler and Baldwin, 2002). Methylated SA is derived through this pathway and is emitted following

herbivory but not mechanical wounding. It, too, likely functions in long-distance signalling (Heil and Ton, 2008). These long-distance signalling molecules function by inducing signal transduction pathways.

Plant signal transduction and transcription factor families

Although growth and development are defined by genetic code, plants show remarkable plasticity in adapting to their environments. Plants are able to regulate their rates of growth and metabolism in response to external cues. These environmental stimuli are transformed into physiological responses through a sequence of receptors, messengers, enzymes, and transcription factors (Sanders, et al., 2002), which are collectively referred to as a signal transduction pathway. Receptors, enzymes, and transcription factors (TF) are generally specific for their physiological stimulus and plant genomes reflect this by the wide array of proteins that serve these functions. Conversely, only a handful of non-protein messengers are known. These include cyclic nucleotides, hydrogen ions, ROS, and Ca^{2+} (Sanders, et al., 2002; Trewavas and Malhó, 1997). Together, these signal transduction pathways exert transcriptional control over genes that will elicit appropriate physiological responses.

Eukaryotic transcription is performed by RNA polymerase II for all protein-coding genes. In order to initiate transcription, general TF are required. These TF, called TFII (TF for RNA polymerase II), form the transcription initiation complex with RNA polymerase II. A short DNA sequence that is comprised of mainly A and T nucleotides and typically found around 25 nucleotides from the transcription start site (the TATA box) recruits the specific TFII protein called TFIID, which in turn is able to recruit other TFII proteins and RNA polymerase II. Other TFII proteins can contain helicase or kinase

domains that are required for transcriptional initiation (Alberts, et al., 2002). The area of the promoter where TFII proteins and RNA polymerase II bind is often referred to as the minimal promoter. The minimal promoter has been defined in the cauliflower mosaic virus 35S promoter (Fang, et al., 1989). It promotes little transcriptional activity on its own, but there will be no transcription if it is absent. Activators and repressors are responsible for transcriptional regulation. Transcriptional activators help attract, position, and modify the TFII proteins and RNA polymerase II so that transcription can begin, while repressors act to suppress transcription (Alberts, et al., 2002). These activators and repressors are predominantly TF that have specific binding sites.

There are predicted to be over 1500 TF genes in the *Arabidopsis* genome (Qu and Zhu, 2006). Large scale expression data has been compiled for 1400 of these genes (Czechowski, et al., 2004). Additionally, expression profiles of a smaller subset of these genes suggest overlap in function in hormone, stress, and senescence signalling (Chen, et al., 2002). There are several large families within *Arabidopsis* that have roles during many different growth and development pathways, as well as during stress response.

The MYB superfamily is the largest TF family in plants consisting of approximately 200 members (Qu and Zhu, 2006). This family is known to have functions in a wide variety of physiological processes as well as during ultra-violet light exposure, wounding, anaerobic stress, and pathogens (Singh, et al., 2002). Other large families such as basic helix-loop-helix (162 members) and MADS (100 members) are responsible for cell proliferation and flower-related physiological and developmental processes, among other things (Qu and Zhu, 2006). TF families that are well known to function during stress responses include the APETALA2/ethylene-responsive-element-binding protein (AP2/EREBP) family, the basic-domain leucine-zipper (bZIP) family, and the WRKY

family. The AP2/EREBP family consists of 147 members in *Arabidopsis*. A subgroup of this family called ethylene-response factors (ERF) is able to bind to two similar promoter elements; one found mainly in pathogenesis-related gene promoters, and the other within dehydration- and cold-responsive genes (Singh, et al., 2002). The ERF genes have been shown to be responsive to wounding, and also to treatment by JA, SA, and ethylene (Rushton and Somssich, 1998). The bZIP family is well characterized and numbers 75 members in *Arabidopsis*. They are known to both positively and negatively regulate many stresses including wounding, pathogens, salt, and drought tolerance. They are also involved in SA, auxin, JA, and hydrogen peroxide signalling (Jakoby, et al., 2002). WRKY TF are unique to plants and are defined by the presence of a WRKY domain (a 60 amino acid region that contains the sequence WRKYGQK) and a zinc-finger-like motif. There are over 70 WRKY TF in *Arabidopsis* (Qu and Zhu, 2006) and they are known to respond to SA treatment, senescence, pathogens, defence signals, and wounding (Eulgem, et al., 2000). WRKY TF bind to a promoter element called a W-box. This W-box is found in the promoters of many defence-related genes, including WRKY TF themselves, indicating transcriptional feedback regulation of these genes (Dong, et al., 2003; Eulgem and Somssich, 2007).

There are obviously also many smaller families of TF. For instance, *Arabidopsis* contains a family of six TF that are activated by a Ca^{2+}-binding protein (Bouché, et al., 2002). One of these TF is known to mediate biotic defences (Galon, et al., 2008). In addition to TF other proteins can also transcriptionally activate genes, as is the case with a specific Ca^{2+}-binding protein (Kushwaha, et al., 2008). Ca^{2+} is also able to regulate transcription without TF through certain promoter elements (Kaplan, et al., 2006; Finkler,

et al., 2007). The messenger Ca^{2+} therefore plays an important role in transcriptional regulation and signal transduction.

Ca^{2+} in plant signal transduction

Ca^{2+} is used as a second messenger by all higher eukaryotes. Due to its tendency to precipitate phosphate, Ca^{2+} is toxic to cells when at high levels in the cytosol (Clapham, 2007). For this reason, the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$) is kept at 10^{-7} M, while extracellular and vacuolar Ca^{2+} concentrations range from 10^{-4} to 10^{-3} M (Bush, 1995). This large gradient allows for a rapid increase in $[Ca^{2+}]_{cyt}$ when channels in the plasma or organelle membranes are opened. $[Ca^{2+}]_{cyt}$ has been shown to increase following the application of systemin (Moyen, et al., 1998) or following wounding (Knight, et al., 1993). Additionally, a Ca^{2+}-binding protein in tomato that is induced by wounding and by systemin may be responsible for the activation of several wound-responsive genes (Bergey and Ryan, 1999), indicating that Ca^{2+} signalling has a role in wound response. In addition to wounding, $[Ca^{2+}]_{cyt}$ elevations are known to be involved in signalling during both abiotic (salt, cold, light, heat, hypoxia, oxidative, drought) and biotic (herbivory, pathogen) stresses (Knight, et al., 1993; Knight, et al., 1991; Snedden and Fromm, 1998; Knight and Knight, 2001; Reddy, 2001; Nayyar, 2003; White and Broadley, 2003; Lecourieux, et al., 2006; Shao, et al., 2008; Song, et al., 2008). Ca^{2+} is also involved in the signal transduction pathways of phytohormones (abscisic acid (ABA), auxin, cytokinins, ethylene, gibberellic acid (GA), brassinosteroids, JA, and SA), as well as in many diverse cellular processes, such as cell growth and differentiation, thigmotropism, photomorphogenesis, gravitropism, nodulation, pollen tube growth, senescence, and circadian rhythm (Reddy, 2001; Nayyar, 2003; White and Broadley,

2003; Lecourieux, et al., 2006; Hahm and Saunders, 1991; Chen, et al., 1997; Hepler, 1997; León, et al., 1998; Yang and Poovaiah, 2000; Du and Poovaiah, 2005; McAinsh and Pittman, 2009; Du, et al., 2009).

Ca^{2+} enters the cytosol through influx channels. In animals, these channels are often Ca^{2+}-specific, but plants generally have influx channels that are permeable to all cations (McAinsh and Pittman, 2009). Influx channels are found in all plant cell membranes and are characterized electrophysiologically into four categories: mechanosensitive Ca^{2+} channels (MCC), depolarization-activated Ca^{2+} channels (DACC), hyperpolarization-activated Ca^{2+} channels (HACC), and voltage-independent Ca^{2+} channels (VICC). DACCs, HACCs, and VICCs have also been shown to be active on the vacuolar membrane (Sanders, et al., 2002; White and Broadley, 2003; McAinsh and Pittman, 2009). MCCs are thought to be key components of mechanical stimuli signalling pathways, but very little information is known about this type of channel (McAinsh and Pittman, 2009). DACCs may be responsible for shaping Ca^{2+} signatures, as depolarization of the plasma membrane is common to many stress stimuli (White and Broadley, 2003). Specific roles for HACCs have been identified, such as stomatal closure during water stress, responses to ROS, and cell growth in root hairs (Sanders, et al., 2002; McAinsh and Pittman, 2009). VICCs include cyclic-nucleotide gated channels and the glutamate receptor family and appear to be the only Ca^{2+}-permeable channels open at the resting state in plant cells, indicating a role in the maintenance of Ca^{2+} homeostasis within the cytosol (Sanders, et al., 2002; White and Broadley, 2003).

Homeostasis of Ca^{2+} in the cytosol is accomplished by actively transporting Ca^{2+} ions against their concentration gradient. Ca^{2+} efflux is achieved through Ca^{2+}-ATPase pumps and H^+/Ca^{2+} antiporters, which are present in the plasma membrane, as well as in

endomembranes of vacuoles and the endoplasmic reticulum (McAinsh and Pittman, 2009). There are two types of Ca^{2+}-ATPases. Both have a high affinity for Ca^{2+}, but one type is defined by its lack of autoregulation, while the other type contains an autoinhibitory amino-terminal domain (White and Broadley, 2003; McAinsh and Pittman, 2009). H^+/Ca^{2+} antiporters have a low affinity for Ca^{2+}, but have a high capacity for efflux. They have roles in maintaining a low $[Ca^{2+}]_{cyt}$ as well as resetting the $[Ca^{2+}]_{cyt}$ following stress induction (McAinsh and Pittman, 2009).

Changes in $[Ca^{2+}]_{cyt}$ have specific spatial and temporal characteristics following stimuli, which are known as "Ca^{2+} signatures". This has led to a Ca^{2+} signature hypothesis that proposes that the Ca^{2+} signature confers signalling specificity (Scrase-Field and Knight, 2003). In guard cells, it has been shown that Ca^{2+} oscillations contain encrypted information that are able to mediate stomatal closure (Allen, et al., 2001). However, other experimental evidence that have shown similar Ca^{2+} signatures with different end responses, similar end responses with different Ca^{2+} signatures, and responses occurring either in the absence of Ca^{2+} or in the presence of a Ca^{2+} agonist have led to a counter-theory that states that Ca^{2+} acts as a chemical switch and that signal specificity lies elsewhere in the signal cascade (Scrase-Field and Knight, 2003). These theories both require more experimental evidence and it may be that both theoretical mechanisms function in different circumstances. Obviously, other elements contribute to Ca^{2+} signalling, and possibly signal specificity, such as Ca^{2+}-binding proteins (CaBP).

CaBP are generally designated by the presence of at least one EF-hand motif; however, several CaBP do not contain EF-hands, such as annexins, C2 domain proteins, and calreticulin (Reddy, 2001; Day, et al., 2002; Bouché, et al., 2005). An EF-hand is a helix-loop-helix that binds one Ca^{2+} ion. Ca^{2+} sensors that are involved in signalling are

CaBP with EF-hand motifs that function by connecting Ca^{2+} signals with downstream effectors (Snedden and Fromm, 1998; Snedden and Fromm, 2001). There are 250 putative EF-hand-containing proteins in *Arabidopsis* (Day, et al., 2002) that fall into three categories: Ca^{2+}-dependent protein kinases (CDPK), calcineurin B-like (CBL), and calmodulin (CaM)/CaM-like.

CDPKs are abundant and ubiquitous in plants. Ca^{2+} binds to the EF-hand motif within the CDPK and stimulates kinase activity (Reddy, 2001). There are over 40 CDPKs in *Arabidopsis*. They have a unique structural arrangement with four EF-hand motifs, a protein kinase catalytic domain, and an autoinhibitory domain that inhibits kinase activity in the absence of Ca^{2+} (Reddy, 2001). CBLs are so named because of their similarity to the B subunit of animal calcineurin. The best-characterized CBL is salt-overly-sensitive 3, indicating a role for these proteins in salt stress signalling. There are at least six *CBL* genes in *Arabidopsis*, several of which have been shown to be upregulated following drought, cold and wounding (Reddy, 2001). However, the most well understood Ca^{2+} sensor is CaM.

CaM and CMLs

CaM is a ubiquitously expressed protein that is highly conserved across all eukaryotes. There is greater than 70% identity between CaM amino acid sequences in plants and animals (Yang and Poovaiah, 2003) and all but one of the Ca^{2+}-coordinating residues are conserved between plants and vertebrates (Zielinski, 1998). Prototypical CaM is a small (16.8 kDa; 148 amino acids), heat stable, acidic protein that contains four EF-hand Ca^{2+}-binding domains that are arranged pair-wise into two globular domains (Zielinski, 1998; Reddy, et al., 2002). Binding to Ca^{2+} causes a conformational change that exposes two

hydrophobic surfaces (one in each globular domain) surrounded by negative amino acid residues (Bouché, et al., 2005). The EF-hands are able to independently bind Ca^{2+} and change conformation (Junker, et al., 2009), although the binding of Ca^{2+} by one EF-hand enhances the binding of Ca^{2+} by the second (Snedden and Fromm, 2001). CaM has no intrinsic enzymatic activity and must function by activating downstream target proteins (CaM binding proteins, CaMBP). It has recently been found that binding to CaMBP stabilizes CaM's folded conformation (Junker, et al., 2009). Interestingly, there does not appear to be a conserved amino acid motif that determines CaM binding. Although there is a motif (IQ motif, IQXXXRGXXXR) that is found in many proteins that interact with CaM in a Ca^{2+}-independent manner (Bähler and Rhoads, 2002), Ca^{2+}-dependent binding mainly occurs through hydrophobic interactions between target proteins and the globular domains (Zielinski, 1998). CaM target sites in CaMBP are 12-30 amino acid residues that have positively charged amphipathic qualities, as well as variability in the primary amino acid sequence and a tendency to form an α-helix when bound to CaM (Bouché, et al., 2005). Proteins from a wide variety of cellular processes have been identified as CaMBP, and many are unique to plants (Bouché, et al., 2005). There are approximately 30 known CaMBP in Arabidopsis, many of which have paralogs making the total number of CaMBP in Arabidopsis closer to 100 (Reddy, et al., 2002). Metabolic, signal transduction, transportation, and structural pathway proteins have been identified as CaMBP (Snedden and Fromm, 1998; Reddy, et al., 2002), and it is confounding that a single protein is able to regulate such different targets.

The way in which CaM regulates so many different processes in plants may be partly explained by the presence of isoforms of CaM as well as CaM-like proteins (CMLs). The Arabidopsis genome contains seven *CaM* genes that encode four isoforms

of CaM (McCormack, et al., 2005), and fifty *CML* genes (McCormack and Braam, 2003). CMLs are defined as proteins that are mostly composed of EF hand domains, that have no other identifiable domains, and that share at least 16% identity with CaM. CMLs range between 83 and 330 amino acids and contain between one and six EF hands, with the majority containing four (McCormack and Braam, 2003). CaM and CMLs have been shown to bind differential targets using protein microarrays (Popescu, et al., 2007) indicating that CMLs likely have unique, unknown functions within the cell. CMLs remain largely unstudied apart from their sequence data, and for most of them, it is unknown whether they are capable of binding Ca^{2+}. However, there are a few examples of CMLs that have been characterized.

CML9 was recently discovered to be expressed in a variety of organs and tissues throughout development and morphogenesis (Magnan, et al., 2008). Expression increased during early stress response and following ABA treatments. Knockout mutants of *CML9* were found to be hypersensitive to ABA and therefore tolerant to drought and salt stresses, which are mediated by ABA. There appears to be a role for CML9 as a negative regulator that is involved in signalling pathways that lead to ABA-dependent gene regulation.

CML24 is also known as touch-induced (TCH)2, a member of the TCH family in Arabidopsis. It was originally chosen for characterization because its transcript levels were upregulated by touch, rain, wound, wind, and darkness (Braam and Davis, 1990). *CML24* displays regulation of expression throughout development and morphogenesis. Transgenic plants with reduced expression of *CML24* were found to have decreased ABA sensitivity specifically during germination. These plants were also defective in long-day induction of flowering, and were more tolerant to ionic stress (Delk, et al., 2005)

indicating roles in these pathways for CML24. Recently, CML23, which shares 71% identity with CML24, has also been studied (Tsai, et al., 2007). Expression analysis shows that CML23 is expressed in a similar, but not identical, fashion to CML24. Double mutants that were deficient in both CML23 and CML24 showed delayed flowering under both long and short day conditions, which was caused by an accumulation of nitric oxide. Loss-of-function, single mutant *cml24* plants also showed an increase in nitric oxide accumulation (Tsai, et al., 2007) as well as an inhibition in both the hypersensitive response and pathogen-induced nitric oxide elevation (Ma, et al., 2008), linking CML24 with nitric oxide evolution and the hypersensitive response during innate immunity and pathogen response.

CML42 and CML43 were definitively shown to bind Ca^{2+} (Chiasson, et al., 2005). Additionally, *CML43* is expressed only in the root tip in regular conditions, but is strongly induced in the leaves by both avirulent and virulent *Pseudomonas syringae* (Chiasson, et al., 2005). Transgenic plants overexpressing CML43 showed acceleration in the hypersensitive response, which suggests a potential role in defence response. Conversely, *CML42* is expressed throughout the plant, notably in trichome support cells (Dobney, et al., 2009). CML42 was found to interact with the protein KIC (kinesin-like CaM-binding protein-interacting Ca^{2+}-binding protein), which is known to regulate the kinesin-like CaM-binding protein during trichome morphogenesis. Interestingly, transgenic plants that were deficient in *CML42* showed increased trichome branching (Dobney, et al., 2009).

CML37, *CML38*, and *CML39*, a subfamily, were found to have differential developmental and stress-responsive expression patterns (Vanderbeld and Snedden, 2007). *CML37* and *CML38* were found to have similar expression patterns. They were

both expressed in young leaves, roots, and floral tissues, but *CML38* was also expressed in guard cells. *CML39* only expressed in elongation and differentiation zones in roots as well as in pollen. These three genes were also found to have dramatic increases following both abiotic and biotic stress (Vanderbeld and Snedden, 2007). All three family members were found to be responsive to drought, salinity, and wounding. Interestingly, *CML37* and *CML38* showed strong induction surrounding wound sites, while *CML39* was induced systemically. *CML37* was also induced by hydrogen peroxide and avirulent *P. syringae*, while *CML39* was dramatically induced throughout the entire seedling following MeJA treatment. The pathways and functions for these three genes are still unknown. There is evidence that CML39 may play a role in programmed cell death (Vanderbeld, Bender, and Snedden, unpublished data), and that CML39 interacts with ATHB21, a member of the zinc finger homeodomain transcription factor family (Vanderbeld, 2007). In order to understand the roles of these genes in stress responses their upstream activators and downstream interactors must be studied. The most common way to find upstream activators of genes is to analyze their promoters.

Promoter analyses

In order to determine the expression patterns of genes, many researchers make use of promoter::reporter constructs. Promoters are the regions within the genome that contain the regulatory sequences (binding sites for RNA polymerase II, TFII, and activator and repressor TF) for their specific gene. Although TF binding sites for any specific gene can be found throughout the genome, studies mainly focus on those that are found in the area directly upstream or within introns of the gene of interest. The most commonly used reporters are green florescent protein (GFP), firefly luciferase (LUC), and β-

glucuronidase (GUS). Although all three reporter genes have pros and cons (de Ruijter, et al., 2003), GUS reporter genes seem to be used most commonly for promoter analysis studies. This is likely because GUS reporter constructs can be examined qualitatively (by histochemical staining) as well as quantitatively (fluorometric assays, Jefferson, et al., 1987). Fluorometric GUS assays can also be performed in real-time by using Q-PCR instruments (Crow, et al., 2006).

Promoters are usually thought of as a linear collection of promoter elements (*cis*-elements), each one recruiting a different TF (Aarts and Fiers, 2003). Characterization of *cis*-elements is extremely important for biotechnological methods of enhancing stress response in plants. For instance, if plants were engineered to constitutively produce volatile organic compounds to attract predators of herbivorous insects, the predators would soon ignore these signals if no insects were present (Kessler and Baldwin, 2002). The most obvious solution is to express these compounds under the control of an herbivory-induced promoter, making the discovery of inducible *cis*-elements of utmost importance.

Classical studies of promoters involve creating a promoter::reporter fusion protein as well as a 5′ deletion series of the promoter. This allows researchers to narrow down the area of the promoter that is responsible for expression under certain conditions. These constructs can be expressed in plants, either stably or transiently (Yang, et al., 2000) or in cultured cells (Berger, et al., 2007). Recently, some studies have even passed over the 5′ deletion of promoters and discovery of *cis*-elements and proceeded directly to the identification of TF. Miao, et al. (2007, 2008) cloned fragments of the *WRKY53* gene promoter into the yeast-one-hybrid system and discovered both novel and known upstream activators that bound specifically to certain areas of the promoter. However, the

5′ deletion approach has proved successful for identifying *cis*-elements as well as the TF that activate them. For example, the *PR-10a* gene promoter was fused to GUS and was found to be upregulated in potato following wounding or treatment by wound elicitors (Matton, et al., 1993). Subsequent 5′ deletion analysis of this promoter identified a *cis*-element (the elicitor response element) that was responsible for the upregulation in GUS following wounding. Electrophoretic mobility shift assays (EMSA) were performed and two binding factors were found to bind the elicitor response element in a phosphorylation-dependent manner (Després, et al., 1995). Further study on one of these binding factors found that it was a novel single-stranded DNA-binding factor (Desveaux, et al., 2000) that was a member of a novel family of TF, called Whirly (Desveaux, et al., 2002).

Another group discovered that the transcripts of a tobacco family of four ethylene responsive TF (ERF) were upregulated following wounding (Suzuki, et al., 1998), and confirmed this expression using a GUS fusion protein (Nishiuchi, et al., 2002). 5′ deletion analysis of the *ERF3* promoter found the element responsible for the wound response, which turned out to be an inverted W-box. NtWRKY1, 2 and 4 were found by EMSA to interact specifically with the inverted W-box, and were found to be responsible for the upregulation of *ERF3* following wounding (Nishiuchi, et al., 2004). Interestingly, there was also an element within the promoter that downregulated the expression of *ERF3*. This element turned out to be a GCC-box, and was found to preferentially bind ERF3, allowing negative feedback. This autorepression by ERF3 coupled with activation by WRKY TF indicates an intricate regulation system (Nishiuchi, et al., 2004).

Another gene in tobacco, *tpoxN1* was also found to be upregulated following wounding, but preferentially in the vascular system (Sasaki, et al., 2002). Several known

defence signal compounds and phytohormones were unable to increase the *tpoxN1promoter::GUS* expression, indicating a novel wound signalling pathway. Successive 5′ deletion of the *tpoxN1* promoter narrowed down the responsive region to a novel *cis*-element termed the vascular system-specific and wound-responsive *cis*-element (Sasaki, et al., 2006). A yeast-one-hybrid screen using this element found two novel TF that bound this *cis*-element. These TF were found to be previously unknown members of the AP2/EREBP family of TF (Sasaki, et al., 2007).

There are many more examples of 5′ deletion analyses of promoters that successfully discovered the *cis*-element or regulatory regions of the promoter responsible for wound response or for JA response (Vignutelli, et al., 1998; Lü, et al., 2007; Liu, et al., 2005; Mason, et al., 1993; Rouster, et al., 1997; Puzio, et al., 2000; Santamaria, et al., 2001; Siebertz, et al., 1989). Due to the successes of these and other studies, online plant promoter databases were created. This, coupled with the increasing amount of microarray data, has led to a newer, *in silico* approach for *cis*-element identification.

The most widespread technique of *cis*-element *in silico* analysis is the clustering of genes by expression profiles and analyzing the promoter regions of these genes for over-represented elements (Aarts and Fiers, 2003). Klok, et al. (2002) used this technique to study genes that responded to low oxygen. Co-regulated genes were identified and the promoter regions of these genes were examined for over-represented elements. Several elements were found, some that had been previously identified as low oxygen-responsive elements, and some novel elements. However, this group did not test the functionality of these novel elements. Another group studied the microarray cluster of genes that were induced shortly after wounding (Walley, et al., 2007). A novel *cis*-element was found to be over-represented and was termed the rapid stress response element. This element was

shown to be sufficient to confer responsiveness to a LUC reporter gene following wounding. This technique of studying microarray data and looking for an over-represented element is likely a good way to find novel elements within a group of genes, but is not useful to find *cis*-elements and upstream activators of a single gene. Online plant promoter databases can also be used to find known *cis*-elements within any given promoter. Tittarelli et al. (2007) combined these two techniques. A plant promoter database was used to search for known *cis*-elements in the promoter of a phosphate transporter from wheat. The promoter was also compared to promoters from phosphate transporters from rice, barley, and Arabidopsis in order to find conserved regions, though none of these *cis*-elements were validated.

It appears that the best approach to characterize a specific promoter is still the classical approach; however, this approach can be aided by *in silico* analysis. Plant promoter databases can help refine the search for *cis*-elements by providing information about the location of putative regulatory sequences but the final proof will always have to come from *in vivo* experiments.

Research objectives

The roles of *CML* genes during stress response are poorly understood. Although it is known that the *CML37*, *CML38*, and *CML39* subfamily show increased expression following various stresses (Vanderbeld and Snedden, 2007), the upstream signalling pathways that result in these increases are completely unknown. With the purpose of shedding light on these signalling pathways, the goal of this project was to discover the *cis*-elements within the *CML37*, *CML38,* and *CML39* promoters that are responsible for their stress-induced expression patterns. The wounding response in *CML37* and *CML38*

as well as the MeJA response in *CML39* were chosen for analysis, as these were the most dramatic responses. In order to find these *cis*-elements, 5′ deletions of the *promoter::GUS* constructs were created and transformed into plants to analyze if they retained the stress responses produced by the full-length promoters. The identification of these *cis*-elements provides a good basis for the future identification of transcription factors that activate these three *CML*s, which will provide a better understanding of the signalling pathways of these stress responses.

CHAPTER 2: Materials and methods

Generation of *CML::GUS* transgenic plants

The genomic region upstream of the ATG translation start site for *CML37*, *CML38*, or *CML39* extending to the predicted UTR of the nearest neighbour gene (1919 bp for *CML37*, 1438 bp for *CML38*, 941 bp for *CML39*) was amplified by PCR and subcloned into the binary vector pBI101 (Clontech, Figure A1.1) by B. Vanderbeld (Vanderbeld and Snedden, 2007). These constructs were used as templates during the PCR amplification of 5′ deletion fragments of these three promoters. Primers used in this study are shown in Table A2.1. PCR products were either cloned into the pCR®8/GW/TOPO® (entry) vector (Invitrogen), or restriction cloned (the BamHI/ SmaI sites were used for *CML37* and *CML38* constructs, while the HindIII/ BamHI sites were used for *CML39* constructs) into the pBI101 vector (see Table A2.1 for construct information). One additional construct was made by restriction digest. *pBI101::CML37* was digested with HindIII, which left a 656 bp fragment of the *CML37* promoter in the pBI101 vector. Constructs in entry vectors were transferred by LR Gateway™ cloning technology (Invitrogen) into the pMDC163 vector (Curtis and Grossniklaus, 2003) or the pGWB203 vector (Nakagawa, et al., 2007). Constructs were confirmed by DNA sequencing. Each construct was transformed into *Agrobacterium tumefaciens* strain C58 pGV3850 and then into *A. thaliana* plants using the floral dip method (Clough and Bent, 1998). Transformants were selected by growth on 0.5x Murashige and Skoog (Caisson Laboratories, Inc., Murashige and Skoog, 1962) media and 0.8% (w/v) agar supplemented with 50 μg/ml hygromycin (pMDC163 and pGWB203 vectors) or 50 μg/ml kanamycin (pBI101 vector).

Hygromycin/ kanamycin resistant seedlings were transplanted to soil and were grown in a growth chamber with a 16 h light (150μmol/m^2/sec)/ 8 h dark photoperiod at 22°C and 70% relative humidity. Plants were watered as necessary and supplemented with 1 g/L 20-20-20 fertilizer every second week until seeds were ready to be collected. T2 generation plants were used for GUS analyses.

Plant material and growth conditions

A. thaliana (ecotype Col-0) seeds were surface sterilized, stratified at 4°C in the dark for 2 d on petri dishes containing sterile MS media and 0.8% (w/v) agar, and then transferred to a growth room with a 24 h photoperiod at 22°C.

Stress treatments

All stress treatments were performed as described by Vanderbeld (2007). For wounding and methyl jasmonate (MeJA) treatments, *A. thaliana* seedlings were allowed to grow on MS plates until stage 1.0-1.04 (Boyes, et al., 2001). At least three independent lines of each construct were analyzed. Wounding experiments used forceps to apply a single pinch to each cotyledon while control plants were not wounded. For fluorometric and histochemical GUS analysis of pBI101 and pMDC163 promoter constructs, samples were collected after 8 h. For fluorometric and histochemical GUS analysis of pGWB203 promoter constructs, samples were collected after 4 h. The response to MeJA was studied by spraying seedlings with 100 μM MeJA (95% solution, Aldrich) or water (control). Samples were collected after 24 h for fluorometric and histochemical GUS analysis.

Histochemical GUS assays

Assays were performed as described by Vanderbeld (2007). The substrate used for histochemical staining of *CML::GUS* transgenic plants was 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-Gluc, Bioshop, Jefferson, et al., 1987). Seedlings were harvested and immediately soaked in ice-cold 90% acetone for 1 h on a rotary shaker, then rinsed with 100 mM Na_2HPO_4-NaH_2PO_4 buffer pH 7.0, then placed in GUS staining solution (100 mM Na_2HPO_4-NaH_2PO_4 buffer pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, and 1 mM X-Gluc) for 10 min at 55°C. Seedlings were then incubated at 37°C for 5 h, and then cleared by washing with 70% ethanol over a period of 24 h. Control plants were always histochemically stained in an identical fashion to treated plants to allow comparison. Less stringent staining was performed in the exact same manner, but the staining solution omitted 2 mM $K_3Fe(CN)_6$ and 2 mM $K_4Fe(CN)_6$. Seedlings were examined with a Zeiss SteREO Discovery.V12 stereomicroscope and digitally photographed.

Fluorometric GUS assays

Multiple replicates of each independent line were analyzed and results were pooled to produce mean GUS activity for each construct. Seedlings were harvested, snap frozen, and homogenized in protein extraction buffer (50 mM Na_2HPO_4-NaH_2PO_4 buffer pH 7.0, 10 mM dithiothreitol (DTT), 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100). Protein extract was added to assay buffer (50 mM Na_2HPO_4-NaH_2PO_4 buffer pH 7.0, 10 mM dithiothreitol (DTT), 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100, 1 mM 4-methylumbelliferyl glucuronide (MUG, Sigma, Jefferson, et al., 1987)) and allowed to incubate at 37°C for 2 h before 0.2

M Na_2CO_3 was added to stop the reaction. Fluorescence readings were taken at an excitation of 365 nm and an emission of 455 nm. Total protein concentration in the extract was calculated as described previously (Bradford, 1976). All seedlings were treated in an identical manner to allow for direct comparison.

Statistical analysis

One-tailed t-tests were used to assess significance between untreated and treated samples. One-way ANOVAs were used to assess significance between untreated samples and between treated samples.

Websites used for *in silico* promoter analyses

The search for known *cis*-elements within the *CML37*, *CML38*, and *CML39* full-length promoters was performed by four online databases.

AGI codes of *CML37*, *CML38*, and *CML39* (At5g42380, At1g76650, and At1g76640, respectively) were entered into the *Arabidopsis* gene regulatory information server (AGRIS) *cis*-element database (Davuluri, et al., 2003). All default conditions were used.

AGI codes of *CML37*, *CML38*, and *CML39* were entered into the visualization section of the *Arabidopsis thaliana* expression network analysis (Athena) database (O'Connor, et al., 2005). All default conditions were used, except that 2000 bp was used as the maximum upstream cut off.

Full-length promoters in FASTA format were entered into the signal scan search of the plant *cis*-acting regulatory DNA elements (PLACE) database (Higo, et al., 1999). All default conditions were used.

Full-length promoters in FASTA format were entered into the CARE search of the plant *cis*-acting regulatory elements (PlantCARE) database (Lescot, et al., 2002). All default conditions were used.

Co-expressed genes were found using the *Arabidopsis thaliana* trans-factor and *cis*-element prediction database (ATTED) II database (Obayashi, et al., 2009). The AGI code for *CML37* was entered into the CoexVersion search engine. Data set version c6.0 was used.

Putative *cis*-elements responsible for wound-responsiveness were found using the multiple em for motif elicitation (MEME) database (Bailey and Elkan, 1994). Regions within the *CML37* promoter found to be responsible for wound-responsiveness (see results) were entered in FASTA format into the MEME search engine to identify common motifs. All default conditions were used. These motifs were examined using the botany array resource (BAR) promomer database (Toufighi, et al., 2005). Motifs generated by MEME were identified in a) all promoters from the genome, and b) promoters of genes that are co-expressed with *CML37* (found using ATTED II) until a motif that was statistically overrepresented in the co-expressed genes was found.

CHAPTER 3: Results

in silico analysis of *CML37*, *CML38*, and *CML39* promoters

Analysis of the promoters of *CML37*, *CML38*, and *CML39* was performed by four online databases: AGRIS (Davuluri, et al., 2003), Athena (O'Connor, et al., 2005), PLACE (Higo, et al., 1999), and PlantCARE (Lescot, et al., 2002). These databases search for known *cis*-elements within the provided sequence. Two of these databases (PLACE and PlantCARE) contain information on *cis*-elements from all plant species, while AGRIS and Athena are databases specific for Arabidopsis *cis*-elements.

Summaries of the raw data collected from each of the four databases for the *CML37* promoter, *CML38* promoter, and *CML39* promoter are shown in Table 3.1, Table 3.2, and Table 3.3, respectively. Unsurprisingly, Arabidopsis-specific databases AGRIS and Athena predicted fewer *cis*-elements than all-plant databases. The *CML37* promoter contained the most predicted *cis*-elements, while the *CML39* promoter contained the least; the AGRIS database was only able to find two putative *cis*-elements within the *CML39* promoter. There was little consensus between the four databases; even elements that are well defined and common, such as the TATA box, were found with different frequency by each database.

All four databases predicted a large number of *cis*-elements that are involved in stress response. This is consistent with previous microarray mining and GUS expression experiments of *CML37*, *CML38*, and *CML39* that have shown increased expression following a multitude of stresses (Vanderbeld and Snedden, 2007).

Table 3.1. Summary of *in silico* analysis of *CML37* promoter.

Description of *cis*-element	Database							
	AGRIS		Athena		PLACE		PlantCARE	
	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique site	total # of *cis*-elements
Elements involved in response to stimuli								
ABA	1	3	2	2	2	17	2	4
Auxin							1	1
Cytokinin					1	1		
GA			2	3	4	6	1	1
MeJA							2	2
Cold					1	2	1	1
Heat							1	1
Light	2	5	1	3	5	43	12	28
Salinity	1	5			1	5		
Drought	2	2	4	9	3	22	1	2
Hypoxia					1	1	1	1
Wounding	1	4	1	4	1	27	2	3
Elicitors					1	2	1	2
Pathogens					1	5		
Copper					1	2		
Iron					1	1		
Sulphur					1	1		
General promoter elements								
TATA box			1	2	1	10	1	77
CAAT box					1	21	1	38
Enhancers	1	4	1	1	3	7	1	1
Initiators					1	3		
Elements involved in cellular regulation					2	2	2	2
Elements involved in tissue specificity					7	60	2	5
Elements found in specific gene promoters	2	5	1	2	17	87	1	1
Known binding sites for TF	5	6	4	11	16	91	1	1
Elements with unknown function							8	19

# of unique sites indicates the total number of different *cis*-elements in each category, while total # of *cis*-elements indicates the total number of times these *cis*-elements are predicted to be present within the promoter. Elements found in specific gene promoters are *cis*-elements that are statistically overrepresented in a promoter or group of promoters. Functions for TF within the known binding sites for TF section were not provided.

Table 3.2. Summary of *in silico* analysis of *CML38* promoter.

Description of *cis*-element	Database							
	AGRIS		Athena		PLACE		PlantCARE	
	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique site	total # of *cis*-elements
Elements involved in response to stimuli								
ABA					2	16		
Auxin	1	2						
Cytokinin					1	2		
Ethylene					1	1	1	1
GA			1	2	3	6		
MeJA							2	4
SA							1	2
Heat							1	1
Light	4	9	1	1	5	36	10	18
Salinity					1	2		
Drought	1	2	1	1	4	23		
Hypoxia					1	7	1	1
Wounding	1	3	1	4	1	22	1	3
Elicitors					1	3	1	3
Pathogens					1	4		
General defence							1	2
Copper					1	8		
Sulphur					1	2		
General promoter elements								
TATA box			1	3	1	10	1	56
CAAT box					1	20	1	29
Enhancers					3	10	1	1
Initiators					1	5		
Elements involved in cellular regulation	1	2	1	1	2	2	1	2
Elements involved in tissue specificity					6	53	2	2
Elements found in specific gene promoters	1	2			19	87		
Known binding sites for TF	5	14	4	10	12	77	2	3
Elements with unknown function							4	8

# of unique sites indicates the total number of different *cis*-elements in each category, while total # of *cis*-elements indicates the total number of times these *cis*-elements are predicted to be present within the promoter. Elements found in specific gene promoters are *cis*-elements that are statistically overrepresented in a promoter or group of promoters. Functions for TF within the known binding sites for TF section were not provided.

Table 3.3. Summary of *in silico* analysis of *CML39* promoter.

Description of *cis*-element	Database							
	AGRIS		Athena		PLACE		PlantCARE	
	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique sites	total # of *cis*-elements	# of unique site	total # of *cis*-elements
Elements involved in response to stimuli								
ABA					1	4		
Cytokinin					1	3		
GA			1	2	2	3	1	1
MeJA							2	2
SA							1	2
Light			1	1	4	19	7	10
Cold			1	1	1	3	1	1
Drought			1	1	3	9		
Hypoxia					1	1		
Wounding			1	3	1	15	1	2
Elicitors					1	2	1	2
Pathogens					1	3		
General defence							1	2
General promoter elements								
TATA box			1	2	1	2	1	47
CAAT box					1	21	1	30
Enhancers					3	4		
Initiators					1	4		
Elements involved in cellular regulation					2	3	1	1
Elements involved in tissue specificity					5	38	1	1
Elements found in specific gene promoters					15	51		
Known binding sites for TF	2	2	1	1	9	47		
Elements with unknown function							3	5

# of unique sites indicates the total number of different *cis*-elements in each category, while total # of *cis*-elements indicates the total number of times these *cis*-elements are predicted to be present within the promoter. Elements found in specific gene promoters are *cis*-elements that are statistically overrepresented in a promoter or group of promoters. Functions for TF within the known binding sites for TF section were not provided.

General promoter elements were predicted in all three promoters, as well *cis*-elements with unknown function. Other types of *cis*-elements found within the three promoters are elements that are involved in cellular regulation and tissue specificity.

Several elements from specific promoters were predicted by the database analysis. These elements were discovered by searching for statistically overrepresented elements in groups of coexpressed genes.

The final category of *cis*-elements predicted in the *CML37*, *CML38*, and *CML39* promoters were elements that are known binding sites for specific TF. These elements, as well as the stress-responsive *cis*-elements, were examined for elements that may have roles in the regulation of *CML37*, *CML38*, and *CML39*.

In order to simplify analysis of data derived from database searching, putative *cis*-elements involved in wounding and MeJA responses were identified in the *CML37*, *CML38*, and *CML39* promoters. WRKY TF are known to function in wound response and bind to W-boxes (Eulgem, et al., 2000). The WUN-motif is a wound responsive *cis*-element that was found in the *WUN1* gene (Siebertz, et al., 1989). Locations of W-boxes and WUN-motifs within the *CML37*, *CML38*, and *CML39* promoters are shown in Figure 3.1 (Aa, Ba, Ca).

Another large family of TF that have been implicated in wound response, and in many other stress responses is the MYB superfamily (Singh, et al., 2002). Conserved MYB-binding motifs are found within many wound-responsive genes (Dong, et al., 2003; Eulgem, 2005), indicating a role in defence. Many MYB binding sites were predicted

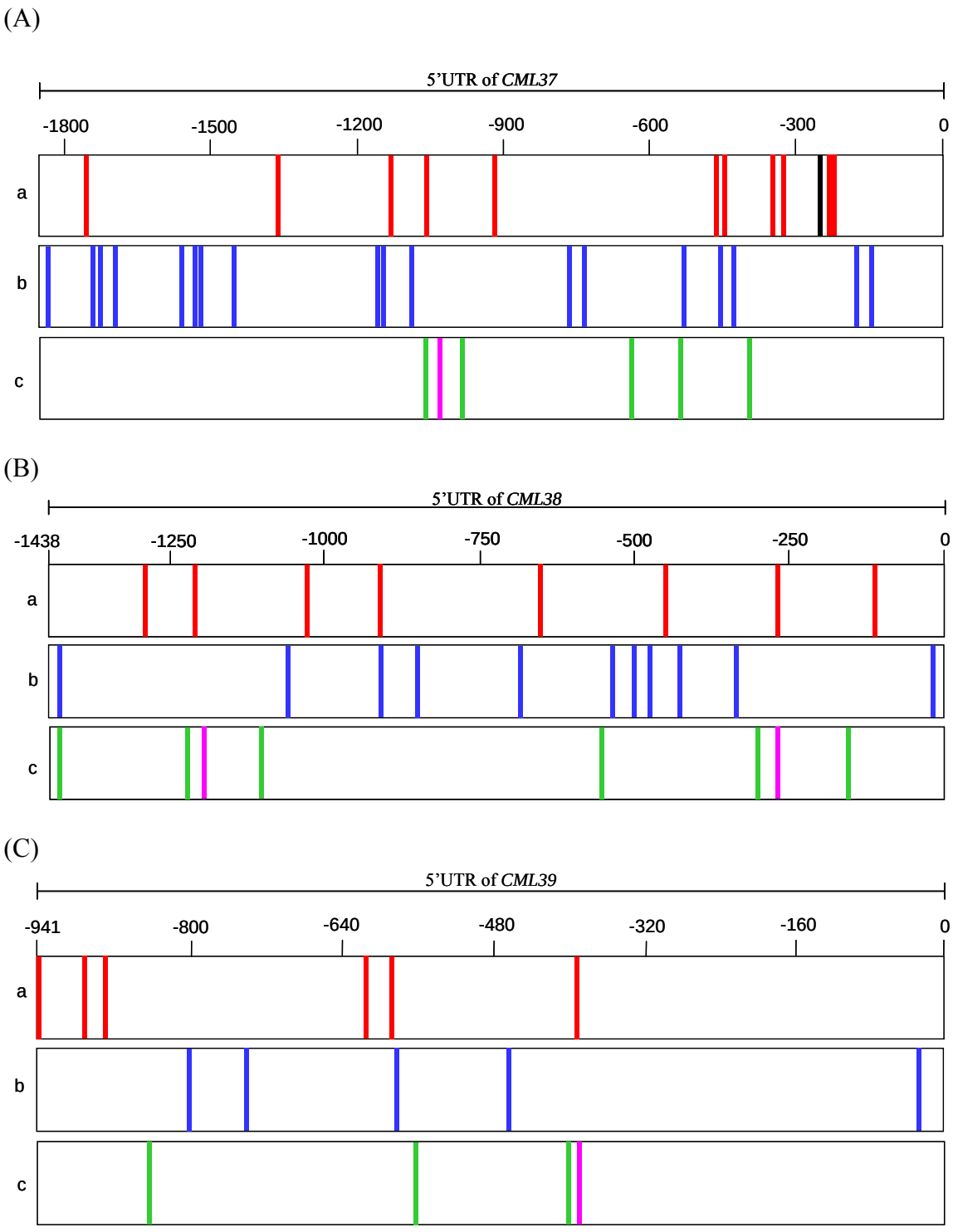


Figure 3.1. Graphical representation of locations of *cis*-elements within the (A) *CML37*, (B) *CML38*, and (C) *CML39* promoters.
Locations of (a) wound-responsive *cis*-elements (W-boxes = red, WUN motif = black), (b) MYB binding sites (blue), and (c) JA-responsive *cis*-elements (MYC binding sites = green, CGTCA motif = pink) within the *CML37*, *CML38*, and *CML39* promoters. Data is pooled from raw *in silico* data.

within the *CML37*, *CML38*, and *CML39* promoters (Figure 3.1 Ab, Bb, Cb).

MYC TF are known to be involved in JA signalling (Boter, et al., 2004). MYC binding sites are defined as CANNTG, and are shown in Figure 3.1 (Ac, Bc, Cc). Also presented in this figure are the locations of the CGTCA motif. This motif was found by the PlantCARE database, but little information is provided except that it is involved in MeJA response.

Analysis of 5′ deletion constructs in the pBI101 and pMDC163 vectors

Full-length promoter constructs for *CML37*, *CML38*, and *CML39* subcloned into the pBI101 vector were provided by B. Vanderbeld (Vanderbeld and Snedden, 2007). While several deletion constructs were also subcloned into pBI101, the majority of the deletion constructs were subcloned into the pMDC163 gateway vector (Curtis and Grossniklaus, 2003) to simplify cloning procedures. For GUS reporter analysis, at least four independent lines of each construct were compared. GUS activity levels were consistent among the transgenic lines.

CML37 promoter analysis

Transgenic seedlings containing either the full-length *CML37* promoter or one of four 5′ deletion constructs (Figure 3.2) were analyzed for GUS activity following wounding. Three of these constructs were created in the pMDC163 vector (-571*, -474*, and -224*) while the full-length construct (-1919^) and the final deletion construct (-656^) were created in the pBI101 vector.

Transgenic seedlings were wounded with forceps and samples were collected 8 h

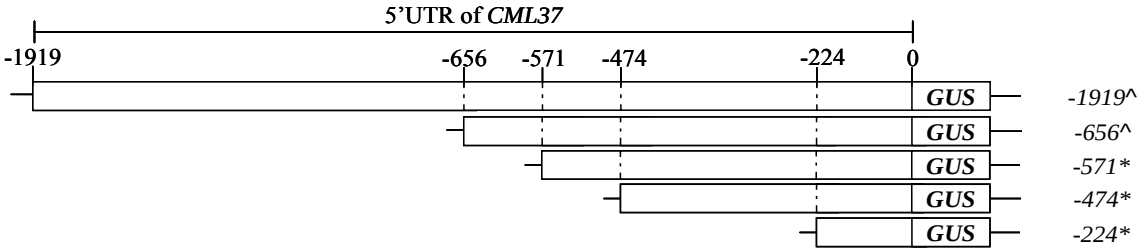


Figure 3.2. Diagram of *CML37* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
-1919^ represents the full-length *CML37* promoter. ^ represents constructs within the pBI101 vector, while * denotes constructs within the pMDC163 vector.

following treatment. Representative patterns of GUS expression following wounding in *CML37* promoter deletion constructs are shown in Figure 3.3. As expected from previous analysis of *CML37::GUS* expression (Vanderbeld and Snedden, 2007), transgenic seedlings containing the full-length promoter construct did not show staining in control conditions (Figure 3.3 Aa) but showed distinct expression surrounding the wound site (Figure 3.3 Ab). Transgenic seedlings containing the -656^ deletion construct showed the same expression pattern as the full-length promoter construct (Figure 3.3 Ba, Bb). Transgenic seedlings containing all of the remaining deletion constructs showed constitutive GUS expression in control conditions (Figure 3.3 Ca, Da, Ea) and there did not appear to be a difference in GUS expression following wounding (Figure 3.3 Cb, Db, Eb). Fluorometric GUS assays were also carried out to quantify GUS activity (Figure 3.4). These assays confirmed the staining patterns. Transgenic seedlings containing either the -1919^ or -656^ constructs showed significant increases in GUS activity following wounding, while transgenic seedlings containing the -571*, -474*, or -224* constructs showed high constitutive activity that was not affected by wounding.

CML38 promoter analysis

Transgenic seedlings containing either the full-length *CML38* promoter or one 5′ deletion construct (Figure 3.5) were analyzed for GUS activity following wounding. This deletion construct (-324*) was in the pMDC163 vector, while the full-length construct (-1438^) was in the pBI101 vector.

Transgenic seedlings were wounded with forceps and samples were collected 8 h following treatment. Representative patterns of GUS expression following wounding are

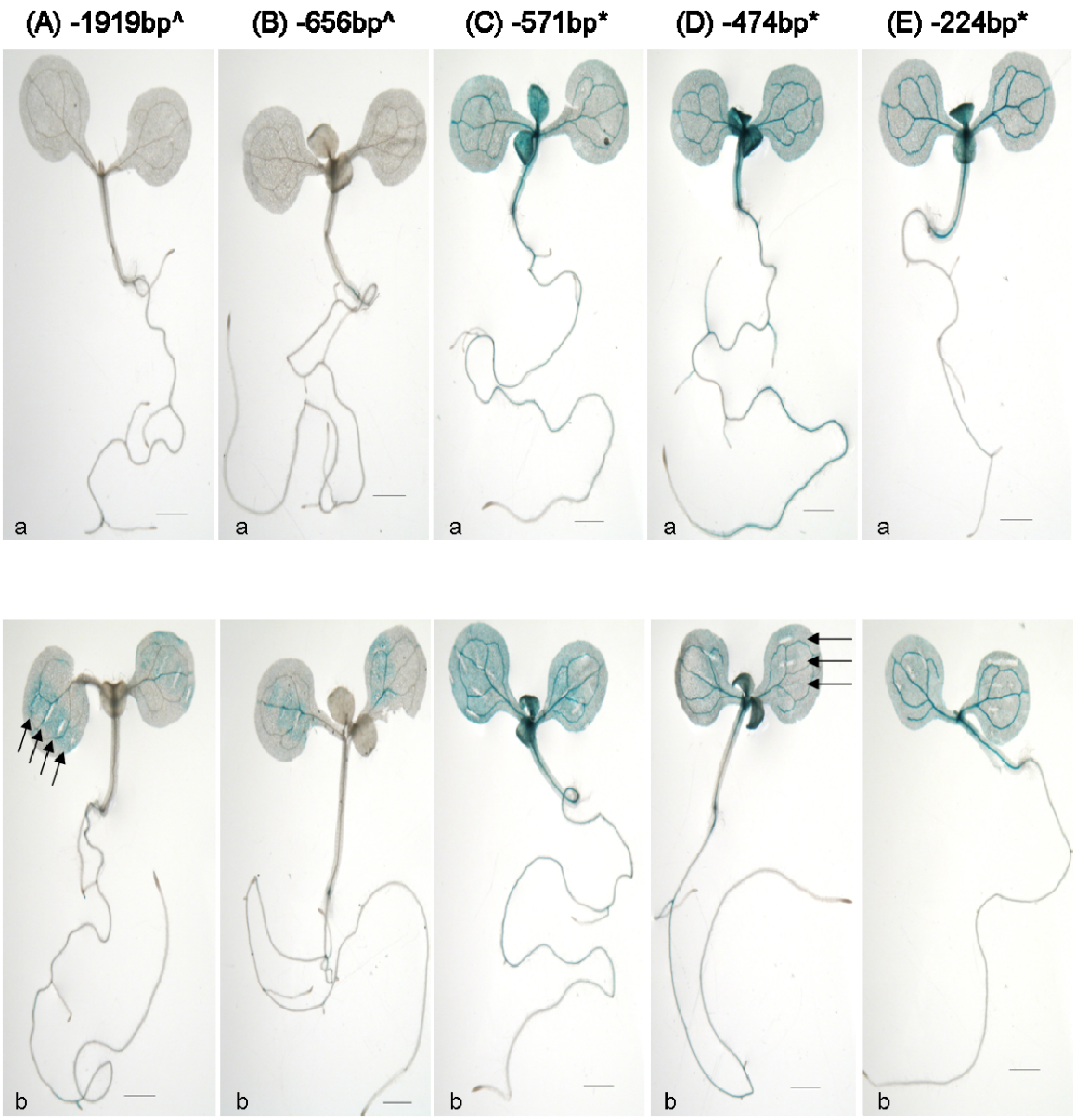


Figure 3.3. Representative patterns of GUS expression in *CML37* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML37* promoter (A) or one of four 5′ deletion GUS constructs (B-E) were either unwounded controls (a) or wounded (b) and assayed for GUS activity 8 h after treatment. ^ indicates constructs within the pBI101 vector, while * denotes constructs within the pMDC163 vector. *Bars* = 1 mm. Arrows denote wound sites.

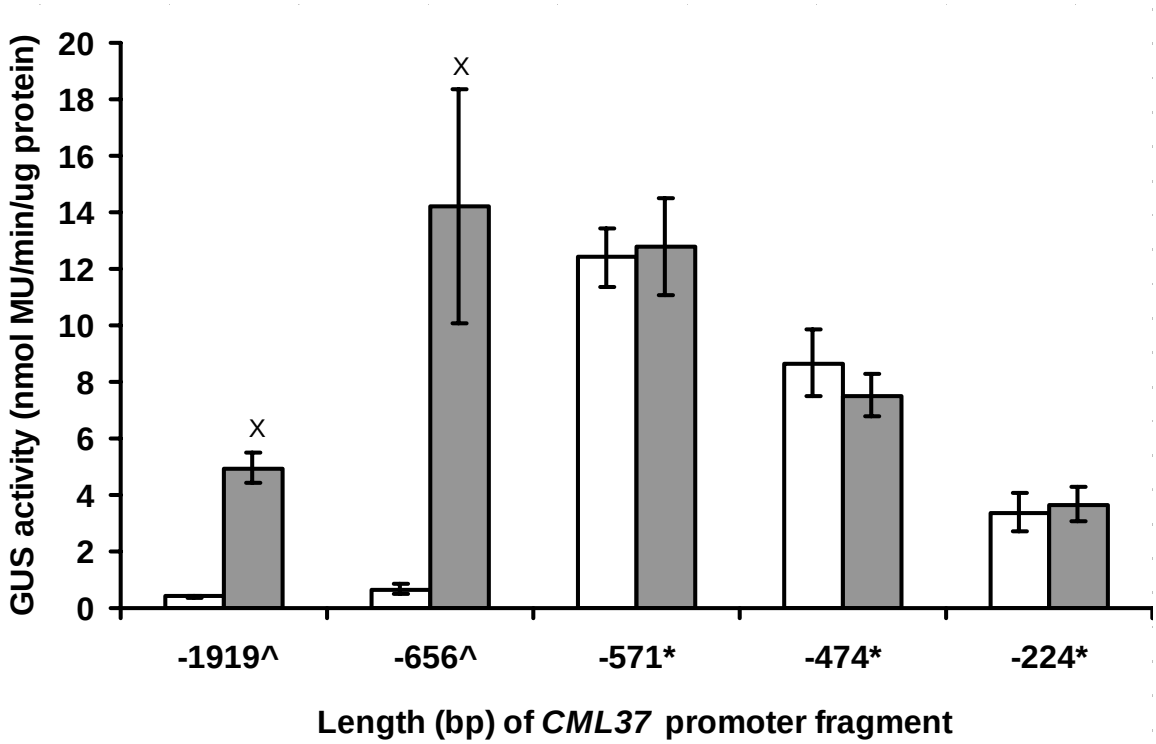


Figure 3.4. Graphical representation of GUS activity in *CML37* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML37* promoter or one of four 5′ deletion GUS constructs were either unwounded controls (white bars) or wounded (grey bars) and assayed for GUS activity 8 h after treatment. ^ indicates constructs within the pBI101 vector, while * denotes constructs within the pMDC163 vector. X indicates that the wounded samples had significantly more GUS activity than their unwounded controls (one-tailed t-test, $p<0.05$, $n>25$). Error bars represent ± standard error of the mean.

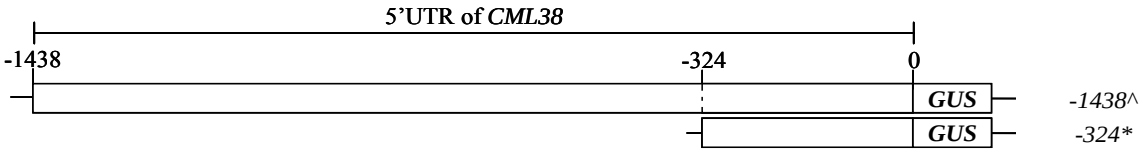


Figure 3.5. Diagram of *CML38* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
-1438^ represents the full-length *CML38* promoter. ^ represents constructs in the pBI101 vector, while * indicates constructs in the pMDC163 vector.

shown in Figure 3.6. As seen previously (Vanderbeld and Snedden, 2007), transgenic seedlings containing the full-length *CML38* promoter construct did not show staining under control conditions (Figure 3.6 Aa) but showed localized expression around the wound site (Figure 3.6 Ab). As seen in the *CML37* promoter deletions in the pMDC163 vector, transgenic seedlings containing the -324* *CML38* deletion construct showed constitutive expression in control conditions (Figure 3.6 Ba). Transgenic seedlings containing this construct showed no apparent change in GUS expression following wounding (Figure 3.6 Bb). Fluorometric assays were carried out to quantify the GUS expression in these constructs (Figure 3.7). These assays showed the same trends as the histochemical staining. Transgenic seedlings containing the -1438^ construct showed a significant increase following wounding, while transgenic seedlings containing the -324* construct showed high constitutive expression that was unaffected by wounding.

CML39 promoter analysis

Transgenic seedlings containing either the full-length *CML39* promoter or one 5′ deletion construct (Figure 3.8) were analyzed for GUS activity following MeJA application. This deletion construct (-301^) was in the pBI101 vector as was the full-length construct (-941^).

Transgenic seedlings were sprayed with 100 μM MeJA and samples were collected 24 h following treatment. Representative patterns of GUS expression following MeJA treatment are shown in Figure 3.9. As previously described (Vanderbeld and Snedden, 2007), transgenic seedlings containing the full-length *CML39* untreated controls showed no GUS expression (Figure 3.9 Aa) while a large increase in GUS expression was seen

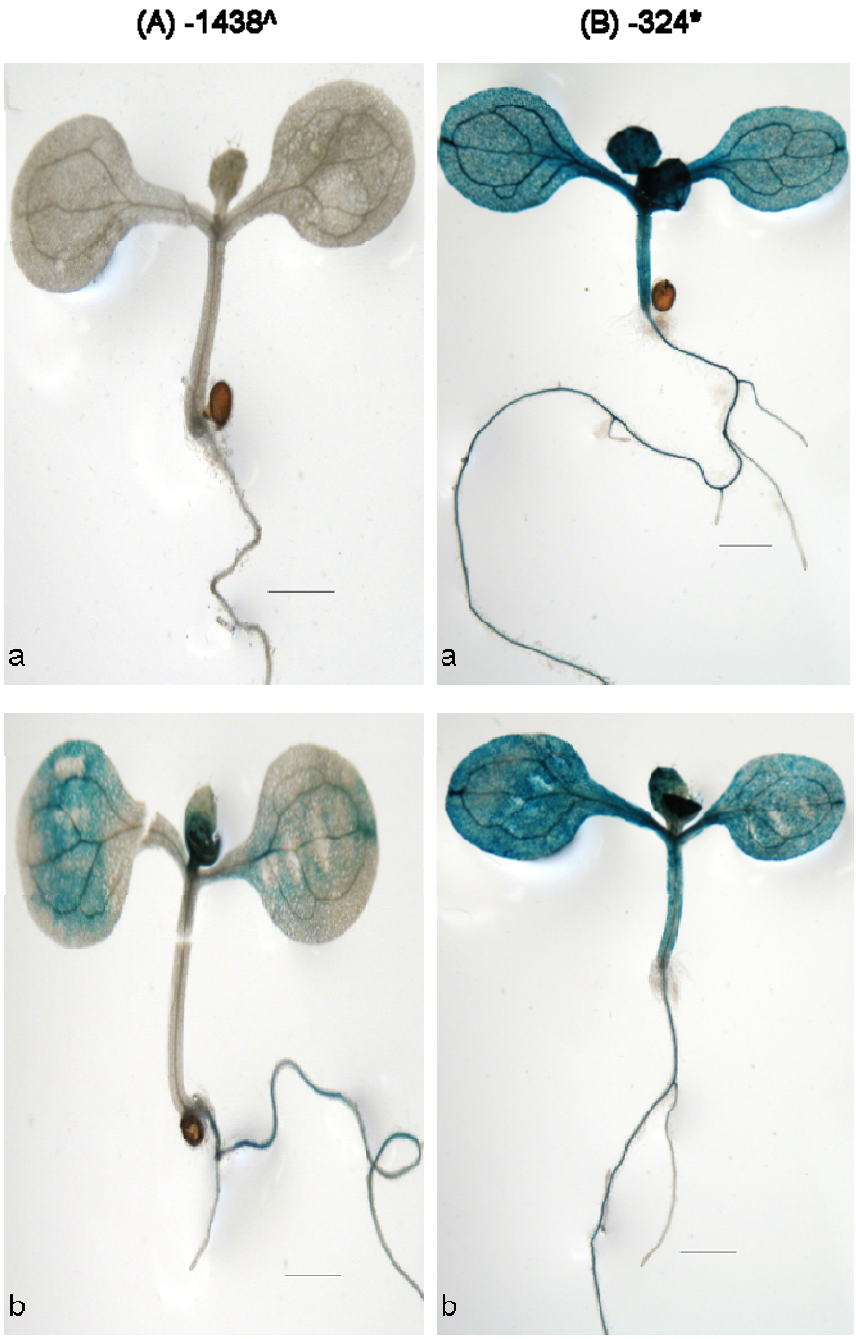


Figure 3.6. Representative patterns of GUS expression in *CML38* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML38* promoter (A) or one 5′ deletion GUS constructs (B) were either unwounded controls (a) or wounded (b) and assayed for GUS activity 8 h after treatment. ^ indicates constructs within the pBI101 vector, while * denotes constructs within the pMDC163 vector. *Bars* = 1 mm.

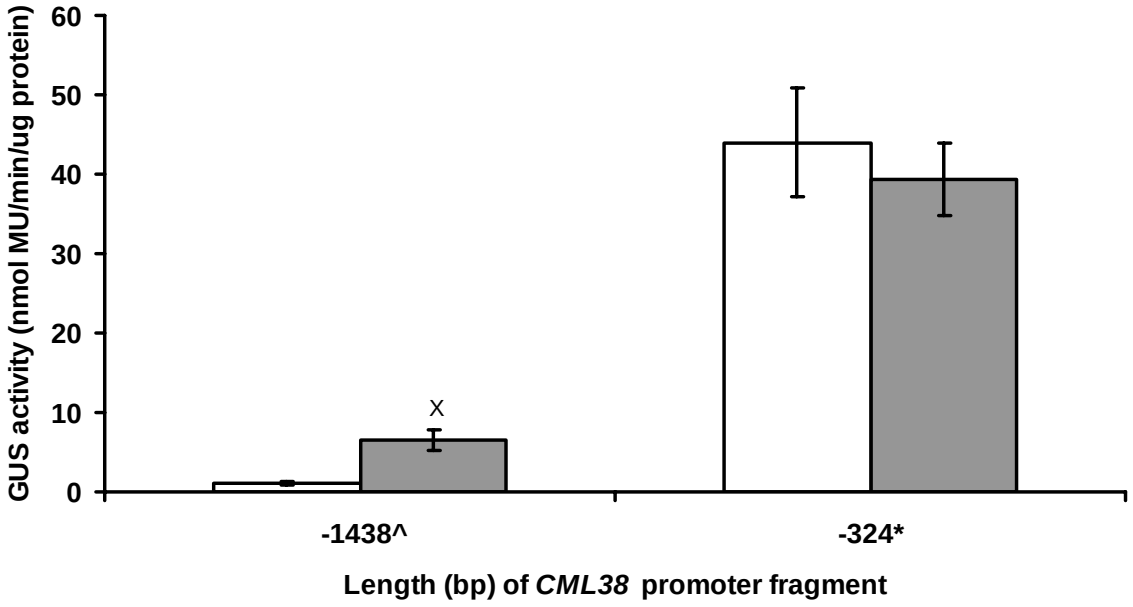


Figure 3.7. Graphical representation of GUS activity in *CML38* 5′ deletion constructs in the pBI101 and pMDC163 vectors.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML38* promoter or a 5′ deletion GUS construct were either unwounded (white bars) or wounded (grey bars) and assayed for GUS activity 8 h after treatment. ^ indicates constructs within the pBI101 vector, while * denotes constructs within the pMDC163 vector. X indicates that the wounded samples had significantly more GUS activity than their unwounded controls (one-tailed t-test, $p<0.05$, $n>25$). Error bars represent ± standard error of the mean.

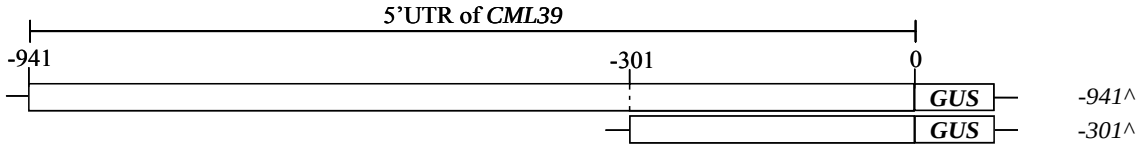


Figure 3.8. Diagram of *CML39* 5′ deletion constructs in the pBI101 vector.
-941^ represents the full-length *CML39* promoter. ^ represents constructs within the pBI101 vector.

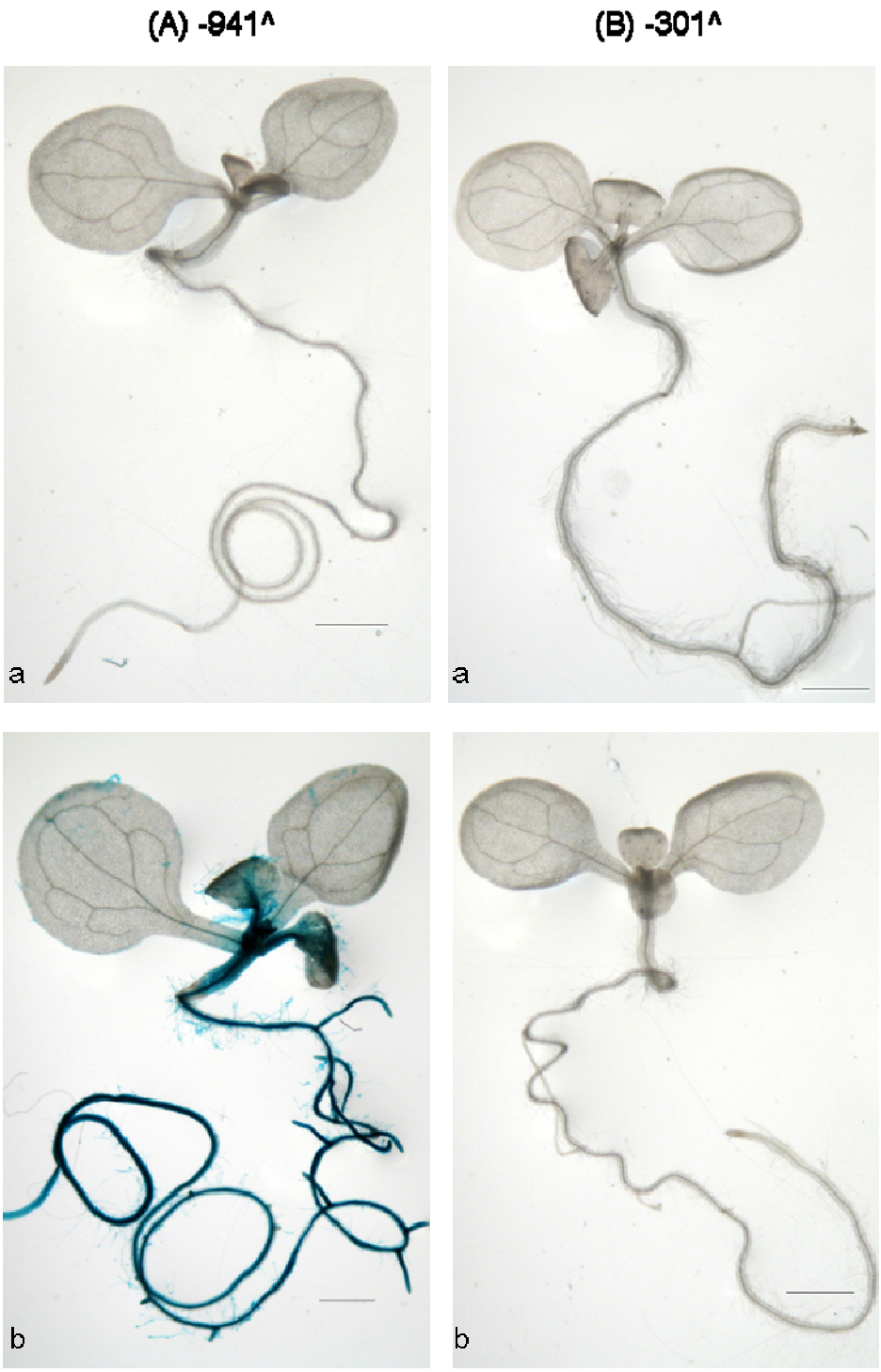


Figure 3.9. Representative patterns of GUS expression in *CML39* 5′ deletion constructs in the pBI101 vector.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML38* promoter (A) or one 5′ deletion GUS constructs (B) were either sprayed with water (a) or sprayed with 100 µM MeJA (b) and assayed for GUS activity 24 h after treatment. ^ indicates constructs within the pBI101 vector. *Bars* = 1 mm.

following MeJA treatment (Figure 3.9 Ab). Transgenic seedlings containing the -301^ deletion construct also showed no expression under control conditions (Figure 3.9 Ba) and levels similar to controls following MeJA treatment (Figure 3.9 Bb). Fluorometric assays were carried out to quantify GUS activity (Figure 3.10). These assays supported the results of the histochemical staining. The GUS activity of transgenic seedlings containing the -941^ construct was very low under control conditions but significantly increased following MeJA application. Transgenic seedlings containing the -301^ construct showed little GUS activity under both control and MeJA treated conditions, indicating that the *cis*-element responsible for MeJA responsiveness within the *CML39* promoter had been removed.

Analysis of *CML37* 5′ deletion constructs in the pGWB203 vector

Given that all transgenic seedlings harbouring deletion constructs within the pMDC163 vector showed constitutive expression (see above) and thus likely artificial, all *CML::GUS* constructs were resubcloned into the pGWB203 vector (Nakagawa, et al., 2007), which is a Gateway vector that uses pBI101 as a backbone.

Promoter analysis

Transgenic seedlings containing one of ten *CML37* promoter constructs (Figure 3.11) were analyzed for GUS activity following wounding. At least four independent lines of each construct were analyzed except for transgenic seedlings containing the -270 or the -164 constructs where only three independent lines were analyzed, and the -370 construct where only one independent line was isolated. GUS activity and expression was consistent across all transgenic independent lines.

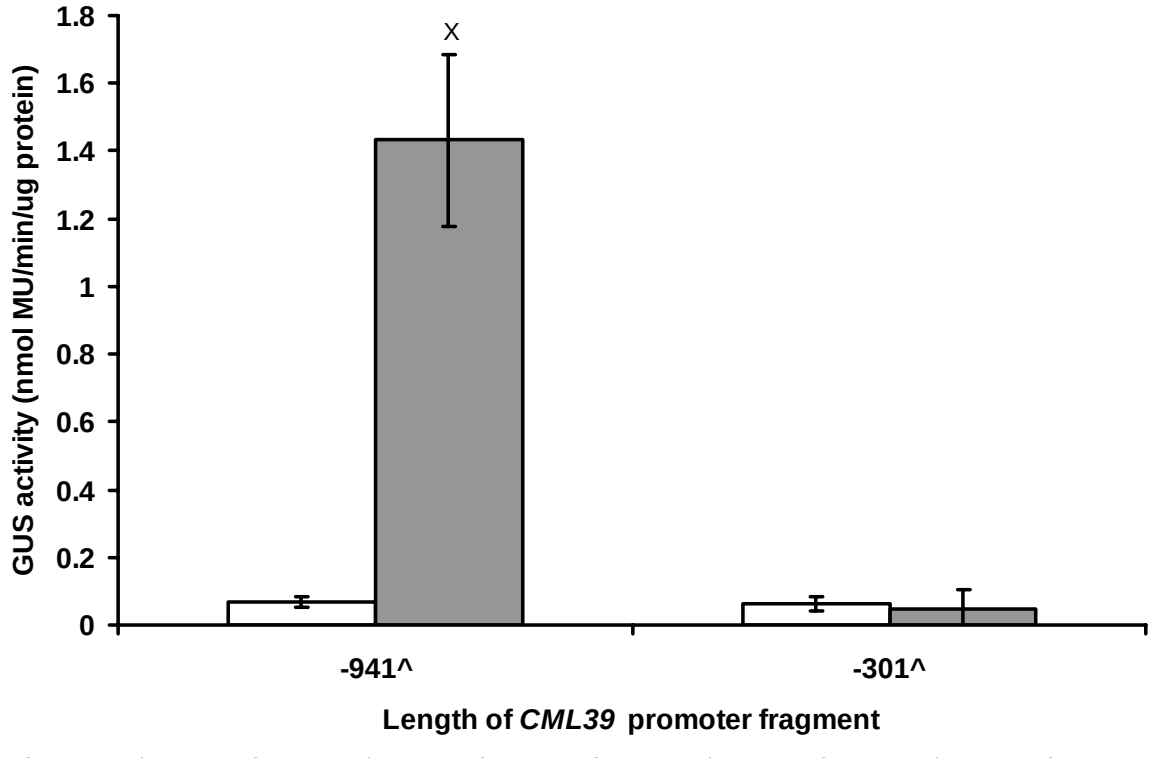


Figure 3.10. Graphical representation of GUS activity in *CML39* 5′ deletion constructs in the pBI101 vector.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML38* promoter or a 5′ deletion GUS construct were either sprayed with water (white bars) or sprayed with 100 μM MeJA (grey bars) and assayed for GUS activity 24 h after treatment. ^ indicates constructs within the pBI101 vector. X indicates that the MeJA treated samples had significantly more GUS activity than their untreated controls (one-tailed t-test, $p<0.05$, $n>25$). Error bars represent ± standard error of the mean.

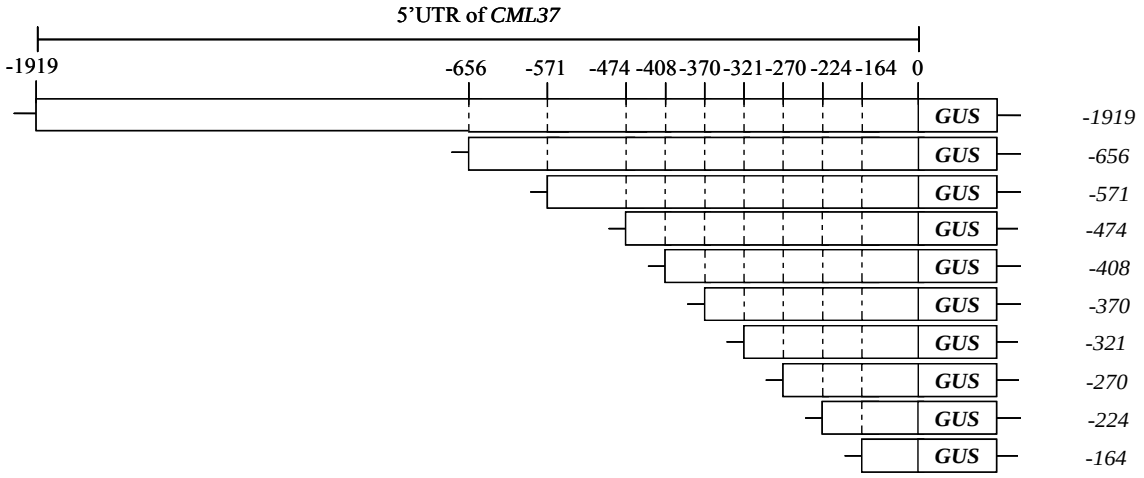
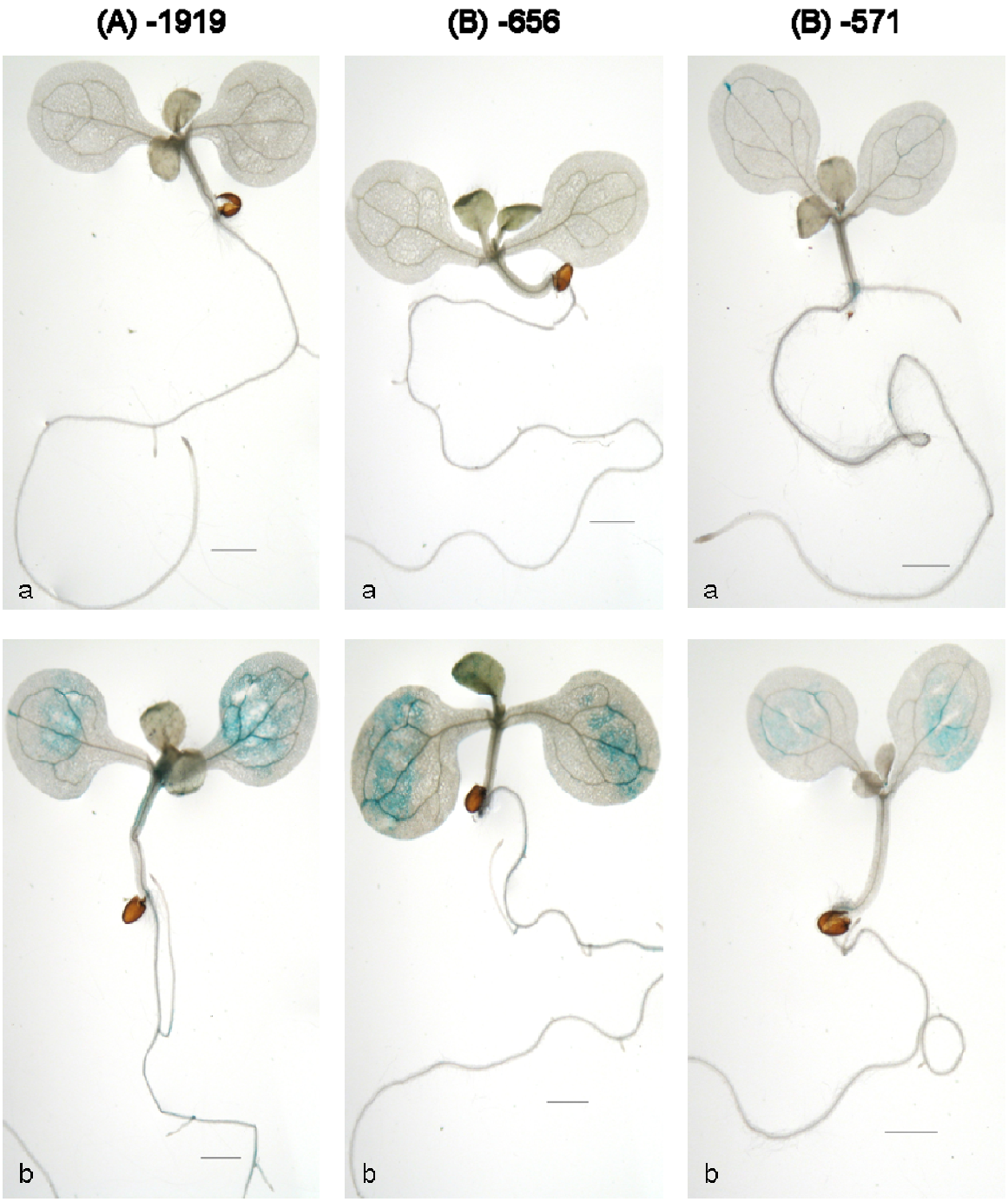


Figure 3.11. Diagram of *CML37* 5′ deletion constructs in the pGWB203 vector. -1919 represents full-length *CML37* promoter construct. All constructs are in the pGWB203 vector.

Transgenic seedlings were wounded with forceps and samples were collected 4 h following treatment. Representative patterns of GUS expression following wounding in transgenic seedlings containing either the full-length (-1919) or one of the five longest (-656, -571, -474, -408, -370) *CML37* promoter deletion constructs are shown in Figure 3.12. Transgenic seedlings containing these constructs showed little staining under control conditions (Figure 3.12 Aa, Ba, Ca, Da, Ea, Fa) but showed GUS expression around the wound site following wounding (Figure 3.12 Ab, Bb, Cb, Db, Eb, Fb). Transgenic seedlings containing on of the four shorter *CML37* promoter deletion constructs (-321, -270, -224, -164) did not show any GUS staining, either under control or wounded conditions (data not shown). This may be due to the stringency of staining conditions. Transgenic seedlings containing one of these four constructs were stained with less stringent staining conditions (see materials and methods), but still did not show any GUS staining (data not shown).

Fluorometric assays were carried out to quantify GUS activity under both control and wounded conditions (Figure 3.13 A). For clarity, the GUS activities of transgenic seedlings containing one of the four shortest promoter constructs are also shown in Figure 3.13 B. Transgenic seedlings containing all constructs except the shortest (-164) showed a significant increase in GUS activity following wounding. Under control conditions, transgenic seedling containing one of the longest six constructs (-1919, -656, -571, -474, -408, -370) showed a general downward trend of GUS activity. This is likely due to the loss of general promoter elements when shortening the promoter. There was a significant decrease in GUS activity under control conditions between transgenic seedlings containing the -370 and the -321 constructs, suggesting that an enhancer element or an


(A) -1919
(B) -656
(B) -571
a
a
a
b
b
b

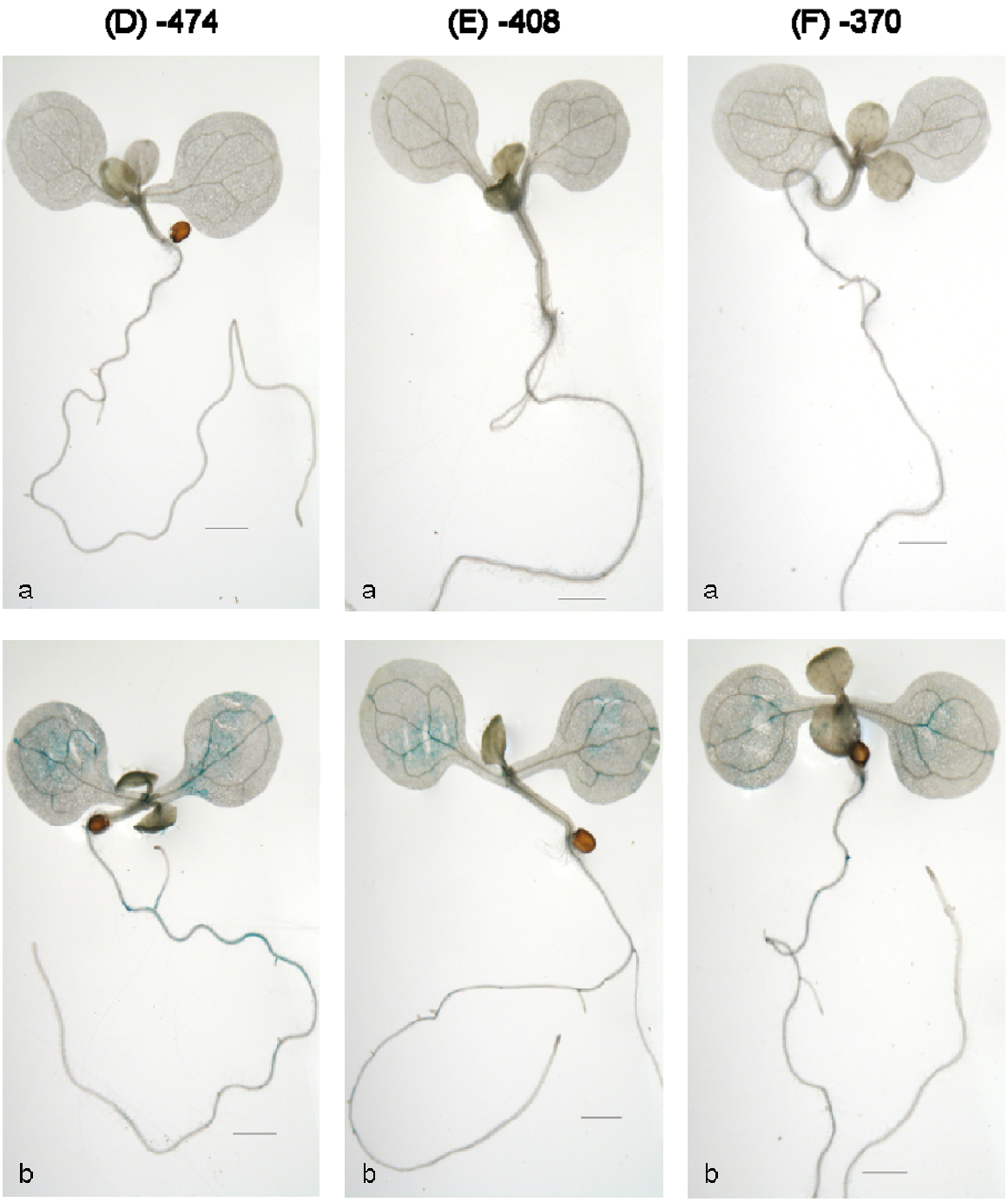


Figure 3.12. Representative patterns of GUS expression in *CML37* 5′ deletion constructs in the pGWB203 vector.
Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML37* promoter (A) or one of the four longest 5′ deletion GUS constructs (B-F) were either unwounded controls (a) or wounded (b) and assayed for GUS activity 4 h after treatment. *Bars* = 1 mm.

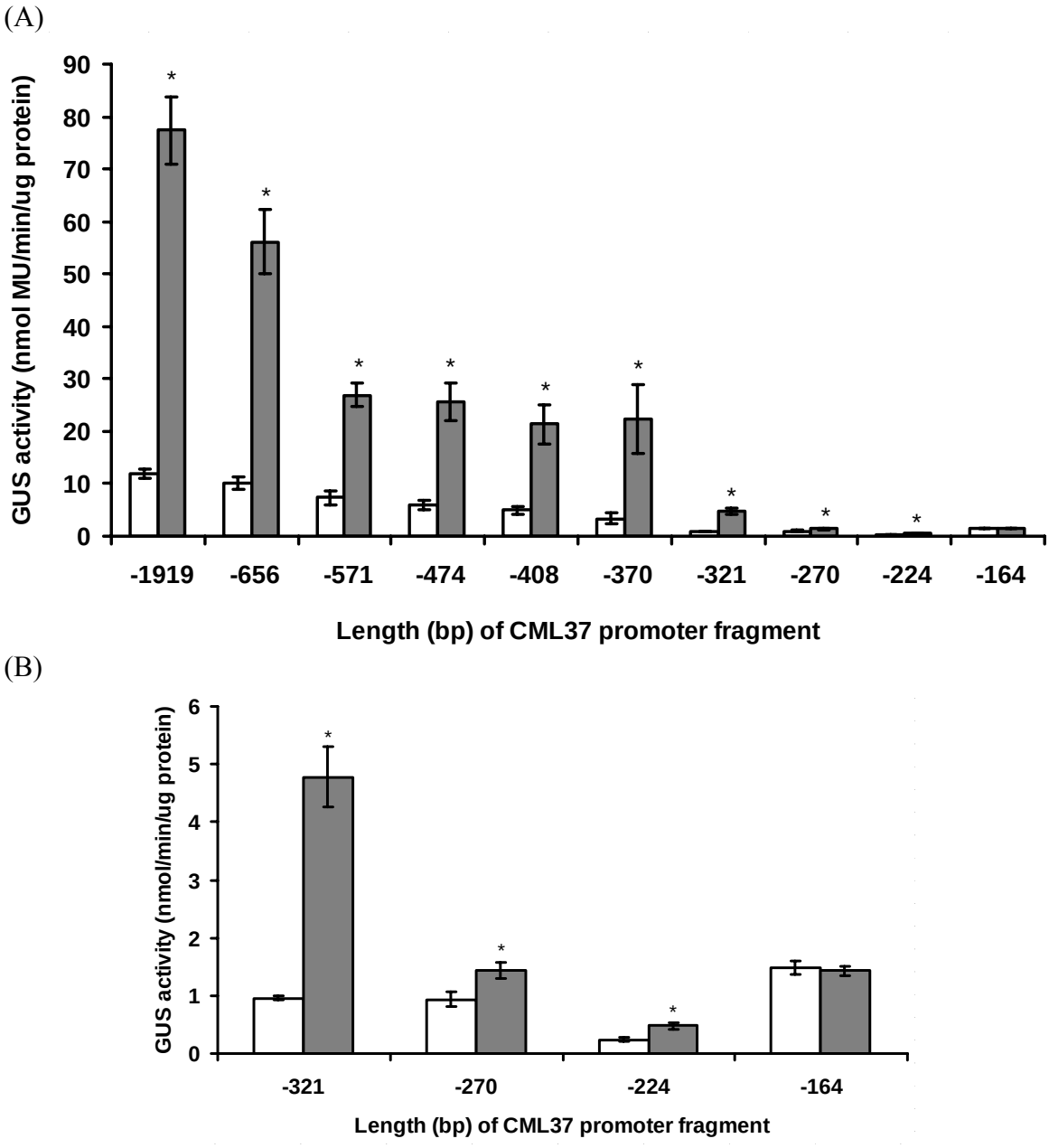


Figure 3.13. Graphical representation of GUS activity in *CML37* 5′ deletion constructs in the pGWB203 vector.

Transgenic Arabidopsis seedlings of stage 1.0-1.04 containing the full-length *CML37* promoter or one of nine 5′ deletion GUS constructs (A) were either unwounded (white bars) or wounded (grey bars) and assayed for GUS activity following 4 h. * indicates that the wounded samples had significantly more GUS activity than their unwounded controls (one-tailed t-test, $p<0.05$, $n>5$). For clarity, (B) also shows the four shortest deletion constructs on an expanded scale. Error bars represent ± standard error of the mean.

element necessary for expression was deleted. There is also a significant decrease in GUS expression under control conditions between the -270 and the -224 constructs.

There appears to be more than one element that is responsible for the wound-responsiveness of *CML37*. There are significant decreases in the magnitude of GUS expression between transgenic seedlings containing the -1919 and the -656 constructs, the -656 and -571 constructs, and the -321 and -270 constructs. These decreases may indicate that the *CML37* promoter has multiple wound-responsive *cis*-elements. There were also significant differences in wound-induced GUS expression between transgenic seedlings containing the -270 and the -321 constructs and between the -270 and the -224 constructs; however, both differences may be explained by the decrease in overall GUS activity, as there were similar decreases under control conditions.

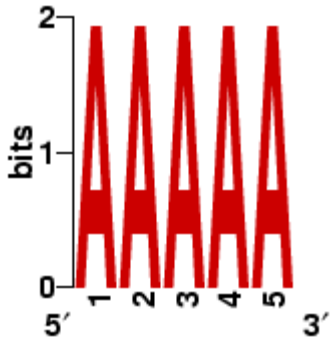
Putative elements responsible for wound response

There are three regions of the promoter that, when deleted, decrease the wound responsiveness of the *CML37* promoter. These regions were examined for known wound-responsive *cis*-elements. Five W-boxes are lost when the promoter is shortened from -1919 to -656. No known wound-responsive element is lost when the promoter is changed from -656 to -571. The putative WUN motif is deleted from the *CML37* promoter when it is shortened from -321 to -270.

In addition to known *cis*-elements, novel motifs were examined. Using the MEME database (Bailey and Elkan, 1994), motifs common to all three wound-responsive regions were examined. A list of genes that are coexpressed with *CML37* during stress response was generated by the ATTED II database (Obayashi, et al., 2009). The promoters of these coexpressed genes were examined for the putative *cis*-elements discovered by MEME by

the BAR (Toufighi, et al., 2005). One putative element, with a sequence of AAAAA (the sequence logo provided by MEME is shown in Figure 3.14 A), was found to be overrepresented in the promoters of genes coexpressed with *CML37* (Figure 3.14 B). This element was found six times within the *CML37* promoter, but only in the regions that were found to be wound-responsive. The AAAAA motif was entered into both the PLACE and PlantCARE databases, but it was not a previously described *cis*-element.

(A)



(B)

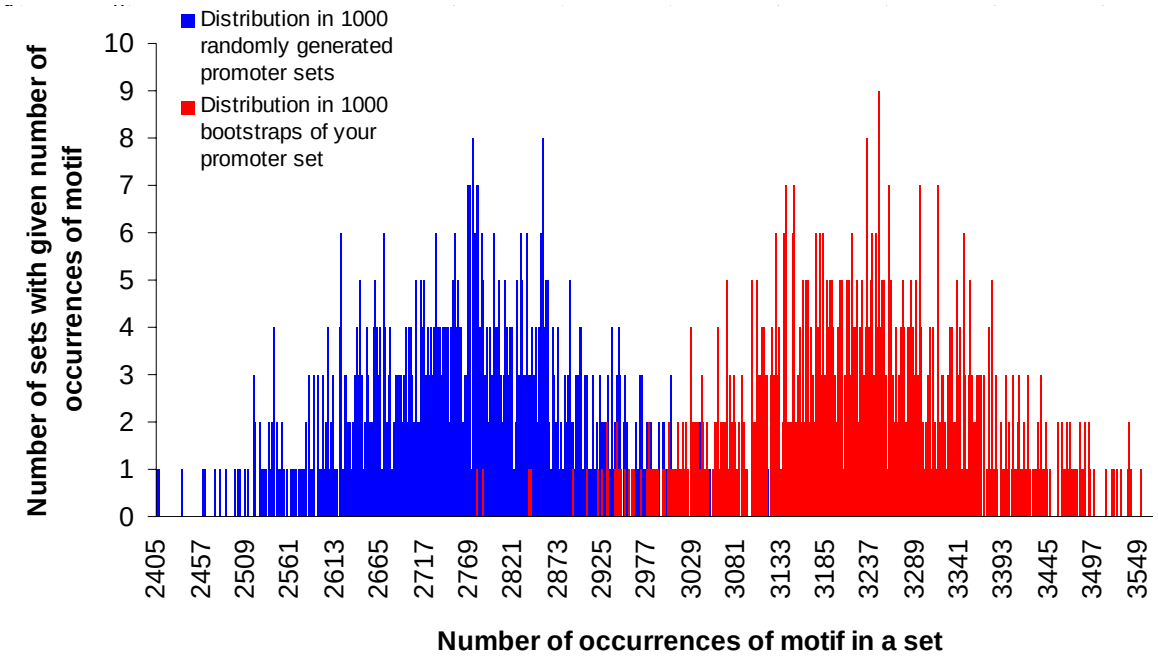


Figure 3.14. Motif common among wound-responsive regions of *CML37* promoter and distribution of the motif in genes coexpressed with *CML37*.
(A) motif common to wound-responsive regions of *CML37* promoter found by MEME. (B) distribution of AAAAA motif in promoters of genes coexpressed with *CML37* (found using ATTED II) compared to distribution of AAAAA motif in all promoters of the Arabidopsis genome (analysis performed by the BAR). Z score = 3.7, p = 0.001.

CHAPTER 4: Discussion

Plants must be able to make transcriptional changes quickly in order to express required genes to respond to external stimuli. Signal transduction pathways are induced so that the plant may elicit the correct response. Ca^{2+} signalling plays an important role in many stress responses, including defence (Lecourieux et al., 2006). Previous research has shown that the transcripts of a subfamily of Ca^{2+}-binding proteins, *CMLs 37-39*, are increased following various stresses, most notably wounding and MeJA application (Vanderbeld and Snedden, 2007). The upstream signalling events that lead to these expression increases are unknown. In order to shed light on the signalling pathways of these CMLs, this study was undertaken to find the *cis*-elements in the 5′ upstream promoter regions of these genes that are responsible for their expression patterns.

in silico analysis of *CML37*, *CML38*, and *CML39* promoters

Full-length *CML37*, *CML38*, and *CML39* promoters were examined for known *cis*-elements using the online databases AGRIS, Athena, PLACE, and PlantCARE. Many putative *cis*-elements were found within the three promoters; the raw data is summarized in Table 3.1, Table 3.2, and Table 3.3.

Among the known *cis*-elements predicted within the three *CML* promoters, a number are implicated in stress response; however, several problems with the raw data become evident under closer inspection. Many of the *cis*-elements that have been entered into the database are not very specific. For instance, an element that is known to have a function in circadian expression has a given sequence of CAANNNNATC (where N

represents any nucleotide). These *cis*-elements with non-specific sequences can likely be found in a wide range of promoter sequences making them unreliable for the purposes of this study. The PLACE database also finds the same elements more than once. Palindromic sequences are found twice: both on the + strand of DNA as well as on the – strand. For instance, the recognition sequence of the MYC transcription factors is provided as CANNTG. Ten of these sequences are reported within the *CML37* promoter; however, there are only five sequences, but the search engine finds each one twice. Additionally, many similar entries in the database cause an increase in the output number of *cis*-elements. There are five separate entries for W-boxes in the PLACE database. W-boxes are known binding sites of WRKY transcription factors (Eulgem, et al., 2000). The given sequences of these W-boxes are TTTGACY, TTGAC, TGACT, TGACY, and TGAC (where Y represents a T or C nucleotide). If the sequence TTTGACT is found within a promoter sequence, the database reports five W-boxes instead of one. Within the *CML37* promoter the database reported 27 distinct W-boxes (shown under wounding in Table 3.1), but closer inspection revealed that there are only eleven unique sites (Figure 3.1 Aa).

Another factor to consider is the location of these genes on the chromosome. The promoter region was considered to be the region upstream of the ATG start site of each gene extending to the UTR of the nearest neighbour gene (see materials and methods). For *CML37* the nearest neighbour gene (At5g42390) is in the opposite orientation as *CML37*, indicating that the 5′ promoter region of At5g42390 is simply the reverse complement of the *CML37* promoter. Since the online database search both the + and – strands of the given promoter, many of the *cis*-elements found for *CML37* may in fact be driving the expression of At5g42390, particularly those at the 5′ end of the *CML37*

promoter. For *CML39*, the UTR of the nearest neighbour gene is the 3′ UTR of *CML38*. The 3′ downstream region of a gene can also contain *cis*-elements, meaning that the *CML39* promoter region may contain *cis*-elements required for *CML38* expression, particularly those at the 5′ end of the *CML39* promoter.

Although a large amount of data were provided by the online *cis*-element search databases, it is impossible to identify with certainty the region or regions of the *CML37*, *CML38*, and *CML39* promoters that are responsible for their expression patterns. In order to identify correctly the region(s) responsible for gene expression, 5′ deletion *promoter::GUS* constructs of the three promoters were generated and then assessed for GUS activity compared to the full-length promoter.

Analysis of 5′ deletion constructs in the pBI101 and pMDC163 vectors

Transgenic seedlings containing either the full-length or 5′ promoter deletion constructs were analyzed for their responses to wounding (*CML37* and *CML38*) or MeJA application (*CML39*). Full-length promoter constructs for *CML37*, *CML38*, and *CML39* were generated by B. Vanderbeld (Vanderbeld and Snedden, 2007). These constructs were produced in the pBI101 vector. Although a couple 5′ deletion constructs were also generated in the pBI101 vector, the majority were produced in the pMDC163 vector to simplify cloning procedures (Curtis and Grossniklaus, 2003). This vector is a gateway-compatible vector that uses a pCAMBIA vector (http://www.cambia.org/) as a backbone (all vector maps are shown in Appendix 1).

Transgenic seedlings containing either the full-length promoter or one of four 5′ deletion constructs of *CML37* (Figure 3.2) were analyzed for GUS activity, both by histochemical staining (Figure 3.3) and by fluormetric assays (Figure 3.4). Transgenic

seedlings containing the full-length *CML37* promoter construct (-1919^) showed the same GUS staining patterns as seen previously (Vanderbeld and Snedden, 2007): no staining was observed under control conditions (Figure 3.3 Aa), but there was distinct staining surrounding the wound site (Figure 3.3 Ab). Transgenic seedlings containing the only deletion construct that was within the pBI101 vector (-656^) showed the same staining pattern (Figure 3.3 Ba, Bb). The other three deletion constructs were in the pMDC163 vector (-571*, -474*, -224*). Transgenic seedlings containing these constructs all showed constitutive expression under control conditions (Figure 3.3 Ca, Da, Ea) that was not affected by wounding (Figure 3.3 Cb, Db, Eb). These staining patterns were verified by fluorometric assays (Figure 3.4). There may be a tissue-specific *cis*-element located between -656 bp and -571 bp of the *CML37* promoter that, when deleted, causes constitutive expression of *CML37*. It is much more likely, however, that the pMDC163 vector itself is responsible for the constitutive expression.

The pCAMBIA vector that the pMDC163 vector uses as a backbone contains a gene that confers hygromycin resistance in plants. This gene is driven by two copies of the cauliflower mosaic virus 35S promoter, which is a strong promoter that shows high constitutive expression (Fang, et al., 1989). It has been reported that the 35S promoter interferes with GUS expression patterns and that this interference is exacerbated in experiments where researchers attempt to analyze trimmed-down versions of their promoters of interest, which lack the natural insulating sequences of full-length promoters (http://www.cambia.org/). An empty vector control was created by transforming promoterless pMDC163 into plants. These plants did not show any GUS activity (data not shown). It may be that the 35S promoter is simply able to enhance GUS expression; without minimal promoter elements transcription cannot be initiated. Therefore, it is

likely that the constitutive expression shown by the -571*, -474*, and -224* constructs is due to 35S interference.

This interference was also seen when analyzing transgenic seedlings containing *CML38* promoter constructs (Figure 3.5). Transgenic seedlings containing the full-length promoter showed no GUS staining under control conditions (Figure 3.6 Aa) but showed increased expression around the wound site (Figure 3.6 Ab), as previously reported (Vanderbeld and Snedden, 2007). Transgenic seedlings containing the 5′ deletion construct (-324*), which was in the pMDC163 vector, showed constitutive activity (Figure 3.6 Ba) that was not affected by wounding (Figure 3.6 Bb). The staining patterns were corroborated by fluorometric assays (Figure 3.7).

Both the full-length and 5′ deletion construct of *CML39* were created in the pBI101 vector (Figure 3.8). Transgenic seedlings containing the full-length promoter behaved as described previously (Vanderbeld and Snedden, 2007): under control conditions no staining was observed (Figure 3.9 Aa), but GUS expression was massively induced following MeJA application (Figure 3.9 Ab). Transgenic seedlings containing the -301^ deletion construct showed no staining under either control or wounded conditions (Figure 3.9 Ba, Bb). Fluorometric assay data showed the same trends (Figure 3.10). Since both of these constructs were in the same vector, it can be inferred that the *cis*-element responsible for the increase in GUS activity following MeJA application had been deleted from the *CML39* promoter. This element appears to lie in the 640 bp that separate the -941^ and -301^ constructs. More 5′ deletion constructs must be created and analyzed to further narrow down the location of this element.

Analysis of *CML37* 5′ deletion constructs in the pGWB203 vector

Due to problems with the pMDC163 vector, it was decided that further promoter constructs would be created in a different vector that did not have the 35S promoter driving a resistance gene. The full-length *CML37* promoter and nine 5′ deletions (Figure 3.11) were subcloned into the pGWB203 vector (Nakagawa, et al., 2007). This vector is a gateway-compatible vector that uses pBI101 as a backbone. Both the gene for resistance to kanamycin in bacteria (*NPTII*) and the gene for hygromycin resistance in plants (*HPT*) are driven by the Nos promoter, which is a weaker promoter than the 35S and does not interfere with GUS expression (Nakagawa, et al., 2007).

Several of the 5′ deletion constructs had fewer plant lines than desirable (especially -370, which only had one independent line). This makes it difficult to account for the effects of location of insertion. More independent lines have been isolated and will be assayed for GUS activity in the future. Transgenic seedlings containing one of the *CML37* promoter constructs were assayed for GUS activity, in both control conditions and following wounding. Transgenic seedlings containing the full-length promoter showed the same staining patterns in the pGWB203 vector as it had in the pBI101 vector (Figure 3.12 Aa, Ab). Transgenic seedlings containing one of the five longest 5′ deletion constructs (-656, -571, -474, -408, -370) also showed no staining in control conditions (Figure 3.12 Ba, Ca, Da, Ea, Fa) but distinct staining around the wound site (Figure 3.12 Bb, Cb, Db, Eb, Fb). Transgenic seedlings containing the four shortest 5′ deletion constructs (-321, -270, -224, -164) did not show any staining under either control or wounded conditions. In order to test whether this lack of staining was due to the stringency of staining conditions, transgenic seedling containing these four constructs were also stained with a less stringent staining solution (data not shown). The transgenic

seedlings still did not show staining at all. It is unclear whether the transgenic plants did not show staining because they do not respond to wounding or because GUS expression is below the threshold of detection by staining.

Fluorometric assays were carried out to quantify GUS activity (Figure 3.13). The quantitative data correlated to the observed staining patterns. Interestingly, the absolute values of GUS activity in the full-length promoter were much higher in the pGWB203 vector than in the pBI101 vector (Figure 3.4). There were several differences between the two experiments so it is difficult to identify the reason for this difference. The most obvious difference is between the vectors. The pGWB203 vector is very similar to the pBI101 vector; the only difference is the addition of a hygromycin resistance gene driven by the Nos promoter (Nakagawa, et al., 2007). Previous studies have determined that the Nos promoter does not interfere with GUS expression (Nakagawa, et al., 2007). Although an empty vector control may be helpful in addressing this question, the lack of a minimal promoter within such a control may compromise its usefulness (see above). This is likely not the reason for the difference between expression in the pGWB203 and pBI101 vectors.

Another possibility to explain the differences may be due to the design of the experiment. Transgenic seedlings containing full-length promoter constructs in the pBI101 vector were wounded and collected 8 h following treatment, while those containing the promoter in the pGWB203 vector were collected 4 h following treatment. *CML37* transcripts are induced 100 fold by 30 minutes after wounding and return to normal levels by 12 h (Vanderbeld and Snedden, 2007). The GUS enzyme may begin to be degraded or lose activity after 8 h. This may account for the lower GUS activity seen

in the pBI101 vector. The activity of GUS should be analyzed at both 4 h and 8 h in both the pBI101 and pGWB203 vectors to test this hypothesis.

The fluorometric data for transgenic seedlings containing the *CML37* 5′ deletion constructs were analyzed (Figure 3.13). Under control conditions, there was a general downward trend of GUS activity. This was likely due to the loss of enhancer elements that are distributed throughout the entire promoter. There were significant decreases in GUS activity between transgenic seedlings containing the -370 or -321 constructs and between the -270 or -224 constructs. These decreases were most likely due to the loss of elements required for normal expression patterns. Transgenic seedlings containing any construct except for the shortest (-164) showed significant increases in GUS activity following wounding. The -270 and -224 constructs only showed approximately a 1.5-fold increase in GUS activity. This, coupled with lack of detectable staining, made it unclear whether seedlings containing these constructs undergo a wound site-specific increase in expression. The large increase in expression following wounding was lost between transgenic seedlings containing the -321 or the -270 construct. There were other areas that, when deleted, caused a decrease in wound response. These areas were between the -1919 and -656 constructs and between the -656 and -571 constructs. Although at first glance there appeared to be a large difference in wound response between the -370 and -321 constructs this difference was likely due to the overall decrease in GUS activity, as both these constructs showed similar increases in activity following wounding (approximately 5-fold).

The regions that, when deleted, caused a decrease in wound responsiveness were analyzed for known wound-responsive elements. The region between the -1919 and the -656 constructs is quite large. There are five putative W-boxes in this region that are

known to bind WRKY TF (Eulgem, et al., 2000). Since WRKY TF are known to function in the regulation of defence response (Eulgem, 2005), it is reasonable to assume that the wound-responsiveness of *CML37* is due to W-boxes. However, many regions in the CML37 promoter contain putative W-boxes that do not cause the wound response to decrease when deleted (between constructs -571 and -474, -474 and -408, -370 and -321, -270 and -224). Also, the other regions that control wound responsiveness (between -656 and -571 and between -321 and -270) do not contain putative W-boxes. It may be that the putative W-boxes in the region between -1919 and -656 act with different wound-responsive elements in the regions between -656 and -571 and between -321 and -270 to confer the total wound responsiveness of the *CML37* promoter. The question remains as to how WRKY TF are able to distinguish between the W-boxes in the -1919 and -656 region and W-boxes in all the other regions of the promoter. It therefore seems unlikely that the wound-responsiveness of the *CML37* promoter is exclusively due to activation by WRKY TF. There are also quite a few predicted MYB-binding *cis*-elements in the region between -1919 and -656, but the same problems arise as when considering W-boxes, indicating that MYB TF are also not likely to control exclusively the wound-response in *CML37*.

No wound-responsive *cis*-elements were predicted within the region between -656 and -571 of the *CML37* promoter. There are very few known elements found in this region. There is a MYC recognition sequence, which is known to confer JA-responsiveness (Boter, et al., 2004). As *CML37* is not responsive to JA (Vanderbeld and Snedden, 2007) it is unlikely that this *cis*-element is involved in its expression. There may be a novel element within this region of the *CML37* promoter that either induces or enhances wound expression. Such a putative element would probably function with other

elements in wound-responsive regions of the promoter to confer the wound-responsiveness displayed by the full-length *CML37* promoter.

The region between the -321 and -270 of the *CML37* promoter contained a putative WUN-motif. This motif was originally found in the potato wound-inducible *WUN1* gene (Siebertz, et al., 1989). This is the only instance of the WUN-motif within the *CML37* promoter. It may be acting with different *cis*-elements in the other wound-responsive regions of to achieve the overall wound-responsiveness of the full-length *CML37* promoter.

The potential for a novel *cis*-element within these three regions was examined. In order to be a candidate for this putative *cis*-element, motifs had to be present in all three of the wound responsive regions of *CML37* but nowhere else in the promoter, and be statistically overrepresented in genes that are coexpressed with *CML37*. Although many putative motifs were found by the motif-searching program MEME (Bailey and Elkan, 1994), only one of these motifs fit the other criteria. This motif (AAAAA, Figure 3.14 A) is found six times within the *CML37* promoter, but only in those regions that confer wound-responsiveness. It is also statistically overrepresented in genes coexpressed with *CML37* (Figure 3.14 B). Although it is very generic, it does not match any known *cis*-elements within the PLACE or PlantCARE databases. There is no precedence in the literature for a mononucleotide element. Although several elements containing mononucleotide repeats, such as the pyrimidine box (sequence: TTTTTCC), which is required for GA induction in barley (Cercos et al., 1999), are known, no elements that consist of a single mononucleotide repeat have been found. The AAAAA motif is therefore very suspect; however, it may still be a part of a larger regulatory element. The regions that confer wound-responsiveness to the full-length *CML37* promoter must be

narrowed down further, perhaps by a gel mobility shift assay. The narrowed down regions must then be tested for their ability to induce wound-responsive expression.

Future directions

This study raises many questions and opens many areas for further study. As stated above, the wound-responsive regions of the *CML37* promoter require further identification. In addition, 5′ deletion constructs of both *CML38* and *CML39* in the pGWB203 vector, many of which have already been transformed into plants (see Appendix 2), must be analyzed for their responses to wounding and MeJA, respectively. Narrowing down the region of wound-responsiveness in *CML38* will likely complement the work performed on the *CML37* promoter; the same *cis*-element(s) may be responsible for the wound-responsiveness in both promoters. Once identified, these *cis*-elements can be used to find upstream TF through yeast one-hybrid studies. This will shed light on the signalling pathways of these three genes.

In summary, this study found that three regions in the *CML37* promoter were responsible for its wound-inducible expression. There are also regions that have essential elements for expression under control conditions. A 600 bp region that confers MeJA-responsiveness to *CML39* was identified. Additionally, it was found that the pMDC163 vector was not useful for studying promoter deletion constructs. It was also not possible to identify conclusively which *cis*-elements conferred wound-responsiveness for the *CML37* promoter using only online databases. These databases were useful for identifying a putative *cis*-element once the regions of wound-responsiveness were known. It remains unclear whether repeats of a single novel element are responsible for the wound-responsiveness of the *CML37* promoter, or whether different elements (both

known and novel) in the wound-responsive regions are working together to produce the overall wound response of the full-length promoter. This study lays the groundwork for future studies that aim to identify upstream signalling pathways of the *CML37*, *CML38*, and *CML39* genes.

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APPENDIX 1: Maps of vectors used in this study

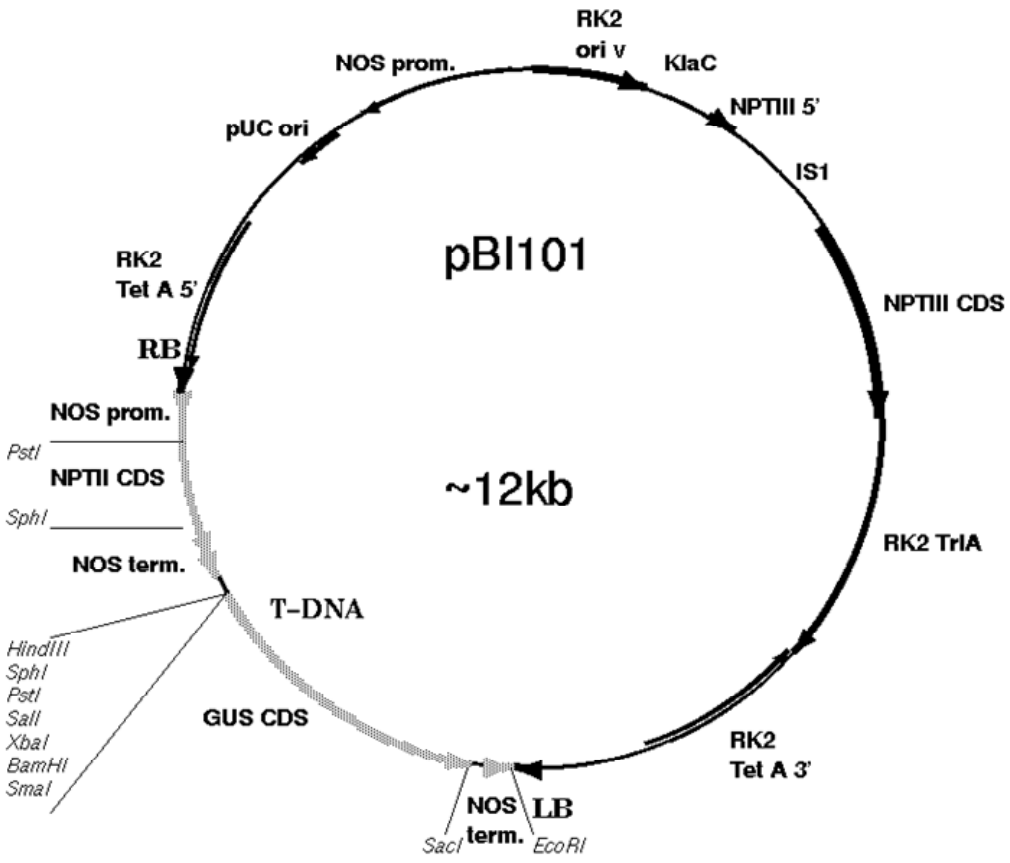

Figure A1.1. Vector map of pBI101 vector.
RB = right border, LB = left border, *NPTII* = confers resistance to kanamycin

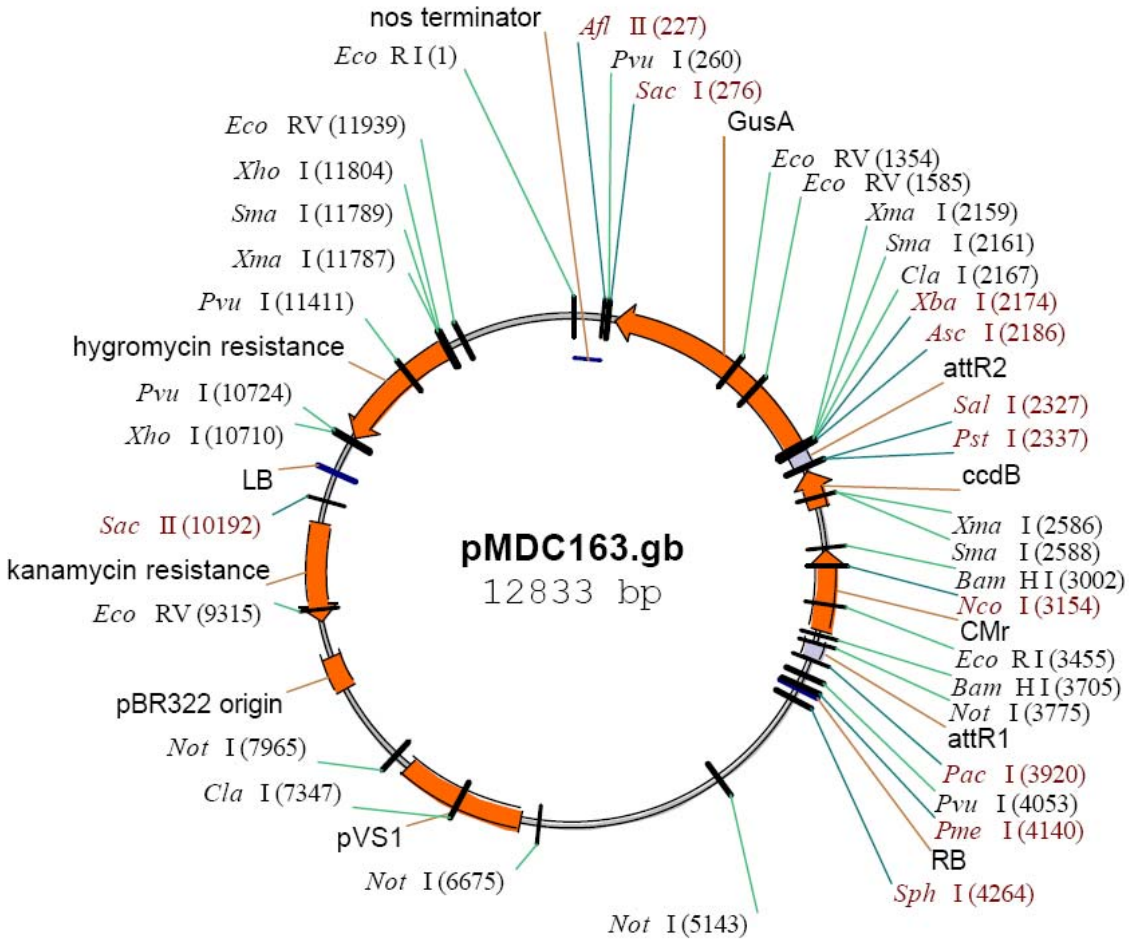

Figure A1.2. Vector map of pMDC163 vector.
RB = right border, LB = left border, CMr = chloramphenicol-resistance marker, *ccdB* = negative selection marker used in bacteria, *attR1* and *attR2* = recombination sites used by LR-Clonase II (Invitrogen)

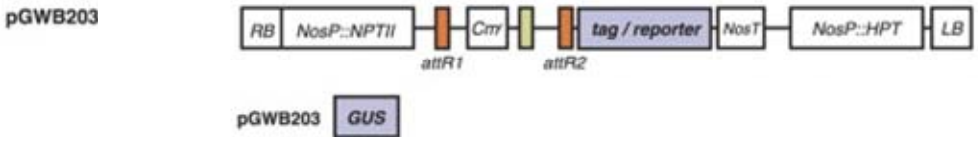

Figure A1.3. Vector map of pGWB203 vector.
RB = right border, LB = left border, Cm^r = chloramphenicol-resistance marker, *ccdB* = negative selection marker used in bacteria, *attR1* and *attR2* = recombination sites used by LR-Clonase II (Invitrogen), *NosP* = Nos promoter, *NPTII* = confers resistance to kanamycin in bacteria, *HPT* = confers resistance to hygromycin in plants.

APPENDIX 2: Creation of 5′ deletion constructs not analyzed in this study

All primers used for the creation of constructs are shown in Table A2.1.

CML37

In addition to the -1919^ and -656^ constructs created in the pBI101 vector, two more constructs were made that had lengths 1598 bp and 1114 bp. These constructs were transformed into agrobacterium and stored as glycerol stocks.

Many other constructs were made in the pMDC163 vector. These constructs had lengths of 408 bp, 370 bp, 321 bp, and 270 bp. These constructs were transformed into plants, but abandoned at the T1 stage when the problems with the pMDC163 vector became apparent. There are also agrobacterium glycerol stocks of these constructs.

CML38

In addition to the full-length *CML38* promoter construct, *pBI101:CML38* was digested with either XbaI or SalI, which resulted in *pBI101::CML38* deletion constructs that were 1067 bp and 1007 bp, respectively. These constructs are available as T3 plants, though only 1-2 lines of each construct are available. Agrobacterium glycerol stocks are also in storage. Another construct of length 715 bp was also created in the pBI101 vector. This construct is stored as an agrobacterium glycerol stock.

Constructs of length 1438 bp, 715 bp, 512 bp, 464 bp, 324 bp, 283 bp, 216 bp, 186 bp, and 127 bp are in the pCR8 entry vector and are ready to be cloned into pGWB203. These constructs are stored as glycerol stocks.

Table A2.1. Sequences of oligonucleotides used throughout this study.

Primer name	Length	5′- Restriction site, Sequence -3′	Vector(s) used when making constructs
CML37 PRO R		tcccccgggttcttgatttgaatatgaatctagaagatt	
CML37 PRO F1	1919 bp	cgggatccagaagagggagatgaggttagcta	pBI101*, pGWB203*
CML37 PRO F2	1598 bp	caccggatccgactatggagtatgagtcgg	pBI101
CML37 PRO F3	1114 bp	caccggatccgtggtgatgtgcaatagtgg	pBI101
CML37 PRO FH	656 bp	aagcttttacatgaagttggc	pGWB203*
CML37 PRO F8	614 bp	aaaataaactctcttttggtcc	
CML37 PRO F5	571 bp	cacctggaaagggaattcaggacc	pGWB203*, pMDC163*
CML37 PRO F9	539 bp	gttagatgaaacttcatcacatg	
CML37 PRO F4	474 bp	caccggatcctcataccactttcgtggagc	pGWB203*, pMDC163*
CML37 PRO F6	408 bp	caccaagaccaaaaccacgtgtcc	pGWB203*, pMDC163
CML37 PRO F10	370 bp	caaagtcaaagagaaggtttgc	pGWB203*, pMDC163
CML37 PRO F11	321 bp	gacatcacatttcacacaatacg	pGWB203*, pMDC163
CML37 PRO F12	270 bp	gaaaacacccctagtcgtgg	pGWB203*, pMDC163
CML37 PRO F7	224 bp	cacctcattgcggaggaaacttcc	pGWB203*, pMDC163*
CML37 PRO F13	164 bp	caccaaaatattcaaacccg	pGWB203*
CML38 PRO R		cccggggagagaaaaataaatggttaagtg	
CML38 PRO F1	1438 bp	ggatcccttctttagtctctttttaatgc	pBI101*
CML38 PRO F2	715 bp	caccggatccatctttctttctcggtccgg	pBI101
CML38 PRO F4	650 bp	aaagcgtaaatcatacctctc	
CML38 PRO F5	512 bp	ctatgtgtgccttggtttgc	
CML38 PRO F6	464 bp	gatattcgtacacatagtcaaac	
CML38 PRO F3	324 bp	caccggatcccaaatacaagtggccggtcc	pMDC163*
CML38 PRO F7	283 bp	ccattaaaaaggaagaaaaagcg	
CML38 PRO F8	216 bp	ctaaacactaatctaccacg	
CML38 PRO F9	186 bp	acacacacacacacttgatc	
CML38 PRO F10	127 bp	taccacaacttgaccacacg	
CML39 PRO R		ggatcctttgagaaagaaaagattgtatttg	
CML39 PRO F1	941 bp	aagcttgtcaatctactaaatatatagtcc	pBI101*, pGWB203
CML39 PRO F2	599 bp	caccaagcttcctcatctttcggttgtagg	pBI101, pGWB203
CML39 PRO F4	525 bp	agccataacaagtttgaggc	pGWB203
CML39 PRO F5	442 bp	aatgattagtagatgaagttacc	pGWB203
CML39 PRO F6	356 bp	atccttcttcctatatttaagac	pGWB203
CML39 PRO F3	301 bp	caccaagcttatggggatgagatatgtaacg	pBI101*, pGWB203
CML39 PRO F7	235 bp	aataatactacttcttctagatc	
CML39 PRO F8	175 bp	gaatctcacacgtttgattcg	
CML39 PRO F9	138 bp	cttattactattctcgacttg	

Asterisks denote constructs that were analyzed in this study.

CML39

In additions to the -941^ and -301^ constructs created in the pBI101 vector, another construct, length 599 bp was created. Transformations into plants yielded no transformants. Agrobacterium glycerol stocks are available.

Constructs of lengths 941 bp, 599 bp, 525 bp, 442 bp, 356 bp and 301 bp were created in the pGWB203 vector. These constructs have all been transformed into plants and are at the T1-T3 stage. These constructs are ready for GUS analyses.

APPENDIX 3: Sequences of promoters and exact locations of *cis*-elements

(A)

AGAAGAGGGAGATGAGGTTAGCTACGTTTATTGTCTTCTCCTTCCTGATTTTTATTTTGTTAAACTCTTTTTATATGAGT
ATATTAATGTTTCTTTCTTTTTTAATACAAACTATATATTTTAATTCCAAAATCAAACCTTCTATTTGTAGCAAAGGGTT
TGAAGTCAAAATCTCAATATTTTGGAAAGATTTTCACCCAGAAGTTTTAACCGGTTGATCATGCGGTAGTTAATGGCAAG
TTTTTTCTGGATTTTTCTGGTTATATTGGTAGATTTAAGACTAAACCTTCACTGGAAATATGTTTCTTGGGTTAATCGTT
CGACTATGGAGTATGAGTCGGACATGAAAACATTGTTTATCTGCTGGCAACTTTGAACTGATCGGTTCAATTAGGTTTAG
GTATTTCGGTTAAGGGCTTAGATAGCAATCTAAAAAAAAAAATTGATGTAATTTTGTGAAATTTGGTTTAAATACGTTGCG
AAAATTATAATTTTTTAAAAAAATGATCGAAAAGTTAATAATTTTATGCATTCATATATTTGCTTATGTTAAGTGTTACA
TGACCCCTAAATTTTTAAAGGATTCATGCCTAATTATAAGAATATTGATTTCGATAGTTAGAAGAAAAGCTATTTGTATG
ATCTCTATTTTTTCTTGTACATATAGTAGACAAGAAAATTGTCTTTCATTCAAACTCGTATAGGTCTCGTTGTATCTCGA
ATATTAAGTTAATTAATGTTTAGATTCTGTTATTTTGGTTTAATCATTTGAATGTGATTAGCTTTCGTCAGATAATGTGT
GTTTTGTGGTGATGTGCAATAGTGGTTACAAGATCGATTGTTTAGGATTTCAAGTGTTTGTCACTCTAAACTAGGCTTTC
TATACTATTTTCACTTTGGCGATCATGAGTTTTTCATAGTAAGTTTATTTGTGAGATTTGATACCAACAATTTAAACTAT
TTGCTCAGAAAGTTTAATTAATGTGAATTAAGTAAGGTCACGATATGCCAGCACCGCATCATGGAAATTCATTTCATCAA
GAATTGAAGATATAGATTAAAGTCTAACATTTTGTTGATATTAGGATCAAATTAAGTATTTATAAGGAGATTTTATGGGG
GGAAACAGAGATGTTTTTGTTAAATAATCTTTGAGCTCGATAATTACGGGGAGAAGTTTCAAAGAATAACTGAATTGTTT
GAACAATATATGCTGGGCCGAGTTAAACCGTCGAGCTTAGAAAGCCGAACTATACATCAGTAAAAGCTTTTACATGAAGT
TGGCATTTGAGATGCAAAAAGATAAAAATAAAACTCTCTTTTGGTCCATATAGAAATATATTTTCTTATGGAAAGGGAAT
TCAGGACCGGGAATGTAACAGTTAGATGAAACTTCATCACATGAAATATTTTATTTTTAATTCAAGTTTCTAGGAACAGA
GGAAGTCATACCACTTTCGTGGAGCATATCTGGACGGTTATATGAAACTTCATGACAGTTTAACAATTTAAAAGACCAAA
ACCACGTGTCCGCACATCGTATACTCAATCAAAGTCAAAGAGAAGGTTTGCTACTTTTCTTAATCTAGTCGTCGATATGA
CATCACATTTCACACAATACGTGAGAAAAACTTACCATCACATTATTACGAAAACACCCCTAGTCGTGGTCAACTTGACC
TTTCCCCACAAACACTCATTGCGGAGGAAACTTCCCGGCCACCGCCGCGCAACCGCCTCTAGCTTTAAATAATAACACCA
AAATATTCAAACCCGTGTTTTAACCGTCGAACAATCAAGAATCTCTCCACCATTACACGCATTCACATACTCATCTATAT
ATAATCTACCACTTCTTGAAAAGAATTTGCATCAGAAATCCATTTCCCAAATCTTCTAGATTCATATTCAAATCAAGAA

(B)

CTTCTTTAGTCTCTTTTTAATGCATTTGTTAACAACAACTTAAGATGTTAGATTGGAGAACTTGTGAATGTTGTTAATTC
TTGATTTTTCTATATCAACACTAAAGTTTTACATTATGTTTTATGTATTATTAGTTGTGTCTACATTGACATGTTCTAGT
AATGTGTTTATCTCCAAACAAAACAATTCAATTCTTGTATTTTTAATCAAACTATTTGCAAATGGTAAACGTCAATTAAT
TCGCCTTTATAATTTAATGGAAGATTACTTTTAAAAAGATGCAAGTTATTAGAATCAAAATAAAAACAAAGACAAAATAT
GTTAACTTGTAGAAAACACTTGGAAGTAAAAATCTGATGAGTTTCTCGTTCTCTAGAATATTGCTGGACCGGATTAGGTC
AATATTATTGGGCCAGATTAGATATTGAATTGTCGACGTTGCTTACGTTACGTTATATCTTGTTTAAGAATTAAACCTAT
CGACTTAGTCTTAATTAAGAAAACATTGCCTTAAATTCTCTGGTCTGCGACCGTTTTTTTGACCGTTAACCCCTAATTAA
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TTTTTTTTTTGTACAAATCTCTAAGAAAAACACTACTACTAATTAAGAAACGTTTCAAACAATTTTATTTTCGTTGGCTC
ATAATCTTTCTTTCTCGGTCCGGGACTAACCGTTGGCAAAAAAAAAAAAAAAAGTTGACAATAATTATTAAAGCGTAAATC
ATACCTCTCAAATAAAAACTTGAATTTGGAAACAAAGACAACTAAAAAACTCGAATTTAAGAGAATTCCTAAAATCAAGT
GAAGTATCATCACTTGGTAAAATTTCATAACCGTTGGCTTCTATTTCTATGTGTGCCTTGGTTTGCAGGAGATAATATTT
CATTTCCAACCAATGATATTCGTACACATAGTCAAACAAATGTTTGTCTTTGTTATTATATTGAGAAAGAAACAAGAAAG
AGAGAGAGAGATAGATAAGACGAAGGAAGTGAAGCTTCCAAGCGCCCACCGTTAAAAATCTCGTGTGCAAGTTTCAAATA
CAAGTGGCCGGTGGTCTCCATAATTTGATCGTCATCCAATTAAAAAGGAAGAAAAAGCGTGTTTTATACAAGAAAACTCA
TTAAAATAAAAGTCCAAAATATCTAAACACTAATCTACCACGTCTATTACACACACACACACACACTTGATCTTAATTTA
TTTTCAAGATTCAAGAAAATACCCATTCCATTACCACAACTTGACCACACGCCTATATATAAAACATAAAAGCCCTTTCC
CCTATTTCTCATTCACTTTTCATTTCAAAAGTAAAACAAGACAAACAAAAAATACACTTAACCATTTATTTTTCTCTC

(C)

GTCAATCTACTAAATATATAGTCCATTGAGGTTTGACCATGATTTGCTCATAGTCATATTATTATTCTCTAGAGCTCACA
ATTCAAGACAAAAGGAATCTAAACAAGATTCAAATGAGCTTTAGTATAAGATCTTCACATAACGGTAACAATTAAGGTAT
CTAATAAGATTTTATTCCTACAAAACAATCTTTTTTTCCAACCAATAAGAGTTTATATATTGTTAGAGTCCTAACACTTT
TGTGAAGAAGAATGTTGGCTTTTATCGATCCATCTCAAACTGAATCTCACTAAGTTACCGACAATAATCCATAGTGTCAA
TGAATTGAATCTTACTATTTGACCTCATCTTTCGGTTGTAGGATTTCTAACACTTATCACTTGTTTTGAATCAACCCAAG
TTGGAGAAATTATAGGAGCCATAACAAGTTTGAGGCATTATATGGACTTCTATGGTATGAAACCTTAGGTTGTGAAGAAA
TTGATTTTTGAAAAAAAGCAATGATTAGTAGATGAAGTTACCATTGTTCTAAATTCAACAAATGGGAGTCGTCAAATATT
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ATGGGGATGAGATATGTAACGAACCAATATACTAATATTTCCTTTCCTAGCTAGATCTAGAAGACTAATAATACTACTTC
TTCTAGATCTAAAGACTAAAAATATTTGTTTTTTTTTTCTAGATCTGAATCTCACACGTTTGATTCGTTTCAATTTCTAT
TTTCTTATTACTATTCTCGACTTGAAACACACCTATATATATAGAGAAACACTTTATACCATTTCTCATTCAAATCCTAA
ATCAAAAAAAGAAAGAACAATACTTGTAACTTATAACAAATACAATCTTTTCTTTCTCAAA

Figure A3.1. Sequences of (A) *CML37*, (B) *CML38*, and (C) *CML39* promoters.

(A)

AGAAGAGGGAGATGAGGTTAGCTACGTTTATTGTCTTCTCCTTCCTGATTTTTATTTTGTTAAACTCTTTTTATATGAGT
ATATTAATGTTTCTTTCTTTTTTAATACAAACTATATATTTTAATTCCAAAATCAAACCTTCTATTTGTAGCAAAGGGTT
TGAAGTCAAAATCTCAATATTTTGGAAAGATTTTCACCCAGAAGTTTTAACCGGTTGATCATGCGGTAGTTAATGGCAAG
TTTTTTCTGGATTTTTCTGGTTATATTGGTAGATTTAAGACTAAACCTTCACTGGAAATATGTTTCTTGGGTTAATCGTT
CGACTATGGAGTATGAGTCGGACATGAAAACATTGTTTATCTGCTGGCAACTTTGAACTGATCGGTTCAATTAGGTTTAG
GTATTTCGGTTAAGGGCTTAGATAGCAATCTAAAAAAAAAAATTGATGTAATTTTGTGAAATTTGGTTTAAATACGTTGCG
AAAATTATAATTTTTTAAAAAAATGATCGAAAAGTTAATAATTTTATGCATTCATATATTTGCTTATGTTAAGTGTTACA
TGACCCCTAAATTTTTAAAGGATTCATGCCTAATTATAAGAATATTGATTTCGATAGTTAGAAGAAAAGCTATTTGTATG
ATCTCTATTTTTTCTTGTACATATAGTAGACAAGAAAATTGTCTTTCATTCAAACTCGTATAGGTCTCGTTGTATCTCGA
ATATTAAGTTAATTAATGTTTAGATTCTGTTATTTTGGTTTAATCATTTGAATGTGATTAGCTTTCGTCAGATAATGTGT
GTTTTGTGGTGATGTGCAATAGTGGTTACAAGATCGATTGTTTAGGATTTCAAGTGTTTGTCACTCTAAACTAGGCTTTC
TATACTATTTTCACTTTGGCGATCATGAGTTTTTCATAGTAAGTTTATTTGTGAGATTTGATACCAACAATTTAAACTAT
TTGCTCAGAAAGTTTAATTAATGTGAATTAAGTAAGGTCACGATATGCCAGCACCGCATCATGGAAATTCATTTCATCAA
GAATTGAAGATATAGATTAAAGTCTAACATTTTGTTGATATTAGGATCAAATTAAGTATTTATAAGGAGATTTTATGGGG
GGAAACAGAGATGTTTTTGTTAAATAATCTTTGAGCTCGATAATTACGGGGAGAAGTTTCAAAGAATAACTGAATTGTTT
GAACAATATATGCTGGGCCGAGTTAAACCGTCGAGCTTAGAAAGCCGAACTATACATCAGTAAAAGCTTTTACATGAAGT
TGGCATTTGAGATGCAAAAAGATAAAAATAAAACTCTCTTTTGGTCCATATAGAAATATATTTTCTTATGGAAAGGGAAT
TCAGGACCGGGAATGTAACAGTTAGATGAAACTTCATCACATGAAATATTTTATTTTTAATTCAAGTTTCTAGGAACAGA
GGAAGTCATACCACTTTCGTGGAGCATATCTGGACGGTTATATGAAACTTCATGACAGTTTAACAATTTAAAAGACCAAA
ACCACGTGTCCGCACATCGTATACTCAATCAAAGTCAAAGAGAAGGTTTGCTACTTTTCTTAATCTAGTCGTCGATATGA
CATCACATTTCACACAATACGTGAGAAAAACTTACCATCACATTATTACGAAAACACCCCTAGTCGTGGTCAACCTTGACC
TTTCCCCACAAACACTCATTGCGGAGGAAACTTCCCGGCCACCGCCGCGCAACCGCCTCTAGCTTTAAATAATAACACCA
AAATATTCAAACCCGTGTTTTAACCGTCGAACAATCAAGAATCTCTCCACCATTACACGCATTCACATACTCATCTATAT
ATAATCTACCACTTCTTGAAAAGAATTTGCATCAGAAATCCATTTCCCAAATCTTCTAGATTCATATTCAAATCAAGAA

(B)

CTTCTTTAGTCTCTTTTTAATGCATTTGTTAACAACAACTTAAGATGTTAGATTGGAGAACTTGTGAATGTTGTTAATTC
TTGATTTTTCTATATCAACACTAAAGTTTTACATTATGTTTTATGTATTATTAGTTGTGTCTACATTGACATGTTCTAGT
AATGTGTTTATCTCCAAACAAAACAATTCAATTCTTGTATTTTTAATCAAACTATTTGCAAATGGTAAACGTCAATTAAT
TCGCCTTTATAATTTAATGGAAGATTACTTTTAAAAAGATGCAAGTTATTAGAATCAAAATAAAAACAAAGACAAAATAT
GTTAACTTGTAGAAAACACTTGGAAGTAAAAATCTGATGAGTTTCTCGTTCTCTAGAATATTGCTGGACCGGATTAGGTC
AATATTATTGGGCCAGATTAGATATTGAATTGTCGACGTTGCTTACGTTACGTTATATCTTGTTTAAGAATTAAACCTAT
CGACTTAGTCTTAATTAAGAAAACATTGCCTTAAATTCTCTGGTCTGCGACCGTTTTTTTGACCGTTAACCCCTAATTAA
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ATACCTCTCAAATAAAAACTTGAATTTGGAAACAAAGACAACTAAAAAACTCGAATTTAAGAGAATTCCTAAAATCAAGT
GAAGTATCATCACTTGGTAAAATTTCATAACCGTTGGCTTCTATTTCTATGTGTGCCTTGGTTTGCAGGAGATAATATTT
CATTTCCAACCAATGATATTCGTACACATAGTCAAACAAATGTTTGTCTTTGTTATTATATTGAGAAAGAAACAAGAAAG
AGAGAGAGAGATAGATAAGACGAAGGAAGTGAAGCTTCCAAGCGCCCACCGTTAAAAATCTCGTGTGCAAGTTTCAAATA
CAAGTGGCCGGTGGTCTCCATAATTTGATCGTCATCCAATTAAAAAGGAAGAAAAAGCGTGTTTTATACAAGAAAACTCA
TTAAAATAAAAGTCCAAAATATCTAAACACTAATCTACCACGTCTATTACACACACACACACACACTTGATCTTAATTTA
TTTTCAAGATTCAAGAAAATACCCATTCCATTACCACAACTTGACCACACGCCTATATATAAAACATAAAAGCCCTTTCC
CCTATTTCTCATTCACTTTTCATTTCAAAAGTAAAACAAGACAAACAAAAAATACACTTAACCATTTATTTTTCTCTC

(C)

GTCAATCTACTAAATATATAGTCCATTGAGGTTTGACCATGATTTGCTCATAGTCATATTATTATTCTCTAGAGCTCACA
ATTCAAGACAAAAGGAATCTAAACAAGATTCAAATGAGCTTTAGTATAAGATCTTCACATAACGGTAACAATTAAGGTAT
CTAATAAGATTTTATTCCTACAAAACAATCTTTTTTTCCAACCAATAAGAGTTTATATATTGTTAGAGTCCTAACACTTT
TGTGAAGAAGAATGTTGGCTTTTATCGATCCATCTCAAACTGAATCTCACTAAGTTACCGACAATAATCCATAGTGTCAA
TGAATTGAATCTTACTATTTGACCTCATCTTTCGGTTGTAGGATTTCTAACACTTATCACTTGTTTTGAATCAACCCAAG
TTGGAGAAATTATAGGAGCCATAACAAGTTTGAGGCATTATATGGACTTCTATGGTATGAAACCTTAGGTTGTGAAGAAA
TTGATTTTTGAAAAAAAGCAATGATTAGTAGATGAAGTTACCATTGTTCTAAATTCAACAAATGGGAGTCGTCAAATATT
ATATTTACCACAATAACATTTCTCAATCCTTCTTCCTATATTTAAGACAGAGAGGTAGAAGAAGAAAACGTGTTTTAAAC
ATGGGGATGAGATATGTAACGAACCAATATACTAATATTTCCTTTCCTAGCTAGATCTAGAAGACTAATAATACTACTTC
TTCTAGATCTAAAGACTAAAAATATTTGTTTTTTTTTTCTAGATCTGAATCTCACACGTTTGATTCGTTTCAATTTCTAT
TTTCTTATTACTATTCTCGACTTGAAACACACCTATATATATAGAGAAACACTTTATACCATTTCTCATTCAAATCCTAA
ATCAAAAAAAGAAAGAACAATACTTGTAACTTATAACAAATACAATCTTTTCTTTCTCAAA

Figure A3.2. Locations of wound-responsive *cis*-elements within the promoters of (A) *CML37*, (B) *CML38*, and (C) *CML39*.

Yellow boxes represent WRKY binding sites on the + strand; red boxes represent WRKY binding sites on the - strand; pink box represents a WUN element on the + strand.

(A)

AGAAGAGGGAGATGAGGTTAGCTACGTTTATTGTCTTCTCCTTCCTGATTTTTATTTTGTTAAACTCTTTTTATATGAGT
ATATTAATGTTTCTTTCTTTTTTAATACAAACTATATATTTTAATTCCAAAATCAAACCTTCTATTTGTAGCAAAGGGTT
TGAAGTCAAAATCTCAATATTTTGGAAAGATTTTCACCCAGAAGTTTTAACCGGTTGATCATGCGGTAGTTAATGGCAAG
TTTTTTCTGGATTTTTCTGGTTATATTGGTAGATTTAAGACTAAACCTTCACTGGAAATATGTTTCTTGGGTTAATCGTT
CGACTATGGAGTATGAGTCGGACATGAAAACATTGTTTATCTGCTGGCAACTTTGAACTGATCGGTTCAATTAGGTTTAG
GTATTTCGGTTAAGGGCTTAGATAGCAATCTAAAAAAAAAATTGATGTAATTTTGTGAAATTTGGTTTAAATACGTTGCG
AAAATTATAATTTTTTAAAAAAATGATCGAAAAGTTAATAATTTTATGCATTCATATATTTGCTTATGTTAAGTGTTACA
TGACCCCTAAATTTTTAAAGGATTCATGCCTAATTATAAGAATATTGATTTCGATAGTTAGAAGAAAAGCTATTTGTATG
ATCTCTATTTTTTCTTGTACATATAGTAGACAAGAAAATTGTCTTTCATTCAAACTCGTATAGGTCTCGTTGTATCTCGA
ATATTAAGTTAATTAATGTTTAGATTCTGTTATTTTGGTTTAATCATTTGAATGTGATTAGCTTTCGTCAGATAATGTGT
GTTTTGTGGTGATGTGCAATAGTGGTTACAAGATCGATTGTTTAGGATTTCAAGTGTTTGTCACTCTAAACTAGGCTTTC
TATACTATTTTCACTTTGGCGATCATGAGTTTTTCATAGTAAGTTTATTTGTGAGATTTGATACCAACAATTTAAACTAT
TTGCTCAGAAAGTTTAATTAATGTGAATTAAGTAAGGTCACGATATGCCAGCACCGCATCATGGAAATTCATTTCATCAA
GAATTGAAGATATAGATTAAAGTCTAACATTTTGTTGATATTAGGATCAAATTAAGTATTTATAAGGAGATTTTATGGGG
GGAAACAGAGATGTTTTTGTTAAATAATCTTTGAGCTCGATAATTACGGGGAGAAGTTTCAAAGAATAACTGAATTGTTT
GAACAATATATGCTGGGCCGAGTTAAACCGTCGAGCTTAGAAAGCCGAACTATACATCAGTAAAAGCTTTTACATGAAGT
TGGCATTTGAGATGCAAAAAGATAAAAATAAAACTCTCTTTTGGTCCATATAGAAATATATTTTCTTATGGAAAGGGAAT
TCAGGACCGGGAATGTAACAGTTAGATGAAACTTCATCACATGAAATATTTTATTTTTAATTCAAGTTTCTAGGAACAGA
GGAAGTCATACCACTTTCGTGGAGCATATCTGGACGGTTATATGAAACTTCATGACAGTTTAACAATTTAAAAGACCAAA
ACCACGTGTCCGCACATCGTATACTCAATCAAAGTCAAAGAGAAGGTTTGCTACTTTTCTTAATCTAGTCGTCGATATGA
CATCACATTTCACACAATACGTGAGAAAAACTTACCATCACATTATTACGAAAACACCCCTAGTCGTGGTCAACTTGACC
TTTCCCCACAAACACTCATTGCGGAGGAAACTTCCCGGCCACCGCCGCGCAACCGCCTCTAGCTTTAAATAATAACACCA
AAATATTCAAACCCGTGTTTTAACCGTCGAACAATCAAGAATCTCTCCACCATTACACGCATTCACATACTCATCTATAT
ATAATCTACCACTTCTTGAAAAGAATTTGCATCAGAAATCCATTTCCCAAATCTTCTAGATTCATATTCAAATCAAGAA

(B)

CTTCTTTAGTCTCTTTTTAATGCATTTGTTAACAACAACTTAAGATGTTAGATTGGAGAACTTGTGAATGTTGTTAATTC
TTGATTTTTCTATATCAACACTAAAGTTTTACATTATGTTTTATGTATTATTAGTTGTGTCTACATTGACATGTTCTAGT
AATGTGTTTATCTCCAAACAAAACAATTCAATTCTTGTATTTTTAATCAAACTATTTGCAAATGGTAAACGTCAATTAAT
TCGCCTTTATAATTTAATGGAAGATTACTTTTAAAAAGATGCAAGTTATTAGAATCAAAATAAAAACAAAGACAAAATAT
GTTAACTTGTAGAAAACACTTGGAAGTAAAAATCTGATGAGTTTCTCGTTCTCTAGAATATTGCTGGACCGGATTAGGTC
AATATTATTGGGCCAGATTAGATATTGAATTGTCGACGTTGCTTACGTTACGTTATATCTTGTTTAAGAATTAAACCTAT
CGACTTAGTCTTAATTAAGAAAACATTGCCTTAAATTCTCTGGTCTGCGACCGTTTTTTTGACCGTTAACCCCTAATTAA
AGAAACAAAATAATTATAGAAAGAGCACTGAAATGTGATTATTTTAACAGTACTCTTATGAGAAAATTCGTACTTTTTAG
TTTTTTTTTTGTACAAATCTCTAAGAAAAACACTACTACTAATTAAGAAACGTTTCAAACAATTTTATTTTCGTTGGCTC
ATAATCTTTCTTTCTCGGTCCGGGACTAACCGTTGGCAAAAAAAAAAAAAAAAGTTGACAATAATTATTAAAGCGTAAATC
ATACCTCTCAAATAAAAACTTGAATTTGGAAACAAAGACAACTAAAAAACTCGAATTTAAGAGAATTCCTAAAATCAAGT
GAAGTATCATCACTTGGTAAAATTTCATAACCGTTGGCTTCTATTTCTATGTGTGCCTTGGTTTGCAGGAGATAATATTT
CATTTCCAACCAATGATATTCGTACACATAGTCAAACAAATGTTTGTCTTTGTTATTATATTGAGAAAGAAACAAGAAAG
AGAGAGAGAGATAGATAAGACGAAGGAAGTGAAGCTTCCAAGCGCCCACCGTTAAAAATCTCGTGTGCAAGTTTCAAATA
CAAGTGGCCGGTGGTCTCCATAATTTGATCGTCATCCAATTAAAAAGGAAGAAAAAGCGTGTTTTATACAAGAAAACTCA
TTAAAATAAAAGTCCAAAATATCTAAACACTAATCTACCACGTCTATTACACACACACACACACACTTGATCTTAATTTA
TTTTCAAGATTCAAGAAAATACCCATTCCATTACCACAACTTGACCACACGCCTATATATAAAACATAAAAGCCCTTTCC
CCTATTTCTCATTCACTTTTCATTTCAAAAGTAAAACAAGACAAACAAAAAATACACTTAACCATTTATTTTTCTCTC

(C)

GTCAATCTACTAAATATATAGTCCATTGAGGTTTGACCATGATTTGCTCATAGTCATATTATTATTCTCTAGAGCTCACA
ATTCAAGACAAAAGGAATCTAAACAAGATTCAAATGAGCTTTAGTATAAGATCTTCACATAACGGTAACAATTAAGGTAT
CTAATAAGATTTTATTCCTACAAAACAATCTTTTTTTCCAACCAATAAGAGTTTATATATTGTTAGAGTCCTAACACTTT
TGTGAAGAAGAATGTTGGCTTTTATCGATCCATCTCAAACTGAATCTCACTAAGTTACCGACAATAATCCATAGTGTCAA
TGAATTGAATCTTACTATTTGACCTCATCTTTCGGTTGTAGGATTTCTAACACTTATCACTTGTTTTGAATCAACCCAAG
TTGGAGAAATTATAGGAGCCATAACAAGTTTGAGGCATTATATGGACTTCTATGGTATGAAACCTTAGGTTGTGAAGAAA
TTGATTTTTGAAAAAAAGCAATGATTAGTAGATGAAGTTACCATTGTTCTAAATTCAACAAATGGGAGTCGTCAAATATT
ATATTTACCACAATAACATTTCTCAATCCTTCTTCCTATATTTAAGACAGAGAGGTAGAAGAAGAAAACGTGTTTTAAAC
ATGGGGATGAGATATGTAACGAACCAATATACTAATATTTCCTTTCCTAGCTAGATCTAGAAGACTAATAATACTACTTC
TTCTAGATCTAAAGACTAAAAATATTTGTTTTTTTTTTCTAGATCTGAATCTCACACGTTTGATTCGTTTCAATTTCTAT
TTTCTTATTACTATTCTCGACTTGAAACACACCTATATATATAGAGAAACACTTTATACCATTTCTCATTCAAATCCTAA
ATCAAAAAAAGAAAGAACAATACTTGTAACTTATAACAAATACAATCTTTTCTTTCTCAAA

Figure A3.3. Locations of MYB-binding *cis*-elements within the promoters of (A) *CML37*, (B) *CML38*, and (C) *CML39*.

Green boxes represent MYB binding sites on the + strand; teal boxes represent MYB binding sites on the - strand; burgundy boxes represent overlap.

(A)

AGAAGAGGGAGATGAGGTTAGCTACGTTTATTGTCTTCTCCTTCCTGATTTTTATTTTGTTAAACTCTTTTTATATGAGT
ATATTAATGTTTCTTTCTTTTTTAATACAAACTATATATTTTAATTCCAAAATCAAACCTTCTATTTGTAGCAAAGGGTT
TGAAGTCAAAATCTCAATATTTTGGAAAGATTTTCACCCAGAAGTTTTAACCGGTTGATCATGCGGTAGTTAATGGCAAG
TTTTTTCTGGATTTTTCTGGTTATATTGGTAGATTTAAGACTAAACCTTCACTGGAAATATGTTTCTTGGGTTAATCGTT
CGACTATGGAGTATGAGTCGGACATGAAAACATTGTTTATCTGCTGGCAACTTTGAACTGATCGGTTCAATTAGGTTTAG
GTATTTCGGTTAAGGGCTTAGATAGCAATCTAAAAAAAAAAATTGATGTAATTTTGTGAAATTTGGTTTAAATACGTTGCG
AAAATTATAATTTTTTAAAAAAATGATCGAAAAGTTAATAATTTTATGCATTCATATATTTGCTTATGTTAAGTGTTACA
TGACCCCTAAATTTTTAAAGGATTCATGCCTAATTATAAGAATATTGATTTCGATAGTTAGAAGAAAAGCTATTTGTATG
ATCTCTATTTTTTCTTGTACATATAGTAGACAAGAAAATTGTCTTTCATTCAAACTCGTATAGGTCTCGTTGTATCTCGA
ATATTAAGTTAATTAATGTTTAGATTCTGTTATTTTGGTTTAATCATTTGAATGTGATTAGCTTTCGTCAGATAATGTGT
GTTTTGTGGTGATGTGCAATAGTGGTTACAAGATCGATTGTTTAGGATTTCAAGTGTTTGTCACTCTAAACTAGGCTTTC
TATACTATTTTCACTTTGGCGATCATGAGTTTTTCATAGTAAGTTTATTTGTGAGATTTGATACCAACAATTTAAACTAT
TTGCTCAGAAAGTTTAATTAATGTGAATTAAGTAAGGTCACGATATGCCAGCACCGCATCATGGAAATTCATTTCATCAA
GAATTGAAGATATAGATTAAAGTCTAACATTTTGTTGATATTAGGATCAAATTAAGTATTTATAAGGAGATTTTATGGGG
GGAAACAGAGATGTTTTTGTTAAATAATCTTTGAGCTCGATAATTACGGGGAGAAGTTTCAAAGAATAACTGAATTGTTT
GAACAATATATGCTGGGCCGAGTTAAACCGTCGAGCTTAGAAAGCCGAACTATACATCAGTAAAAGCTTTTACATGAAGT
TGGCATTTGAGATGCAAAAAGATAAAAATAAAACTCTCTTTTGGTCCATATAGAAATATATTTTCTTATGGAAAGGGAAT
TCAGGACCGGGAATGTAACAGTTAGATGAAACTTCATCACATGAAATATTTTATTTTTAATTCAAGTTTCTAGGAACAGA
GGAAGTCATACCACTTTCGTGGAGCATATCTGGACGGTTATATGAAACTTCATGACAGTTTAACAATTTAAAAGACCAAA
ACCACGTGTCCGCACATCGTATACTCAATCAAAGTCAAAGAGAAGGTTTGCTACTTTTCTTAATCTAGTCGTCGATATGA
CATCACATTTCACACAATACGTGAGAAAAACTTACCATCACATTATTACGAAAACACCCCTAGTCGTGGTCAACTTGACC
TTTCCCCACAAACACTCATTGCGGAGGAAACTTCCCGGCCACCGCCGCGCAACCGCCTCTAGCTTTAAATAATAACACCA
AAATATTCAAACCCGTGTTTTAACCGTCGAACAATCAAGAATCTCTCCACCATTACACGCATTCACATACTCATCTATAT
ATAATCTACCACTTCTTGAAAAGAATTTGCATCAGAAATCCATTTCCCAAATCTTCTAGATTCATATTCAAATCAAGAA

(B)

CTTCTTTAGTCTCTTTTTAATGCATTTGTTAACAACAACTTAAGATGTTAGATTGGAGAACTTGTGAATGTTGTTAATTC
TTGATTTTTCTATATCAACACTAAAGTTTTACATTATGTTTTATGTATTATTAGTTGTGTCTACATTGACATGTTCTAGT
AATGTGTTTATCTCCAAACAAAACAATTCAATTCTTGTATTTTTAATCAAACTATTTGCAAATGGTAAACGTCAATTAAT
TCGCCTTTATAATTTAATGGAAGATTACTTTTAAAAAGATGCAAGTTATTAGAATCAAAATAAAAACAAAGACAAAATAT
GTTAACTTGTAGAAAACACTTGGAAGTAAAAATCTGATGAGTTTCTCGTTCTCTAGAATATTGCTGGACCGGATTAGGTC
AATATTATTGGGCCAGATTAGATATTGAATTGTCGACGTTGCTTACGTTACGTTATATCTTGTTTAAGAATTAAACCTAT
CGACTTAGTCTTAATTAAGAAAACATTGCCTTAAATTCTCTGGTCTGCGACCGTTTTTTTGACCGTTAACCCCTAATTAA
AGAAACAAAATAATTATAGAAAGAGCACTGAAATGTGATTATTTTAACAGTACTCTTATGAGAAAATTCGTACTTTTTAG
TTTTTTTTTTGTACAAATCTCTAAGAAAAACACTACTACTAATTAAGAAACGTTTCAAACAATTTTATTTTCGTTGGCTC
ATAATCTTTCTTTCTCGGTCCGGGACTAACCGTTGGCAAAAAAAAAAAAAAAAGTTGACAATAATTATTAAAGCGTAAATC
ATACCTCTCAAATAAAAACTTGAATTTGGAAACAAAGACAACTAAAAAACTCGAATTTAAGAGAATTCCTAAAATCAAGT
GAAGTATCATCACTTGGTAAAATTTCATAACCGTTGGCTTCTATTTCTATGTGTGCCTTGGTTTGCAGGAGATAATATTT
CATTTCCAACCAATGATATTCGTACACATAGTCAAACAAATGTTTGTCTTTGTTATTATATTGAGAAAGAAACAAGAAAG
AGAGAGAGAGATAGATAAGACGAAGGAAGTGAAGCTTCCAAGCGCCCACCGTTAAAAATCTCGTGTGCAAGTTTCAAATA
CAAGTGGCCGGTGGTCTCCATAATTTGATCGTCATCCAATTAAAAAGGAAGAAAAAGCGTGTTTTATACAAGAAAACTCA
TTAAAATAAAAGTCCAAAATATCTAAACACTAATCTACCACGTCTATTACACACACACACACACACTTGATCTTAATTTA
TTTTCAAGATTCAAGAAAATACCCATTCCATTACCACAACTTGACCACACGCCTATATATAAAACATAAAAGCCCTTTCC
CCTATTTCTCATTCACTTTTCATTTCAAAAGTAAAACAAGACAAACAAAAAATACACTTAACCATTTATTTTTCTCTC

(C)

GTCAATCTACTAAATATATAGTCCATTGAGGTTTGACCATGATTTGCTCATAGTCATATTATTATTCTCTAGAGCTCACA
ATTCAAGACAAAAGGAATCTAAACAAGATTCAAATGAGCTTTAGTATAAGATCTTCACATAACGGTAACAATTAAGGTAT
CTAATAAGATTTTATTCCTACAAAACAATCTTTTTTTCCAACCAATAAGAGTTTATATATTGTTAGAGTCCTAACACTTT
TGTGAAGAAGAATGTTGGCTTTTATCGATCCATCTCAAACTGAATCTCACTAAGTTACCGACAATAATCCATAGTGTCAA
TGAATTGAATCTTACTATTTGACCTCATCTTTCGGTTGTAGGATTTCTAACACTTATCACTTGTTTTGAATCAACCCAAG
TTGGAGAAATTATAGGAGCCATAACAAGTTTGAGGCATTATATGGACTTCTATGGTATGAAACCTTAGGTTGTGAAGAAA
TTGATTTTTGAAAAAAAGCAATGATTAGTAGATGAAGTTACCATTGTTCTAAATTCAACAAATGGGAGTCGTCAAATATT
ATATTTACCACAATAACATTTCTCAATCCTTCTTCCTATATTTAAGACAGAGAGGTAGAAGAAGAAAACGTGTTTTAAAC
ATGGGGATGAGATATGTAACGAACCAATATACTAATATTTCCTTTCCTAGCTAGATCTAGAAGACTAATAATACTACTTC
TTCTAGATCTAAAGACTAAAAATATTTGTTTTTTTTTTCTAGATCTGAATCTCACACGTTTGATTCGTTTCAATTTCTAT
TTTCTTATTACTATTCTCGACTTGAAACACACCTATATATATAGAGAAACACTTTATACCATTTCTCATTCAAATCCTAA
ATCAAAAAAAGAAAGAACAATACTTGTAACTTATAACAAATACAATCTTTTCTTTCTCAAA

Figure A3.4. Locations of JA-responsive *cis*-elements within the promoters of (A) *CML37*, (B) *CML38*, and (C) *CML39*.

Purple boxes represent MYC binding sites; blue boxes represent the CGTCA motif.