The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.
YU-HUNG WEI

Dissecting the jasmonate signalling pathway in Arabidopsis thaliana

Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Molecular and Cell Biology

UNIVERSITY OF CAPE TOWN

December 2008
ACKNOWLEDGEMENT

Firstly, I’d like to thank Dr. Katherine Denby, for being my supervisor during all these years. The strength and success she showed in her career together with her friendly and caring attitude made her the perfect role model for me as a graduate student. No matter how daunting the task at hand was Dr. Denby’s interest in my project and her words of encouragement and advice guided me to the completion of my project. She was not only my scientific compass but also my friend. Katherine made life in the lab pleasant and was always available for academic or social support.

Secondly, I’d like to thank Dr. Thomas Eulgem, for being my mentor during the last stages of my project. Scientific research can be both invigorating and frustrating, especially so for an international student so far away from home. But Dr. Eulgem always managed to turn frustration from failed experiments into curiosity of the unknown with his patience and enthusiasm. I’d like to thank him especially for making me feel so welcomed in his laboratory.

Lastly, I’d like to thank my co-workers who have all become my dear friends for their participation in scientific discussions and social chit-chats; Shane Murray, Kirrily Peters, Maryke Carstens, Lara Donaldson, Colleen Knoth, Alexander Aevrard, Tokuji Tsuchiya, Mercedes Schroeder, Mindy Salus, Ayesha Baig, Linda Saetern and Mindy Nguyen. I would like to especially thank Lindsay Petersen and Nicolette Adams for their friendship and camaraderie through the trials of scientific life. They sat through the ups and downs of this scientific roller coaster ride screaming, crying and laughing besides me. I would also like to thank my family for their sacrifices and support in making all this possible.
DEDICATION

To my dearest mother....who gave me life and wisdom,

my loving husband...who I cannot live without,

and the new life I'm about to give any day now.
# TABLE OF CONTENTS

Table of Contents ........................................... i
Abstract ...................................................... iii

## CHAPTER 1: INTRODUCTION ................................ 1
1.1) Jasmonate biosynthesis .................................. 5
1.2) Jasmonates in development ............................ 15
1.3) Jasmonates in defence ................................. 17
1.4) Regulation of jasmonate biosynthesis .............. 19
1.5) Regulation of jasmonate responses ................ 20
1.6) Jasmonate-responsive palindromic promoters and transcription factors .......................... 25
1.7) Jasmonate-responsive GCC-box and GCC-box-like promoters and transcription factors .. 27

## CHAPTER 2: MATERIALS AND METHODS .................. 31
2.1) Plant material growth conditions .................... 32
2.2) Methyl jasmonate treatment ........................ 32
2.3) DNA isolation ........................................ 32
2.4) RNA isolation, blots and probes .................... 33
2.5) *Botrytis cinerea* infection ........................ 35
2.6) Promoter analysis .................................... 35
2.7) Promoter deletion construction ..................... 36
2.8) Cloning and bacterial transformation .............. 37
2.9) Plant transformation and transgenic plant selection ........................................ 38
2.10) Luciferase and Bradford assays ................... 39
2.11) Electrophoretic mobility Shift Assays ............ 40
2.12) Yeast one-hybrid “Bait” construction ............ 40
2.13) Library construction ................................ 41
2.14) Yeast one-hybrid assay ............................. 41
CHAPTER 3: UTILISING PYK10 AS A REPORTER TO ELUCIDATE THE JA SIGNALLING PATHWAY

3.1) Introduction 44
3.2) Results 48
3.2.1) The MeJA inducibility of PYK10 48
3.2.2) PYK10 expression is not induced in response to Botrytis cinerea infection 53
3.2.3) PYK10 expression in JA mutants 55
3.2.4) MeJA-responsive motifs in the promoter region of PYK10, COI-dependent MeJA-inducible genes and genes co-expressed with PYK10 58
3.2.5) PYK10 promoter-deletion analysis 68
3.3) Discussion 76

CHAPTER 4: THE HUNT FOR TRANSCRIPTION FACTORS 81

4.1) Introduction 82
4.2) Results 85
4.2.1) The specific DNA-binding activity of a W-box is MeJA inducible and dependent on its 3’ flanking sequence 85
4.2.2) Yeast one-hybrid: The Wbox::pOne-1 “bait” and the cDNA library “prey” 91
4.2.3) Screening the yeast one-hybrid libraries 99
4.3) Discussion 103

CHAPTER 5: CONCLUSION 106

CHAPTER 6: REFERENCES 115
Jasmonic acid and derivatives, such as the volatile methyl jasmonate, are collectively known as jasmonates. Jasmonates are important signalling molecules which have a developmental role, as well as a role in insect defence and disease resistance. COI1, an F-box protein, forms part of the receptor for jasmonic acid. COI1 is vital for the transduction of the jasmonate signal in gene regulation but the only targets of COI1 identified thus far are three JAZ repressor proteins which have been shown in vitro to bind the MYC2 transcription factor. Degradation of the JAZ proteins in response to jasmonic acid may release MYC2 enabling its binding to jasmonate-responsive promoters. However, MYC2 only regulates a subset of jasmonate-responsive promoters and the involvement of other transcription factors and specificity mechanisms of different jasmonates remain elusive. Gene expression is regulated by transcription factors that bind to specific *cis*-elements. To date, the G-box, C-box, T/G-box and GCC-box-like motifs have been found to confer jasmonate responsiveness to plant promoters. The only transcription factors shown to bind jasmonate-responsive *cis*-elements and regulate gene expression are AtMYC2, JAMYC2 and JAMYC10, binding G-box and T/G-box motifs, and ORCA2 and ORCA3, binding GCC-like-box motifs. The work outlined in this thesis aimed to further our understanding of jasmonate signalling by identifying specific promoter elements and associated transcription factors important for gene regulation by methyl jasmonate. A cDNA encoding a β-glucosidase, *PYK10*, was previously isolated using the differential display technique in an attempt to isolate methyl jasmonate-induced genes in *Arabidopsis thaliana*. β-glucosidases catalyse the breakdown of glucosinolates into glucose and sulphate releasing toxic by-products which play a role in plant defence against insects and pathogens. *PYK10* induction by methyl jasmonate was found to be inhibited in the jasmonate mutant, *coi1*, but not to require MYC2 expression. Through luciferase assays important genomic regions in the *PYK10* promoter directing methyl jasmonate expression of *PYK10* were delineated. A
W-box motif -975 bp upstream from the transcription start site was found to display specific DNA-binding activity with Arabidopsis nuclear proteins. Moreover, the observed DNA-binding activity was enhanced upon methyl jasmonate treatment and its specificity was attributed to both the W-box core sequence and flanking 3’ nucleotides. Tandem repeats of the identified W-box motif were used as bait in yeast one-hybrid assays but despite repeated efforts, an interacting transcription factor could not be isolated. This may indicate that an interacting transcription factor requires coupling with other proteins for binding to this W-box motif. W-box motifs are bound by WRKY transcription factors which have been predominately associated with the salicylic acid pathway, only a few WRKY transcription factors have been implicated in the jasmonate signalling pathway. This study demonstrates the important role of a W-box motif (and a as yet unidentified WRKY transcription factor) in the jasmonate signalling pathway of plants.
CHAPTER 1

INTRODUCTION
Living organisms are vulnerable to the environment and face constant pathogen attack. This vulnerability is enhanced in plants due to their sessile nature. It is therefore imperative that plants develop defence mechanisms that are both sensitive and effective. Plants are challenged by numerous microorganisms daily and for protection against the majority of microorganisms they are equipped with non-host resistance, the resistance of all member of a plant species against all members of a given parasitic species and basal resistance, a general early plant defence response (Heath, 2000b). Two type of non-host resistance exist which results from both preformed and inducible defence mechanisms: Type I which shows no symptoms, and Type II which results in cell death (Mysore and Ryu, 2004). At the onslaught of an attack the plant’s first line of defence are preformed defences such as its physiology in the form of a waxy cuticle which forms a physical barrier against penetrating microorganisms (Chassot and Métraux, 2005). The topography of stomatal guard cells can also affect fungal cell differentiation upon host infection (Hoch et al., 1987). Even if the invading microorganism overcomes these physical barriers it still has to combat the plant’s chemical arsenal, which consists of toxic products produced by the plant’s intrinsic secondary metabolism (Mikkelsen et al., 2003). Saponins are examples of these preformed secondary metabolites which are constitutively produced in healthy plants and act as antimicrobial compounds (Osbourn, 1996). Oat sad mutants deficient in saponins have compromised disease resistance (Papadopoulou et al., 1999).

The next line of defence is inducible plant defence and it differs from preformed defence due to its specific recognition of the invader and energy expenditure in actively producing compounds and proteins against it (Ferreira et al., 2007). Plants are able to recognise microorganisms by the perception of microbial- or pathogen-associated molecular patterns, such as flagellin and lipopolysaccharides of bacteria and chitin of fungi, via transmembrane pattern recognition receptors (Lipka et al., 2008). Resulting plant defences include rearrangement of plant actin microfilaments to protect plants against fungal infections as the application of cytochalasins, inhibitors of actin polymerisation, allows for fungal penetration and growth of haustoria in plant tissues (Kobayashi et al., 1997). Increases in the accumulation of phytoalexins, which are low-
molecular weight molecules that act as antimicrobial compounds, also occur. However, these compounds are synthesised de novo and are not to be confused with antimicrobial preformed compounds mentioned earlier which are synthesised from preformed precursors. Mutants that are phytoalexin deficient, such as the Arabidopsis pad3-1 mutant deficient in the phytoalexin camalexin, show increased susceptibility to pathogens, for example, Alternaria brassicicola, compared to wildtype (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997; Thomma et al., 1999a). Preformed and Inducible plant defences are associated with Type I non-host resistance and can efficiently deny an infection. Although no visible symptoms are observed several molecular events partake in this resistance such as the induction of pathogenesis-related (PR) genes. Defence-responsive genes such as those encoding for PR proteins will be expressed locally following necrosis and also systemically throughout the plant (Durrant and Dong, 2004) contributing to systemic-acquired resistance (SAR) to render long-lasting enhanced resistance against subsequent microbial invasions (Maleck and Dietrich, 1999; Lu et al., 2001). Type II non-host resistance is what the pathogen encounters after bypassing preformed and elicitor-induced plant defence responses. Specific elicitors are detected by the plant’s surveillance system leading to the hypersensitive response (HR) resulting in cell death and local containment of the infection (Heath, 2000a).

Plant induced defences not only serve non-host and basal resistance but also contribute to $R$ gene-mediated resistance, which results in an accumulation of PR proteins and the setup of SAR upon pathogen infection (Maleck and Dietrich, 1999), and rhizobacteria-mediated induced systemic resistance (ISR) upon infection by nonpathogenic, rhizosphere-colonizing bacteria (van Loon et al., 1998). ISR is effective against different types of pathogens and is phenotypically similar to SAR but do not involve the accumulation of PR proteins (Vallad and Goodman, 2004). $R$ gene-mediated resistance is often associated with the production of reactive oxygen species (ROS) and HR, and is activated upon recognition of pathogen avirulence factors (Avr) by corresponding $R$ gene products in the host plant (Glazebrook, 2005). This resistance is race-specific and adopted mainly by biotrophs, pathogens that require living cells to complete their life
cycles, whose Avr gene product is recognised by a corresponding R protein in the host plant resulting in local resistance and SAR (Baker et al., 1997). Whether the interaction between Avr and R proteins leading to plant defence activation is direct or indirect remains unclear as evidence for both interactions have been observed. Direct interaction was proposed as the “gene-for-gene” concept by Flor in the 50’s and has been supported by evidence over time (Flor, 1955; Tang et al., 1996; Jia et al., 2000; Dodds et al., 2006). The guard hypothesis was proposed much later (Dangl and Jones, 2001), also supported by evidence, and puts forward the idea that the R protein (the guard) recognises changes in another plant protein (the guardee) that interacts with Avr (Mackey et al., 2002; Shao et al., 2003).

The activation of these induced defences is mediated by signalling molecules which include the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). SAR is mediated by the SA signalling pathway (Durrant and Dong, 2004) but ISR on the other hand, does not involve the accumulation of PR proteins or SA but instead is mediated by the JA and ET signalling pathways (Pieterse et al., 1998). Other plant hormones are growth regulators such as auxin, gibberellin, cytokinin and abscisic acid (Hedden and Thomas, 2006). Abscisic acid and ET also respond to various biotic and abiotic stresses as do the hormones brassinosteroids, SA and JA (Davies, 2004). For the purpose of this dissertation, the focus will be on JA signalling.

Jasmonates are structural and functional analogues of oxylipin signalling molecules found in mammals. Oxylipins are biologically active molecules derived from oxygenated polyunsaturated fatty acids called eicosanoides in mammals which include prostaglandins (Stanley, 2006; Kazan and Manners, 2008). The fragrant, volatile methyl derivative, methyl jasmonate was discovered in as early as the 60’s in jasmine flowers and drew the interest of scientists in the 80’s by its ability to retard root growth and promote senescence (Ueda and Kato, 1980; Yamane et al., 1981).
1.1) Jasmonate biosynthesis

Jasmonic acid (JA), its precursors and its derivatives, such as the volatile methyl jasmonate (MeJA), are cyclopentone derivatives synthesised via the octadecanoid pathway and are collectively known as jasmonates (JAMs). Elucidation of the octadecanoid pathway is attributed to Vick and Zimmerman who pioneered much of the research in the 1980’s (Vick and Zimmerman, 1983; Farmer et al., 1998). The term “octadecanoid pathway” refers to $\text{C}_{18}$ $\alpha$-linolenic acid (18:3), also known as octadecatrienoic acid. Membrane-bound fatty acid desaturases convert oleic acid (18:1) to linoleic acid (18:2) and then to linolenic acid (18:3) in cellular lipid bilayers such as the chloroplast membrane where the early steps of the octadecanoid pathway takes place (Somerville and Browse, 1991) (Fig. 1). In plants, 18:2 and 18:3 are oxygenated at C-9 and C-13, respectively, and these products are precursors for the formation of oxylipins which include JA and other biologically active compounds. Three $\omega$3 fatty acid desaturases (FAD) are present in Arabidopsis, FAD3, FAD7 and FAD8. FAD3 is an endoplasmic reticulum-localised desaturase that converts dienoic fatty acid 18:2 to trienoic fatty acid 18:3 while FAD7 and FAD8 are chloroplast-localised desaturases that convert both 16:2 and 18:2 to 16:3 and 18:3, respectively (Howe, 2004). Functional redundancy exists between these enzymes and single mutations produced an undetectable change in trienoic fatty acid accumulation, however, the triple mutant $fad3fad7fad8$ produced no trienoic fatty acids and consequently no JA (McConn and Browse, 1996; Wallis and Browse, 2002).

Oxylipins originate from 18:3 released from the chloroplast membrane by lipid-hydrolysing enzymes belonging to at least 5 different families (Dormann, 2005): (1) phospholipase A$_1$ which cleaves the acyl moiety in the $sn1$ position; (2) phospholipase A$_2$ which cleaves the acyl moiety in the $sn2$ position; (3) patatin-like acyl hydrolases which have phospholipase and glycolipase activities; (4) DAD (delayed anther dehiscence)-like lipases which are involved in phospholipid and galactolipid acyl hydrolysis; and (5) SAG (senescence-associated gene) 101-like acyl hydrolases. However, only the tomato wound-induced phospholipase (PLA)$_2$ A$_2$, PLA$_2$ (Narvaez-
Vasquez et al., 1999), an Arabidopsis DAD-like phospholipase A₁, DAD1 (Ishiguro et al., 2001), and an Arabidopsis patatin-like acyl hydrolase, AtPLA1 (Yang et al., 2007), have been linked to JA biosynthesis. Evidence for this was provided by the phenotype of the dad1 (delayed anther dehiscence 1) mutant where a decrease of JA content in the flower buds was observed compared to wildtype in addition to defects in flower opening, anther dehiscence and pollen maturation (Ishiguro et al., 2001). The mutant Atpla1-1 also showed a decrease in basal JA levels and higher susceptibility to Botrytis cinerea compared to wildtype (Yang et al., 2007).

Localisation studies have shown that the three initial reactions of the octadecanoid pathway are catalysed by lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) in the chloroplast (Laudert et al., 1996; Froehlich et al., 2001; Stenzel et al., 2003) (Fig. 1). 18:3 is the substrate for 13-LOX which catalyses C-13 oxygenation to form (13)-hydroperoxyoctadecatrienoic acid (13-HPOT), the substrate for JA biosynthesis (Wasternack and Hause, 2002). In Arabidopsis, six genes encoding 13-LOX (AtLOX1 to AtLOX6) are present, of which LOX2 appears to be involved in JA biosynthesis in response to wounding (Bell et al., 1995) and LOX3 and LOX4 to senescence (He et al., 2002). AOS is encoded by a single gene copy in Arabidopsis which converts 13-HPOT to a highly unstable allene oxide that either decays into α- and γ-ketols or racemic cis-12-oxophytodienoic acid via a non-enzymatic reaction or is converted to the enantiomerically pure cis-(+)-12-oxophytodienoic (OPDA) by AOC. The aos mutant is male sterile and when compared to wildtype, exhibits a decrease in endogenous JA levels and VSP and LOX gene expression upon wounding (Park et al., 2002). AOC is of particular importance as it produces the first pathway intermediate having the characteristic pentacyclic ring structure of JAMs. It is encoded by 4 genes in Arabidopsis, AOC1-4, whose gene expression levels are increased upon wounding (Stenzel et al., 2003).

OPDA is the final product of the chloroplast-localised reactions and further steps of the JA biosynthesis pathway occur in the peroxisome (Fig. 1). How OPDA is transported from the chloroplast to the peroxisome is unknown but Theodoulou et al. in 2005
observed a decrease in basal JA levels in a fertile cts mutant, whose wildtype gene encodes the ABC transporter COMATOSE, compared to wildtype even after wounding (Theodoulou et al., 2005). They also proposed an alternative leaky pathway in which OPDA enters the peroxisome via ion trapping hence avoiding male-sterility in the cts mutant. Once inside the peroxisome, OPDA is converted by OPDA reductase (OPR) from the cyclopentenone OPDA to cyclopentanones such as JA and MeJA. OPR is encoded by a small gene family of five members in Arabidopsis, of which three have been characterised (OPR1-3) (Biesgen and Weiler, 1999; Schaller et al., 2000). Only OPR3 shows activity towards OPDA and is targeted to the peroxisome while OPR1 and OPR2 are found in the cytosol (Strassner et al., 2002). OPR3 is thus the only OPR currently known to be involved in JA biosynthesis as mutants defective in OPR3, opr3 and dde1 (delayed dehiscence 1), are JA deficient, male sterile and their function cannot be substituted by other OPR isoforms (Sanders et al., 2000; Stintzi and Browse, 2000). Regulation of OPR activity is important as OPDA displays signalling roles distinct from JA (Stintzi et al., 2001).

The next and final step in the peroxisome leading to the production of JA in the octadecanoid pathway is three rounds of β-oxidation on the cyclopentanone OPC8. Another cyclopentenone, dinor OPDA (dnOPDA) from a parallel hexadecanoid pathway could also be another substrate for OPR in the formation of cyclopentanones (Weber et al., 1997). However, β-oxidation acts on CoA esters and it has been suggested that 2 peroxisomal 4-coumarate:CoA ligase (4-CL)- like enzymes that have a strong preference for OPDA and dnOPDA in vitro prepare JA intermediates for β-oxidation (Schneider et al., 2005). The β-oxidation step is carried out by three core enzymes, an acyl-CoA oxidase (ACX), a multifunctional protein (MFP) and a L-3-ketoacyl-CoA thiolase (KAT) encoded by 6, 2 and 3 genes in Arabidopsis, respectively, which catalyse the repeat removal of acetate units from acyl-CoA. In Arabidopsis, antisense mutants for ACX1 and KAT2 showed reduced JA levels and reduced expression of JA-related genes upon wounding (Castillo et al., 2004). Also, recombinant ACX1A has been shown to metabolise OPDA-CoA in vitro (Li et al., 2005). Delker et al. in 2007 observed reduced JA levels in response to wounding in the aim1 mutant, defective in one of the
two MPF proteins, and the *pex6* mutant, defective in peroxisomal function (Richmond and Bleecker, 1999; Zolman and Bartel, 2004; Delker et al., 2007). In tomato, an *acx1* mutant is JA deficient and upon wounding, has reduced expression of proteinase inhibitor (PI) genes and increased susceptibility to the tobacco hornworm (*Manduca sexta*) (Li et al., 2005). The reduced wound-induced JA accumulation seen in the single *acx1* mutant was totally abolished in the *acx1/5* double mutant, which also displayed low pollen viability and fecundity, suggesting redundant function of these enzymes (Schilmiller et al., 2007).
Figure 1: The jasmonic acid biosynthesis pathway. Pathway intermediates: $\alpha$-LA, $\alpha$-Linolenic acid; 13-HPOT, 13(S)-hydroperoxy linolenic acid; AO, 12,13(S)-epoxy-9(2),11,15(Z)-octadecatrienoic acid; OPDA, 12-oxo-phytodienoic acid; OPC8, 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid. Pathway enzymes are shown in red: LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, OPDA reductase 3.
The final product of the JA biosynthetic pathway proposed by Vick and Zimmerman is (+)-7-iso-JA (Vick and Zimmerman, 1983) but 7 metabolic routes have been shown for JA according to products identified in plants to date (Fig. 2). However, few of the enzymes responsible for these conversions have been identified.

(1) Amino acid conjugation: JA conjugated to various amino acids has been found in higher plants such as barley, tomato and Arabidopsis (Kramell et al., 1995; Hause et al., 2000; Staswick and Tiryaki, 2004). JAR1, an enzyme encoding JA-amino synthetase, was first identified by Staswick et al. in 2002 and is the defective gene responsible for the JA insensitive jar1 mutant (Staswick et al., 1992; Staswick et al., 2002). They later showed that recombinant JAR1 was able to form JA-isoleucine (JA-Ile), -valine, -leucine and -phenylalanine in vitro and that only JA-Ile was able to complement the jar1-1 mutant (Staswick and Tiryaki, 2004). By expressing wildtype JAR1 in jar1-1 which contained low levels of JA-Ile, they were able to restore JA sensitivity to wildtype levels by raising the endogenous level of JA-Ile.

(2) 1-aminocyclopropane-1-carboxylic acid (ACC) conjugation: ACC, a metabolic precursor of ET, was first found conjugated to JA in Arabidopsis by Staswick and Tiryaki in 2004 (Staswick and Tiryaki, 2004). In this study, JA-ACC was found at higher levels in jar1-1 than wildtype plants, the opposite situation to another amino conjugate JA-Ile. Even though JA-ACC is an ineffective root inhibitor and therefore probably not a biologically active JA like JA-Ile, it may act as a control point for the release of active JA and ACC.

(3) Methylation: The volatile methyl ester of JA is ubiquitous throughout the plant kingdom (Hamberg and Gardner, 1992). Seo et al. in 2001 isolated a gene encoding S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) which when expressed constitutively in Arabidopsis led to increased resistance to B. cinerea, increased endogenous MeJA levels and increased expression of JA-responsive genes. JA levels remained constant (Seo et al., 2001). Biologically active MeJA is a compound released in copious volumes when plants are damaged and may act as a volatile signal in plant defence as in Artemesia tridentate (Baldwin et al., 2002). Endogenous levels of MeJA
accumulate in wounded stems of soybean seedlings and exogenous application of MeJA lead to increased expression of wound-responsive genes such as chalcone synthase (CHS) and vegetative storage protein (VSP) (Staswick et al., 1991; Creelman et al., 1992).

(4) Decarboxylation to cis-jasmone: cis-Jasmone is another volatile compound, like MeJA, and has been shown to be an important active compound in blackcurrant Ribes nigrum which not only prevents herbivory by lettuce aphids but also attracts predators of these grazers (Birkett et al., 2000). Interestingly, MeJA was excluded from the list of active compounds in this volatile profile which suggests different roles in signalling for these 2 volatile JA metabolites.

(5) Hydroxylation: Hydroxylation of JA at C11 or C12 to 11-hydroxyjasmonic acid (11-OH-JA) or 12-hydroxyjasmonic acid (12-OH-JA), respectively, and its subsequent O-glycosylation to 12-hydroxyjasmonoylglucosid (12-O-Glc-JA) or sulfonation to 12-hydroxyjasmonic acid sulphate (12-HSO₄-JA) occurs in many plant species (Miersch et al., 2008). 12-OH-JA was first discovered as a tuber-inducing compound in the potato, thus commonly known as tuberonic acid (Yoshihara et al., 1989). Wounding of tomato leaves results in the JA-dependent accumulation of 12-OH-JA and its glycosylated and sulfonated derivatives (Miersch et al., 2008). Also, 12-OH-JA and MeJA were both able to induce a gene for hydroxyjasmonate sulfotransferase (AtST2a) in Arabidopsis and cause an increase in endogenous 12-HSO₄-JA (Gidda et al., 2003). Despite their JA-dependent accumulation, these compounds have not been shown to be bioactive as they have no effect on processes that are usually affected by bioactive JAMs such as the inhibition of root growth, tendril coiling in Bryonia dioica and induction of JA-responsive genes (Blechert et al., 1999; Miersch et al., 1999; Gidda et al., 2003; Miersch et al., 2008). Hydroxylation and sulfonation may therefore serve as regulators of JA levels in the plant.

(6) Formation of glucose and gentiobiose esters of JA: Jasmonoyl-1-β-glucose, jasmonoyl-1-β-gentiobiose and hydroxyjasmonoyl-1-β-glucose were found to accumulate in tobacco Bright Yellow-2 suspension cells after exogenous application of JA and MeJA (Swiatek et al., 2004). However, they have not been
found to occur naturally in plants. These compounds can inhibit Arabidopsis root growth to the same extent as JA, have high solubility in water and are able to release free JA (Swiatek et al., 2004). Therefore they may act as reserves or a transport system for JA.

(7) Reduction at the keto group of JA to form cucurbic acid: Cucurbic acid is a naturally occurring JA metabolite first identified in broad bean *Vicia faba* (Miersch et al., 1989; Sembdner and Parthier, 1993). Like MeJA but to a lesser extent, it is able to affect plant development by inhibiting anther extrusion in barley (Honda et al., 2006).
Figure 2: Different metabolites of jasmonic acid. The carboxylic side chain can be glucosylated (+ Glucose) to form jasmonoyl-1-β-glucose (JA-O-Glc), conjugated with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to form JA-ACC, methylated (+ CH₃) by S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) to form MeJA, decarboxylated (-CO₂) to form cis-jasmone or conjugated with amino acids such as isoleucine (+ Ile) by JA amino acid synthetase (JAR1). The pentenyl side chain can be hydroxylated (+ OH) and subsequently glucosylated to form 12-hydroxyjasmonoylglucosid (12-O-Glc-JA) or sulfonated (+PAPS) by hydroxyjasmonate sulfotransferase (St2a) to form 12-hydroxyjasmonic acid sulphate (12-HSO₄-JA). The pentenone ring can be reduced to form cucurbic acid. The solid red ovals represent enzymes that have been cloned in Arabidopsis while the open ovals represent unknown enzymes.
Table 1: Mutants affected in JA biosynthesis and signalling.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Altered process</th>
<th>Affected gene and its corresponding AGI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Defective in JA biosynthesis and metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fad3fad7fad8</td>
<td>α-LA formation</td>
<td>FAD3; At2g29980</td>
<td>(McConn and Browse, 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FAD7; At3g11170</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FAD8; At5g05580</td>
<td></td>
</tr>
<tr>
<td>dad1</td>
<td>α-LA formation</td>
<td>DAD1; At2g44810</td>
<td>(Ishiguro et al., 2001)</td>
</tr>
<tr>
<td>Atpl1-1</td>
<td>α-LA formation</td>
<td>ATPL1; At1g61850</td>
<td>(Yang et al., 2007)</td>
</tr>
<tr>
<td>aos</td>
<td>OPDA conversion</td>
<td>AOS; At5g42650</td>
<td>(Park et al., 2002)</td>
</tr>
<tr>
<td>dde1</td>
<td>OPDA conversion</td>
<td>OPR3; At2g06050</td>
<td>(Sanderson et al., 2000)</td>
</tr>
<tr>
<td>opr3</td>
<td>OPDA conversion</td>
<td>OPR3; At2g06050</td>
<td>(Stintzi and Browse, 2000)</td>
</tr>
<tr>
<td>acx1/acx5</td>
<td>β-oxidation</td>
<td>ACX1; At4g16780</td>
<td>(Schilmiller et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACX5; At2g35630</td>
<td></td>
</tr>
<tr>
<td>aim1</td>
<td>β-oxidation</td>
<td>MPF; At4g29010</td>
<td>(Richmond and Bleeker, 1999)</td>
</tr>
<tr>
<td>cts</td>
<td>β-oxidation</td>
<td>COMATOSE; At4g39850</td>
<td>(Theodoulou et al., 2005)</td>
</tr>
<tr>
<td>pex6</td>
<td>Phenylalanine</td>
<td>ATPase; At1g03000</td>
<td>(Zolman and Bartel, 2004)</td>
</tr>
<tr>
<td>jar1</td>
<td>JA conjugation</td>
<td>JAR1; At2g46370</td>
<td>(Staswick and Tiryaki, 2004)</td>
</tr>
<tr>
<td><strong>JA insensitive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mpk4</td>
<td>Altered JA- and SA-responsive gene expression</td>
<td>MPK4; At4g01370</td>
<td>(Petersen et al., 2000)</td>
</tr>
<tr>
<td>jin1</td>
<td>Transcription factor</td>
<td>MYC2; At1g32640</td>
<td>(Berger et al., 1996; Lorenzo et al., 2004)</td>
</tr>
<tr>
<td>cot1</td>
<td>Proteolysis via SCF</td>
<td>COI1; At2g39940</td>
<td>(Fey et al., 1994; Xu et al., 2002)</td>
</tr>
<tr>
<td>jai4</td>
<td>Proteolysis via SCF</td>
<td>SG1T1b; At4g11260</td>
<td>(Lorenzo et al., 2004)</td>
</tr>
<tr>
<td>axr1</td>
<td>Proteolysis via SCF</td>
<td>AXR1; At1g05180</td>
<td>(Tiryaki and Staswick, 2002)</td>
</tr>
<tr>
<td><strong>Constitutive JA responses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cev1</td>
<td>Cell wall synthesis</td>
<td>CeS3; At5g05170</td>
<td>(Ellis et al., 2002)</td>
</tr>
<tr>
<td>cet1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(Hilpert et al., 2001)</td>
</tr>
<tr>
<td>joe1</td>
<td>Increased LOX expression</td>
<td>Unknown</td>
<td>(Jensen et al., 2002)</td>
</tr>
<tr>
<td>joe2</td>
<td>Increased LOX expression</td>
<td>Unknown</td>
<td>(Jensen et al., 2002)</td>
</tr>
</tbody>
</table>
1.2) Jasmonates in development

The most obvious response of plants to exogenous JA application is the inhibition of root growth along with stunted growth and anthocyanin production (Staswick et al., 1992). Various genetic screens have used JA inhibition of root growth for isolating mutants defective in JA signalling such as jin1, jar1 and coi1 (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996; Sagi and Fluhr, 2001). Mutants that have constitutive JA signalling such as cev1 and cet1 have wildtype phenotypes (Ellis and Turner, 2001; Hilpert et al., 2001). Despite the insensitivity of these mutants to JA inhibition of root growth, only coi1 is male sterile (Xie et al., 1998). JAMs are usually applied above the physiological concentration and do not target specific cell types, therefore, mutants defective in JA biosynthesis and signalling are therefore better tools for analysing roles of JA in development (Table 1). JA biosynthesis mutants produce no JA and as a result dad1 which hydrolyses lipids, fad3fad7fad8 which produces no trienoic fatty acids, and opr3 and dde2-2 which are responsible for the reduction of OPDA, are male sterile (McConn and Browse, 1996; Stintzi and Browse, 2000; Ishiguro et al., 2001). Conversely, mutants that have increased expression of the JA biosynthesis LOX2 gene such as joe1 and joe2 have increased accumulation of anthocyanins and increased inhibition of root growth, respectively (Jensen et al., 2002).

JA plays a vital role in male gametophyte development as the development of viable pollen, correct anther dehiscence and anther filament elongation were affected in fad3-2fad7-2fad8, opr3 and dde1 whose sterility could be reversed by exogenous JA treatment (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000). The coi1 mutant is male sterile but interestingly, its mutant homologue in tomato, jai1, has reduced pollen viability but is predominantly female-sterile (Li et al., 2004b), suggesting JA plays different roles in different species. In addition to its role in female organ development, JAI1 is also important in trichome development (Li et al., 2004b).

Senescence is the last stage of the plant life cycle in which JA has an obvious role as reviewed by Parthier in 1990 where he highlighted observed senescence symptoms
after JA treatment such as decreases in photosynthetic and house-keeping protein activity and increases in proteolytic activity (Parthier, 1990). In 2001, He et al. identified several SAGs using the enhancer-trap system of which SAG12, encoding a cysteine protease, was later shown to be JA inducible (He et al., 2001; He et al., 2002). The onset of senescence in Arabidopsis correlates with the induction of several JA biosynthetic genes which results in an increase in JA levels (He et al., 2002). With this in mind, it is therefore not surprising for He et al. to have found SAG101, encoding an acyl hydrolase, in 2002 with his enhancer trap system for SAG12 could provide 18:3 substrate for JA production (He and Gan, 2002). Other evidence which links JA to senescence include the senescent phenotype exhibited by transgenic barley constitutively over-expressing LOX2 which resulted in increased levels of JA (Sharma et al., 2006). Conversely, a negative regulator of senescence such as Oryza sativa delay of the onset of senescence (OsDOS), a novel nuclear-localised CCCH-type zinc finger protein in rice, also negatively regulates the JA pathway again linking the JA pathway to senescence (Kong et al., 2006). In Arabidopsis, exogenous application of JA induces premature senescence and requires COI1 as the coi1 mutant is insensitive to this JA-induced premature senescence (He et al., 2002). A mutation in ORE9, a gene encoding an F-box protein similar to COI1, also exhibits delayed senescence (Woo et al., 2001; He et al., 2002).

JA analogues such as OPDA have been linked to tendril coiling in B. dioica as tendril coiling was induced upon exogenous application of OPDA and endogenous levels of OPDA accumulated transiently during the progression of mechanically stimulated tendrils (Falkenstein et al., 1991; Blechert et al., 1999). Tuberisation in potato was linked to tuberonic acid 12-OH-JA after radioactivity was found to accumulate in tubers as a result of exogenously applied radioactively-labelled JA being metabolised to 12-OH-JA (Yoshihara et al., 1996). Anti-sense suppression of a potato LOX, POTLX-1, compromised JA biosynthesis by reducing LOX activity and hence, resulted in retarded tuber formation (Kolomiets et al., 2001), thus highlighting specific roles played by JA analogues.
1.3) Jasmonates in defence

Enemies of the plant have to overcome preformed and inducible plant defences in order to set up a successful attack. The JA signal transduction pathway plays an important role in inducible plant defences via direct and indirect defence responses. Direct defence involves the synthesis of phytochemicals/antimicrobial peptides induced by the JA pathway that negatively interfere with the feeding habits, reproduction or development of invaders (Walling, 2000; Chen et al., 2005). Such phytochemicals include proteinase inhibitors and polyphenol oxidases detrimental to herbivores (Constabel et al., 1995; Chen et al., 2005), and antimicrobial peptides such as thionins and defensins which act against pathogens (Epple et al., 1995; Penninckx et al., 1996). JAMs are required for direct defence as mutants defective in JA biosynthesis such as acx1/5 and fad3fad7fad8 or signalling such as coi1 are vulnerable to herbivore (Stintzi et al., 2001; Schilmiller et al., 2007) and pathogen attacks (Vijayan et al., 1998; Ellis et al., 2002a; Thaler et al., 2004). Conversely, mutants that have constitutive activation of the JA signalling pathway such as cev1 or constitutive activation of the JA biosynthesis JMT gene are more resistant to pathogen attack (Seo et al., 2001; Ellis et al., 2002a; Ellis et al., 2002b). OPDA is able to induce a set of wound-related genes (Taki et al., 2005) and an AOS mutant (dde2), which is deficient in both OPDA and JA is more susceptible to B. cinerea than the opr3 mutant, which is deficient only in JA, suggesting a potential role of OPDA in defence signalling against Botrytis infection (Raacke et al., 2006).

Indirect defence by JA usually occurs via a tritrophic interaction involving the host plant, the herbivore and the natural predator of the herbivore. Elicitors found in the herbivore’s oral secretions induce the production of volatile organic compounds and extrafloral nectar via the JA signalling pathway, which the predator uses to locate its prey (Kessler and Baldwin, 2002; Heil, 2008). For example, damaged wildtype tomato plants are able to produce more volatile compounds than damaged mutant def1, which is JA-deficient (Thaler et al., 2002). Def1 mutants supported a higher survival rate of
herbivorous *Spodoptera exigua* caterpillars than wildtype plants as it was less effective at attracting their predatory mite, *Phytoseiulus persimilis*. However, attraction of *P. persimilis* can be restored to wildtype levels after JA treatment (Thaler et al., 2002).

JAMs also play a role in the systemic immunity of plants where they induce protective measures in systemic or distal parts of the plant after the initial assault against further attacks. This ISR requires both JA and ET signalling pathways. *PDF1.2* is a JA-responsive gene encoding an antifungal plant defensin whose protein levels increase concomitantly with JA levels both in *Alternaria brassicicola*-infected and non-infected systemic Arabidopsis leaves. There is also an increase in mRNA levels which is absent in the JA mutant *coi1* and ET mutant *ein2* (Penninckx et al., 1996). Rhizobacteria-mediated ISR has also been demonstrated to require JA and ET signalling pathways as ISR mediated by Rhizobacteria *Pseudomonas fluorescens* protected Arabidopsis plants against subsequent infection by the bacteria *Pseudomonas syringae pv tomato*. This induced protection by *P. fluorescens* could be simulated by the exogenous application of MeJA and ACC but both treatments were unable to protect subsequent infection by *P. syringae pv tomato* in the JA mutant *jar1* and ET mutant *etr1* (Pieterse et al., 1998). Recently, JAMs have also been surprisingly implicated in the initiation of SAR, which scientists previously thought involves exclusively the SA signalling pathway (Truman et al., 2007). Truman et al. in 2007 provided evidence of JA presence in the phloem, increased expression of JA biosynthetic genes in systemic leaves together with accumulation of JA levels after treatment with an avirulent strain of *P. syringae*. This together with a decrease in SAR in the JA mutant *jin1*, provided convincing evidence that JAMs play a central role in systemic resistance (Truman et al., 2007).

In 1983, Baldwin and Schultz showed data for the support of interplant communication via volatile compounds by wounding (Baldwin and Schultz, 1983). MeJA is such a compound that the plant uses for “warning” surrounding plants of potential attacks (Farmer, 2001). Farmer and Ryan in 1990 provided evidence of such interplant communication when they demonstrated the ability of MeJA-treated tomato leaves to induce synthesis of proteinase inhibitors in untreated leaves of surrounding tomato
plants. Interestingly, they also showed that communication via MeJA can also occur interspecifically when MeJA produced by leaves of sagebrush *Artemisia tridentata* induced the synthesis of proteinase inhibitors in leaves of surrounding tomato plants (Farmer and Ryan, 1990; Farmer, 2001). However, instead of showing neighbourly love MeJA can also act as an allelopathic agent by a plant to promote its own presence in an environment. *A. tridentate* uses MeJA to inhibit the germination of seeds of the tobacco species *Nicotiana attenuate* in order to exert its dominance in the Great Basin Desert (Preston et al., 2002).

Wounding of plant tissues leads to the production of JAMs, which act as long-range signals either between plants or within the plant itself, for the induction of systemic immunity. This phenomenon is well illustrated in tomato by a classic grafting experiment showing wound-induced proteinase inhibitor (*PI-II*) gene expression in distal regions using wildtype, JA biosynthetic mutant *spr2* and JA response mutant *jai1* (Li et al., 2002). In this experiment, the authors were able to show that by grafting wildtype stock leaves onto *spr2* scions, which lacked wound-inducible systemic *PI-II* expression due to its defect in JA biosynthesis but responded similarly to MeJA as wildtype, they were able to induce systemic *PI-II* expression in *spr2* scions by wounding wildtype stock leaves. When the authors grafted wildtype stock leaves onto *jai1* scions, which lacked wound-inducible systemic *PI-II* expression due to its defect in JA signalling and MeJA insensitivity, they were unable to induce systemic *PI-II* expression in *jai1* scions by wounding wildtype stock leaves. To further demonstrate JA as the long-range signalling molecule in the wound response, they grafted *jai1* stock leaves, which had wildtype JA levels, onto *spr2* scions, which had wildtype JA sensitivity, and were able to induce systemic *PI-II* expression in *spr2* scions by wounding *jai1* stock leaves.

1.4) Regulation of jasmonate biosynthesis

Substrate availability in the octadecanoid pathway appears to regulate JA biosynthesis as bioactive JA and OPDA are formed only after wounding in spite of the constitutive
presence of biosynthetic enzymes such as LOX, AOS and AOC in developed Arabidopsis rosette leaves (Stintzi et al., 2001; Stenzel et al., 2003). Even in transgenic plants overexpressing AOS constitutively there is no increased level of JA compared to wildtype, which only occurs upon wounding (Park et al., 2002). There is a positive feedback on JA biosynthesis by JA in Arabidopsis as all JA biosynthetic genes could be induced by exogenous JA application (Stenzel et al., 2003; Castillo et al., 2004). Tissue specificity may also regulate JA biosynthesis as specific spatial patterns of AOS activity were detected in leaves and floral organs of transgenic Arabidopsis and tobacco plants harbouring the GUS gene under the control of the AOS promoter after treatment with JA, OPDA and coronatine, a mimic of JA, suggesting local synthesis of JA (Kubigsteltig et al., 1999).

1.5) Regulation of jasmonate responses

As noted, the JA signalling pathway plays an important role in the life of a plant from development to defence in activating appropriate genes in response to internal and external stimuli. A defective component in the pathway will alter the JA response, sometimes with detrimental effects. COI1 is such a component because it is necessary for most known JA responses. coi1 mutants are defective in wound-induced gene expression, reproductive development and resistance to pathogens and insects (Feys et al., 1994; Xie et al., 1998; Devoto et al., 2005). COI1 encodes an F-box protein that is part of the SCF^{COI1} complex, an E3 ubiquitin ligase, which recruits proteins for degradation via the 26S proteasome (Devoto et al., 2002). In vitro binding assays led scientists recently to the discovery of JAZ (Jasmonate ZIM-domain) repressor proteins as targets of COI1 that have remained elusive for many years (Chini et al., 2007; Thines et al., 2007). In the presence of JA-Ile JAZ proteins through interaction with COI1 may indirectly repress JA-responsive genes by negatively regulating JA-inducible transcription factors such as MYC2 (Chini et al., 2007; Thines et al., 2007). Regulation of JA-responsive genes is therefore necessary for the desired response and can be achieved via direct interactions by TFs such as MYC2.
A transcription factor (TF) is a protein that binds to a specific DNA sequence, termed cis-element, via its DNA-binding domain and either activates or represses gene transcription (Latchman, 1997). DNA has several levels of structural organisation, with the basic unit being the nucleosome, which has 146 base pairs (bp) of DNA wrapped around a histone octamer (Garrett and Grisham, 1995). Nucleosomes affect the accessibility of TFs to DNA. DNA access by TFs can be gained by chromatin remodelling, which involves histone acetylation and nucleosome disruption (Singh, 1998). Proteins that affect gene regulation via interactions with transcription activators or repressors are called transcription coactivators or corepressors, respectively. Coactivators and corepressors do not bind DNA directly but they have the potential to affect gene regulation via chromatin remodelling and protein-protein interactions (Fig. 3).

**Figure 3:** Model of transcription activation via direct-DNA binding of a transcription activator to a cis-element and indirect-DNA binding of a transcription coactivator. Interaction between the transcription activator and co-activator remodels DNA for access of the transcription machinery. Communication between the transcription coactivator and the RNA polymerase II (Pol II) transcription-initiation complex via the
mediator promotes Pol II transcription-initiation binding to the core promoter (TATA box and transcription start site) thereby initiating gene expression.

Transcription of eukaryotic genes to synthesise RNA begins by the binding of transcription activators (TFs) to cis-elements and through interaction with co-activators remodel the chromatin to expose the promoter (Garrett and Grisham, 1995). An activator will promote the assembly of RNA polymerase II (Pol II) transcription-initiation complex to the core promoter element via interaction with a mediator (Cantin et al., 2003). The core promoter element consists of the transcription start site and the TATA box and is sufficient for directing transcription initiation. The Pol II transcription-initiation complex consists of Pol II and proteins IID, IIB, IIF, IIE and IIH, collectively termed general TFs. The regulation of transcription is, however, carried out by TFs, either alone or in complex with coactivators or corepressors and controls the assembly of the Pol II transcription-initiation complex or the catalytic efficiency of Pol II during the initiation, elongation or termination process (Nikolov and Burley, 1997). In addition to the regulation displayed by COI1 and JAZ proteins outlined earlier transcription regulation plays an important role in JA responses.

TFs contain DNA-binding domains that recognise specific DNA sequences and are therefore grouped into families according to their different forms of DNA-binding domain. In plants, there are currently 64 families of TFs according to the plant TF database DATF consisting of members belonging to the superclasses zinc-coordinating, basic domains, helix-turn-helix and β-scaffold (Guo et al., 2005). There are some TF families that are found exclusively in plants and these include TFs from the AP2/ERF, NAC, ARF and trihelix DNA binding protein families (Riechmann et al., 2000).

Not much is known about TFs involved in the regulation of JA-responsive genes and only a few have been isolated. TFs identified in JA- and ET-dependent gene activation are from one of the largest plant TF families, the APETALA2/ET Response Factor (AP2/ERF) family, and include ERF1, Octadecanoid-Responsive Arabidopsis AP2/ERF 59 (ORA59), Octadecanoid-Responsive Catharanthus AP2-domain 2 and 3 (ORCA2
and ORCA3). JA and ET signalling pathways co-operatively induce ERF1 in response to necrotrophic pathogen attack and transgenic Arabidopsis plants constitutively expressing \( ERF1 \) show increased defence gene expression levels of \( PDF1.2, b-CHI \) and increased resistance to pathogens \( B. \) \( cinerea \) and \( Plectosphaerella \) \( cucumerina \) compared to wildtype (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). Similarly to ERF1, transgenic Arabidopsis plants overexpressing \( ORA59 \) also showed increased gene expression levels of \( PDF1.2, HEL, b-CHI \) and increased resistance to \( B. \) \( cinerea \) compared to wildtype (Pre et al., 2008). Conversely, transgenic plants where \( ORA59 \) is silenced showed decreased expression levels of \( PDF1.2, HEL \) and increased susceptibility to \( B. \) \( cinerea \) compared to wildtype (Pre et al., 2008). ORCA2 and ORCA3 in \( Catharanthus \) \( roseus \) regulate secondary metabolic biosynthetic genes involved in terpenoid indole alkaloid (TIA) biosynthesis, many of which are JA-responsive (Memelink et al., 2001). Both \( ORCA2 \) and \( ORCA3 \) are JA and elicitor inducible (Menke et al., 1999a; van der Fits and Memelink, 2001). It appears that members of the AP2/ERF family of TFs have conserved roles in JA- and ET-dependent gene activation.

One of the earliest JA-insensitive mutants in Arabidopsis, \( jin1 \), encodes the MYC2 TF belonging to the basic helix-loop-helix (bHLH) leucine zipper family (Berger et al., 1996; Lorenzo et al., 2004). In Arabidopsis, MYC2 works antagonistically to ERF1 in regulating 2 branches of the JA signalling pathway, one in positively regulating JA- and wound-responsive genes such as \( VSP2 \) and \( JR1 \) and the second in negatively regulating JA- and ET-responsive genes against pathogens such as \( PDF1.2 \) and \( b-CHI \) (Boter et al., 2004; Lorenzo et al., 2004). This is the only TF shown to be repressed by JAZ proteins thus far (Chini et al., 2007).

Another TF family involved in the JA signalling pathway is that of the WRKY TF family. WRKY70 has been implicated in the antagonistic crosstalk between the SA and JA signalling pathways. It acts as an activator of SA-responsive genes such as \( PR1 \) (pathogenesis-related 1) and repressor of JA-responsive genes such as \( PDF1.2 \) (Li et al., 2004a). Transgenic Arabidopsis plants overexpressing \( WRKY70 \) also show increased resistance to SA-mediated \( Erysiphe cichoracearum \) and increased
susceptibility to JA-mediated *A. brassicicola*. Conversely, *wrky70* knockout mutants show increased resistance to *A. brassicicola* and increased susceptibility to *E. cichoracearum* (Li et al., 2006). *WRKY62* may also play a role in the antagonistic crosstalk between SA and JA signalling downstream of the SA signalling component NPR1, whose mutant do not express *WRKY62* upon SA and JA treatment (Dong et al., 2003; Mao et al., 2007). Transgenic Arabidopsis plants overexpressing *WRKY62* and *wrky62* mutants suggest *WRKY62* to be a negative regulator of JA-responsive genes such as *LOX2* and *VSP2* (Mao et al., 2007). Unlike *WRKY62* and *WRKY70* which are negative regulators of the JA signalling pathway, *WRKY33* is the only positive regulator known to date and unlike *WRKY62* and *WRKY70*, it does not require endogenous SA for its basal or induced gene expression (Mao et al., 2007). Transgenic Arabidopsis plants overexpressing *WRKY33* showed increased resistance to necrotrophic fungal pathogens *B. cinerea* and *A. brassicicola*, and increased susceptibility to the bacterial pathogen *P. syringae* which was associated with reduced gene expression levels of SA-responsive *PR1* compared to wildtype (Zheng et al., 2006). Meanwhile, *wrky33* mutants demonstrated increased susceptibility to *B. cinerea* and *A. brassicicola* which was associated with a reduced expression of the JA-responsive *PDF1.2* gene compared to wildtype.

Little is known about TFs involved in the JA signalling pathway, and what is known is mostly based on knockouts and overexpressor lines with few TFs shown to actually bind the genes they regulate. The AP2/ERF family contains ORCA2 and ORCA3 which have been shown to directly interact with a GCC-box-like motif in the jasmonate- and elicitor-responsive element (JERE) of the strictosidine synthase (*STR*) promoter (Menke et al., 1999a; van der Fits and Memelink, 2001). MYC2 from the bHLH leucine zipper TF family is responsible for the regulation of 2 branches of the JA signalling pathway via T/G-box motifs (Boter et al., 2004).
1.6) Jasmonate-responsive palindromic promoters and transcription factors

Palindromic DNA sequences, short DNA sequences that are able to undergo complementary pairing, are often found in cis-elements where they act as target recognition sites for TF binding. Palindromic cis-elements in the promoters of JA-responsive genes that have been shown to be important in JA regulation include the G-box motif and the C-box motif. The G-box motif was first identified in the promoter of the light-regulated ribulose 1,5-bisphosphate small subunit (RBCS) gene (Giuliano et al., 1988) and contains the hexameric sequence CACGTG (Menkens et al., 1995). JA-responsive promoters that have been shown to possess functional G-box motifs identified through promoter deletions fused to reporter genes are the soybean vegetative storage protein-acid phosphatase (VSPB) promoter (Mason et al., 1993) and the potato PI-II promoter (Kim et al., 1992b). T/G-box motifs, similar to the G-box motifs with DNA sequence AACGTG, that also confer JA responsiveness have been identified through promoter deletions fused to reporter genes in the Arabidopsis VSP1 (Guerineau et al., 2003) promoter, the tobacco putrescine N-methyltransferase (PMT) promoter (Xu and Timko, 2004) and the tomato leucine aminopeptidase (LAP) promoter in conjunction with hydroxyl radical interference experiments (Boter et al., 2004).

In 2004, Boter et al. isolated two bHLH leucine zipper TFs from tomato in the JA signalling pathway that bound the G-box motif in the tomato PIN2 (proteinase inhibitor II) and the T/G-box motif in the LAP promoter, JAMYC2 and JAMYC10, using yeast one-hybrid assays (Boter et al., 2004). MeJA was able to induce the expression of JAMYC2 and JAMYC10 whose expression preceded that of PIN2 and LAP upon MeJA treatment. Transgenic potato plants individually overexpressing JAMYC2 and JAMYC10 also resulted in induced expression of PIN2 and LAP in response to JA compared to wildtype. MYC TFs belong to the family of bHLH proteins and the homolog of JAMYC2 in Arabidopsis is AtMYC2, a key regulator of JA responsive genes, which is encoded by the wildtype gene of the mutant jin1 (Boter et al., 2004; Lorenzo et al., 2004). Another MYC protein, CrMYC1, was isolated from periwinkle via yeast one-hybrid assays using the G-box motif found in the promoter of the MeJA-inducible STR
gene (Chatel et al., 2003). Its binding to the G-box motif could be demonstrated in the yeast but further studies are needed to elucidate the role of this element in the regulation of STR in response to MeJA as it does not seem to function as a JA-responsive element in periwinkle. Unfortunately, no TFs have been isolated that bind to JA-responsive C-box motifs.

The C-box motif comprises sequence TGACG and variations thereof, NNGACGTCNN (Izawa et al., 1993) and was first found in the octopine synthase enhancer (OCS) gene, also through promoter deletions fused to reporter genes (Ellis et al., 1987). C-box motifs reside in promoters of the JA-responsive barley grain LOX1 (Rouster et al., 1997), the Agrobacterium nopaline synthase (NOS) promoter (Kim et al., 1993) and the rice OPR1 promoter (Sobajima et al., 2007). To date, these C-box motifs are the only C-box motifs that have been shown to confer JA responsiveness to their corresponding genes.

Although the only TFs in the JA signalling pathway which have been shown to bind one of these elements and direct JA-responsive gene expression are JAMYC2 and JAMYC10 to the T/G-box motif, some light can be shed on others as the DNA-binding preference of some TF families is known. The G-box motif is bound by the G-box binding factor (GBF) family of basic-region leucine-zipper (bZIP) proteins of which there are approximately 75 members in Arabidopsis (Siberil et al., 2001; Jakoby et al., 2002). Another TF family which may also bind G-box motifs are bHLH proteins as they bind to the DNA consensus sequence GANNTG (Meshi and Iwabuchi, 1995). Most bZIP proteins bind palindromic sequences with an ACGT core located in the cis-element and have a preference for G-box and C-box motifs (Izawa et al., 1993). However, not all bZIP proteins have this sequence preference as bZIP proteins have been shown to bind to a palindromic sequence without an ACGT core in a seed–specific element of the pea lectin (PSL) promoter (de Pater et al., 1994). Also, not all bZIP proteins bind G-box motifs as the TGA family of TFs bind TGACG(T/C) motifs, which are homologous to the C-box motif (Meshi and Iwabuchi, 1995).
1.7) Jasmonate-responsive GCC-box and GCC-box-like promoters and transcription factors

The $PDF1.2$ gene serves as a common molecular marker for JA-dependent defence responses and encodes a plant defensin (Manners et al., 1998). A GCC-box motif with DNA sequence AGCCGCC was found to regulate the JA-responsiveness of this gene (Brown et al., 2003). GCC-box motifs have been previously shown to function as ET-responsive elements (Ohme-Takagi and Shinshi, 1995). Interestingly, the simultaneous addition of ET and JA synergistically induced $PDF1.2$ (Penninckx et al., 1998), which could be the result of convergent action of the two signals on the GCC-box. The JERE present in the promoter of the $STR$ gene that is involved in TIA biosynthesis confers JA- and elicitor-responsive transcriptional activation (Menke et al., 1999). This element bears resemblance to the GCC-box motif and is therefore referred to as the GCC-box-like motif.

The GCC-box sequence is bound by the AP2/ERF TF family found exclusively in plants and whose members possess a highly conserved DNA-binding domain (Ohme-Takagi and Shinshi, 1995; Hao et al., 1998). Although there is no direct evidence of any members physically binding to the GCC-box motif in response to MeJA, the JA-inducible ORA59 and ERF1 have been shown to be activators of $PDF1.2$ expression (Pre et al., 2008). Two JA-inducible AP2-domain TFs have been isolated in the Madagascar periwinkle, $C. roseus$, ORCA2 and ORCA3 that bind in vitro to the JERE of the $STR$ promoter transactivating its expression (Menke et al., 1999; van der Fits and Memelink, 2000). Therefore, AP2/ERFs may be involved in the JA signalling pathway via interactions with GCC-box and GCC-box-like motifs.
Table 2: JA-responsive cis-elements and their corresponding transcription factors. These cis-elements were identified in the promoters of several genes from different organisms. * indicate putative TFs whose direct interaction with a cognate cis-element needs to be demonstrated.

<table>
<thead>
<tr>
<th>Motif</th>
<th>Organism</th>
<th>Gene</th>
<th>Associating TF</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-box (CACGTG)</td>
<td>Soybean</td>
<td>VSPB (Mason et al., 1993)</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Potato</td>
<td>PIN2 (Kim et al., 1992)</td>
<td>JAMYC (Boter et al., 2004)</td>
</tr>
<tr>
<td>T/G-box (AACGTG)</td>
<td>Tomato</td>
<td>LAP (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)</td>
<td>JAMYC (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Arabidopsis</td>
<td>VSP1 (Guerineau et al., 2003)</td>
<td>AtMYC2* (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)</td>
</tr>
<tr>
<td>C-box (TGACG)</td>
<td>Barley</td>
<td>LOX1 (Rouster et al., 1997)</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Agrobacterium</td>
<td>NOS (Kim et al., 1993)</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>OPR1 (Sobajima et al., 2007)</td>
<td>?</td>
</tr>
<tr>
<td>GCC-box (AGCCGCC)</td>
<td>Arabidopsis</td>
<td>PDF1.2 (Brown et al., 2003)</td>
<td>AtERF2* (Brown et al., 2003)</td>
</tr>
<tr>
<td>GCC-box-like</td>
<td>Periwinkle</td>
<td>STR (Menke et al., 1999a)</td>
<td>ORCA2 &amp; ORCA3 (Menke et al., 1999a; van der Fits and Memelink, 2001)</td>
</tr>
</tbody>
</table>
The JA signal transduction pathway is responsible for numerous plant developmental and defence responses. In addition to their response to JA, JERE and GCC-box motifs can also respond to different stimuli such as elicitors and ET, respectively (Ohme-Takagi and Shinshi, 1995; Menke et al., 1999a). This supports the fact that TFs from different signalling pathways can regulate the expression of genes through binding of the same cis-element such as ERFs which respond not only to the stimulus ET but also to JA and pathogen infection (Fujimoto et al., 2000). This opens the door to the complex world of signal crosstalk and provides the plant with a highly effective communication network to respond accurately and efficiently to environmental cues. As it has been mentioned above, AtMYC2 acts to positively regulate JA-responsive genes in response to wounding and participates in the ET signalling pathway by negatively regulating JA-and ET-responsive genes after infection with necrotrophic pathogens (Lorenzo et al., 2004). The SCFcoat complex is the central regulator of all JA-dependent responses and is regulated by genes that encode components of the SCF complex such as SGT1b and AXR1 (Gray, 2002). SGT1b and AXR1 may regulate other SCF complexes involved in other signalling pathways as the jai4 mutant, a JA-insensitive mutant isolated in an ein3 (ET-insensitive) background, is defective in the expression of SGT1b (Lorenzo et al., 2004) and another JA-insensitive mutant axr1 is defective in AXR1 expression, a positive regulator of auxin responses (Tiryaki and Staswick, 2002).

Also mentioned above is the regulation of negative crosstalk between SA and JA signalling pathways by WRKY TFs; WRKY 62 and WRKY70 acting as negative regulators while WRKY33 as a positive regulator. Signalling pathways often utilise MAP kinases (MAPK), downstream of MAPK kinases (MAPKK) which operate downstream of MAPKK kinases in a series of phosphorylation events to amplify signals and may work as regulators of signal crosstalk (Garrett and Grisham, 1995). MPK4 is such a MAPK that regulates the negative crosstalk between SA and JA signalling pathways. Arabidopsis mpk4 mutants show constitutive SA-mediated SAR with increased SA levels, increased resistance to biotrophic pathogens such as Hyaloperonospora parasitica and virulent P. syringae and increased PR gene expression (Petersen et al., 2000). On the other hand, mpk4 mutants lack JA-responsive PDF1.2 and THI2.1
induction after JA treatment and therefore act as a positive regulator of JA signalling and a negative regulator of SA signalling. WRKY33 has been shown to be an in vitro substrate of MPK4 and thus fits nicely with the positive regulation of JA responses (Andreasson et al., 2005).

Currently, only the G-box, T/G-box, C-box, GCC-box and GCC-box-like motif have been shown to respond to JA with JA-inducible MYC TFs binding directly to G-box and T/G-box motifs and ORCA TFs to GCC-box-like motifs (Table 2). However, JA-responsive promoters that do not contain any of the above motifs have also been found, such as the Arabidopsis FAD7 promoter (Nishiuchi et al., 1999) and the tomato LOX promoter (Beaudoin and Rothstein, 1997). This suggests that other unidentified motifs may be the targets of TFs involved in the JA signalling pathway.

In this study, a 1306 bp JA-inducible promoter region was identified through promoter-deletion analysis using the Arabidopsis PYK10 promoter fused to the luciferase reporter gene. Further analysis of this JA-inducible promoter region via electromobility shift assays (EMSAs) demonstrated the ability of nuclear proteins to bind in a sequence-specific manner to a W-box motif whose DNA-binding activities were enhanced in response to MeJA. W-box motifs are bound by the WRKY TF family whose gene expression is usually SA-mediated (Eulgem et al., 2000). Only WRKY33 has been demonstrated to be a positive regulator of JA responses (Zheng et al., 2006). No studies have demonstrated the use of W-box motifs in the regulation of JA-responsive genes. It is therefore novel to find a W-box motif whose binding activity to nuclear proteins was increased upon MeJA treatment. Attempts at isolating the TF responsible for this JA-induced binding activity were also made in this study.
2.1) Plant material growth conditions

*Arabidopsis thaliana* ecotype Col-0 and Ws-2, and mutants *coi1*, *jar1* and *jin1* were obtained from the Arabidopsis Biological Resource Centre. Arabidopsis seeds were sterilised in 70% (v/v) Ethanol for 7 minutes followed by 10% (v/v) bleach, 0.02% (v/v) Triton for 15 minutes and washed 5 times with sterile water. The seeds were stratified for at least 3 days at 4 °C. For northern analyses and luciferase assays, the seeds were grown on Murashige and Skoog (MS) media (Murashige and Skoog, 1962) in Petri dishes under fluorescent light of 80-100 µmol photon/sec/m² in a controlled environment room at 21°C with ambient humidity and a 16 hour-light and 8 hour-dark cycle. Peat plugs (Jiffy Products International AS, Stange, Norway) were used for the growth of plants for *B. cinerea* experiments and transgenic plants. They were covered with clingfilm for 1 week to ensure 100% humidity and were grown under the same growth conditions as mentioned above.

2.2) Methyl jasmonate treatment

A stock solution of 95% MeJA (Sigma-Aldrich, St. Louis, USA) was dissolved in either absolute ethanol or dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) and diluted with sterile water to a concentration of 450 µM in 0.1% (v/v) ethanol (Sigma-Aldrich, St. Louis, USA) or 0.05% (v/v) DMSO, respectively. Taking into account the volume of MS media in each Petri dish, the volume added was adjusted to give a final concentration of 45 µM MeJA, 0.01% (v/v) Ethanol and 0.005% (v/v) DMSO.

2.3) DNA isolation

DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, USA) as instructed by the manufacturer.
2.4) RNA isolation, blots and probes

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, USA) as instructed by the manufacturer. RNA blots were prepared using Hybond-N+ membrane (GE Life Sciences, Piscataway, USA) as described by Ausubel et al. (Ausubel et al., 1987). The *PYK10* (At3g09260) probe was amplified from Col-0 genomic DNA via polymerase chain reaction (PCR) using *PYK10* primers, BioTaq DNA Polymerase (Bioline, Taunton, USA) and PCR reactions and conditions as instructed by the manufacturer. All primer sequences and their respective annealing temperatures are given in Table 1. The *PDF1.2* (At5g44420) probe was excised from the pZL1 plasmid (Denby et al., 2005) with restriction enzymes *Sal*I and *Not*I (New England Biolabs, Ipswich, USA). The PCR product of *PYK10* and the restriction enzyme-digested fragment of *PDF1.2* were resolved by electrophoresis on a 1% (v/v) agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) and used as probes. Probes were labelled with [α-32P]dCTP (GE Life Sciences, Piscataway, USA) using the Megaprime DNA Labelling System (GE Life Sciences, Piscataway, USA) as instructed by the manufacturer. The probes were purified using SigmaSpin Post-Reaction Purification Columns (Sigma-Aldrich, St. Louis, USA) as instructed by the manufacturer. Prehybridisation, hybridisation and wash conditions were performed as described by McCaughern-Carucci (McCaughern-Carucci, 1997). Radioactivity from hybridised probes was visualized by a Personal Molecular Imager FX (Biorad, Hercules, USA) and quantitated using the Quantity One Quantification Software Version 4.1 (Biorad, Hercules, USA).
Table 1: PCR primers. Letters in small caps represent additional nucleotides added for increased cleavage efficiency. Letters highlighted in yellow represent the *SacI* restriction site. Letters highlighted in blue represent the *EcoRI* restriction site. Note: All PCR reactions were carried out using Taq DNA polymerase (New England Biolabs, Ipswich, USA) as instructed by the manufacturer using standard PCR conditions unless specified otherwise.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA Sequence (5’ – 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PYK10 Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PYK10 forward</td>
<td>AAGCTTCCTCTCATGAGGC</td>
<td>55</td>
</tr>
<tr>
<td>PYK10 reverse</td>
<td>CTTGGCATTTAGCATTGAC</td>
<td>55</td>
</tr>
<tr>
<td><strong>Deletion Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1 forward</td>
<td>ATTAGCTCTCCCTCAGATGTCATGCTATC</td>
<td>60</td>
</tr>
<tr>
<td>D2 forward</td>
<td>ATTAGCTCATGACATGCTGTTGTCG</td>
<td>60</td>
</tr>
<tr>
<td>D3 forward</td>
<td>ATTAGCTCAGAGAGGTGGATGCGTTCGTC</td>
<td>60</td>
</tr>
<tr>
<td>D reverse</td>
<td>ATTGGATGTTTACCATATCGA</td>
<td>60</td>
</tr>
<tr>
<td><strong>T3 Selection Primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 reverse</td>
<td>ATTACACGCGATCTTTCCG</td>
<td>57</td>
</tr>
<tr>
<td><strong>NPTII Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPTII forward</td>
<td>GAGGCTATTCCGGCTATGACTG</td>
<td>60</td>
</tr>
<tr>
<td>NPTII reverse</td>
<td>ATCGGGAGCGGCGATACCCTG</td>
<td>60</td>
</tr>
<tr>
<td><strong>pOne-1 primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pOne-1 forward</td>
<td>GGTGTTTCCAGTCACGAC</td>
<td>57</td>
</tr>
<tr>
<td>pOne-1 reverse</td>
<td>CAAAATCATGCGTCCGCTG</td>
<td>57</td>
</tr>
<tr>
<td><strong>Library primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Library forward</td>
<td>AGGGATGTCTTATACCACTAC</td>
<td>48</td>
</tr>
<tr>
<td>Library reverse</td>
<td>GCACAGTTGAGTGACTTGC</td>
<td>48</td>
</tr>
</tbody>
</table>
2.5) *Botrytis cinerea* infection

Plants were infected with 10 to 14 day-old spores from the *B. cinerea* isolate GLUK-1 (Kliebenstein et al., 2005). The isolate was maintained on tinned-apricot halves and subcultured onto fresh apricot halves every 2 to 3 weeks. Spores were dislodged from infected apricots into sterile water by gentle force using a glass rod until the water turned translucent. The spore concentration was determined by a haemocytometer and adjusted to $5 \times 10^4$ spores/ml by dilution with 50% (v/v) grape juice (Ceres, Ceres, South Africa). Leaves of 3 week-old plants were excised, placed on 0.8% (v/v) agar plates and their adaxial surfaces drop-inoculated with 4 µl of either spore suspension or 50% (v/v) grape juice. The agar plates were then sealed and returned to the growth room. 3 leaves were harvested for each time point and used for northern analyses.

2.6) Promoter analysis

1kb TAIR loci upstream sequence of those sets of genes found in Chapter 3, Table 1 were downloaded from the TAIR website (http://www.arabidopsis.org/tools/bulk/sequences/index.jsp). To search for JA-responsive *cis*-element and novel *cis*-elements the Patmatch and Motif Analysis tool from the TAIR website was used, respectively (http://www.arabidopsis.org). The genome frequency was either calculated as the *cis*-element of interest having at least one occurrence in all 33282 Arabidopsis genes in their 1kb TAIR Loci upstream sequence (Chapter 3, Table 1) or in upstream sequences 1kb from the start codon (Chapter 3, Table 3). This value was then used to calculate the expected frequency of the *cis*-element in the different sets of genes. *P*-Values were calculated using Microsoft Excel’s Poisson distribution function with the following parameters: number of events = number of occurrences of the *cis*-element in all promoters of its gene set; mean = expected number of occurrences of this *cis*-element in this gene set based on its average frequency in all Arabidopsis promoters; cumulative = false.
2.7) Promoter deletion construction

Promoter deletions D1, D2 and D3 were amplified from Col-0 genomic DNA via PCR using Deletion primers (Table 1). The PCR primers were designed to generate PCR products with SacI at the 5’ end and EcoRI at the 3’ end (Table 1). The PCR products were resolved by electrophoresis on a 1% (w/v) agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) as instructed by the manufacturer. The purified PCR products were digested by restriction enzymes SacI and EcoRI as instructed by the manufacturer (New England Biolabs, Ipswich, USA) and the resulting fragments resolved by electrophoresis on a 1% (w/v) agarose gel. The fragment of interest was then purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) as instructed by the manufacturer. The DNA sequence of D1, D2 and D3 were validated by the Sequencing Unit (University of Cape Town, Cape Town, Western Cape) on an ALFexpress DNA Automated Sequencer (GE Life Sciences, Piscataway, USA). D1 fragment was ligated to SacI and EcoRI–digested p7LUC (Murray et al., 2002) and transformed into Escherichia. coli JM109 as described below. Colonies that contained D1::p7LUC were identified via colony PCR using D1 Deletion primers (Table 1).

D1::p7LUC plasmid was excised by SacI and NotI as instructed by the manufacturer (New England Biolabs, Ipswich, USA) and the resulting fragments resolved by electrophoresis on a 1% (w/v) agarose gel. The fragment of interest was then purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) as instructed by the manufacturer. D1::LUC::OCS fragment was ligated to binary vector pART27 (Gleave, 1992) after both fragment and vector were digested with SacI and NotI as instructed by the manufacturer (New England Biolabs, Ipswich, USA), and transformed into E. coli JM109 as described below. Colonies that contained D1::LUC::OCS::pART27 were identified via colony PCR using the same PCR reactions as mentioned above for D1 amplification. D1::LUC::OCS::pART27 plasmid was excised by SacI and EcoRI as instructed by the manufacturer (New England Biolabs, Ipswich, USA) to release the D1 fragment and replaced with D2 and D3 fragments via ligation to generate
D2::LUC::OCS::pART27 and D3::LUC::OCS::pART27. All ligations were validated by the Sequencing Unit (University of Cape Town, Cape Town, South Africa) on an ALFexpress DNA Automated Sequencer (GE Life Sciences, Piscataway, USA).

2.8) Cloning and bacterial transformation

D1::p7LUC and D1/D2/D3::LUC::OCS::pART27
Ligation reactions were performed at a insert:vector ratio of 3:1 using the equation:

\[
\text{Vector (ng)} \times \text{Insert size (kb)} \times 3 = \text{insert (ng)}
\]

\[
\frac{\text{Vector size (kb)}}{\text{50 ng of vector was used for all ligations. Reaction volumes were 10 µl consisting of 1X Ligase Buffer (New England Biolabs, Ipswich, USA), and 400 units of T4 DNA Ligase (New England Biolabs, Ipswich, USA). All molecular biology techniques and procedures were followed as described by Ausubel et al. (Ausubel et al., 1987) and are briefly outlined below. 2 µl of ligation reaction was transformed into 50 µl of Escherichia coli strain JM109 via the heat-shock method. Successfully transformed E. coli cells were selected for growth on Luria-Bertani (LB) agar plates containing the antibiotic ampicillin (100 µg/ml) for D1::p7LUC or kanamycin (50 µg/ml) for D1/D2/D3::LUC::OCS::pART27 and their DNA plasmids isolated using the alkaline lysis method. 1 µg each of D1/D2/D3::LUC::OCS::pART27 were transformed into 100 µl of Agrobacterium tumefaciens strain GV3101 via the freeze-thaw method described by Weigel and Glazebrook (Weigel and Glazebrook, 2002). Successfully transformed Agrobacterium cells were selected for growth on yeast extract phosphate (YEP) agar plates containing antibiotics streptomycin (25 µg/ml), rifampicin (150 µg/ml) and gentamycin (15 µg/ml), and confirmed for the presence of each of the D1/D2/D3::LUC::OCS::pART27 plasmids via colony PCR using the same PCR reactions as mentioned above for D1, D2 and D3 amplification and used for plant transformation.
**Wbox::pOne-1**

100 ng of pOne-1 vector was used for all ligations. Reaction volumes were 10 µl consisting of 1X Ligase Buffer (Takara, Shiga, Japan), and 50 units of DNA Ligase (Takara, Shiga, Japan). The Wbox insert was added to make the volume up to a total of 10 µl. For religation of the Wbox::pOne-1 plasmid after a portion of the MCS has been excised, the same procedure was followed without the addition of the Wbox insert. 5 µl of ligation reaction was transformed into 100 µl of *E. coli* strain DH5α via the heat-shock method. Successfully transformed *E. coli* cells were selected for growth on LB agar plates containing the antibiotic tetracycline (15 µg/ml) for Wbox::pOne-1. Colonies were screened for the presence of insert via colony PCR with pOne-1 primers covering the insertion site (Table 1). DNA plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Valencia, USA) and its sequence confirmed by the Sequencing Unit (University of California-Riverside, Riverside, USA) on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, USA).

**cDNA::pAD-GAL4-2.1**

100 ng of vector was transformed into *E. coli* DH5α via the heat-shock method. Successfully transformed *E. coli* cells were selected for growth on LB agar plates containing ampicillin (100 µg/ml) and DNA plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Valencia, USA).

### 2.9) Plant transformation and transgenic plant selection

Arabidopsis Col-0 plants were transformed with D1/D2/D3::LUC::OCS::pART7 using *Agrobacterium tumefaciens* via the floral dip method (Clough and Bent, 1998) and allowed to set seed. T₁ transgenic plants were subjected to antibiotic screening on MS plates containing 50 µg/ml kanamycin to select successful transformants. Successful transformants surviving the kanamycin screen were transplanted onto soil and allowed to self-fertilise. Seeds from individual plants were harvested separately. T₂ transgenic plants from each line were once again subjected to the same kanamycin screen and
those that had a 75% or more survival rate were transplanted individually onto soil and allowed to self-fertilise. Stable $T_3$ homozygous transgenic lines were identified from each of the representative $T_2$ lines by 100% growth on kanamycin selection media and confirmed by 2 sets of PCR for the insertion gene. The first set with the forward primer used to amplify each deletion fragment and the reverse primer against the $LUC$ gene, and the second set with primers directed against the $NPTII$ gene (Table 1).

2.10) Luciferase and Bradford assays

Crude protein extracts were prepared from transgenic plants by grinding 2 or 3 seedlings in 100 µl of extraction buffer (0.1 M sodium phosphate buffer, pH 7.2; 5 mM DTT). Samples were centrifuged to pellet debris at 12 000 rpm for 5 minutes at 4°C and the supernatant saved for luciferase and Bradford assays. Bradford assays were used to determine the amount of protein in the crude protein extracts using bovine serum albumins (BSA) to construct a standard curve (Bradford, 1976). Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, USA) were combined with the sample and the BSA standard and measured using a Titertrek Multiskan Plus microtitre plate reader (Labsystems and Flow Laboratories, Helsinki, Finland) as instructed by the manufacturers. Luciferase activity was measured using a Turner Designs TD-20/20 Luminometer (Turner Biosystems, Sunnyvale, USA) with the following settings; Mode: signal; Time: 20 seconds; Interval: 0; N points: 1; Delay: 0; Columns: 1; Dispenser: manual. The luminometer was prefushed with distilled water. 100 µl of assay buffer (60 mM Tris-HCl, pH 8; 20 mM MgCl$_2$; 20 mM DTT; 2 mM EDTA, pH 8; 2 mM ATP) was added to 100 µl of protein extract in a 5 ml plastic luminometer tube and the background luminescence was measured after 20 seconds. 100 µl of luciferin buffer (60 mM Tris-HCl, pH 8; 20 mM MgCl$_2$; 20 mM DTT; 2 mM EDTA, pH 8; 1 mM luciferin) was injected into the mixture and the luminescence was measured at 20 seconds thereafter. Luciferase activity was calculated as relative light units per µg of protein per ml after subtracting the background luminescence. Luciferase imaging was performed using the
services of the Plant Transformation Research Facility (University of California-Riverside, Riverside, USA)

2.11) Electrophoretic mobility shift assays

EMSAs were performed as described by Knoth and Eulgem (Knoth and Eulgem, 2008) with nuclear protein extracts isolated as described by Desveaux et al. (Desveaux et al., 2004) and synthesized oligonucleotide probes (Invitrogen, Carlsbad, USA). Bradford assays were used to determine the amount of protein in the nuclear protein extracts using BSA as a standard curve (Bradford, 1976). EMSA gels were vacuum-dried and exposed to HyBlot CL autoradiography films (Denville Scientific, South Plainfield, USA) at -80°C until the desired exposure was achieved. The films were developed on an AFP Mini-Medical 90 Film Processor (AFP Imaging, Elmsford, USA).

2.12) Yeast one-hybrid “Bait” construction

The DNA “bait” fragment was synthesised (Invitrogen, Carlsbad, USA) to consist three tandem repeats of W-box motif (TTGACT) including flanking sequences (six nucleotides immediately upstream and downstream) separated by a 4-bp spacer. An EcoRI and XhoI site were integrated at the 5’ and 3’ end, respectively, with additional nucleotides to facilitate restriction enzyme binding. The bait fragment was ligated to the shuttle pOne-1 vector (Cormack et al., 2002) following XhoI and EcoRI (New England Biolabs, Ipswich, USA) restriction enzyme digests of both the bait fragment and shuttle vector as instructed by the manufacturer and transformed into E. coli DH5α as described above. The resulting plasmid was then cut by EcoRI (New England Biolabs, Ipswich, USA) as instructed by the manufacturer to remove 54 bp in the multiple cloning site (MCS) and religated as described above. The Wbox::pOne-1 construct containing the “bait” was transformed into Saccharomyces cerevisiae strain YM4271 via the lithium acetate method and successful transformants were screened for their ability to grow in synthetic
dropout (SD) media lacking tryptophan. 1 mM 3-aminotriazole (3-AT) was added to SD media lacking tryptophan and histidine to lower the false positive rate arising from leaky HIS3 expression.

2.13) Library construction

cDNA was synthesised from 2.5 µg of mRNA isolated from 2 week-old whole Col-0 seedlings that had been treated for 3, 6, 12 and 24 hours with MeJA using the HybriZAP-2.1 XR cDNA Synthesis Kit (Statagene, La Jolla, CA) as instructed by the manufacturer. The resulting cDNAs were pooled and used to construct the MeJA cDNA library or “prey” for the yeast one-hybrid screen using the HybriZAP-2.1 XR Library Construction Kit (Stratagene, La Jolla, USA) as instructed by the manufacturer. PCR analysis of inserts was also performed using Library primers (Table 1).

2.14) Yeast one-hybrid assay

Yeast one-hybrid assays were performed as described by Cormack et al. (Cormack et al., 2002). Positive interactions between bait and prey were observed via uninhibited growth on SD media lacking tryptophan, leucine and histidine with increasing concentrations of 3-AT. These colonies were subjected to PCR using library primers bordering the cDNA inserts in the pAD-GAL4-2.1 vector (Table 1). The resulting PCR products were resolved by electrophoresis on a 1% (w/v) agarose gel and DNA fragments of interest were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA) and sequenced by the Sequencing Unit (University of California-Riverside, Riverside, USA).

To confirm transcriptional activity, prey vector DNA was isolated from the yeast clone of interest as instructed by the manufacturer of HybriZAP-2.1 XR Library Construction Kit (Stratagene, La Jolla, USA) and transformed into E.coli DH5α for amplification. The
prey vector was transformed into yeast YM4271 containing either the Wbox::pOne-1 construct or an empty pOne-1 vector via the lithium acetate method (Ausubel et al., 1987) and analysed on SD media lacking tryptophan; tryptophan and leucine; or tryptophan, leucine and histidine.
CHAPTER 3

UTILISING *PYK10* AS A REPORTER TO ELUCIDATE

THE JA SIGNALLING PATHWAY
3.1) Introduction

A large repertoire of genes is induced via the JA signalling pathway by the exogenous application of MeJA, a biologically active JA (Seo et al., 2001). cDNA microarray and macroarray studies have allowed researchers to investigate the simultaneous global expression of genes after a specific stimulus. To this end, many genes involved in plant defence encoding proteins involved in cell-wall synthesis and modification (such as cellulose synthase), antioxidant enzymes involved in the oxidative burst surrounding the infection (such as catalases), antimicrobial proteins (such as defensins) and even signalling and developmental proteins (such as senescence-associated proteins) were induced simultaneously in response to MeJA (Parthier, 1990; Penninckx et al., 1996; Lamb and Dixon, 1997; Schenk et al., 2000; Sasaki et al., 2001; Ellis et al., 2002b). Several of these genes had similar profiles to each other in global expression studies in response to wounding, pathogen attack and other hormones, suggesting JA-mediated regulation and crosstalk in these pathways (Reymond et al., 2000; Schenk et al., 2000; Sasaki et al., 2001; Cheong et al., 2002). MeJA is an important signalling molecule as the constitutive expression of \textit{JMT}, the only known gene encoding an enzyme converting JA to MeJA, in transgenic Arabidopsis led to constitutive expression of JA-responsive genes and enhanced resistance to \textit{B. cinerea} (Seo et al., 2001). The volatile MeJA can also act as a signal between plants and may also be a systemic signal within a plant (Baldwin and Schultz, 1983; Farmer and Ryan, 1990; Li et al., 2002).

The many genes induced in response to MeJA include those encoding enzymes involved in the glucosinolate biosynthetic pathway such as anthranilate synthase \(\alpha\)-subunit (ASA), Trp synthase \(\alpha\)-subunit (TSA), Trp synthase \(\beta\)-subunit (TSB), a cytochrome P-450 monooxygenase (CYP79B3) and UDPG-thiohydroximate glucosyltransferase (SGT) (Brader et al., 2001). Glucosinolate accumulation, which plays a role in anti-herbivory in Arabidopsis, is induced in response to MeJA and involves the JA signalling pathway as this induction is abolished in the JA-insensitive
coi1 mutant (Rask et al., 2000; Brader et al., 2001). PYK10 (At3g09260) was discovered to be a MeJA inducible gene in an undirected differential display study (Wei, 2002). PYK10 encodes β-D-glucoside glucohydrolase (β-glucosidase) which catalyses the hydrolysis of glucosinolates leading to the production of toxic by-products thereby contributing to the plant’s arsenal against insects and pathogens (Rask et al., 2000).

In oat cells, β-glucosidases are compartmentalised in plastids and their substrate avenocoside in vacuoles. These two organelles have been shown to fuse upon stress to produce toxic aglycons and separate toxic by-products formed by β-glucosidase from healthy plant cells (Nisius, 1988). Similarly, PYK10 may also be compartmentalised separately from its substrate, which is currently unknown, in order to protect healthy cells from toxic by-products. PYK10 has been found to accumulate in endoplasmic reticulum (ER) bodies in response to wounding and exogenous MeJA treatment (Matsushima et al., 2003). ER bodies are structures derived from the ER surrounded by ribosomes. They have been shown to contain proteinases that fuse with each other and lytic vacuoles, which may house the substrate of PYK10, under osmotic stress (Hayashi et al., 2001). When the plant cell is wounded by chewing insects its water potential is altered by feeding and leads to osmotic stress (Cabrera et al., 1995). ER bodies could then fuse to the compartment housing the substrate thereby releasing toxic compounds for defence. Recently, PYK10 was shown to be necessary for the beneficial interactions between Arabidopsis and Piriformospora indica in the promotion of growth and higher seed yields induced by P. indica by keeping the fungal population in check (Sherameti et al., 2008).

The JA signalling pathway is of paramount importance in plant development and defence and some of its components act as TFs regulating the expression of genes in response to stimuli. For example, the MYC2 TF in Arabidopsis and tomato plays a key role in JA-induced defence gene activation by binding to G-box and T/G-box motifs (Boter et al., 2004; Lorenzo et al., 2004). Each eukaryotic gene typically possesses discrete sets of appropriate cis-elements in its promoter for the regulation of its gene expression by TFs. There are a total of 64 families listed in the Arabidopsis Database.
of Transcription Factors, however thus far, only MYC2, which binds G-box and T/G-box motifs, in the bHLH family and ORCA2 and ORCA3, which binds GCC-box-like motifs, in the AP2-domain TF family have been shown to participate in JA signalling by actively binding to their respective *cis*-elements to regulate gene expression (Menke et al., 1999a; van der Fits and Memelink, 2001; Boter et al., 2004; Lorenzo et al., 2004; Guo et al., 2005). TFs within the same family often bind to the same core sequence within *cis*-elements as the large plant-specific AP2-domain TF family to which the ORCAs belong to also contain ERF TFs that bind GCC-box core motifs (GCCGCC) which are strikingly similar to the core sequence found in GCC-box-like motifs (ACCGCC) (Hao et al., 1998). It is unclear which ORCA binds to which GCC-box-like motif as a complete *cis*-element includes not only its core sequence but also its flanking sequences which dictates TF binding specificity. Usually, the core sequence is elucidated first within highly similar TFs. Members of large TF families such as the AP2-domain family consisting of 146 members, and the bHLH family consisting of 127 members, require recruitment to their specific *cis*-elements not only by the core sequence but also by flanking nucleotides, demonstrated by the loss of activity using mutated motifs in reporter gene assays and EMSAs (van der Fits and Memelink, 2001; Boter et al., 2004; Guo et al., 2005). To date, approximately 21 *cis*-elements, ranging from 4 to 8 nucleotides, have been identified in Arabidopsis, however, more than half are known to bind certain TF families rather than specific TFs (Yoshiharu et al., 2007). Our knowledge of transcriptional regulation is limited to the recognition of TFs to their corresponding *cis*-elements via core sequences and their specificity is dictated by their flanking sequences but exactly how is yet unknown.

Not much is known about the JA signalling pathway, especially in the area of transcriptional regulation as only the G-box and T/G-box motif bound by MYC2, and the GCC-box-like motif bound by ORCA2 and ORCA3 have been identified (Menke et al., 1999a; van der Fits and Memelink, 2001; Boter et al., 2004; Lorenzo et al., 2004; Guo et al., 2005). Even though the C-box and GCC-box motif have been shown to be JA responsive no binding TFs have been isolated despite their similarities to G-box and GCC-box-like motifs through overlapping sequences (Rouster et al., 1997; Brown et al.,
2003). This suggests a tight control over transcriptional regulation via flanking sequences or perhaps even other interacting cis-elements. Since PKY10 gene expression is induced by MeJA it was used in this study as a representative MeJA-induced gene for the investigation of transcriptional gene regulation by the JA signalling pathway.
3.2) Results

3.2.1) The MeJA inducibility of PYK10

PYK10 was originally identified as a seedling- and root-specific gene in Arabidopsis by Nitz et al. who observed PYK10 expression exclusively in the roots when compared with the stems and leaves of 3 week-old Arabidopsis plants via northern analysis (Nitz et al., 2001). Differential display then identified PYK10 as a MeJA-inducible gene in a previous study (Wei, 2002). Hence, PYK10 expression in 2 week-old seedlings and older 3 week-old Arabidopsis plants as well as in their shoots and roots will be investigated to compare to the Nitz observations.

A northern analysis was performed using the 1.5 kb PYK10 probe against RNA isolated from roots and aerial parts (shoots) of untreated 2 week-old Col-0 plants and plants treated with MeJA for 24 hours (Fig. 1A). Harvested plants had been grown on MS plates and treatment involved the exogenous application of 45 µM MeJA solution to plates. An increase in the steady state PYK10 mRNA levels in both roots and shoots upon MeJA treatment was observed with a 4-fold induction in the roots and a higher 8-fold induction in the shoots. This MeJA-inducible PYK10 expression was also observed to be higher in leaves compared to roots of older 3 week-old plants grown on MS plates and therefore not seedling specific, albeit reduced with age. Although basal levels of PYK10 was higher in roots than shoots PYK10 expression increased by 3 fold in 6 hours and 6 fold in 12 hours of MeJA treatment in shoots compared to 2.2 fold in 6 hours and 2 fold in 12 hours in roots (Fig. 1B). PYK10 expression is higher in the shoots and is usually masked by the high basal expression level in roots when whole plants are used as can be observed in Figure 1B where overall PYK10 expression increased by 5 fold in 6 hours and 3 fold in 12 hours. In summary, MeJA-induced PYK10 expression is also apparent in older 3 week-old Arabidopsis plants and therefore not seedling specific, and PYK10 induction by MeJA is even more prominent in shoots than it is in roots in both 2 week- and 3 week-old plants.
Figure 1: RNA gel blot analysis of *PYK10* in (A) 2 week-old roots and shoots, (B) 3 week-old roots (R), leaves (L) and whole plants (W) of untreated Col-0 plants and those treated with 45 µM MeJA/0.005% DMSO and 0.005% DMSO for (A) 24 hours and for (B) 6 and 12 hours. 10 µg of total RNA was loaded onto each lane. rRNA (A) and RNA staining of the transfer membrane using methylene blue (B) were used as a control for equal loading. Similar results were obtained from 2 independent experiments.
MeJA is commercially available as a hydrophobic solution and has to be dissolved in an appropriate solvent prior to usage. Both ethanol and DMSO have powerful dissolving powers that dissolve polar and non-polar compounds. Ethanol was the solvent for MeJA that was initially used to treat Arabidopsis plants analysed by northerns for the MeJA-inducibility of *PYK10* (Wei, 2002). However, ethanol proved to be an erratic solvent for MeJA as alone it would occasionally induce *PYK10* expression as shown in Figure 2A. The suitability of DMSO as a solvent for MeJA was hence investigated. The MeJA-responsiveness of *PYK10* and its response to the solvent DMSO were analysed by a RNA gel blot. The same 1.5 kb portion of *PYK10* was probed against RNA isolated from untreated 2 week-old Col-0 plants and plants treated with 45 µM MeJA for 6, 12 and 24 hours (Fig. 2B). In untreated plants, *PYK10* expression was expressed but its steady state mRNA level increased approximately 36 fold 6 hours following MeJA treatment and this increase was maintained even after 24 hours. No increase in *PYK10* steady state mRNA level was observed in the DMSO control. These results therefore proved that DMSO is a more suitable solvent for MeJA than ethanol as it alone did not induce *PYK10* expression and consistently gave reproducible results.

These experiments demonstrated a strong induction of *PYK10* by MeJA treatment after 24 hours, however, *PYK10* had been previously identified via a differential display experiment to find MeJA-induced genes in Arabidopsis after 2 and 6 hours of treatment with MeJA (Wei, 2002). To investigate the timing of *PYK10* induction, Arabidopsis plants were grown on MS plates for 2 weeks and untreated plants and those treated with the exogenous application of 45 µM MeJA solution to plates for 3, 6, 12 and 24 hours were harvested and analysed via northern analysis. *PYK10* was not induced by MeJA at earlier time points of 5 and 15 minutes (data not shown) but its induction was evident at 3, 6, 12 and 24 hours after MeJA treatment (Fig. 2B and 2C; Fig 2C is part of the northern blot in Fig 5 but shown here for easy reference).

Analysis of *PYK10* expression across 22,000 genes from Arabidopsis, as represented on the Affymetrix ATH1 GeneChip, in the Bio-Array Resource (BAR) using the Arabidopsis electronic Fluorescent Pictograph (eFP) Browser (Winter et al., 2007)
showed similar patterns of expression for PYK10 in development (i.e. seedlings versus older plants) and root specificity as the northerns above. The eFP Browser showed higher PYK10 expression in 1 week-old plants and 2 week-old Col-0 plants compared to older plants which showed lower or no PYK10 expression. However, no MeJA induction of PYK10 was observed for the data set as represented by 1 week-old Col-0 plants treated with 10 µM MeJA for 30 minutes, 1 and 3 hours compared to those of mock treated plants. This is in contrast to the PYK10 expression observed at 3 hours mentioned above and could be the result of the lower 10 µM concentration of MeJA used in those experiments as opposed to 45 µM used in this study. Alternatively, MeJA induction of PYK10 could also occur at a later time point such as 24 hours in 1 week-old Col-0 plants as shown later.
Figure 2: RNA gel blot analysis of *PYK10* in 2 week-old Col-0 plants. Untreated plants (X) and plants treated with (A) 45 µM MeJA/0.01% EtOH or 0.01% EtOH, (B) 45 µM MeJA/0.005% DMSO or 0.005% DMSO and (C) 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane and membranes were probed with a 1.5 kb portion of the *PYK10* gene. rRNA (A) and RNA staining of the transfer membrane using methylene blue (B) and (C) were used as a control for equal loading. Similar results were obtained from 2 independent experiments.
3.2.2) *PYK10* expression is not induced in response to *Botrytis cinerea* infection

The JA signalling pathway is involved in defence against the necrotrophic fungus *B. cinerea*. Evidence for this has been provided by Thomma et al. (1998; 2000) where they showed increased susceptibility in the JA mutant *coi1* to Botrytis infection and also an increase in resistance against Botrytis by pre-treating Arabidopsis with MeJA. In addition, endogenous levels of MeJA increased approximately 2-fold in transgenic Arabidopsis plants over-expressing *JMT*, which encodes an enzyme responsible for converting JA into MeJA, and these plants were more resistant to Botrytis when compared to wildtype plants (Seo et al., 2001). Given the tight link between the activation of the JA signalling pathway and defence against Botrytis the expression of *PYK10* after Botrytis infection was investigated.

Northern analyses of *PDF1.2* and *PYK10* expression were carried out on the excised leaves of 3 week-old untreated Col-0 plants drop-inoculated with *B. cinerea* spores and those treated with grape juice only, the medium for Botrytis spore suspension, over a period of 3 days. The Arabidopsis MeJA-inducible *PDF1.2* gene encodes a plant defensin and is commonly used as a characteristic marker for JA- and ethylene-dependent defence responses (Penninckx et al., 1996; Penninckx et al., 1998). *PDF1.2* expression is also induced after Botrytis infection (Manners et al., 1998). To determine that an efficient infection with Botrytis had been achieved the RNA gel blot was probed with a 400 bp fragment of the *PDF1.2* gene to monitor its induction (Fig. 3). The Botrytis infection was a success due to a 51-fold increase observed in the steady state level of *PDF1.2* mRNA 48 hours after infection with an even higher increase of 661-fold 72 hours after infection. Minimal or no induction was observed in untreated leaves and those treated with grape juice over the time course of infection. The same RNA gel blot was probed with the 1.5 kb *PYK10* gene and no *PYK10* expression was observed (Fig. 3).
As earlier data observed MeJA-induced PYK10 expression in leaves of 3 week-old plants (Fig. 1B), these results suggest that PYK10 expression can be induced in older leaves by MeJA but not by Botrytis infection, a stimulus that induces JA accumulation.

**Figure 3:** RNA gel blot analysis of PDF1.2 and PYK10 in 3 week-old Col-0 leaves. Untreated excised leaves and leaves treated with half-strength grape juice (Grape J) and Botrytis cinerea (Botrytis) at 5X10^4 spores/ml were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane. RNA staining of the transfer membrane using methylene blue was used as a control for equal loading. Similar results were obtained from 2 independent experiments.
3.2.3) **PYK10 expression in JA mutants**

The JA signalling pathway is an intricate network of components that interact to bring about a specific response to a biotic or abiotic stimulus. They affect differential responses and some components affect more JA responses than others. For example, mutations in the gene encoding COI1, a part of the vital JA signalling component SCF\(^{COI1}\) (Xu et al., 2002), affect all JA responses known thus far while mutations in the gene encoding JAR1, an amino acid synthetase, affects only some JA responses eg. \(\textit{coi1}\) is male-sterile while \(\textit{jar1}\) is fertile (Staswick et al., 1992; Feys et al., 1994). These mutants can therefore be used to shed light on which components of the JA signalling pathway is involved in regulating **PYK10** expression.

**PYK10** expression was examined in \(\textit{jar1}, \textit{jin1}\) and \(\textit{coi1}\) because all 3 mutants are insensitive to exogenous MeJA treatment and have altered JA responses such as reduced inhibition of root growth (Staswick et al., 1992; Berger et al., 1996; Xie et al., 1998a). These mutants are in a Col-0 background. \(\textit{Coi1}\) mutants are defective in reproductive development, wound-induced gene expression and resistance to pathogens and insects (Feys et al., 1994; Xie et al., 1998b; Devoto et al., 2005). COI1 is an F-box protein that is part of the SCF\(^{COI1}\) E3 ubiquitin ligase complex which recruits proteins for degradation via the 26S proteasome (Devoto et al., 2002; Feng et al., 2003; Katsir et al., 2008). Targets for COI1 remained undiscovered until scientists recently isolated JAZ repressor proteins, which appear to repress JA-mediated responses by repressing JA-inducible TFs such as MYC2 (Chini et al., 2007; Thines et al., 2007). JAZ proteins are targeted for degradation upon interaction with COI1, thereby releasing TFs from repression. JAR1 is an upstream component of JA signalling as it conjugates isoleucine to jasmonic acid producing a bioactive JA (Staswick and Tiryaki, 2004; Melotto et al., 2008). However, not all JA responses are mediated by JA-Ile as other bioactive JAMs such as MeJA and OPDA are also important signalling molecules (Stintzi and Browse, 2000; Seo et al., 2001). \(\textit{JIN1}\) encodes a transcription factor, AtMYC2, which differentially regulates 2 branches of the JA signalling pathway. AtMYC2 was shown to positively regulate the branch that is required for the expression
of wound-related genes such as VSP2 and negatively regulate the branch that is required for the expression of pathogen-related genes such as PDF1.2 (Lorenzo et al., 2004). JAZ repressor proteins, such as JAZ3, may repress MYC2 and this repression is possibly alleviated by the degradation of JAZ3 by COI1, the central regulator of JA signalling, which must regulate other JA TFs besides MYC2 (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008).

PYK10 gene expression following MeJA treatment was first examined in the JA mutant, coi1-1. Coi1-1 is a recessive mutation that causes male sterility and therefore has to be maintained as a segregating population. The inhibition of root growth after MeJA treatment is significantly compromised in this mutant (Xie et al., 1998). This phenotype was used to isolate homozygous coi1-1 mutants on plates of MS media containing MeJA for one week and then these seedlings were transplanted to normal MS for a further growth of one week. Upon initial growth on MeJA-containing MS media coi1-1 mutants segregated in a 1:3 ratio (coi1-1 mutant: wildtype) and wildtype plants were a mixture of wildtype COI1 and heterozygous coi1 (collectively termed WTCOI1) (data not shown). These wildtype plants served as a positive control. As shown in Figure 4, steady state levels of PYK10 mRNA increased 3 fold in 2 week-old WTCOI1 plants 6 hours following MeJA treatment. The observed fold induction is much lower in WTCOI1 compared to Col-0 (Fig 2B). This could be due to the prior exposure of WTCOI1 to MeJA during the selection of homozygous coi1-1 mutants thereby activating the JA signalling pathway leading to the induction of PYK10. PYK10 induction may be nearing saturation levels before the second exposure to MeJA and hence the induction observed 6 hours following treatment was dampened. However, the induction of PYK10 expression was totally abolished in the coi1 mutant with basal PYK10 expression levels below that of WTCOI1. The fact that some basal expression is still present in coi1-1 mutants suggests that the seedling expression is independent of COI1, and perhaps JA, as all known JA responses are absent in the coi1-1 mutant. The lack of MeJA-inducibility in coi1-1 indicates that protein(s) targeted by COI1 for degradation normally inhibit PYK10 expression.
Figure 4: RNA gel blot analysis of *PYK10* in 2 week-old JA mutant *coi1-1* and wildtype *WTCOI1*. Untreated plants (0) and plants treated with 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane and membranes were probed with a 1.5 kb portion of the *PYK10* gene. RNA staining of the transfer membrane using methylene blue was used as a control for equal loading. Similar results were obtained from 3 independent experiments.

*PYK10* gene expression was also examined in the JA mutants *jar1* and *jin1* after MeJA treatment as shown in Figure 5. As expected, in 2 week-old wildtype Col-0 plants grown on MS plates, *PYK10* expression increased dramatically (approximately 40 fold) following 45 µM MeJA treatment. This increase in steady state levels of mRNA was observed as early as 3 hours and maintained for at least 24 hours. This induction pattern was repeated with similar intensity in the *jar1* mutant and also in the *jin1* mutant from 6 to 24 hours. These results show that isoleucine conjugation of JA by JAR1 and the signalling branches regulated by AtMYC2 are not required for *PYK10* expression in the JA signalling pathway after MeJA application.
Figure 5: RNA gel blot analysis of PYK10 in 2 week-old Col-0 and the JA signalling mutants jar1 and jin1. Untreated plants (0) and plants treated with 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane and membranes were probed with a 1.5 kb portion of the PYK10 gene. RNA staining of the transfer membrane using methylene blue was used as a control for equal loading. Similar results were obtained from 3 independent experiments.

3.2.4) MeJA-responsive motifs in the promoter region of PYK10, COI-dependent MeJA-inducible genes and genes co-expressed with PYK10

Gene transcription regulation occurs via proximal cis-regulatory elements upstream of the transcription start site as well as via long-range regulatory elements termed either enhancers or repressors which can be positioned kilo-base pairs upstream or even downstream of the transcriptional start site. However, most regulatory elements are found within 1kb upstream sequence from the transcription start site in Arabidopsis (Maleck et al., 2000). DNA-binding factors or transcription factors bind these regulatory elements and as a result lead to the induction or the repression of gene expression. Coactivators and corepressors are additional regulatory proteins that do not bind DNA directly but have the potential to affect gene regulation via chromatin remodelling and protein-protein interactions. It is clear that PYK10 expression is regulated by the JA signalling pathway due to the ability of MeJA to induce PYK10 expression and the lack of MeJA-induced PYK10 expression in coi1-1, the male sterile mutant defective in all JA-dependent defence responses including JA-dependent fertility. Therefore, regulatory elements must be present in the PYK10 promoter region that binds
transcription factors that play a role in the JA signalling pathway. This doesn’t appear to be MYC2 as its mutant jin1 still showed MeJA-inducible PYK10 expression.

Promoter deletion analysis has been widely used to identify cis-elements responsible for gene regulation. Such methods have led to the identification of JA-responsive cis-elements such as the G-box motif (CACGTG) in the soybean VSPB promoter (Mason et al., 1993) and the potato PI-II promoter (Kim et al., 1992a), the C-box motif (TGACG) in the barley grain LOXI promoter (Rouster et al., 1997) and the Agrobacterium NOS promoter (Kim et al., 1993), the GCC-box (GCGGCC) in the Arabidopsis PDF1.2 promoter (Brown et al., 2003), the GCC-box-like motif (ACCGCC) in the periwinkle STR promoter (Menke et al., 1999a), and more recently the T/G-box (AACGTG) in the tomato LAP promoter (Ruiz-Rivero and Prat, 1998; Boter et al., 2004) and the Arabidopsis AtVSP promoter (Guerineau et al., 2003). In addition, a common stretch of DNA sequence such as AATGTT found in the JA-responsive region of the soybean VSPB (Mason et al., 1993) and barley LOXI (Rouster et al., 1997) gene promoter may also play a role in the JA-responsiveness of its associating gene.

The transcription start site of PYK10 has been experimentally determined to be 48 bp upstream of the start codon (Nitz et al., 2001). A region 2169 bp upstream from the PYK10 transcription start site was analysed for JA-responsive cis-elements as this region should contain most if not all of the regulatory elements. Previous studies have shown that 1 kb is often sufficient (Maleck et al., 2000). Two C-box motifs at positions -236 bp and -1278 bp, and 3 T/G-box motifs at positions -1600 bp, -1863 bp and -2120 bp were identified (Fig. 6). These motifs could be responsible for MeJA regulation of PYK10. However, the statistical relevance of these cis-elements cannot be determined since only one gene is analysed. Furthermore, this analysis only provides known JA-responsive cis-elements.

With the recent bloom in DNA microarray technology global expression analysis of genes is now possible using various web-based tools to answer, confirm or discover genes involved in biological processes. Co-expressed genes are often involved in the
same or related pathways as similar expression profiles may be the result of common transcriptional regulation. Such an approach has been successful in the identification of genes required for cellulose synthesis co-expressed with known cellulose synthase genes, and brassinosteroid (BR)-related genes co-expressed with the BR signalling components BRI1 and BAK1 (Lisso et al., 2005; Persson et al., 2005). Since transcriptional regulation involves the binding of TFs to *cis*-elements, the promoters of co-regulated genes in the same or related pathways must share identical *cis*-elements in order to recruit these TFs. Analysis on promoters of genes co-expressed in response to JA could therefore attribute possible JA responsiveness to these known motifs and perhaps also to novel JA-responsive *cis*-elements.

**Figure 6:** Diagrammatic representation of the *PYK10* promoter region with known JA-responsive *cis*-elements highlighted. C-box motifs are represented in yellow and T/G-box motifs in blue. D1, D2 and D3 represent the deletion fragments used for generating promoter-deletion constructs.
1kb TAIR loci upstream sequence of 155 COI1-dependent MeJA-inducible genes (Devoto et al., 2005) were analysed using Patmatch available on the TAIR website (www.arabidopsis.org) because the expression of PYK10 is induced by MeJA treatment and is COI1-dependent (Fig. 4, Table 1A). 1kb TAIR loci upstream sequence is defined by 1kb of sequences preceding the 5' end of each transcription unit. Sequences immediately upstream of 5'UTR are used for those genes with annotated UTRs otherwise sequences upstream of translational start site are used. This analysis demonstrated the enrichment of known JA-responsive cis-elements G-box and T/G-box by 2.01 (P-Value = 5.63E-06) and 1.67 (P-Value = 4.68E-05) times, respectively, in these DNA sequences compared to 33282 1kb TAIR loci upstream sequences of the entire Arabidopsis genome.

The Expression Angler on the Botany Array Resources website (http://bar.utoronto.ca/) was used to find genes co-expressed with PYK10 across 392 experiments from the NASCArrays database (Toufighi et al., 2005). This is the most comprehensive dataset available for the Expression Angler tool and thus it was chosen to provide more meaningful correlation data. A total of 32 genes were found to correlate with PYK10 at an r-value greater than 0.67 (Fig. 7 and Table 2). Among these genes are MeJA-inducible PBP1 and NAI1 which have been previously shown to co-express with PYK10 after MeJA treatment (Matsushima et al., 2004). Analysis of 1kb TAIR loci upstream sequence of these genes using Patmatch available on the TAIR website (www.arabidopsis.org) demonstrated the enrichment of known JA-responsive cis-elements G-box and T/G-box by 2.01 (P-Value = 0.018) and 1.52 (P-Value = 0.045) times, respectively, in their promoters compared to 33282 1kb TAIR loci upstream sequences of the entire Arabidopsis genome (Table 1B).
Table 1: Known Jasmonate-responsive cis-elements in (A) 155 genes that are COI1-dependent and MeJA inducible, (B) PYK10 and 32 co-expressed genes, and (C) PYK10 and 16 of these 32 co-expressed genes that are MeJA-inducible. a is the genome frequency of all 33282 Arabidopsis genes, having at least one occurrence of the respective motif in their 1kb TAIR Loci upstream sequence. b is the expected frequency of the DNA motif in their 1kb TAIR Loci upstream sequence. c is the number of genes containing the respective motif in their 1kb TAIR Loci upstream sequence. P-Value represents the significance of the difference between the observed number of DNA sequences containing the respective motif and the expected frequency calculated by the Poisson distribution.

(A) 155 genes that are COI1-dependent and MeJA inducible

<table>
<thead>
<tr>
<th>Motif</th>
<th>DNA sequence</th>
<th>Genome frequency a</th>
<th>Expected frequency b</th>
<th>No. of genes c</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-box</td>
<td>CACGTG</td>
<td>0.15</td>
<td>23.34</td>
<td>47</td>
<td>5.63E-06</td>
</tr>
<tr>
<td>T/G-box</td>
<td>AACGTG</td>
<td>0.24</td>
<td>37.03</td>
<td>62</td>
<td>4.68E-05</td>
</tr>
<tr>
<td>C-box</td>
<td>TGACG</td>
<td>0.54</td>
<td>84.25</td>
<td>81</td>
<td>4.16E-02</td>
</tr>
<tr>
<td>GCC-box</td>
<td>GCCGCC</td>
<td>0.09</td>
<td>13.48</td>
<td>5</td>
<td>5.18E-03</td>
</tr>
<tr>
<td>GCC-box-like</td>
<td>ACCGCC</td>
<td>0.10</td>
<td>14.87</td>
<td>6</td>
<td>5.23E-03</td>
</tr>
</tbody>
</table>

(B) PYK10 and 32 co-expressed genes

<table>
<thead>
<tr>
<th>Motif</th>
<th>DNA sequence</th>
<th>Genome frequency a</th>
<th>Expected frequency b</th>
<th>No. of genes c</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-box</td>
<td>CACGTG</td>
<td>0.15</td>
<td>4.97</td>
<td>10</td>
<td>0.018</td>
</tr>
<tr>
<td>T/G-box</td>
<td>AACGTG</td>
<td>0.24</td>
<td>7.88</td>
<td>12</td>
<td>0.045</td>
</tr>
<tr>
<td>C-box</td>
<td>TGACG</td>
<td>0.54</td>
<td>17.94</td>
<td>15</td>
<td>0.079</td>
</tr>
</tbody>
</table>

(C) PYK10 and 16 MeJA-inducible co-expressed genes

<table>
<thead>
<tr>
<th>Motif</th>
<th>DNA sequence</th>
<th>Genome frequency a</th>
<th>Expected frequency b</th>
<th>No. of genes c</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-box</td>
<td>CACGTG</td>
<td>0.15</td>
<td>2.56</td>
<td>6</td>
<td>0.030</td>
</tr>
<tr>
<td>T/G-box</td>
<td>AACGTG</td>
<td>0.24</td>
<td>4.06</td>
<td>7</td>
<td>0.062</td>
</tr>
<tr>
<td>C-box</td>
<td>TGACG</td>
<td>0.54</td>
<td>9.24</td>
<td>9</td>
<td>0.131</td>
</tr>
</tbody>
</table>
Figure 7: Thumbnail graphic of Expression Angler output (median-centred and normalised) showing genes whose expression correlates with that of PYK10, At3g09260, at a r-value of 0.67 or higher across 392 samples in the NASCArrays database. The colour scale indicates the log2-level of expression above or below the median. Red indicates folds above the median and blue indicates folds below the median.
Table 2: List of genes whose expression correlates with that of *PYK10* at an r-value of 0.67 or higher across 392 experiments in the NASCArrays database with their corresponding AGI and annotation. MeJA inducibility is derived from gene expression data in the PathoPlant® database.

<table>
<thead>
<tr>
<th>AGI-ID</th>
<th>r-value</th>
<th>Annotation</th>
<th>MeJA-induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>At3g09260</td>
<td>1.000</td>
<td>PYK10</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g16420</td>
<td>0.927</td>
<td>PBP1</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g16460</td>
<td>0.895</td>
<td>jacalin lectin family protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At5g26280</td>
<td>0.854</td>
<td>MATH domain-containing protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g15950</td>
<td>0.804</td>
<td>TSA1-LIKE</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g20370</td>
<td>0.802</td>
<td>MATH domain-containing protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At1g54000</td>
<td>0.798</td>
<td>myrosinase-associated protein, putative</td>
<td>No</td>
</tr>
<tr>
<td>At5g64100</td>
<td>0.741</td>
<td>peroxidase, putative</td>
<td>No</td>
</tr>
<tr>
<td>At3g16450</td>
<td>0.737</td>
<td>jacalin lectin family protein</td>
<td>No</td>
</tr>
<tr>
<td>At4g27860</td>
<td>0.729</td>
<td>integral membrane family protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At2g22170</td>
<td>0.726</td>
<td>lipid-associated family protein</td>
<td>No</td>
</tr>
<tr>
<td>At2g22770</td>
<td>0.725</td>
<td>NAI1</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g16390</td>
<td>0.725</td>
<td>jacalin lectin family protein</td>
<td>No</td>
</tr>
<tr>
<td>At2g18980</td>
<td>0.724</td>
<td>peroxidase, putative</td>
<td>No</td>
</tr>
<tr>
<td>At2g39310</td>
<td>0.722</td>
<td>jacalin lectin family protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At5g44380</td>
<td>0.721</td>
<td>FAD-binding domain-containing protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At5g44020</td>
<td>0.720</td>
<td>acid phosphatase class B family protein</td>
<td>No</td>
</tr>
<tr>
<td>At4g23680</td>
<td>0.713</td>
<td>major latex protein-related</td>
<td>No</td>
</tr>
<tr>
<td>At2g38310</td>
<td>0.712</td>
<td>unknown protein</td>
<td>No</td>
</tr>
<tr>
<td>At1g03870</td>
<td>0.710</td>
<td>FLA9</td>
<td>No</td>
</tr>
<tr>
<td>At4g30670</td>
<td>0.705</td>
<td>unknown protein</td>
<td>No</td>
</tr>
<tr>
<td>At1g62660</td>
<td>0.703</td>
<td>beta-fructosidase (BFRUCT3)</td>
<td>Yes</td>
</tr>
<tr>
<td>At5g65020</td>
<td>0.703</td>
<td>annexin Arabidopsis 2 (ANAT2)</td>
<td>Yes</td>
</tr>
<tr>
<td>At4g21600</td>
<td>0.702</td>
<td>bifunctional nuclease, putative</td>
<td>Yes</td>
</tr>
<tr>
<td>At1g04250</td>
<td>0.697</td>
<td>Auxin resistant 3 (AXR3)</td>
<td>No</td>
</tr>
<tr>
<td>At3g16440</td>
<td>0.689</td>
<td>Myrosinase-binding protein-like protein</td>
<td>No</td>
</tr>
<tr>
<td>At1g28580</td>
<td>0.688</td>
<td>GDSL-motif lipase, putative</td>
<td>Yes</td>
</tr>
<tr>
<td>At5g23820</td>
<td>0.687</td>
<td>ML domain-containing protein</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g09220</td>
<td>0.682</td>
<td>Laccase7 (LAC7)</td>
<td>No</td>
</tr>
<tr>
<td>At1g64200</td>
<td>0.679</td>
<td>VHA-E3</td>
<td>Yes</td>
</tr>
<tr>
<td>At1g28130</td>
<td>0.678</td>
<td>GH3.17</td>
<td>No</td>
</tr>
<tr>
<td>At1g17860</td>
<td>0.678</td>
<td>trypsin and protease inhibitor family</td>
<td>Yes</td>
</tr>
<tr>
<td>At3g05890</td>
<td>0.671</td>
<td>rare-cold-inducible 2B (RCI2B)</td>
<td>No</td>
</tr>
</tbody>
</table>
Using PathoPlant®, a database on plant-pathogen interactions and signal transduction reactions (Bulow et al., 2007), the MeJA-inducibility of these co-expressed genes was analysed in Arabidopsis thaliana microarray gene expression data from TAIR (Garcia-Hernandez et al., 2002). One week-old whole Col-0 plants were subjected to MeJA treatment for 30 minutes to 3 hours. 16 genes co-expressed with PYK10 were induced by MeJA, including PBP1 and NAI1 (Table 2). Analysis of 1kb TAIR loci upstream sequence of these MeJA-inducible genes using Patmatch available on the TAIR website (www.arabidopsis.org) demonstrated the enrichment of known JA-responsive cis-element G-box and T/G-box by 2.34 (P-Value = 0.03) and 1.67 (P-Value = 0.062) times, respectively, in their promoters compared to 33282 1kb TAIR loci upstream sequences of the entire Arabidopsis genome (Table 1C). Interestingly, PYK10 expression is not induced under these conditions in PathoPlant® even though its expression was observed at 3 hours after MeJA treatment by northern analysis (Fig. 5). Plants were treated with 10 µM MeJA for the microarray experiments whereas for northern experiments, they were treated with 45 µM MeJA. MeJA induction of PYK10 in Arabidopsis may require a higher concentration of MeJA as Matsushima et al. in 2004 also observed an increase in PYK10 expression using 50 µM MeJA in their RT-PCR analysis (Matsushima et al., 2004). Similar to PYK10, some of the other 16 co-expressed genes could also be inducible with a higher concentration of MeJA. Also, only one gene out of the 16 MeJA-inducible genes was represented in the 155 COI1-dependent MeJA-inducible genes. This highlights certain restrictions with microarray analysis; meaningful correlation data can only be derived from large datasets, researchers are limited to the experimental conditions offered by available datasets and microarray analyses are not as sensitive as northern or RT-PCR analyses, which can detect subtle changes.

From the above statistical analysis, there was a similar frequency of C-box motifs in the 1kb TAIR loci upstream sequence of the analysed genes as in the whole genome, which could possibly suggest that this JA-responsive motif may not be necessary in the recruitment of TFs in the JA signalling pathway in these sets of genes. The recruitment of such TFs may be the role of G-box and T/G-box motifs due to their high presence in
the promoters of these genes thereby making them better candidates for conferring MeJA-responsiveness. These co-expressed and co-regulated MeJA-induced genes were further analysed for over-represented 6-mer DNA motifs compared to the overall Arabidopsis genome, all having at least one occurrence of the respective motif in their DNA sequence 1kb upstream from the start codon using Motif Analysis available on the TAIR website (www.arabidopsis.org). This analysis would hopefully reveal novel TF cis-elements that may bind to the *PYK10* promoter and regulate its expression in response to MeJA.

The DNA sequence 1kb upstream from the start codon of these MeJA-inducible co-expressed/-regulated genes were compared to the Arabidopsis genome and over-represented 6-mer DNA motifs that are also present in the *PYK10* promoter are shown in Table 3 and Figure 8. Three motifs from the set of 155 COI1-dependent MeJA-inducible genes had 1.6 times higher than the expected frequency (Table 3A) and six motifs from the set of 16 MeJA-inducible co-expressed with *PYK10* had twice the expected frequency (Table 3B). The binding of TFs to all of these motifs has not been previously shown except for the sunflower *DC3* promoter binding factors (DPBF) 1 and 2 which have been shown to bind motif CACGAG (Kim et al., 1997). DPBF1 and 2 were first isolated via a yeast one-hybrid assay binding to a consensus G-box-like motif ACACNNG from the carrot *DC3* promoter (Kim et al., 1997). These motifs thereby represent potential cis-elements involved in the MeJA regulation of *PYK10*. 
Table 3: 6-mer DNA motifs present in *PYK10* and over-represented in (A) 155 genes that are COI1-dependent and MeJA inducible, and (B) 16 MeJA-inducible co-expressed genes, compared to the Arabidopsis genome.  

\( a \) is the genome frequency of all 33282 Arabidopsis genes, having at least one occurrence of the respective motif in their 1kb upstream DNA sequence.  

\( b \) is the expected frequency of the DNA motif in upstream DNA sequence 1kb from the start codon.  

\( c \) is the number of genes containing the respective motif in their DNA sequence 1kb upstream from the start codon.  

\( P \)-Value represents the significance of the difference between the observed number of genes containing the respective motif and the expected frequency calculated by the Poisson distribution. Only confidence levels higher than 99.8\% in (A) and 98\% in (B) were considered.

### (A) 155 genes that are COI1-dependent and MeJA inducible

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>Genome frequency( ^a )</th>
<th>Expected frequency( ^b )</th>
<th>No. of genes( ^c )</th>
<th>( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACACG</td>
<td>0.17</td>
<td>26.66</td>
<td>53</td>
<td>2.30E-06</td>
</tr>
<tr>
<td>CCATGCG</td>
<td>0.16</td>
<td>25.24</td>
<td>41</td>
<td>9.97E-04</td>
</tr>
<tr>
<td>TACGTA</td>
<td>0.15</td>
<td>23.86</td>
<td>39</td>
<td>1.14E-03</td>
</tr>
</tbody>
</table>

### (B) PYK10 AND 16 MeJA-inducible co-expressed genes

<table>
<thead>
<tr>
<th>DNA sequence</th>
<th>Genome frequency( ^a )</th>
<th>Expected frequency( ^b )</th>
<th>No. of genes( ^c )</th>
<th>( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACGAG</td>
<td>0.17</td>
<td>2.94</td>
<td>9</td>
<td>2.37E-03</td>
</tr>
<tr>
<td>ACACGA</td>
<td>0.25</td>
<td>4.31</td>
<td>9</td>
<td>1.91E-02</td>
</tr>
<tr>
<td>GGATAC</td>
<td>0.19</td>
<td>3.24</td>
<td>9</td>
<td>4.28E-03</td>
</tr>
<tr>
<td>CAACGA</td>
<td>0.32</td>
<td>5.36</td>
<td>11</td>
<td>1.24E-02</td>
</tr>
<tr>
<td>CCATGG</td>
<td>0.11</td>
<td>1.95</td>
<td>6</td>
<td>1.09E-02</td>
</tr>
<tr>
<td>CGGATC</td>
<td>0.17</td>
<td>2.86</td>
<td>7</td>
<td>1.77E-02</td>
</tr>
</tbody>
</table>
Figure 8: Diagrammatic representation of the PYK10 promoter region with 6-mer DNA motifs that were found to be over-represented in MeJA-inducible co-expressed/-regulated genes compared to the Arabidopsis genome 1 kb from the start codon.

3.2.5) PYK10 promoter-deletion analysis

Transcription factors in the JA signalling pathway could bind to any of the numerous cis-elements identified in the 2169 bp PYK10 promoter region. For the regulation of PYK10 they could bind to the T/G-box and C-box motifs already identified to be JA-responsive or one of the novel motifs identified. Hence, deletion analysis was employed to identify important MeJA-responsive regions of the promoter.

Three sets of primers were designed to separate this 2169 bp region into 3 different promoter fragment lengths, each having sequential deletions of the above-mentioned cis-elements (Fig. 6 and 8). These promoter fragments were cloned upstream from the luciferase gene (LUC) in the luciferase reporter vector p7LUC (Fig. 9).
D1/D2/D3::LUC::OCS fragments were then introduced into the pART27 binary plasmid between its left and right border upstream of the kanamycin-resistant gene, NPTII, for transformation into A. tumefaciens. Agrobacterial transformation of Arabidopsis resulted in the generation of stable transgenic lines harbouring these promoter-deletion constructs. Transgenic lines harboured the dominant kanamycin-resistant gene and homozygous lines were then selected via antibiotic screens with kanamycin.

Figure 9: A schematic representation of generation of the reporter system.
T₁ heterozygous plants were selected on MS media containing kanamycin for one week and those seedlings that were resistant to kanamycin (as seen by their healthy display of colour and size), compared to susceptible siblings that were blanched and stunted in growth, were transplanted onto soil. They were allowed to self-fertilise and set seed. The T₂ generation were subjected to the same kanamycin screen and those that survived the kanamycin screen (75% or more per screening plate) were either homozygous or heterozygous for the insertion gene (data not shown). In order to select appropriate lines for further analysis initial luciferase assays were performed on a total of 10, 6 and 4 independently transformed 2 week-old T₂ lines from D1, D2 and D3 transgenic plants, respectively, after growth for one week on MS media with kanamycin for selection (Fig. 9). These plants were then transplanted onto normal MS media for recovery from the effects of kanamycin and grown for another week before treatment with MeJA. Three samples of total protein extracts from untreated plants and those treated 24 hours with MeJA were used for luciferase assays to measure the relative light units per µg of protein. Each sample contained 3 to 5 plants. Luciferase activity in plants after 24 hours of MeJA treatment was higher than in plants treated for 6 hours so this time point was used for all luciferase assays. Luciferase activity as a direct response of LUC gene expression driven by the different PYK10 promoter deletion fragments is shown in Figure 10.

Two representative transgenic lines for each of the transgene constructs were selected from these assays, A5-1 and A7-1 for D1, A2-2 and A3-2 for D2 and A1-1 and A1-12 for D3. Stable T₃ homozygous transgenic lines were isolated from each of the representative T₂ lines shown by 100% growth on kanamycin selection media and rigorously confirmed by 2 sets of PCR for the insertion gene (data not shown). The first set with the forward primer directed against the 3'-end of the respective deletion fragments and the reverse primer against the LUC gene, and the second set with primers directed against the NPTII gene. As expected, all homozygous progenies tested harboured the respective deletion fragment fused to the LUC gene 3' of the NPTII gene.
Figure 10: Luciferase assays on untreated 2 week-old T2 D1, D2 and D3 transgenic plants and those treated with 45 μM MeJA/0.005% DMSO for 24 hours. The error bars shown represent standard error across 3 samples for each line, each sample containing 3-5 plants. These experiments were repeated twice with similar results.
Luciferase assays were performed on untreated T₃ homozygous 2 week-old D1, D2 and D3 transgenic lines (the chosen representative lines) and those treated with MeJA and DMSO for 24 hours. Luciferase activity as a direct response of LUC gene expression driven by the different PYK10 promoter deletions is shown in Figure 11. The relative light units of D1 A5-1 and D1 A7-1 increased 4.6 fold and 3 fold, respectively, after MeJA treatment with no comparable increases observed after DMSO treatment. This induction profile mirrors the expression of PYK10, which confirms the presence of cis-elements that control MeJA responsiveness within the largest promoter fragment. D2 A2-2 and D2 A3-2 transgenic lines which have a further deletion of this promoter region both showed a smaller increase in luciferase activity after MeJA treatment with D2 A2-2 and D2 A3-2 having a 1.7 fold and 3 fold increase, respectively. This increase compared to untreated was higher than the increase observed with the DMSO control treatment. This suggests that MeJA-responsive cis-elements are present in the D2 promoter and may work synergistically with MeJA-responsive cis-elements in the D1 promoter region absent in D2 to enhance MeJA induction of PYK10. Since only approximately half of the 5’ upstream sequence separating PYK10 from its adjacent gene was used for promoter analysis it cannot be excluded that had the whole sequence been used a stronger response to MeJA may have been observed. Untreated D3 A1-12 transgenic plants have low relative light units close to the baseline level and treatment with either MeJA or DMSO did not significantly increase luciferase activity. Untreated D3 A1-1 transgenic plants have a 8-fold higher luciferase activity compared to untreated D3 A1-12 transgenic lines but this is likely due to a position effect i.e. where the transgene inserted. No significant increase in luciferase activity was observed with either MeJA or DMSO treatment. D3 promoter region therefore showed no MeJA inducibility. Therefore, MeJA-responsive cis-elements in PYK10 appear to be located outside of the D3 promoter region.
**Figure 11:** Luciferase assays on untreated 2 week-old homozygous D1, D2 and D3 transgenic plants and those treated with 45 µM MeJA/0.005% DMSO and 0.005% DMSO for 24 hours. The error bars shown represent standard error across 3 samples for each line, each sample containing 3-5 plants. These experiments were repeated 3 times with similar results.
Additionally, untreated homozygous 1 week-old transgenic lines of similar size and those treated with MeJA and DMSO for 24 hours were subjected to chemiluminescence imaging (Fig. 12). The overall pattern of MeJA induction observed closely mirrors that of luciferase assays performed on these transgenic lines at 2 weeks as no luciferase activity could be observed for D3 1-1 and D3 1-12, while the increase in luciferase activity observed in D2 2-2 and D2 3-2 after MeJA treatment is enhanced in D1 5-1 (Fig. 12(I)) and D1 7-1 (Fig. 12(II)). Since PYK10 expression is seedling specific basal levels are probably much higher in 1 week-old plants compared to 2 week-old and this appears to be the case in these imaging experiments. The image could be improved by removing background luciferase activity with prior luciferin treatment before treating with MeJA to just visualise luciferase production in response to stimulus. From these luminometer and imaging experiments, it can be concluded that the region of the genome present in D1 but not in D3 seems to account for the strongest MeJA inducibility but there may be additional elements in the region present in D1 and not D2 which enhance the response.
Figure 12: Luciferase imaging on untreated (a) 1 week-old homozygous transgenic plants from lines (I) D1 5-1, D2 2-2, D2 3-2, D3 1-1, D3 1-12 and (II) D1 7-1, and those treated with 0.005% DMSO (b) and 45 µM MeJA/0.005% DMSO (c) for 24 hours. The scale bar is shown in the upper left-hand corner ranging from (I) black being the least intense to red being the most intense and (II) black being the least intense to white being the most intense. This experiment was repeated twice with similar results.
3.3) Discussion

*PYK10* was initially characterised as a seedling and root-specific gene (Nitz et al., 2001) and induced by MeJA within 6 hours in 2 week-old Col-0 plants (Wei, 2002). In this work, MeJA induction of *PYK10* was shown to occur as early as 3 hours and last up to at least 24 hours in whole Col-0 plants of the same age with the induction more prominent in shoots than root. Moreover, activating the JA signalling pathway induced *PYK10* expression even in leaves of 3 week-old Col-0 plants. Analysis of *PYK10* expression using the Arabidopsis eFP Browser tool in BAR showed similar patterns of expression for *PYK10* in development, higher *PYK10* expression in 1 week-old plant and 2 week-old Col-0 plants and lower or no *PYK10* expression from older plants. However, no MeJA induction of *PYK10* was observed for the data set as represented by 1 week-old Col-0 plants treated with MeJA for 30 minutes, 1 and 3 hours compared to those of mock treated plants. This is in contrast to the *PYK10* expression observed at 3 hours mentioned above and could be the result of the lower concentration of MeJA used. In the microarray experiments the concentration of MeJA used was 10 µM and for northern experiments 45 µM MeJA. MeJA induction of *PYK10* in Arabidopsis may require a higher concentration of MeJA as Matsushima et al. also observed an increase in *PYK10* expression using 50 µM MeJA in their RT-PCR analysis (Matsushima et al., 2004). Also, whole plants were used and the large root basal levels may mask induction.

*B. cinerea* is a necrotrophic pathogen able to infect Arabidopsis (Thomma et al., 1999b). Following infection, an army of defence-related genes are expressed by activation of the JA signalling pathway (Tabor and Richardson, 1987; Thomma et al., 1998; Thomma et al., 2000). However, despite the ability of *B. cinerea* infection to activate the JA signalling pathway *PYK10* was not induced as a result of *B. cinerea* infection. Therefore, not all JA-responsive genes are activated in response to stimuli which induces the accumulation of JAMs.
Since *PYK10* expression in response to MeJA is unaffected in *jar1*, JA conjugated to amino acids, mediated by JAR1, leading to the production of bioactive molecules such as JA-Ile do not play a signalling role in this pathway. However, the lack of MeJA-inducibility in *coi1-1* indicates the targets of COI1 for degradation are proteins normally inhibiting *PYK10* expression. One such target could be the JAZ proteins that have recently been discovered as they have been shown to repress JA-mediated responses by repressing JA-inducible TFs (Chini et al., 2007; Thines et al., 2007). One TF which was put to the test is AtMYC2, whose affect on *PYK10* expression was investigated in the *jin1* mutant. However, wildtype *PYK10* expression patterns observed in *jin1* in response to MeJA suggest that AtMYC2 may not be the TF involved. However, it could still play a role without revealing a phenotype if a redundant MYC protein is also involved. COI1 could also be targeting another TF, probably through interaction with JAZ proteins, in the regulation of *PYK10* expression in the JA signalling pathway.

There are 12 plant-specific JAZ proteins identified to date, but interactions with COI1 have only been shown for JAZ1 and JAZ3 and these interactions are dependent on JA-Ile (Chini et al., 2007; Thines et al., 2007). Due to the small number of JAZ proteins and their sequence diversity they may have different roles in JA signalling. Disrupting the conserved Jas motif in JAZ1 made its corresponding transgenic plants male-sterile while its disruption in JAZ3 and JAZ10 had no effect on fertility of their corresponding transgenic plants (Chini et al., 2007). However, these JAZ proteins may possess partially redundant functions as mutant plants overexpressing wildtype JAZ or lack thereof display no obvious defects in JA-related responses (Chini et al., 2007; Thines et al., 2007). Also, despite disruption of the JAZ3 gene MYC2, a target of JAZ3, was still inactive prior to JA treatment suggesting repression of MYC2 by other JAZ proteins such as JAZ1 and JAZ9 (Chini et al., 2007). The mechanism of interaction between COI1 and JAZ proteins is thus complex and not well understood. JAZ proteins are most likely involved in the regulation of *PYK10* via repression of an associating TF, but not MYC2, which has been shown in this study not to be required for *PYK10* induction in response to MeJA. Although JA-Ile appears to be the active hormone for inducing interaction of JAZ1, JAZ3 and JAZ9 with COI1 rather than MeJA, *PYK10* expression
appears unaltered in the jar1 mutant whose wildtype gene encodes the JAR1 enzyme responsible for the conjugation of isoleucine to JA (Staswick and Tiryaki, 2004; Thines et al., 2007; Melotto et al., 2008). This may suggest the involvement of other active JAMs in inducing interaction between JAZ proteins and COI1. Coronatine, a JA analogue similar to JA-Ile, has been shown to promote JAZ3 degradation in jar1 mutant extracts (Chini et al., 2007) and interactions between Arabidopsis COI1 and multiple JAZ proteins (Melotto et al., 2008). JA sensitivity could be restored in jar1, which contains low levels of JA-Ile, by complementing it with wildtype JAR1 to raise endogenous levels of JA-Ile (Staswick and Tiryaki, 2004). However, the induction of PYK10 expression in response to MeJA is JAR1-independent perhaps due to its minor role in the transcriptional regulation of JA-responsive genes. Recently, it has been shown that despite the low levels of JA-Ile observed in wounded jar1 tissue compared to wildtype Arabidopsis tissue many JA-dependent wound-induced genes such as PDF1.2 and LOX2 were unaffected (Suza and Staswick, 2008). In tomato, PDF1.2 induction by JA has also been shown to be independent of jar1 (Onate-Sanchez and Singh, 2002). PYK10 expression could be solely MeJA-dependent or if it does require JA-Ile the low levels of JA-Ile still present in jar1, perhaps due to other unidentified conjugating enzymes, could be sufficient for signalling as COI1-JAZ1 interaction was shown to occur in the presence of 50 nM JA-Ile in vitro (Thines et al., 2007).

JIN1 is the TF AtMYC2 which differentially regulates 2 branches of the JA signalling pathway, it has been shown to positively regulate the branch that is required for the expression of wound-related genes and negatively regulate the branch that is required for the expression of pathogenesis-related genes (Lorenzo et al., 2004). AtMYC2, a target of JAZ proteins degraded by COI1 mentioned above does not play a role in PYK10 induction in response to MeJA. The AtMYC2 mutant jin1 is fertile while the coi1 mutant is sterile and therefore MYC2 derepression is not responsible for all COI1-mediated JA responses. Hence, other targets of JAZ proteins may be involved in the MeJA regulation of PYK10 or its regulation may be JAZ-independent but still COI-dependent.
Well characterised JA-responsive motifs present in the *PKY10* promoter region are three T/G-box motifs and two C-box motifs. The T/G-box motif is over-represented in the 1kb promoter region of 155 genes that are COI1-dependent and MeJA inducible, and *PYK10* and 32 co-expressed genes, 16 of which are MeJA inducible, compared to the whole Arabidopsis genome, further enhancing the probability of it directing MeJA-responsiveness. Nine over-represented DNA sequences were also identified in these promoter regions (Table 3) with two of them, CACACG and CACGAG, displaying G-box-like motif sequence of CACNNG. The CACGAG motif is present in the *DC3* promoter, in the region responsible for seed-specificity and ABA responsiveness, show binding to sunflower DPBF1 and DPBF2 belonging to a novel class of bZIP TFs (Kim et al., 1997). This motif is not known to be JA responsive and this group of bZIP TFs are not dictated by the ACGT core sequence found in their DNA-binding motifs but prefer the consensus sequence ACACNNG (Kim et al., 1997). More complicated motif discovery tools are available which take slight variations of motifs into account and even orthologous promoter regions because it is thought that functional motifs evolve slower under evolutionary pressure (Das and Dai, 2007). However, identifying statistically over-represented motifs in the promoter region of co-expressed genes was useful for *PYK10* promoter-deletion design.

To test whether any of the potential elements identified are important in regulating *PYK10* induction by JA, the luciferase reporter assay system was utilised to delineate the MeJA-responsive promoter regions. The advantages of the luciferase (LUC) assay system is that 1) LUC activity is tightly coupled to protein synthesis and therefore closely mimics that of transcriptional activity; 2) sensitivity is the highest for chemiluminescent reactions due to the high quantum efficiency of its light production (Wood, 1998); 3) each sample takes a few seconds to run; and if low-light video imaging is used 4) it is non-invasive; and 5) spatial information can be obtained. However, disadvantages of the LUC assay system lies in the expensive luminometer or imaging equipment and the sensitivity of LUC to degradation by proteases. Other reporter genes are also available such as those encoding β-glucuronidase and green fluorescent protein however, LUC reporter activity mimics transcriptional activity the
closest due to its short half-life (de Ruijter et al., 2003). There are other ways of delineating cis-elements such as site mutagenesis but that would require prior knowledge of cis-elements and could be time consuming if the cis-elements have to be mutated one by one to observe an effect. By generating promoter deletions one can analyse numerous cis-elements simultaneously without any prior knowledge of the cis-elements of interest and if more than one cis-element is required for function this may be easily elucidated if they are separated by the promoter deletions. Nine novel motifs were identified but only 4 exist outside of D3. Through luciferase assays important elements were identified to be present in D1 and enhanced by elements present in D2 but not D3. D1 specific region is therefore the most crucial region for MeJA responsiveness and is found between -2196 and -1471 upstream of the PYK10 transcription start site. JA-responsive cis-element such as the T/G-box motif or other novel elements such as CCATGC and ACACGA present in this region could be the DNA-binding site of TFs involved in the JA signalling pathway.

PYK10 is a good candidate gene for the elucidation of the JA signalling pathway because its expression is induced by MeJA and is COI1-dependent, COI1 being a vital JA signalling component. A genomic region containing signals directing MeJA expression of PYK10 was delineated in this study. Future experiments would be required to conclude that this region is sufficient for JA regulation and can be performed by generating transgenic lines housing luciferase constructs with the D1 specific region fused to the 35S minimal CaMV promoter. However, by the use of other molecular techniques this region was subsequently dissected to identify potentially important cis-elements required for the regulation of PYK10 in response to JA.
CHAPTER 4

THE HUNT FOR TRANSCRIPTION FACTORS
4.1) Introduction

Regulation of JA-responsive genes is necessary for rapid and appropriate response to abiotic and biotic stresses. The regulation of transcription is carried out by TFs, either alone or in complex with coactivators or corepressors, which control the assembly of the Pol II transcription-initiation complex or the catalytic efficiency of Pol II during the initiation, elongation or termination process (Nikolov and Burley, 1997). TFs contain DNA-binding domains that recognise specific DNA sequences, cis-elements, which are usually less than 15 bp in plant promoters (Higo et al., 1999). Each TF family often has a specific sequence they recognise located in the core region of cis-elements but sequences flanking these conserved core regions are what dictate binding specificity; this is important especially in large TF families. In plants, there are currently 64 families of TFs according to the plant TF database DATF (Guo et al., 2005) whose members have key roles in gene regulation networks. These networks are complex and far from complete, it is therefore important to identify TFs interacting with known or novel cis-elements that respond to a specific stimulus in order to place them in the network for better understanding of gene regulation.

One method of examining DNA-protein binding is via electromobility shift assays (EMSAs). EMSAs are widely used by researchers to identify protein-DNA interactions and exploit the characteristic migration pattern of DNA on gels. Protein-DNA complexes will migrate slower through a non-denaturing polyacrylamide gel than free DNA thereby causing a shift in the migration pattern. This shift can then be visualised according to the method of choice for DNA labelling, such as autoradiography for a radio-labelled DNA probe or fluorescence for a biotinylated DNA probe. There are several advantages of performing EMSAs; they are time-saving as crude extracts from the nucleus or whole cell can be used rather than a purified preparation and the proteins are in their native state allowing them to bind their DNA targets as they would in vivo. Moreover, protein-DNA interactions which require coupling of other proteins and usually go undetected in one-dimensional systems, are allowed in this environment and cause
a “supershift” in the migration pattern. Finally target DNA sequences of cis-elements bound by transcription factors can be identified down to a couple of base pairs with the aid of mutagenesis.

Possible candidates for the binding of TFs involved in JA signalling are the five cis-elements identified to date that have been found via promoter deletion analyses in promoters of JA-responsive genes and in PYK10; the G-box (Kim et al., 1992b; Mason et al., 1993), the C-box (Kim et al., 1993; Rouster et al., 1997), the GCC-box (Maleck et al., 2000), the GCC-box-like (Menke et al., 1999a) and the T/G-box motif (Ruiz-Rivero and Prat, 1998; Guerineau et al., 2003; Boter et al., 2004). Their corresponding TFs have been elusive but with yeast one-hybrid technology, TFs such as ORCA2 in Catharanthus, associated with the GCC-box-like motif in the STR promoter (Menke et al., 1999a) and JAMYC2 and JAMYC10 in tomato, associated with the T/G-box motif in the LAP promoter (Boter et al., 2004), have been shown to bind their cognate cis-elements.

The yeast one-hybrid system takes advantage of the bipartite structure of certain TFs where the DNA-binding domain (DBD) and the transcription activation domain (AD) can be engineered to function separately. cDNAs encoding proteins are fused to the transcription AD which acts as the “prey” while the cis-element of interest is usually fused to a nutritional reporter gene, such as HIS3, and acts as “bait”. Transcription of the reporter gene is activated upon bait recognition and binding by the prey, whose cDNA can be isolated and identified. Since the yeast one-hybrid system has proved successful in the identification of TFs this approach was adopted, once a suitable cis-element was identified, in the hope of identifying the transcription factor responsible for PYK10 gene regulation in the JA signalling pathway.

Initial promoter deletions narrowed down the MeJA-responsive region of the PYK10 promoter from 2196 bp to 1306 bp which may contain several independent or interacting regions necessary for JA regulation. This region was further dissected to
narrow down the regions required for MeJA regulation via EMSA and attempts were made to identify proteins binding to these regions using the yeast one-hybrid system.
4.2) Results

4.2.1) The specific DNA-binding activity of a W-box is MeJA inducible and dependent on its 3’ flanking sequence

Two different types of known JA-responsive motifs are present in the 1306 bp region examined, one C-box in the region demarcated as D2 and three T/G-boxes in D1 (Fig. 1) (Kim et al., 1993; Rouster et al., 1997). A conserved motif found in several JA-responsive promoters is also present in D1 as well as a sequence which confers JA-responsiveness in the CDI and LAP promoters (Ishikawa et al., 1994; Ruiz-Rivero and Prat, 1998). In addition to these motifs that may confer MeJA-responsiveness to the PYK10 promoter, three W-box motifs were identified in this region, one in D1 and two in D2 (Fig. 1). Defence-associated genes often have W-box motifs ((T)TGAC(C/T)) in their promoters which are specifically recognised by WRKY transcription factors (Eulgem et al., 2000). A paper by Maleck et al. (Maleck et al., 2000) showed that all of the 26 available promoters from 31 genes that correlated with PR1 during SAR contained W-box motifs. These known motifs are all possible binding sites for TFs regulating PYK10 gene expression in the JA signalling pathway and were thus tested for possible interactions with DNA-binding proteins via EMSAs.
Figure 1: The MeJA-inducible region of the PYK10 promoter. This region is located between -2196 and -890 bp upstream from the transcription start site with the sequences used for promoter deletion constructs demarcated as D1 and D2, respectively.
Each of the W-box and JA-responsive motifs incorporating 6 nucleotides of upstream and downstream sequence were radioactively-labelled and used as DNA probes to detect possible interactions with nuclear proteins after MeJA treatment. Surprisingly, no specific DNA-binding interactions were observed for any of the JA-responsive motifs after MeJA treatment (data not shown). However, one of the three W-box motifs (Wbox3) (Fig. 2A) showed a modest increase in DNA-binding activity after MeJA treatment (Fig. 2B and 2C). The weak DNA-binding activity observed between the radio-labelled Wbox3 probe and the nuclear proteins from untreated plants was slightly enhanced when nuclear proteins from MeJA-treated plants were used. Upon the addition of unlabelled Wbox3 as competitor, 50 times in molar excess, the observed DNA-binding activity was strongly reduced both in the untreated and MeJA-treated nuclear protein samples (Fig. 2B). The disappearance of DNA-binding activity with the addition of specific competitor (Wbox3) in the presence of non-specific competitor (poly dl-dC) indicates that this interaction is specific. Also, the modest increase in DNA-binding activity after MeJA treatment of 2 week-old Arabidopsis plants suggests a higher presence of MeJA-responsive TFs specific for the Wbox3 motif.

The core nucleotide sequence TGAC is vital for WRKY TF binding and altering its nucleotide sequence will lead to the loss of WRKY binding activity (Eulgem et al., 1999; Yu et al., 2001; Miao et al., 2004). This is demonstrated in Figure 2C whereupon mutation of the core nucleotide sequence TGAC to GTTA (m1, Fig. 2A) abolished its ability to compete with the WT radio-labelled Wbox3 motif for interaction with DNA-binding proteins in the nuclear extract.

Only one of the three W-box motifs identified in the MeJA-responsive region of the PYK10 promoter interacted differentially and specifically with nuclear proteins after activating the JA signalling pathway. It is well known that WRKY transcription factors bind their cognate W-box motifs with high specificity through their flanking sequence (Eulgem et al., 2000). All three W-box motifs have an invariant sequence TTGACT but differ in their flanking sequences (Fig. 1) which were examined further as a potential mechanism for conferring specificity. To investigate the significance of sequences
flanking the active W-box motif, Wbox1 and Wbox2 were added as competitors to compete with the radio-labelled Wbox3 for interaction with DNA-binding proteins. They failed to compete with radio-labelled Wbox3 as DNA-binding activity was still present (Fig. 3B). Since these three DNA sequences differ only in the nucleotides surrounding the W-box motif, flanking sequences are therefore important for the specificity of TF binding. To identify which flanking sequences were responsible for specificity three nucleotides were altered immediately upstream and downstream, m2 and m3 respectively (Fig. 2A), and used as competitors for DNA-binding activity. As observed in Figure 2B, by mutating the three nucleotides immediately upstream (m2) the probe retained its ability to compete with the radio-labelled Wbox3 probe and therefore these nucleotides do not play a part in transcription factor binding specificity. However, mutation of the three nucleotides immediately downstream (m3) resulted in loss of ability to compete with the radio-labelled Wbox3 motif for interaction with DNA-binding proteins. One or more of the three thymidines immediately downstream of the W-box motif is thus important for the specificity of transcription factor binding.

Co-regulated genes have similar cis-elements in their promoters to subject them to the same transcriptional control by a common set of TFs (Maleck et al., 2000) and upon inspection, 1kb TAIR loci upstream sequence of 32 genes co-regulated with PYK10 (Chapter 3, Table 2) were enriched for thymine or adenine nucleotides immediately downstream of the W-box motif TGACT (Table 1). Interestingly, the frequency of these nucleotides was higher than expected in the promoters of genes co-regulated with PYK10, with confidence levels greater than 95%, thereby suggesting a role for them in recruiting the same or similar WRKY TFs.

In utilising EMSAs it was possible to decipher that the W-box motif present -975 bp from the transcription start site of PYK10 interacts specifically with a TF(s) and that this DNA-binding activity is enhanced after MeJA treatment. In conjunction with mutagenesis, specificity of TF(s) for this W-box motif is determined by the TGAC core motif as well as three nucleotides downstream of the active W-box motif.
A)

Wbox1: 5’- TATATTTGACTCAAAAA -3’
Wbox2: 5’- AACAACTTGAGATT -3’
Wbox3: 5’- ATTGTTTTGACCTTTTTC -3’
m1: 5’- ATTGTTTgttaTTTTTC -3’
m2: 5’- ATTtggTTGACCTTTTTC -3’
m3: 5’- ATTGTTTTGACTaggTTC -3’

B)

![Probe: Wbox3](image)

Nuclear extract: None, Untreated, Untreated, MeJA*, MeJA*
Figure 2: MeJA-inducibility of the DNA-binding activity between nuclear proteins and the Wbox3 motif and the effect of different competitors. A) The nucleotide sequence of the probes used for the EMSA. The W-box is in bold type and the mutated nucleotides are in small letters. The EMSA was performed with nuclear extracts from 2 week-old untreated Col-0 plants and plants treated with 45 µM MeJA/0.005% DMSO for 24 hours incubated with probes consisting of B) radioactively-labelled Wbox3 motif and unlabelled Wbox3 motif 50 times in molar excess as the competitor where indicated (●), and C) radioactively-labelled Wbox3 motif and various unlabelled competitors 50 times in molar excess as denoted in (A) and above the lanes. WT = Wbox3 motif. The blue arrow indicates DNA-binding activity and the purple arrow indicates free probe. This experiment has been repeated twice with similar results.
Table 1: The expected frequency of W-box motifs with additional 3’ W (thymine/adenine) nucleotides and the actual observed number of those motifs in 1kb TAIR loci upstream sequence from 32 genes co-expressed with PYK10. \(^a\) is the genome frequency of all 33282 Arabidopsis genes, having at least one occurrence of the respective DNA sequence in their 1kb TAIR loci upstream sequences. \(^b\) is the expected frequency of the DNA sequence in 1kb TAIR loci upstream sequence of the 32 genes. \(^c\) is the number of promoters containing the respective DNA sequence. \(P\)-Value represents the significance of the difference between the observed number of promoters containing the respective motif and the expected frequency calculated by the Poisson distribution.

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>Genome Frequency(^a)</th>
<th>Expected Frequency(^b)</th>
<th>No. of Promoters(^c)</th>
<th>(P)-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGACTW</td>
<td>0.68</td>
<td>21.71</td>
<td>28</td>
<td>0.033</td>
</tr>
<tr>
<td>TGACTWW</td>
<td>0.52</td>
<td>16.73</td>
<td>22</td>
<td>0.040</td>
</tr>
<tr>
<td>TGACTWWW</td>
<td>0.39</td>
<td>12.47</td>
<td>17</td>
<td>0.046</td>
</tr>
</tbody>
</table>

4.2.2) Yeast one-hybrid: The Wbox::pOne-1 “bait” and the cDNA library “prey”

Regulatory cis-elements are bound specifically by TFs and the observed increase in DNA-binding activity between TF(s) and the W-box motif in the PYK10 promoter after MeJA treatment in EMSAs suggests that this is an important site for PYK10 regulation. This W-box motif could be a potential binding site for TF(s) in the JA signalling pathway. The yeast one-hybrid system was used as a method to reveal the identity of these TF(s). The genotype and features of the yeast strains used in this system are shown in Table 2.
Three tandem repeats of the MeJA-inducible W-box motif (TTGACT) including flanking sequences (six nucleotides immediately upstream and downstream) separated by a 4-bp spacer was used as the DNA “bait” fragment. Three tandem repeats of cis-elements approximately the same size as the latter bait have been utilised successfully on numerous occasions to isolate TFs via the yeast one-hybrid system (Kizis and Pages, 2002; Shen et al., 2003; Ryu et al., 2005). In order to clone this fragment into the shuttle vector pOne-1, an EcoRI site was placed at the 5’ end and a Xhol site at the 3’ end with additional nucleotides to facilitate restriction enzyme cleavage (Fig. 3). This pOne-1 vector has been used successfully in a yeast one-hybrid screen with a similar W-box motif repeat in parsley (Cormack et al., 2002).

Table 2: Yeast strain used in yeast one-hybrid screens

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Genotype</th>
<th>Reporter</th>
<th>Transformation selection markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM4271</td>
<td>MATa, ura3-52, his3-200, ade2-101, ade5, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4D, gal8D, ade5::hisG</td>
<td>N/A</td>
<td>trp1 leu2 ura3 his3</td>
<td>(Liu et al., 1993)</td>
</tr>
</tbody>
</table>
Figure 3: DNA “bait” fragments used in the yeast one-hybrid system. DNA fragments consisting of (A) three repeats of the wildtype W-box motif, Wbox, and (B) three repeats of the mutated W-box motif, \( W_m \)box. W-box motifs are in bold; 4-bp spacers in red; \( EcoR \)I restriction site in green, \( XhoI \) restriction site in orange, nucleotides facilitating restriction enzyme binding in blue; mutated nucleotides in small print.

The bait fragment was ligated into the \( XhoI/EcoR \)I restriction enzyme site in the MCS of the pOne-1 vector following \( XhoI \) and \( EcoR \)I restriction enzyme digests of both the bait fragment and shuttle vector (Fig. 4A). The ligation was transformed into \( E. coli \) DH5\( \alpha \) competent cells and successful transformants were selected for with the antibiotic tetracycline against the tetracycline-resistant gene \( tet \) in pOne-1. Presence of the insert was confirmed by PCR with primers located in the pOne-1 vector bordering the MCS and by sequencing (data not shown). In order to reduce the possibility of nonspecific binding to DNA sequences in the MCS a nucleotide length of 54 bp, consisting of restriction enzyme sites between the insert and the Gal 1-10 minimal promoter in the MCS, was excised by \( EcoR \)I digestion and the plasmid religated (Fig. 4B). The resulting Wbox::pOne-1 construct was transformed into \( E. coli \) DH5\( \alpha \) competent cells and successful transformants were again selected for with tetracycline and confirmed by PCR and sequencing (data not shown).
Figure 4: Graphical representation of pOne-1 vector (A) before insertion of DNA fragment and (B) after insertion of DNA fragment. ARS/CEN6, sequence for stable and low-copy number replication in yeast; TRP1, yeast selectable marker for tryptophan; MCS, multiple cloning site; Gal 1-10, Gal 1-10 minimal promoter; HIS, yeast assay marker for histidine; Ori, origin of replication in *E. coli*; Tet, tetracycline resistant selection; INSERT, DNA “bait” fragment. The restriction sites used for the insertion of DNA fragments are highlighted in yellow.
The Wbox::pOne-1 construct was transformed into yeast (S. cerevisiae) YM4271 competent cells, which are tryptophan and histidine auxotrophs and therefore require the presence of both amino acids in the media to grow. Successful transformants harbouring the Wbox::pOne-1 construct were thus selected for their ability to grow in media lacking tryptophan (Fig. 5A) and presence of the correct plasmid was further confirmed by PCR with primers in the pOne-1 vector. PCR analysis of 16 yeast clones able to grow on SD media lacking tryptophan resulted in the expected DNA band of approximately 280 bp, which includes the bait fragment (Fig. 5C). The initial Wbox::pOne-1 construct with the original MCS was used as a template for the positive control and therefore yielded a larger DNA band than the band of interest as it still contained the full repertoire of restriction enzyme sites (Fig. 4A) Sterilised water was the template for the negative control and yielded no products. Some yeast reporter strains express low background levels of the HIS3 gene and may cause a problem in the selection of positive clones in the yeast one-hybrid assay. To overcome this problem SD media lacking tryptophan and histidine was supplemented with a low dose of 1 mM 3-AT (3-aminotriazole), a competitive inhibitor of the yeast HIS3 protein. Yeast clones selected from the transformation with Wbox::pOne-1 constructs were unable to grow on SD media with 1mM of 3-AT (Fig. 5B) and therefore this concentration of 3-AT served as a good starting point to lower the false positive rate arising from leaky HIS3 expression.
Figure 5: Yeast YM4271 cells harbouring the Wbox::pOne-1 construct. SD media without (A) tryptophan; (B) without tryptophan and histidine and supplemented with 1 mM 3-AT; (C) PCR confirming the presence of the Wbox::pOne-1 construct. + is the positive control and – is the negative control. * represents the 100 bp ladder. The numbers correspond to the individual colonies propagated from the original yeast transformation plate.
Since 90% of yeast colonies picked from the original yeast transformation plate contained the Wbox::pOne-1 construct, competent yeast cells were generated from a representative clone and used to screen a cDNA library. cDNA was synthesised from tissue isolated from 2 week-old Col-0 seedlings that had been treated for 3, 6, 12 and 24 hours with MeJA using the HybriZAP-2.1 XR cDNA Synthesis Kit (Statagene, La Jolla). These time points were chosen to cover the window range where the level of \textit{PYK10} gene expression was maximal and together with the MeJA treatment there should be an enrichment of the mRNA of interest. The resulting cDNAs were pooled and used to construct the MeJA cDNA library for the yeast one-hybrid screen using the HybriZAP-2.1 XR Library Construction Kit (Stratagene, La Jolla). To amplify the library \(5 \times 10^4\) pfu were used as recommended by the manufacturer (Stratagene, La Jolla). The pAD-GAL4-2.1 phagemid vector excised \textit{in vivo} from the HybriZAP-2.1 vector that had been transformed into \textit{E.coli} DH5\(\alpha\) competent cells was analysed by PCR for insert percentage and size. PCR analysis with primers bordering the cDNA insertion site of 30 single rescued colonies arising from the transformation showed that 73\% contained cDNA insert sizes approximately 500 bp or larger (Fig. 6). Therefore, the MeJA cDNA library generated was satisfactory for the yeast one-hybrid screen because 73\% of pAD-GAL4-2.1 phagemid vectors not only showed the presence of cDNA inserts but were also enriched for longer cDNA inserts which will increase the chances of encoding functional DNA-binding proteins. Since the EMSA results show basal DNA-binding activity in untreated plants a cDNA library from untreated 2 week-old Col-0 plants, kindly provided by Dr. Thomas Eulgem, was also included in the yeast one-hybrid screens.
Figure 6: PCR analysis of single rescued colonies each containing a pAD-GAL4-2.1 phagemid vector with varying DNA inserts. Lanes 1 to 30 represent individual colonies, a is the 1 kb molecular weight marker and b is the 100 bp molecular weight marker. The blue lines are an indication of marker sizes.
4.2.3) Screening the yeast one-hybrid libraries

A total of 16 million clones were screened by transforming yeast YM4271 cells harbouring the Wbox::pOne-1 construct with the cDNA libraries. The transformed yeast cells were selected on SD media lacking tryptophan, leucine and histidine. Positive clones will be able to produce their own tryptophan as a result of the Wbox::pOne-1 construct and leucine as a result of the cDNA::pAD-GAL4-2.1 construct but histidine will only be produced via the transcription of the $\text{HIS3}$ gene as a result of interaction between a protein encoded by the cDNA library (prey) and the W-box motifs within the Wbox::pOne-1 construct (bait).

Approximately 60 positive interactions were observed via uninhibited growth on SD media lacking tryptophan, leucine and histidine (data not shown). In order to exclude false positives these positive clones were grown on increasing concentrations of 3-AT, a competitive inhibitor of HIS. Cells that contain higher levels of HIS, indicative of tighter binding of prey to bait resulting in higher $\text{HIS}$ transcription levels, will survive higher concentrations of 3-AT. However, 3-AT at high concentrations slows the rate of yeast cell growth. All positives clones were reproducible on SD media lacking tryptophan, leucine and histidine without 3-AT. However, with increasing concentrations of 3-AT the growth of clones became limited and out of the approximately 60 clones resulting from the screens 38 clones were able to grow on at least 15 mM 3-AT (data not shown). In one screen with the MeJA cDNA library, clone numbers 4, 8 and 19 were able to grow on 30 mM 3-AT (Fig. 7A-F). In order to reveal the identity of the proteins encoded by the cDNAs harboured by these 38 clones these colonies were subjected to PCR using primers bordering the cDNA inserts in the pAD-GAL4-2.1 vector (data not shown). The resulting PCR products were gel-purified and sequenced.
Figure 7: Screening for positive clones. (A) to (F), positive clones on SD media with increasing concentrations of 3-AT and lacking tryptophan, leucine and histidine. White spots and red boxes represent yeast growth while translucent spots represent no growth.
WRKY transcription factors bind W-box motifs with high fidelity (Eulgem et al., 1999; Eulgem et al., 2000) and therefore their cDNAs were expected to be contained in the majority of the 38 clones. However, no cDNAs sequenced showed any similarity to known WRKY transcription factors. One interesting candidate cDNA (At3g04590) encoded a protein containing an AT hook motif, which is a DNA-binding domain with a preference for A/T rich regions (Aravind and Landsman, 1998) and is similar to the Arabidopsis AT-hook protein 1 (AHP1). The remaining 37 clones were identified as false positives as they encoded ribosomal proteins, ribulose-bisphosphate carboxylases, glutathione transferases and the photosystem I subunit, which have no reported transcriptional activity.

In order to confirm the transcriptional activity rendered by the AT hook motif-containing protein, vector DNA was isolated from the yeast clone of interest and transformed into *E.coli* DH5α for amplification. To select for the cDNA::pAD-GAL4-2.1 vector transformed *E.coli* DH5α cells were grown in the presence of the antibiotic ampicillin (the pAD-GAL4-2.1 vector contains an ampicillin resistance gene), and transformed into yeast YM4271 cells already harbouring the Wbox::pOne-1 construct. Competent yeast cells containing the Wbox::pOne-1 construct were generated from a representative clone that was used to screen cDNA libraries. As a negative control, the vector was also transformed into yeast YM4271 cells already harbouring the empty pOne-1 bait vector. Growth of yeast cells independently transformed with the bait vectors pOne-1 and Wbox::pOne-1 was observed upon media lacking tryptophan due to their inherent tryptophan selectable marker. When these yeast cells were transformed with the prey cDNA::pAD-GAL4-2.1 vector with its inherent leucine selectable marker, growth was also observed upon media lacking tryptophan and leucine. As expected, the negative control was unable to grow on media lacking tryptophan, leucine and histidine as the HIS3 gene lacked an upstream driver. However, the positive interaction observed in the MeJA cDNA library screen using the same strain of yeast cells with the same bait and prey vectors was not able to grow in the absence of histidine. The lack of interaction between the bait Wbox::pOne-1 and the prey cDNA::pAD-GAL4-2.1 encoding AT hook
motif-containing protein was disappointing, and this cDNA was therefore regarded as yet another false positive clone.
4.3) Discussion

The Wbox3 motif is important in JA regulation as it was identified in the MeJA-responsive region of the *PYK10* promoter via promoter deletion analyses, and this motif showed an increase in DNA-binding activity after MeJA treatment which was abolished by mutation of the core region. The invariant core of Wbox3 is conserved in W-box motifs and is required for binding of WRKY TFs (Eulgem, 2005). WRKY proteins are a 74-member family of TFs which bind to the W-box motif with high specificity (Eulgem, 2005) and therefore different modes of sequence recognition must be in place for members to differentiate and discriminate between W-box motifs within the promoter of the same gene or different genes.

The recruitment of specific WRKY TFs to its corresponding W-box motif is determined by the choice of flanking nucleotides immediately upstream or downstream of the motif (Ciolkowski et al., 2008) or maybe by its numbers and its orientation relative to each other (Rushton et al., 1996; Fukuda, 1997; Eulgem et al., 1999; Xu et al., 2006). The three W-box motifs present in the MeJA-inducible region of the *PYK10* promoter are distantly located from each other and DNA-binding activity was shown only for Wbox3. Therefore, nucleotide spacing and motif clustering attributed to the recruitment of WRKY TFs to the promoters of certain genes such as Arabidopsis and parsley *PR1*, and tobacco *CHN50* probably do not influence recruitment in this case (Rushton et al., 1996; Fukuda, 1997; Eulgem et al., 1999). Binding specificity was instead conferred by the immediate downstream flanking nucleotide sequence of Wbox3.

Motif flanking sequences play an important role in TF specificity, especially when dealing with large TF families, such as the WRKY TF family. For a given motif, the nucleotide combination of flanking sequences is $4^x$, where $x$ is the number of nucleotides and thus increases exponentially with each additional nucleotide. If one assumes a TF recognises nucleotides either immediately upstream or downstream of a motif then the first immediate nucleotide would result in 4 combinations, the second 16,
the third 64 and so forth, providing sufficient combinations to satisfy large TF families. Kirsch et al. scanned nucleotides immediately surrounding a W-box motif found in the parsley *CMPG1* promoter using point mutations and found that not only is the core motif important for regulating transcriptional activity but also the nucleotides surrounding it (Kirsch et al., 2001). Other motifs such as the G-box motif also employ flanking nucleotides for the accurate recruitment of bZIP transcription factors that are known to bind G-box motifs (Schindler et al., 1992; Williams et al., 1992). Likewise, the W-box motif identified in this study utilises one or more of its 3' flanking nucleotides to direct specific binding. W-box motifs with different combinations of flanking nucleotides were unable to compete with the W-box motif of interest for DNA-binding activity when used in EMSA studies. Therefore, due to the observed enhancement of specific DNA-binding activity of nuclear proteins to the W-box motif of interest in response to MeJA, together with the importance of the core and flanking sequences, DNA-binding protein are highly likely to contain WRKY TF(s).

Due to the high specificity of WRKY TFs for W-box motifs it was surprising that no WRKY transcription factors were isolated in yeast one-hybrid assays with Wbox3 as bait as several WRKY transcription factors have been isolated by this method (Cormack et al., 2002; Xu et al., 2004; Heidenreich et al., 2006). EMSA results showed that the Wbox3 motif was constitutively occupied by TF(s) whose DNA-binding activity increased upon MeJA treatment. A problem of the yeast one-hybrid assay is its inability of isolating TFs that require dimerisation for DNA-binding activity. A plausible explanation for the lack of positive clones could be that the transcription factor responsible for binding the Wbox3 motif actually requires the binding of other proteins for its action. WRKY transcription factors are able to dimerise with other proteins via their N-terminal leucine zipper (Eulgem et al., 2000). A good example of this was shown by Xu et al. in 2006 where they showed that AtWRKY18, AtWRKY40 and AtWRKY60 can form homodimers and heterodimers with each other which dramatically influences their DNA-binding capabilities *in vitro* (Xu et al., 2006).
Another problem of the yeast one-hybrid assay is its inability of isolating TFs that require post-translational modification, absent in the yeast, for activity. Potential modifications include phosphorylation as members of the WRKY family possess serine-proline residues in their N-terminal D-domains which could be potential phosphorylation sites (Eulgem et al., 2000). Recombinant WRKY25 and WRKY33 have been shown to be phosphorylated by MPK4 \emph{in vitro} (Andreasson et al., 2005). Several WRKY TFs involved in the SA signalling pathway have been isolated using yeast one-hybrid assays (Cormack et al., 2002; Xu et al., 2004; van Verk et al., 2008). However, it is likely that WRKY is activated post-translationally in JA signalling pathways as no WRKY factors were isolated by direct binding to the W-box motif in this or other studies. WRKY TFs participating in the JA signalling pathway by regulating gene transcription may have gone unnoticed as they may not require \emph{de novo} synthesis and thus are activated from a pool of preformed WRKY factors. It has been suggested that phosphorylation of the JA signalling pathway activator WRKY33 by MPK4 requires coupling of MAP kinase 4 substrate 1 (MKS1) and hence direct interactions between MPK4 and WRKY33 failed to be detected by yeast two-hybrid assays (Andreasson et al., 2005). Similarly, if WRKY TFs require post-translational modification by coupling with other proteins for activation then yeast one-hybrid assays will be unsuitable for the isolation of such TFs.

WRKY33 is an activator of the JA signalling pathway required for resistance against necrotrophic pathogens and EMSAs have demonstrated the ability of recombinant WRKY33 to bind W-box motifs (Journot-Catalino et al., 2006). However, this W-box motif TTGACC is the standard W-box motif (T)TGAC(C/T) and with the suggestion of constitutive and indiscriminate occupation of W-box motifs by WRKY factors (Turck et al., 2004) the DNA-binding activity observed by WRKY33 may not be specific. This may be the reason why DNA-binding activity was observed in untreated nuclear extracts and only a modest increase of DNA-binding activity upon MeJA treatment was observed. This modest increase may be due to existing WRKY TF at the W-box becoming specific and active and more WRKY TFs being recruited to the W-box or replacement by a different specific and active WRKY TF.
CHAPTER FIVE

CONCLUSION
PYK10 is a β-glucosidase of 65kDa found predominantly in ER bodies which are ER-derived structures found in epidermal cells and surrounded by ribosomes (Hayashi et al., 2001; Matsushima et al., 2003). It has been shown that these ER bodies accumulate under conditions of stress such as wounding and with MeJA treatment (Matsushima et al., 2003), which correlates with the MeJA inducibility of PYK10 previously observed (Wei, 2002). PYK10 could potentially be involved in defence as β-glucosidases are known to produce compounds that may be toxic to insects and pathogens via the cleavage of glucosinolates (Rask et al., 2000). Also, another β-glucosidase BGL1, with 70% homology to PYK10, has been shown to increase its gene expression upon exposure to the diamondback moth and MeJA (Stotz et al., 2000).

The substrate for PYK10 has not yet been identified.

PYK10 expression occurs predominantly in roots with minimal expression in aerial parts of 2 and 3 week-old Arabidopsis. Consistent with this data, Matsushima et al. observed the presence of PYK10 exclusively in roots of 15 day-old Arabidopsis via immunocytochemistry and they reported a nai1 mutant which had minimal ER bodies and failed to accumulate PYK10 (Matsushima et al., 2003). PYK10 does not seem to be required for development, a function of JA, as nai1 had a normal growth phenotype (Matsushima et al., 2003).

PYK10 most likely functions in plant defence via the JA signalling pathway through COI1 as the MeJA inducibility of PYK10 was totally abolished in the coi1 mutant. Coi1 is the only mutant known to date that lacks all JA responses (Berger, 2002). Other JA-insensitive mutants such as jin1 and jar1 accumulated normal PYK10 transcript levels similar to wildtype levels upon MeJA treatment and therefore are not required for MeJA-inducible PYK10 expression. Other JA-induced genes that are JAR1-independent are those involved in the production of parasitoid attracting volatiles in Arabidopsis, which involves the JA signalling pathway (Van Poecke and Dicke, 2002). Van Poecke and Dicke in 2002 showed that in Arabidopsis, JA conjugation to amino acids by JAR1 is not required for production of herbivory-induced parasitoid attracting volatiles as jar1-1
mutants were just as capable of attracting *Cotesia rubecula* to *Pieris rapae*-infested plants as wildtype Col-0 (Van Poecke and Dicke, 2002).

Mining the bioinformatics data has further supported the role of *PYK10* in defence as many co-expressed genes encode putative myrosinase-associating proteins, jacalin lectin proteins, peroxidises and also PBP1 and NAI1, which have all been linked to defence (Taipalensuu et al., 1997; Kawano, 2003; Matsushima et al., 2004; Van Damme et al., 2004). Moreover, genes encoding PBP1 and the transcription factor NAI1 have been shown to be co-expressed with *PKY10* after MeJA treatment (Matsushima et al., 2004; Nagano et al., 2005).

JA signalling pathways protect the plant against a wide spectrum of pathogens, especially necrotrophic fungi such as *B. cinerea* (Thomma et al., 2000). An expression profile of Arabidopsis plants infected with *B. cinerea* done by AbuQamar et al. in 2006 showed an induction of over 600 genes of which 181 genes were dependent on a functional COI1 (AbuQamar et al., 2006). The plant responds to *B. cinerea* infection by activating several MeJA-induced genes encoding plant defence proteins such as *PDF1.2* and *THI2.1* encoding defensins and thionins, respectively (Thomma et al., 1998; Hilpert et al., 2001; AbuQamar et al., 2006). However, unlike the MeJA induction of *PYK10* in 2 and 3 week-old leaves, *PYK10* expression was unaltered in response to *B. cinerea* infection and therefore the JA accumulation in response to *B. cinerea* infection is unable to induce *PYK10*.

Gene regulation occurs at the transcription level via the binding of TFs to distal enhancers or repressors or to proximal regulatory *cis*-elements that ultimately lead to gene induction or repression. Promoter deletion analysis have led to the identification of all JA-responsive *cis*-elements known to date, the G-box motif in soybean *VSPB* promoter (Mason et al., 1993) and potato *Pl-Il* promoter (Kim et al., 1992a), the C-box motif in barley *LOXI* promoter (Rouster et al., 1997) and Agrobacterium *NOS* promoter (Kim et al., 1993), the GCC-box in Arabidopsis *PDF1.2* promoter (Brown et al., 2003), the GCC-box-like motif in periwinkle *STR* promoter (Menke et al., 1999a), and the T/G-
box in tomato *LAP* promoter (Ruiz-Rivero and Prat, 1998; Boter et al., 2004) and Arabidopsis *AtVSP* promoter (Guerineau et al., 2003). Promoter deletion analysis have also led to the isolation of a recurring stretch of DNA sequence, AATGTT, found in JA-responsive region of soybean *VSPB* (Mason et al., 1993) and barley *LOXI* (Rouster et al., 1997) promoter which may play a role in the JA-responsiveness of its associating gene.

Due to the MeJA inducible nature of *PYK10*, promoter deletions fused to the luciferase reporter gene showed that D2 contained *cis*-elements that were responsive to MeJA and D1 contained *cis*-elements that further enhanced promoter activity to MeJA. The only *cis*-elements known to respond to JA present in the MeJA-responsive region of the *PYK10* promoter are 3 T/G-box motifs in D1 and a C-box motif in D2. The AATGTT DNA sequence found in other JA-responsive promoters is also present once in D1. Furthermore, another *cis*-element, the W-box motif is present once in D1 and twice in D2. W-box motifs (T)TGAC(C/T) are usually found within promoters of defence-associated genes which are specifically recognised by WRKY TFs (Eulgem et al., 2000). These motifs are all possible binding sites for TFs regulating *PYK10* gene expression in the JA signalling pathway and were thus tested for possible interactions via EMSAs. EMSAs showed that none of the possible JA-responsive *cis*-elements found in D1 and D2 were able to bind TFs DNA-specifically in Arabidopsis plant extracts in response to MeJA. However, the second W-box motif -976 bp from the transcription start site in D2 showed constitutive DNA-binding activity which was DNA sequence specific and induced by MeJA. EMSAs performed with mutated probe sets conferred DNA-binding specificity to the W-box motif core (TGAC) and three nucleotides immediately downstream of this W-box motif (TGACT).

W-box motifs are present in the promoters of genes involved in SA-mediated plant defence such as *RLK4* (Du and Chen, 2000), *NPR1* (Yu et al., 2001), *AtTRXh5* (Laloi et al., 2004) and *SFR2* (Rocher et al., 2005) where they have been shown to positively regulate gene expression. SAR at the onset of pathogen attack requires the accumulation of SA (Dong, 1998), and Maleck et al. showed that the W-box motif was
enriched in promoters of genes required for SAR with similar regulatory profile as *PR1* (Maleck et al., 2000), a common molecular marker for SAR (Uknes et al., 1993). The W-box motif consensus sequence (T)TGAC(C/T) is recognised and bound by a family of WRKY TFs consisting of 74 members (Eulgem et al., 2000). Clearly, W-box motifs play a role in the transcriptional regulation of genes involved in the SA signalling pathway. However, no W-box motifs have been implicated in the JA signalling pathway to date. In this study, a W-box motif TTGACT was found to bind TFs in a constitutive and sequence-specific manner with an increase in DNA-binding activity after MeJA treatment.

The W-box motif responsible for enhanced DNA-binding activity in response to MeJA behaves similarly to typical W-box motifs required for transcriptional regulation due to the importance of the invariant TGAC core for WRKY TF binding and its flanking sequence for WRKY TF discrimination (Eulgem et al., 1999; Eulgem et al., 2000). The core motif is vital for WRKY TF binding as DNA-binding activity was drastically reduced upon its mutation. However, it has been demonstrated that a single nucleotide change could abolish WRKY TF binding and therefore to attribute the observed DNA-binding activity exclusively to WRKY TFs single nucleotide disruptions in the TGAC core should be unable to compete with Wbox3 for binding. Also, one or more thymine nucleotides immediately downstream of the W-box motif TGACT dictated binding specificity of the WRKY TFs to the W-box motif and the frequency of these nucleotides was higher than expected in the promoters of genes co-expressed with *PYK10*, thereby suggesting a role for them in recruiting the same or similar WRKY TF.

Although no MeJA-inducible W-box motifs have been identified, a small number of WRKY proteins have been implicated in the JA signalling pathway. Mutant and over-expression studies have shown Arabidopsis WRKY11, WRKY17 and WRKY33 to be activators of the JA signalling pathway while WRKY70 acts as a repressor (Li et al., 2004a; Journot-Catalino et al., 2006; Zheng et al., 2006). Interestingly, these WRKY proteins appear to participate in the antagonistic crosstalk between JA and SA signalling pathways as activation of defence gene expression attributed to one pathway
simultaneously leads to repression of defence gene expression in the other pathway (Fig. 1). JA-mediated defence responses are generally activated in response to necrotrophic pathogens and SA-mediated in response to biotrophic pathogens (Glazebrook, 2005). Wrky33 mutants have reduced expression levels of the JA molecular marker gene PDF1.2 and increased susceptibility to necrotrophic B. cinerea and A. brassicicola compared to wildtype plants. Conversely, plants overexpressing WRKY33 were more resistant to these necrotrophic pathogens. While wrky33 mutants had no response to virulent P. syringae, plants overexpressing WRKY33 were more susceptible to P. syringae and had reduced expression levels of the SA molecular marker gene, PR1 (Zheng et al., 2006). WRK11 and WRKY17 have also been shown to be activators of the JA signalling pathway by negatively regulating the SA signalling pathway leading to basal resistance against P. syringae (Journot-Catalino et al., 2006). Mutations in these WRKY genes increased resistance to both avirulent and virulent strains of P. syringae and lead to downregulation of JA-responsive genes such as LOX2, AOS and JMT (Journot-Catalino et al., 2006). Since WRKY11 and WRKY17 gene expression is unaffected by JA treatment and mutations within these genes resulted in the downregulation of JA biosynthetic genes LOX2, OAS and JMT, WRKY11 and WRKY17 must act upstream of JA by positively regulating P. syringae-stimulated accumulation of JAMs. Only one WRKY has been shown to be induced by MeJA, the cotton WRKY1 (Xu et al., 2004).

With more evidence of signalling crosstalk coming to light, WRKY70 is perhaps the best example thus far of a signalling component acting in both pathways, an activator in SA signalling and a repressor in JA signalling. Li et al. in 2004 demonstrated the ability of WRKY70 to activate the expression of SAR-related genes, mediated by SA, and repress the expression of JA-responsive genes (Li et al., 2004a). Mutations in the WRKY70 gene rendered plants more resistant towards necrotrophic A. brassicicola and more susceptible to biotrophs E. cichoracearum and P. syringae. Conversely, overexpressors of WRKY70 were more susceptible to A. brassicicola and more resistant to biotrophs E. cichoracearum and P. syringae (Li et al., 2006). Not only were typical JA-mediated defence responses downregulated in WRKY70 overexpressors,
inhibition of root growth in response to JA were also obliterated, and so were JA-
induced accumulation of anthocyanin (Li et al., 2006). The above-mentioned WRKY
factors must work in concert to bring about an equilibrated response upon pathogen
infection in order to provide the host plant with an efficient and accurate defence
response. WRKY11 and WRKY17 may positively regulate JA biosynthetic enzymes
LOX2, OAS and JMT to activate JA-responsive genes through the JA signalling
pathway as they appear to be upstream signals, unlike WRKY70 and WRKY33
(Journot-Catalino et al., 2006). They may inhibit the SA signalling pathway through
WRKY70 inhibition by COI1 as WRKY70 is negatively regulated by WRKY11 and
WRKY17 (Journot-Catalino et al., 2006). JA accumulation may induce WRKY33 to
positively regulate the JA signalling pathway while negatively regulating the SA
signalling pathway by some unknown mechanisms. On the other hand, upon JA
accumulation COI1 may target WRKY70 for degradation thus alleviating the repression
it has on JA-responsive genes thereby activating the JA signalling pathway for an
appropriate defence response to pathogens. WRKY70 which is induced by SA and
repressed by JA is activated by SA accumulation to induce SA-responsive genes for a
SA-mediated defence response (Li et al., 2004a) (Fig. 1). However, despite the
elucidation of these WRKY factors in the JA signalling pathway WRKY TFs have yet to
be shown to directly bind to W-box motifs and regulate the expression of genes in
response to JA.

The yeast one-hybrid assay is commonly used to identify TFs binding to a specific DNA
sequence or cis-element. This assay has been successfully employed for the isolation
of TFs involved in the JA signalling pathway such as JAMYC2 and JAMYC10 binding
specifically to the G-box and T/G-box motifs (Boter et al., 2004), and ORCA2 and
ORCA3 binding to the GCC-box-like motif (Menke et al., 1999a; van der Fits and
Memelink, 2001). However, yeast one-hybrid assays performed in this study failed to
identify any TFs from the WRKY or other TF families. This could be due to the TF in
question requiring dimerisation for its DNA-binding activity as WRKY TFs are able to
dimerise with other proteins via their N-terminal leucine zipper (Eulgem et al., 2000).
Also, the TF may require post-translational modification for its activity such as
phosphorylation as members of the WRKY family possess potential phosphorylation serine-proline residues in their N-terminal D-domains (Eulgem et al., 2000) and thus may have gone unnoticed as they may not require de novo synthesis and are activated from a pool of preformed WRKY factors.

**Figure 1:** Working model for WRKY action in JA and SA signal crosstalk. Upon pathogen infection either JA or SA or both are synthesised. WRKY11 and WRKY17 positively regulate JA biosynthetic enzymes LOX2, OAS and JMT to activate JA-responsive genes through the JA signalling pathway. They may inhibit the SA signalling pathway through WRKY70. JA accumulation may induce WRKY33 to positively regulate the JA signalling pathway while negatively regulating the SA signalling pathway by unknown mechanisms. On the other hand, upon JA accumulation COI1 may target WRKY70 for degradation thus alleviating the repression it has on JA-responsive genes thereby activating the JA signalling pathway for an appropriate defence response to
pathogens. Upon SA accumulation WRKY70 is activated to induce SA-responsive genes for a SA-mediated defence response.

Other alternative methods of using cis-elements to isolate TFs include affinity chromatography where cis-elements are immobilised on affinity columns and used to purify DNA-binding proteins whose identities are revealed following mass spectrometry (MS) (Kroeger and Abraham, 1997). Cis-elements could also be affixed to a DNA affinity chip and used to purify DNA-binding proteins whose identities are revealed following surface enhanced laser desorption/ionization mass spectrometry (SELDI-MS) (Forde et al., 2002).

In conclusion, PYK10 promoter deletions fused to the luciferase reporter gene were used to identify MeJA-inducible regions within the promoter. Potential cis-regulatory elements were identified in the isolated MeJA-inducible region and their DNA-binding abilities were analysed by EMSAs. This led to the identification of a W-box motif TTGACT, which bound nuclear proteins in a constitutive and sequence-specific manner, and responded to MeJA with an increase in DNA-binding activity. Future work to show that this W-box motif is sufficient for MeJA regulation include generating transgenic plants with copies of this motif, with mutations and with/without flanking regions. This would demonstrate whether this W-box motif can confer MeJA responsiveness and whether the flanking regions identified as essential for binding are essential in vivo. It would be interesting to generate and test the MeJA responsiveness of D1 transgenic plants containing a mutant Wbox3 motif. Although attempts at isolating its corresponding WRKY TF(s) failed with the yeast one-hybrid system it is a reminder that transcription regulation is a complex three-dimensional system that involves not only interactions between DNA and TFs but also other coupling proteins and that the regulation of DNA binding by TFs may be controlled post-translationally.
CHAPTER 6

REFERENCES


Matsushima, R., Fukao, Y., Nishimura, M., and Hara-Nishimura, I. (2004). NAI1 gene encodes a basic-helix-loop-helix-type putative transcription factor that regulates the formation of an endoplasmic reticulum-derived structure, the ER body. Plant Cell 16, 1536-1549.


