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 Saeturn 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

Dissecting the jasmonate signalling pathway in *Arabidopsis thaliana*

Yu-Hung Wei

Thesis presented for the degree of Doctor of Philosophy, December 2008

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 C_{18} α-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 ω3 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 *sn*1 position; (2) phospholipase A_2 which cleaves the acyl moiety in the *sn*2 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) A_2, PLA_2 (Narvaez-

Vasquez et al., 1999), an Arabidopsis DAD-like phospholipase A_1, 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 *AtplaI-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 hormworm (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).

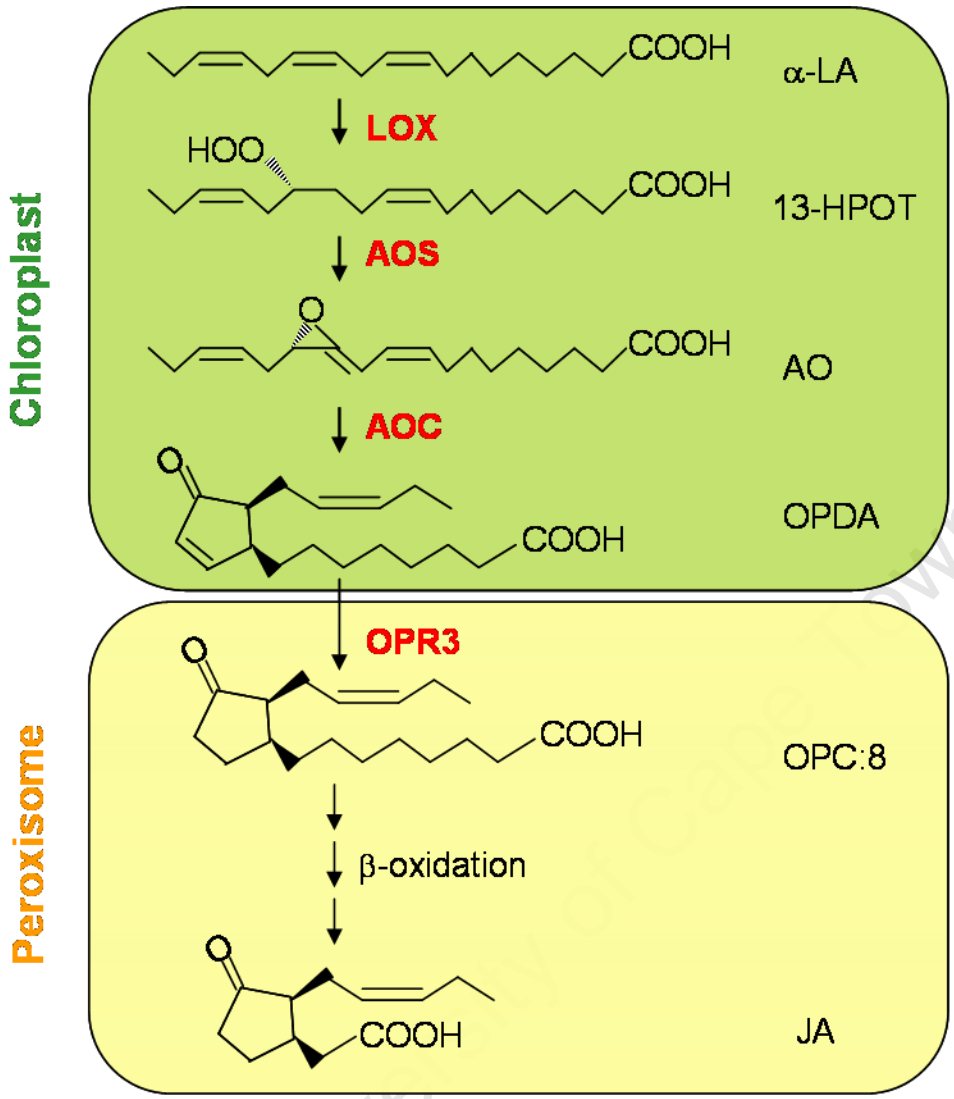


Figure 1: The jasmonic acid biosynthesis pathway. Pathway intermediates: α-LA, α-Linolenic acid; 13-HPOT, 13(S)-hydroperoxy linolenic acid; AO, 12,13(S)-epoxy-9(2),11,15(Z)-octadecatrienoic acid; OPDA, 12-oxo-phytodienoic acid; OPC8, 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid. Pathway enzymes 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-HSO_4-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-HSO_4-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).

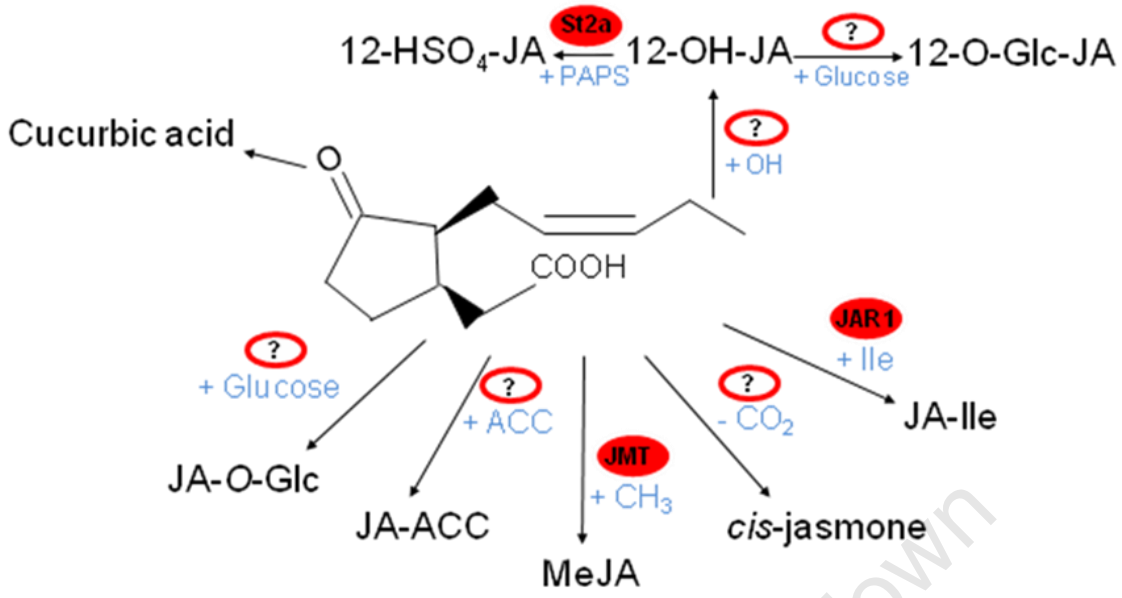


Figure 2: Different metabolites of jasmonic acid. The carboxylic side chain can be glucosylated (+ Glucose) to form jasmonoyl-1-β-glucose (JA-O-Glc), conjugated with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to form JA-ACC, methylated (+ CH_3) by S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) to form MeJA, decarboxylated (-CO_2) 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-HSO_4-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.

Mutant	Altered process	Affected gene and its corresponding AGI	Reference
Defective in JA biosynthesis and metabolism			
fad3fad7fad8	α-LA formation	*FAD3*; At2g29980 *FAD7*; At3g11170 *FAD8*; At5g05580	(McConn and Browse, 1996)
dad1	α-LA formation	*DAD1*; At2g44810	(Ishiguro et al., 2001)
Atplal-1	α-LA formation	*ATPLA1*; At1g61850	(Yang et al., 2007)
aos	OPDA conversion	*AOS*; At5g42650	(Park et al., 2002)
dde1	OPDA conversion	*OPR3*; At2g06050	(Sanders et al., 2000)
opr3	OPDA conversion	*OPR3*; At2g06050	(Stintzi and Browse, 2000)
acx1/acx5	ß-oxidation	*ACX1*; At4g16760 *ACX5*; At2g35690	(Schilmiller et al., 2007)
aim1	ß-oxidation	*MPF*; At4g29010	(Richmond and Bleecker, 1999)
cts	ß-oxidation	*COMATOSE*; At4g39850	(Theodoulou et al., 2005)
pex6	Perixomal function	ATPase; At1g03000	(Zolman and Bartel, 2004)
jar1	JA conjugation	*JAR1*; At2g46370	(Staswick and Tiryaki, 2004)
JA insensitive			
mpk4	Altered JA- and SA-responsive gene expression	*MPK4*; At4g01370	(Petersen et al., 2000)
jin1	Transcription factor	*MYC2*; At1g32640	(Berger et al., 1996; Lorenzo et al., 2004)
coi1	Proteolysis via SCF	*COI1*; At2g39940	(Feys et al., 1994; Xu et al., 2002)
jai4	Proteolysis via SCF	*SGT1b*; At4g11260	(Lorenzo et al., 2004)
axr1	Proteolysis via SCF	*AXR1*; At1g05180	(Tiryaki and Staswick, 2002)
Constitutive JA responses			
cev1	Cell wall synthesis	*CeS3*; At5g05170	(Ellis et al., 2002)
cet1	Unknown	Unknown	(Hilpert et al., 2001)
joe1	Increased *LOX* expression	Unknown	(Jensen et al., 2002)
joe2	Increased *LOX* expression	Unknown	(Jensen et al., 2002)

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).

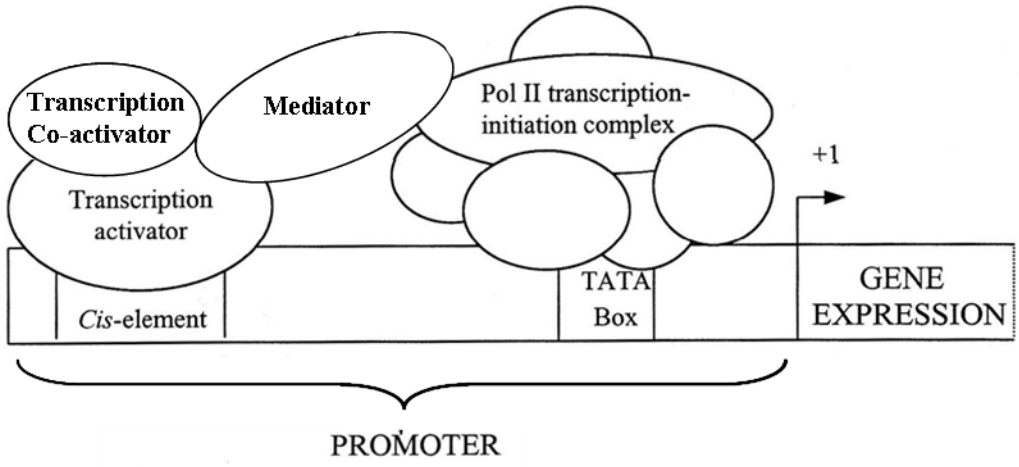


Figure 3: Model of transcription activation via direct-DNA binding of a transcription activator to a *cis*-element and indirect-DNA binding of a transcription coactivator. Interaction between the transcription activator and co-activator remodels DNA for access of the transcription machinery. Communication 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 Arabidospis, 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 posses 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., 1999b). 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 posses 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., 1999a; 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.

Motif	Organism	Gene	Associating TF
G-box (CACGTG)	Soybean	*VSPB* (Mason et al., 1993)	?
	Potato	*PIN2* (Kim et al., 1992)	JAMYC (Boter et al., 2004)
T/G-box (AACGTG)	Tomato	*LAP* (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)	JAMYC (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)
	Arabidopsis	*VSP1* (Guerineau et al., 2003)	AtMYC2* (Ruiz-Rivero and Prat, 1998; Boter et al., 2004)
C-box (TGACG)	Barley	*LOX1* (Rouster et al., 1997)	?
	Agrobacterium	*NOS* (Kim et al., 1993)	?
	Rice	*OPR1* (Sobajima et al., 2007)	?
GCC-box (AGCCGCC)	Arabidopsis	*PDF1.2* (Brown et al., 2003)	AtERF2* (Brown et al., 2003)
GCC-box-like	Periwinkle	*STR* (Menke et al., 1999a)	ORCA2 & ORCA3 (Menke et al., 1999a; van der Fits and Memelink, 2001)

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 SCF^{coi1} 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.

CHAPTER 2

MATERIALS AND METHODS

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 ^{o}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/m^2 in a controlled environment room at 21^{o}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-^{32}P]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 *Sac*I restriction site. Letters highlighted in blue represent the *EcoR*I 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.

Primer	**DNA Sequence (5' – 3')**	**Tm (°C)**
***PYK10* Primers**		
PYK10 forward	AAGCTTCCTCTCATTGGGC	55
PYK10 reverse	CTTGGCATTTAGCATTGACG	55
Deletion Primers		
D1 forward	ATTAGAGCTCTCCCTCAGATGGTCATGCTATC	60
D2 forward	ATTAGAGCTCCAAGCATGTACATGCTGTGGTG	60
D3 forward	ATTAGAGCTCGAAAGAGGAGTGGTGTCGCTC	60
D reverse	ATTGAATTCGGATGGTTCTTTTACATATCGAAG	60
T_3 Selection Primer		
T_3 reverse	ATTACACGGCGATCTTTCCG	57
***NPTII* Primers**		
NPTII forward	GAGGCTATTCGGCTATGACTG	60
NPTII reverse	ATCGGGAGCGGCGATACCGTA	60
pOne-1 primers		
pOne-1 forward	GGTTTTCCCAGTCACGACG	57
pOne-1 reverse	CAAAAATCATCGCTTCGCTG	57
Library primers		
Library forward	AGGGATGTTTAATACCACTAC	48
Library reverse	GCACAGTTGAAGTGAACTTGC	48

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 $5X10^4$ 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 *Sac*I at the 5' end and *EcoR*I 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 *Sac*I and *EcoR*I 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 *Sac*I and *EcoR*I–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 *Sac*I and *Not*I 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 *Sac*I and *Not*I 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 *Sac*I and *EcoR*I 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:

$$\frac{\text{Vector (ng)} \times \text{Insert size (kb)} \times 3}{\text{Vector size (kb)}} = \text{insert (ng)}$$

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 T_4 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. T_1 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. T_2 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^{o}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 preflushed 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^{o}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 *EcoR*I and *Xho*I 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 *Xho*I and *EcoR*I (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 *EcoR*I (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 *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 *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 α-subunit (ASA), Trp synthase α-subunit (TSA), Trp synthase β-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.

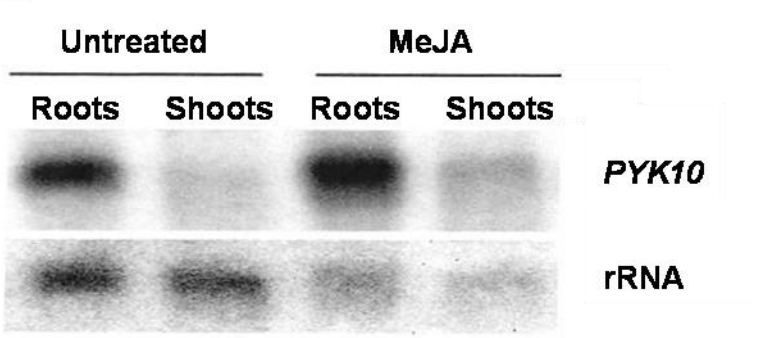
3.2) Results

3.2.1) The MeJA inducibilty 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.

(A)



(B)

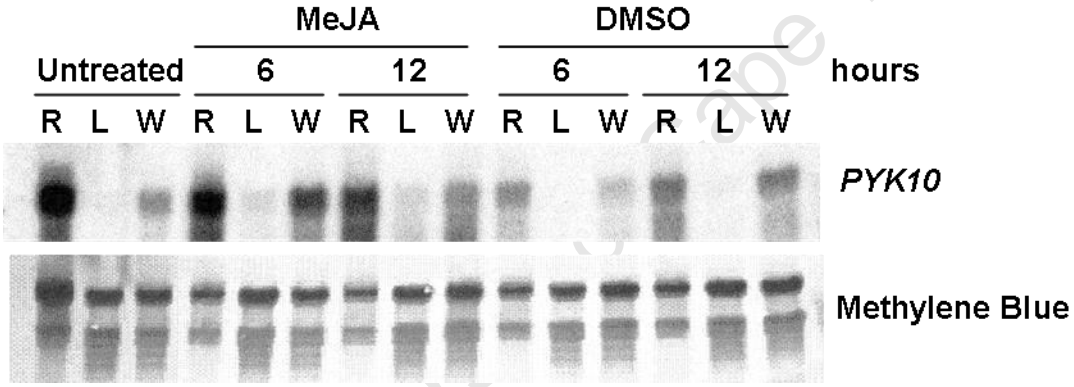


Figure 1: RNA gel blot analysis of *PYK10* in (A) 2 week-old roots and shoots, (B) 3 week-old roots (R), leaves (L) and whole plants (W) of untreated Col-0 plants and those treated with 45 µM MeJA/0.005% DMSO and 0.005% DMSO for (A) 24 hours and for (B) 6 and 12 hours. 10 µg of total RNA was loaded onto 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.

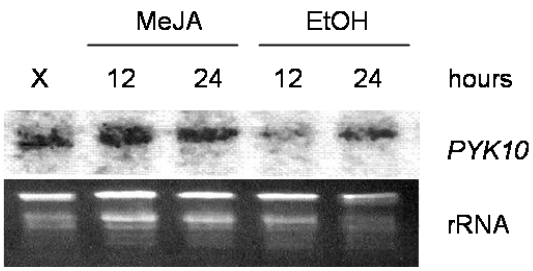
MeJA is commercially available as a hydrophobic solution and has to be dissolved in an appropriate solvent prior to usage. Both ethanol and DMSO have powerful dissolving powers that dissolve polar and non-polar compounds. Ethanol was the solvent for MeJA that was initially used to treat Arabidopsis plants 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).

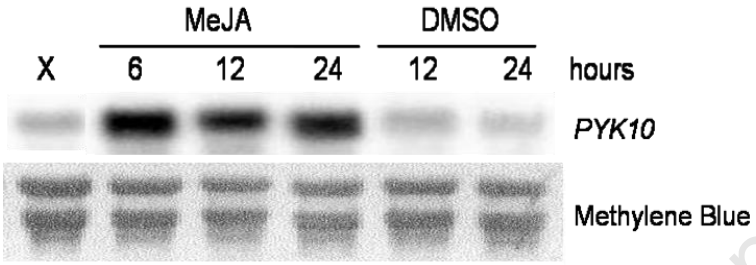
Analysis of *PYK10* expression across 22,000 genes from Arabidopsis, as represented on the Affymetrix ATH1 GeneChip, in the Bio-Array Resource (BAR) using the Arabidopsis electronic Fluorescent Pictograph (eFP) Browser (Winter et al., 2007)

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.

(A)



(B)



(C)

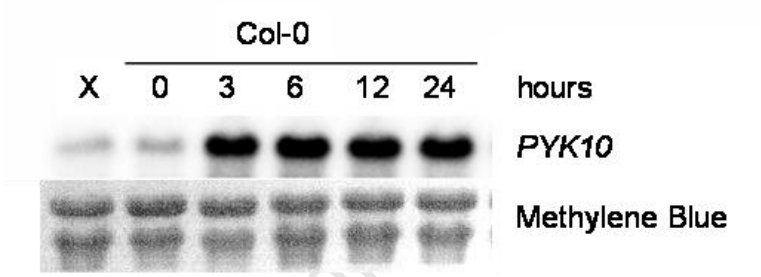


Figure 2: RNA gel blot analysis of *PYK10* in 2 week-old Col-0 plants. Untreated plants (X) and plants treated with (A) 45 µM MeJA/0.01% EtOH or 0.01% EtOH, (B) 45 µM MeJA/0.005% DMSO or 0.005% DMSO and (C) 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto 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.

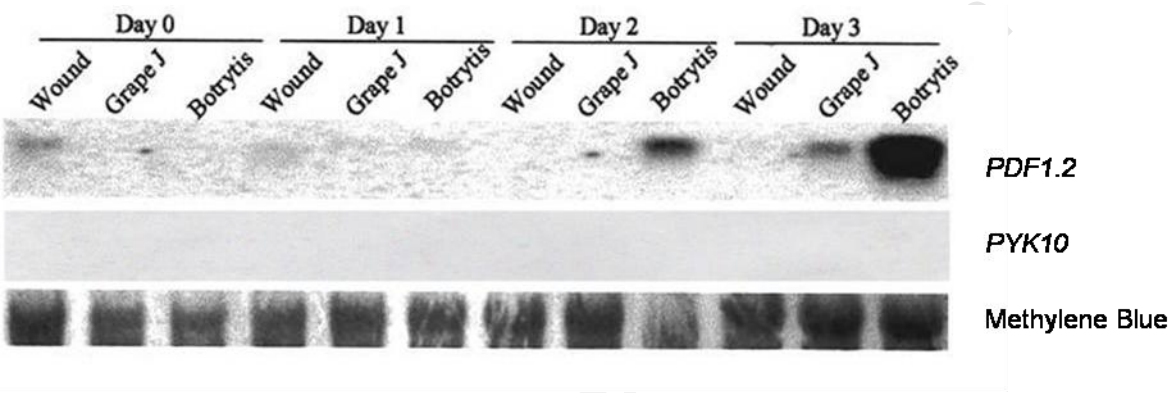


Figure 3: RNA gel blot analysis of *PDF1.2* and *PYK10* in 3 week-old Col-0 leaves. Untreated excised leaves and leaves treated with half-strength grape juice (Grape J) and *Botrytis cinerea* (Botrytis) at $5X10^4$ spores/ml were harvested at the indicated times. 20 µg of total RNA was loaded onto each 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. *coi1* is male-sterile while *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 *jar1*, *jin1* and *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. *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). *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.

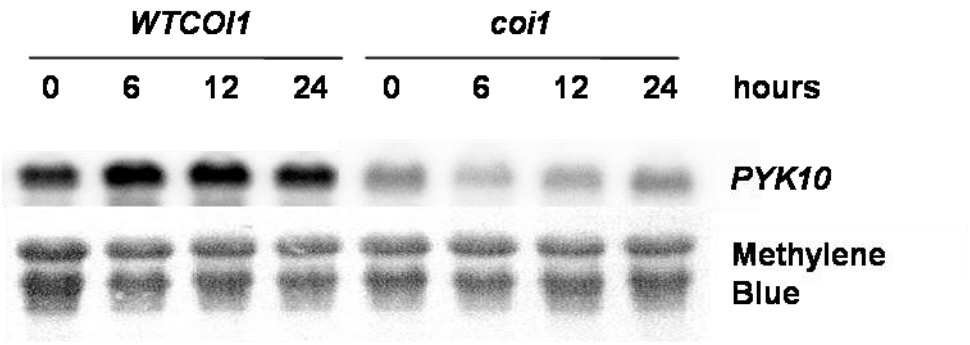


Figure 4: RNA gel blot analysis of *PYK10* in 2 week-old JA mutant *coi1-1* and wildtype *WTCOI1*. Untreated plants (0) and plants treated with 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane and membranes were probed with a 1.5 kb portion of 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.

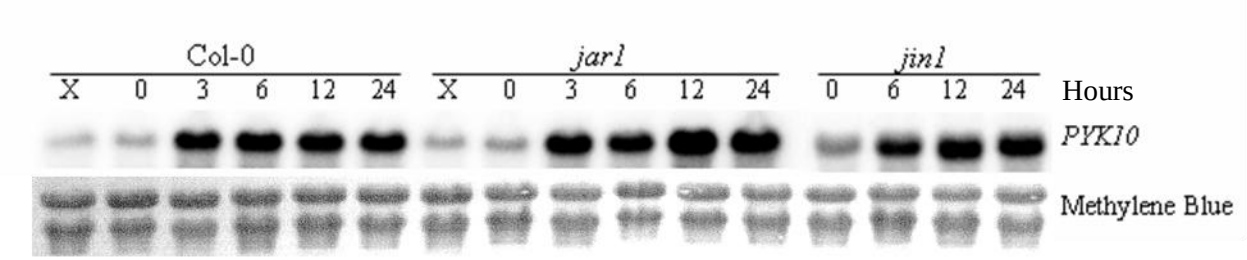


Figure 5: RNA gel blot analysis of *PYK10* in 2 week-old Col-0 and the JA signalling mutants *jar1* and *jin1*. Untreated plants (0) and plants treated with 45 µM MeJA/0.005% DMSO were harvested at the indicated times. 20 µg of total RNA was loaded onto each lane and membranes were probed with a 1.5 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 (GCCGCC) in the Arabidopsis *PDF1.2* promoter (Brown et al., 2003), the GCC-box-like motif (ACCGCC) in the periwrinkle *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.

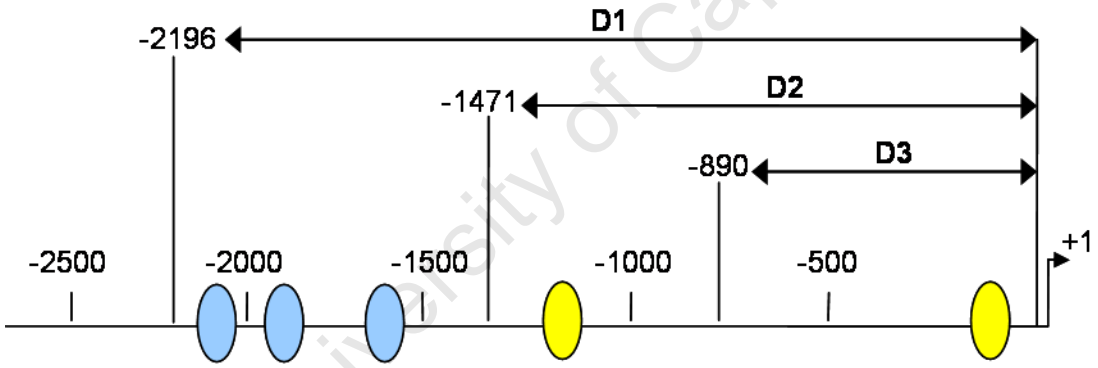


Figure 6: Diagrammatic representation of the *PYK10* promoter region with known JA-responsive *cis*-elements highlighted. C-box motifs are represented in yellow and T/G-box motifs in blue. D1, D2 and D3 represent the deletion fragments used for generating promoter-deletion constructs.

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 upsteam 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

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

(B) PYK10 and 32 co-expressed genes

Motif	DNA sequence	Genome frequency[a]	Expected frequency[b]	No. of genes[c]	*P*-Value
G-box	CACGTG	0.15	4.97	10	0.018
T/G-box	AACGTG	0.24	7.88	12	0.045
C-box	TGACG	0.54	17.94	15	0.079

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

Motif	DNA sequence	Genome frequency[a]	Expected frequency[b]	No. of genes[c]	*P*-Value
G-box	CACGTG	0.15	2.56	6	0.030
T/G-box	AACGTG	0.24	4.06	7	0.062
C-box	TGACG	0.54	9.24	9	0.131

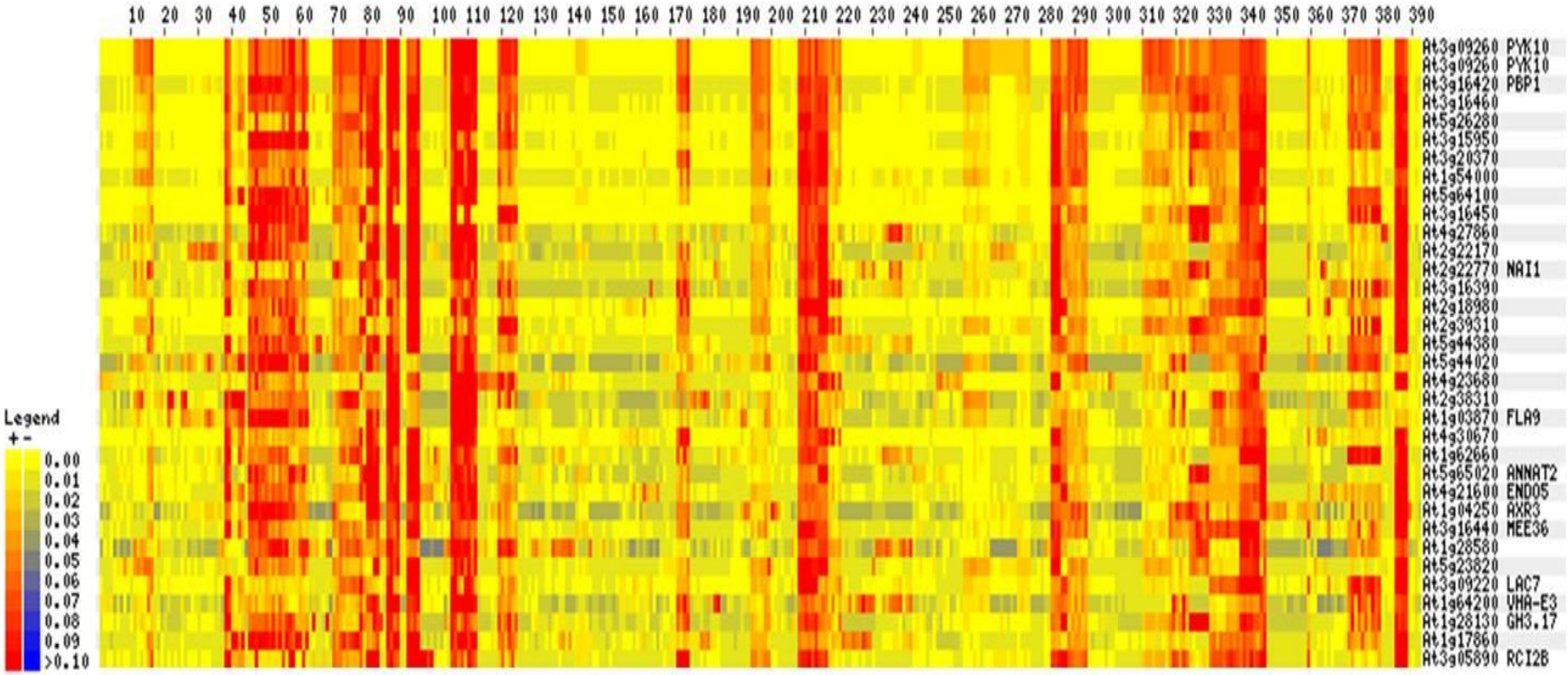


Figure 7: Thumbnail graphic of Expression Angler output (median-centred and normalised) showing genes whose expression correlates with that of *PYK10*, At3g09260, at a r-value of 0.67 or higher across 392 samples in the NASCArrays database. The colour scale indicates the $\log_2$-level of expression 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.

AGI-ID	r-value	Annotation	MeJA-induced
At3g09260	1.000	PYK10	Yes
At3g16420	0.927	PBP1	Yes
At3g16460	0.895	jacalin lectin family protein	Yes
At5g26280	0.854	MATH domain-containing protein	Yes
At3g15950	0.804	TSA1-LIKE	Yes
At3g20370	0.802	MATH domain-containing protein	Yes
At1g54000	0.798	myrosinase-associated protein, putative	No
At5g64100	0.741	peroxidase, putative	No
At3g16450	0.737	jacalin lectin family protein	No
At4g27860	0.729	integral membrane family protein	Yes
At2g22170	0.726	lipid-associated family protein	No
At2g22770	0.725	NAI1	Yes
At3g16390	0.725	jacalin lectin family protein	No
At2g18980	0.724	peroxidase, putative	No
At2g39310	0.722	jacalin lectin family protein	Yes
At5g44380	0.721	FAD-binding domain-containing protein	Yes
At5g44020	0.720	acid phosphatase class B family protein	No
At4g23680	0.713	major latex protein-related	No
At2g38310	0.712	unknown protein	No
At1g03870	0.710	FLA9	No
At4g30670	0.705	unknown protein	No
At1g62660	0.703	beta-fructosidase (BFRUCT3)	Yes
At5g65020	0.703	annexin Arabidopsis 2 (ANNAT2)	Yes
At4g21600	0.702	bifunctional nuclease, putative	Yes
At1g04250	0.697	Auxin resistant 3 (AXR3)	No
At3g16440	0.689	Myrosinase-binding protein-like protein	No
At1g28580	0.688	GDSL-motif lipase, putative	Yes
At5g23820	0.687	ML domain-containing protein	Yes
At3g09220	0.682	Laccase7 (LAC7)	No
At1g64200	0.679	VHA-E3	Yes
At1g28130	0.678	GH3.17	No
At1g17860	0.678	trypsin and protease inhibitor family	Yes
At3g05890	0.671	rare-cold-inducible 2B (RCI2B)	No

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

DNA sequence	Genome frequency[a]	Expected frequency[b]	No. of genes[c]	*P*-Value
CACACG	0.17	26.66	53	2.30E-06
CCATGC	0.16	25.24	41	9.97E-04
TACGTA	0.15	23.86	39	1.14E-03

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

DNA sequence	Genome frequency[a]	Expected frequency[b]	No. of genes[c]	*P*-Value
CACGAG	0.17	2.94	9	2.37E-03
ACACGA	0.25	4.31	9	1.91E-02
GGATAC	0.19	3.24	9	4.28E-03
CAACGA	0.32	5.36	11	1.24E-02
CCATGG	0.11	1.95	6	1.09E-02
CGGATC	0.17	2.86	7	1.77E-02

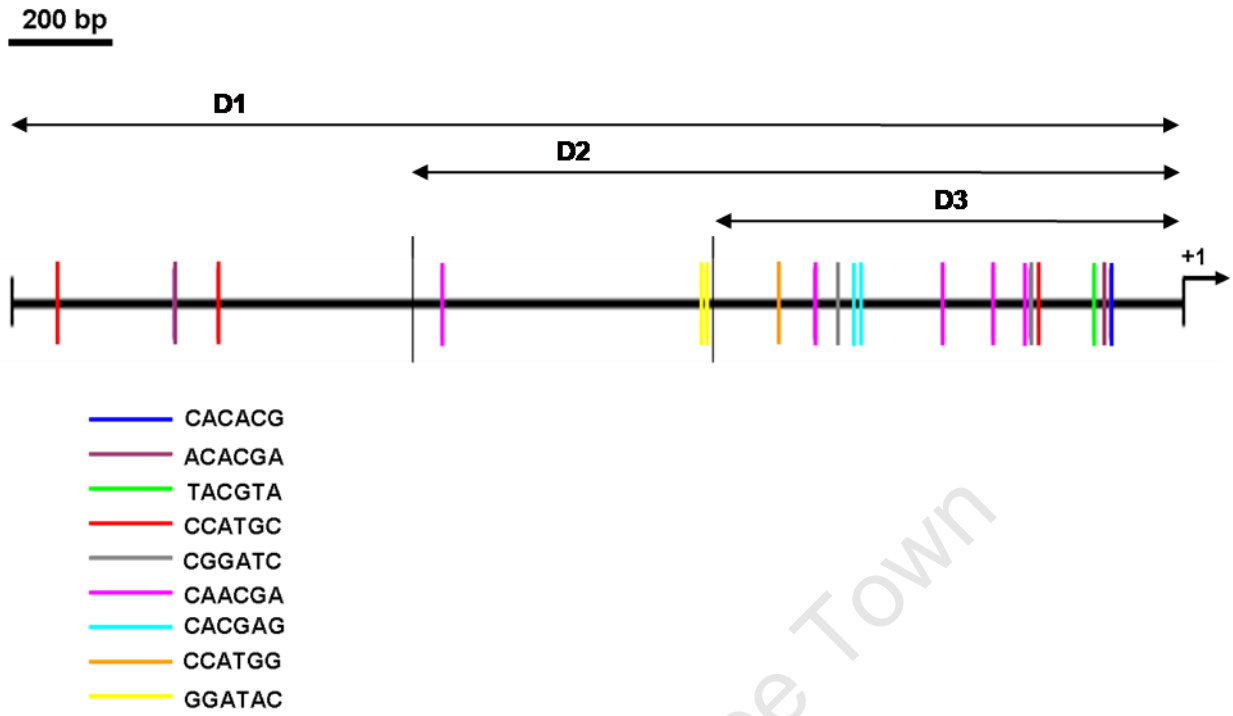


Figure 8: Diagrammatic representation of the *PYK10* promoter region with 6-mer DNA motifs that were found to be over-represented in MeJA-inducible co-expressed/-regulated genes compared to the Arabidopsis genome 1 kb from the start codon.

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.

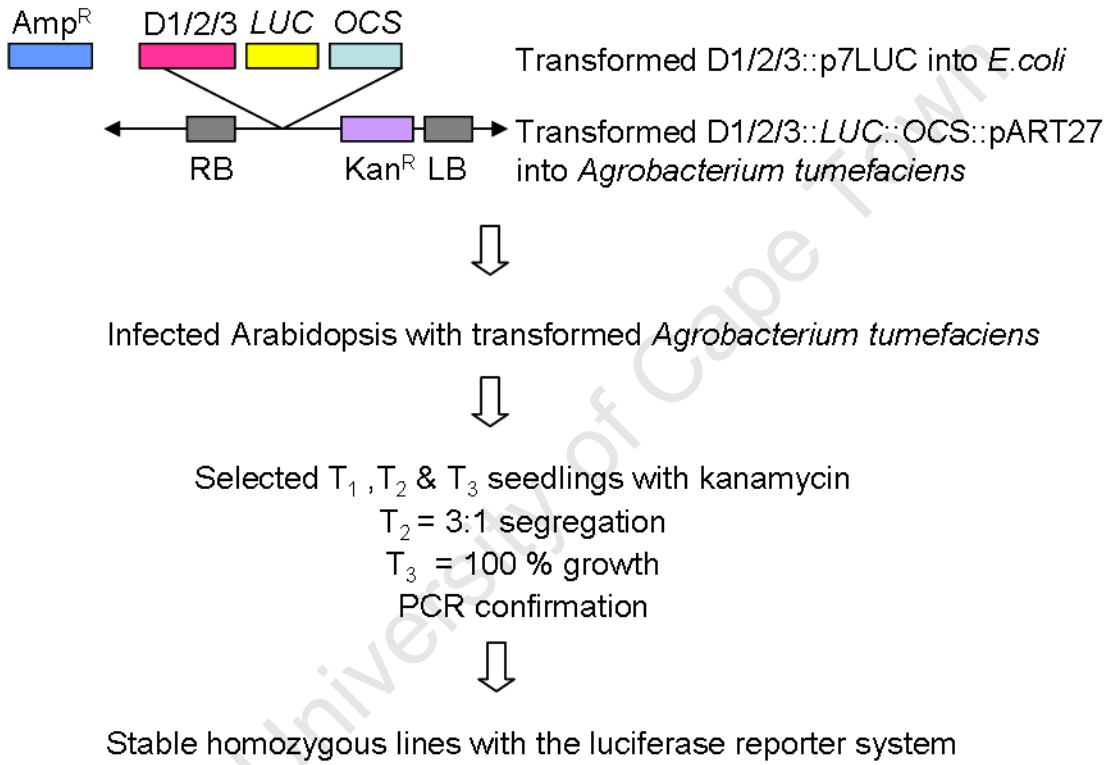


Figure 9: A schematic representation of generation of the reporter system.

T_1 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 T_2 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 T_2 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 T_3 homozygous transgenic lines were isolated from each of the representative T_2 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.

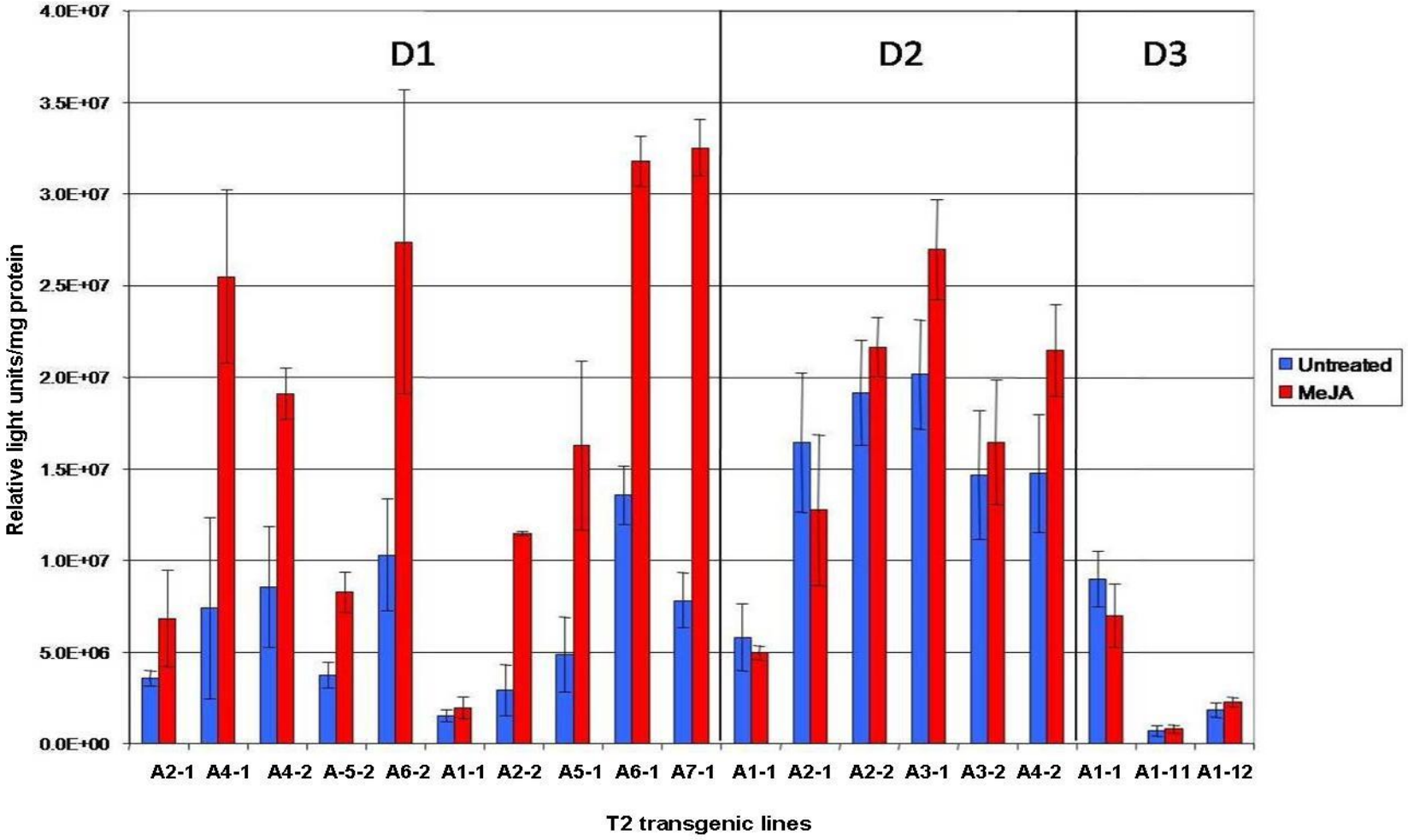


Figure 10: Luciferase assays on untreated 2 week-old T_2 D1, D2 and D3 transgenic plants and those treated with 45 µM MeJA/0.005% DMSO for 24 hours. The error bars shown represent standard error across 3 samples for each line, each sample containing 3-5 plants. These experiments were repeated twice with similar results.

Luciferase assays were performed on untreated T_3 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.

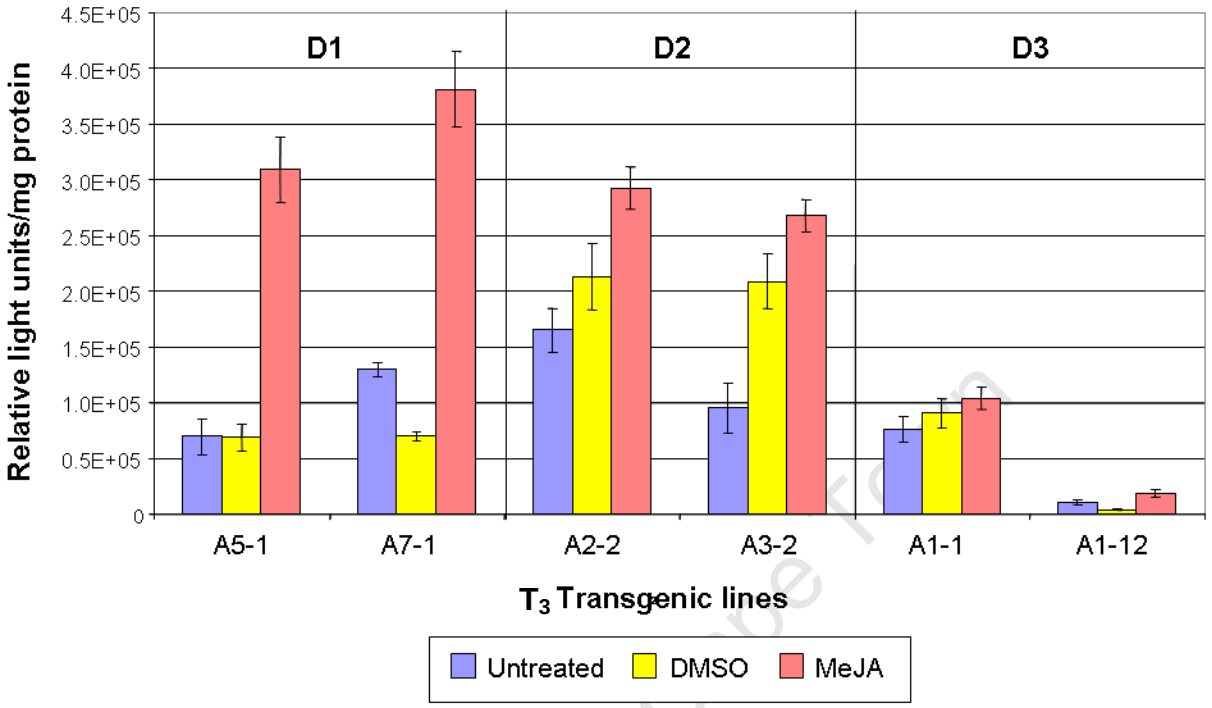
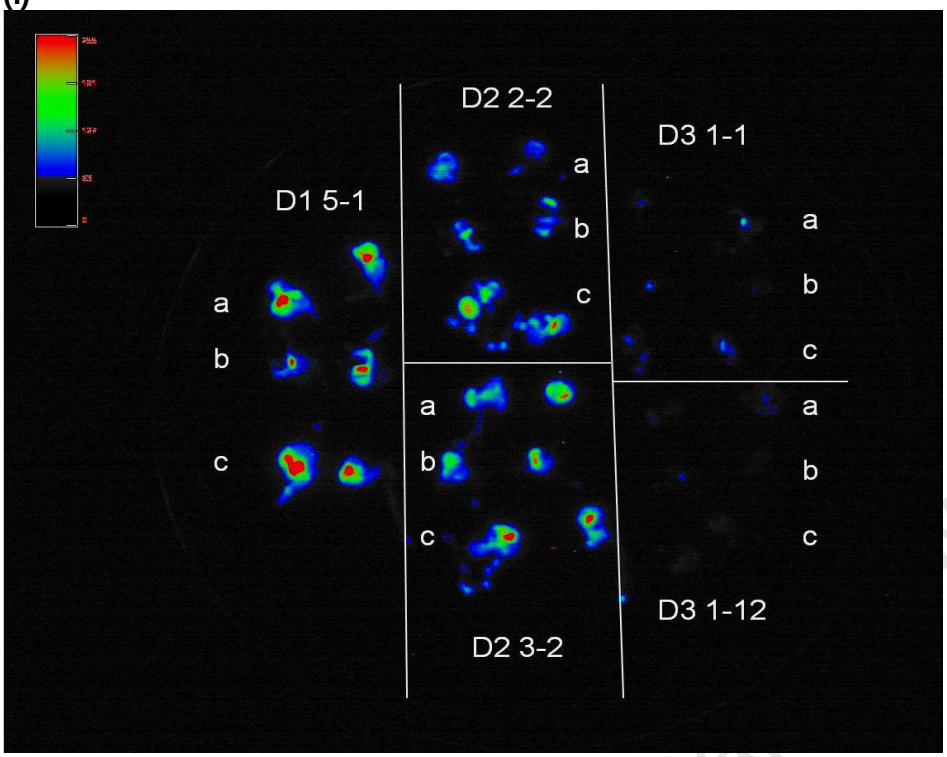


Figure 11: Luciferase assays on untreated 2 week-old homozygous D1, D2 and D3 transgenic plants and those treated with 45 µM MeJA/0.005% DMSO and 0.005% DMSO for 24 hours. The error bars shown represent standard error across 3 samples for each line, each sample containing 3-5 plants. These experiments 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.

(I)



(II)

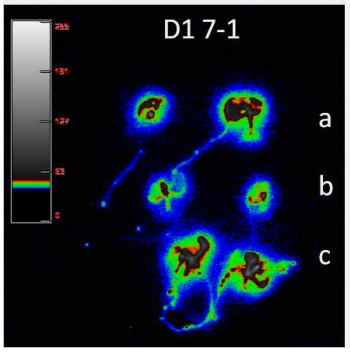


Figure 12: Luciferase imaging on untreated (a) 1 week-old homozygous transgenic plants from lines (I) D1 5-1, D2 2-2, D2 3-2, D3 1-1, D3 1-12 and (II) D1 7-1, and those treated with 0.005% DMSO (b) and 45 µM MeJA/0.005% DMSO (c) for 24 hours. The scale bar is shown in the upper left-hand corner ranging 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.

[D1]TCCCTCAGATGGTCATGCTATCATTTTCGCTTTCAAATAGCGCGACCTAATTTTTTATAT
AATAAAATTACTAACGTGGATCGCATGGGATATTTTAATATAATAAAAATGTTTTAAGAAAATA
AGGAAATGGAAGAGCCCACCGTCCACCAATAAATTACCGAGTAAACGATTTATACGACCGTCGA
AATGAACTGAGAAGATAACGAGAAAAAAAGAATCGGAATTATATATTTTGACTCAAAAACGAGA
AAATAATTCGTAGCGATTCTAACTCCTACTTTATACCTTAAGGAACACGAAACTTATGAGATTT
TATGGAAGTTACAACGTGGTTAGTTTTTTTTTCTTTCTATTGGACCAGTGTTAAATTTTCAATT
TGGCATGGTGTAAAACTACACAAAACAGCCTTTCTTTCTCTGACCCGTAAAACTACTATTTTAT
CTTATTTCAAATCTAACAGATTTTCATTATGGCGATAGATATAGTCCTTAAAAATTATATTGGA
TTCATTAGCAAAACATAACTATACATTGAAATTGTATTGATAAAATTTATATTATTACATGCAA
CCAAGCAAGAGCGGATGTACACGTTTTGGTGTGGGTGCGAGTTCCACATCAGAATTTGTTTGTC
TATATAAGTAATTGTGAGAGACAATCGGAATAATTGGCTAGAATCAGTCTTTTTTTCCTAGTGG
ATCTTTAAAAACCATTCTTTTATAC[D2]CAAGCATGTACATGCTGTGGTGTGGGTGTAAGTAA
ATCCTGCCCCAATGAAAATTGTTTTTGGACTCGCCACTGCAACGAAGTGTACCAACAACTTGAC
TAGGATTCTAAGTTCTTTTATGTATAGGATGTCTATATTAAACTACCATGACTAACATATATAT
AGTAGTTCCATATGCTCGATAAACTATGATAGATCAACAATTTTAAACATATAGTTTAACACTA
TTTATTTGTTCAACGTCAATAGTTTATAGTTCGCATGCGCTCGGCTTAGATTTGGTCCCCAACA
GTCGAAATTGTCAAATAATATAAAATAAAAGTTTCATTGTTAGGATTCATTTATTCTTCGGGTG
GTTATTGTAATAAAAGGCAAAAGAAAAAGAAGAACAAAATTCACAAGTAAAAAAAAAGATAACA
TCATTCTTTTAGTCGACAAAAAAAAAAAAAAAATCAAAAAGATTTATTCAGTACTACAGTTTAA
TATTGTTTTGACTTTTTTCTTTTTCTTTATATTATCTGAAAATTCTAGACTGCAGCTGAAACAT
GTGATATGGATTAAAGGCGTATCCAGTATCCACA

TTGACT	W-box
TGACG	C-box
AACGTG	T/G-box (MYC sites)
AATGTT	Conserved motif of several JA-responsive promoters

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 dI-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.

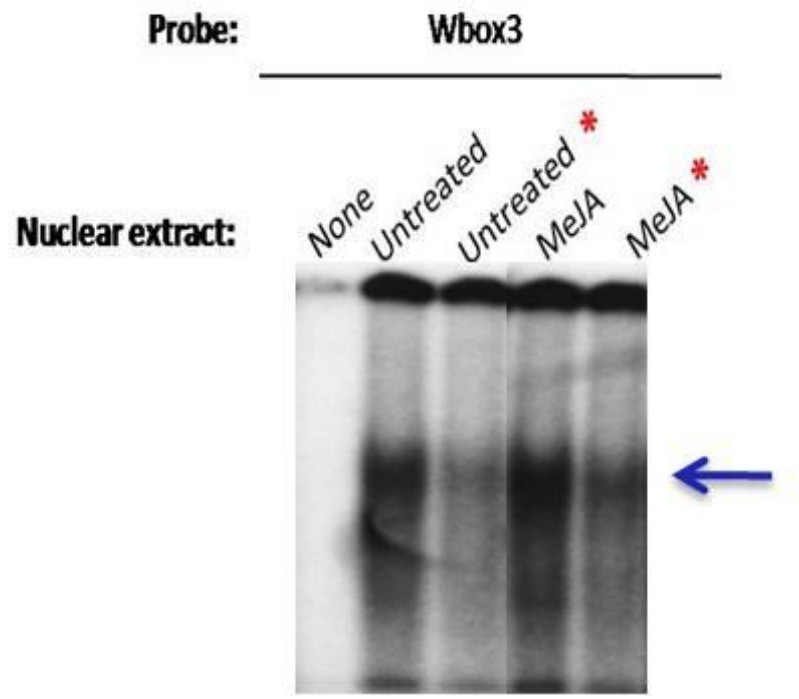
Co-regulated genes have similar *cis*-elements in their promoters to subject them to the same transcriptional control by a common set of TFs (Maleck et al., 2000) and upon inspection, 1kb TAIR loci upstream sequence of 32 genes co-regulated with *PYK10* (Chapter 3, Table 2) were enriched for thymine 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′- TATATT**TTGACT**CAAAAA -3′
Wbox2:	5′- AACAAC**TTGACT**AGGATT -3′
Wbox3:	5′- ATTGTT**TTGACT**TTTTTC -3′
m1:	5′- ATTGTT**T**gtta**T**TTTTTC -3′
m2:	5′- ATTtgg**TTGACT**TTTTTC -3′
m3:	5′- ATTGTT**TTGACT**aggTTC -3′

B)



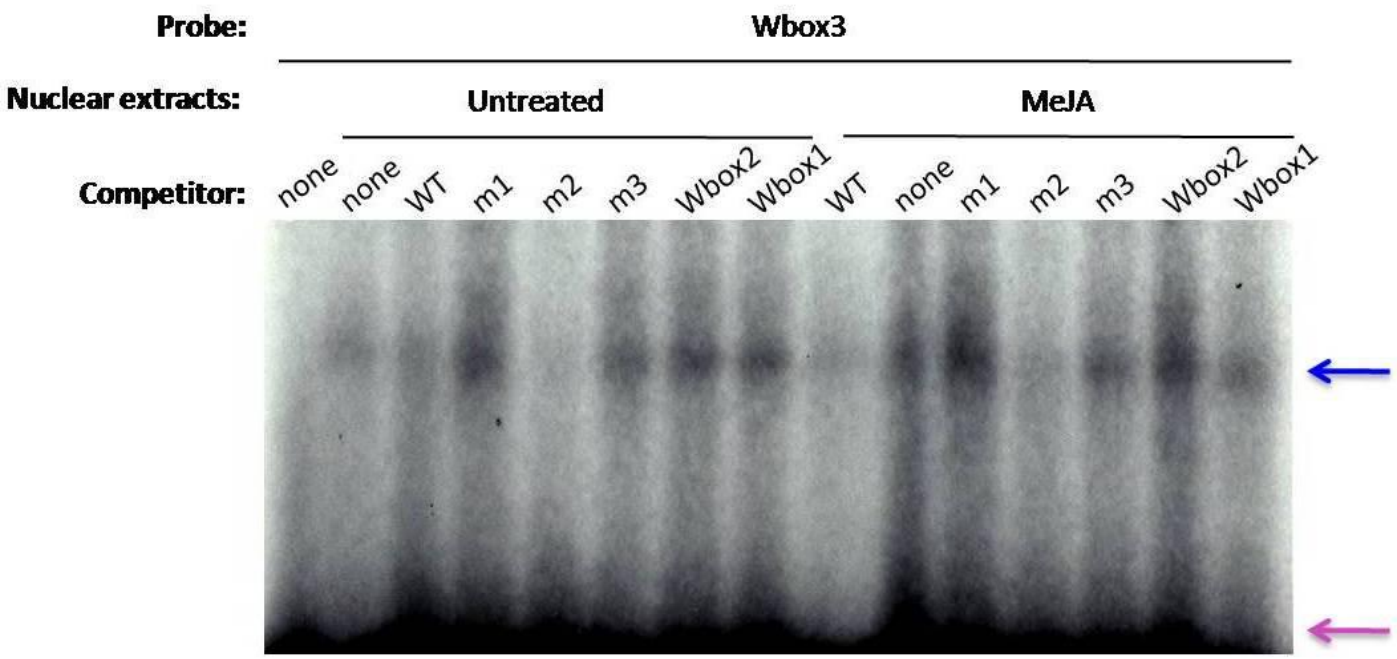


Figure 2: MeJA-inducibilty of the DNA-binding activity between nuclear proteins and the Wbox3 motif and the effect of different competitors. A) The nucleotide sequence of the probes used for the EMSA. The W-box is in bold type and the mutated nucleotides are in small letters. The EMSA was performed with 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.

DNA Sequence	Genome frequency[a]	Expected frequency[b]	No. of Promoters[c]	*P*-Value
TGACTW	0.68	21.71	28	0.033
TGACTWW	0.52	16.73	22	0.040
TGACTWWW	0.39	12.47	17	0.046

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 *EcoR*I site was placed at the 5' end and a *Xho*I 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

Yeast strain	Genotype	Reporter	Transformation selection markers	Reference
YM4271	MATa, ura3-52, his3-200, ade2-101, ade5, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4D, gal8D, ade5::hisG	N/A	trp1 leu2 ura3 his3	(Liu et al., 1993)

(A)

5′-AATTAATTGAATTCATTGTT**TTGACT**TTTTTCAAAAATTGTT**TTG**
ACTTTTTTCAAAAATTGTT**TTGACT**TTTTTCCTCGAGAATTAATT-3′

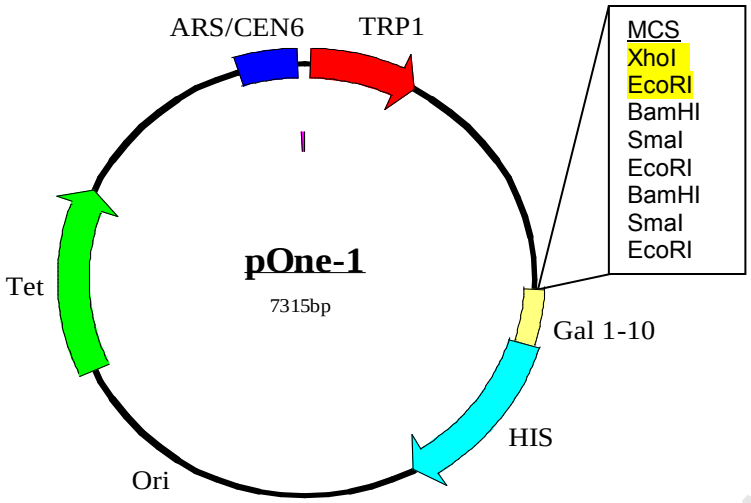
(B)

5′-AATTAATTGAATTCATTGTT**TTGACT**aggTTCAAAAATTGTT**TTG**
ACTaggTTCAAAAATTGTT**TTGACT**aggTTCCTCGAGAATTAATT-3′

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, *Xho*I restriction site in orange, nucleotides facilitating restriction enzyme binding in blue; mutated nucleotides in small print.

The bait fragment was ligated into the *Xho*I/*EcoR*I restriction enzyme site in the MCS of the pOne-1 vector following *Xho*I and *EcoR*I restriction enzyme digests of both the bait fragment and shuttle vector (Fig. 4A). The ligation was transformed into *E. coli* DH5α 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* DH5α competent cells and successful transformants were again selected for with tetracycline and confirmed by PCR and sequencing (data not shown).

A)



B)

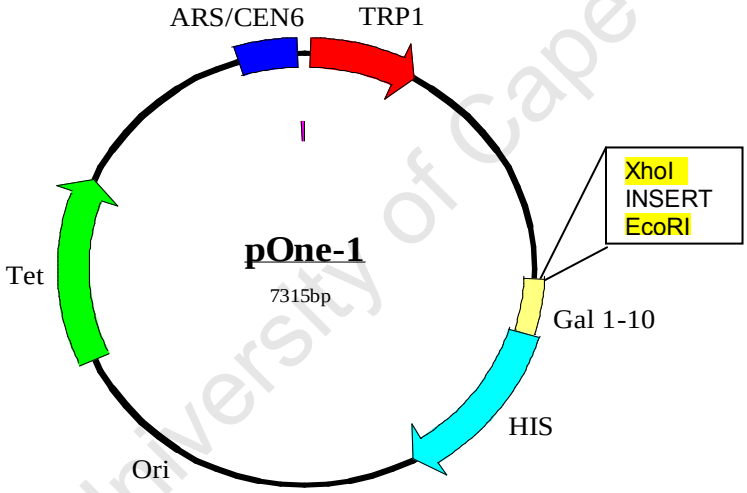
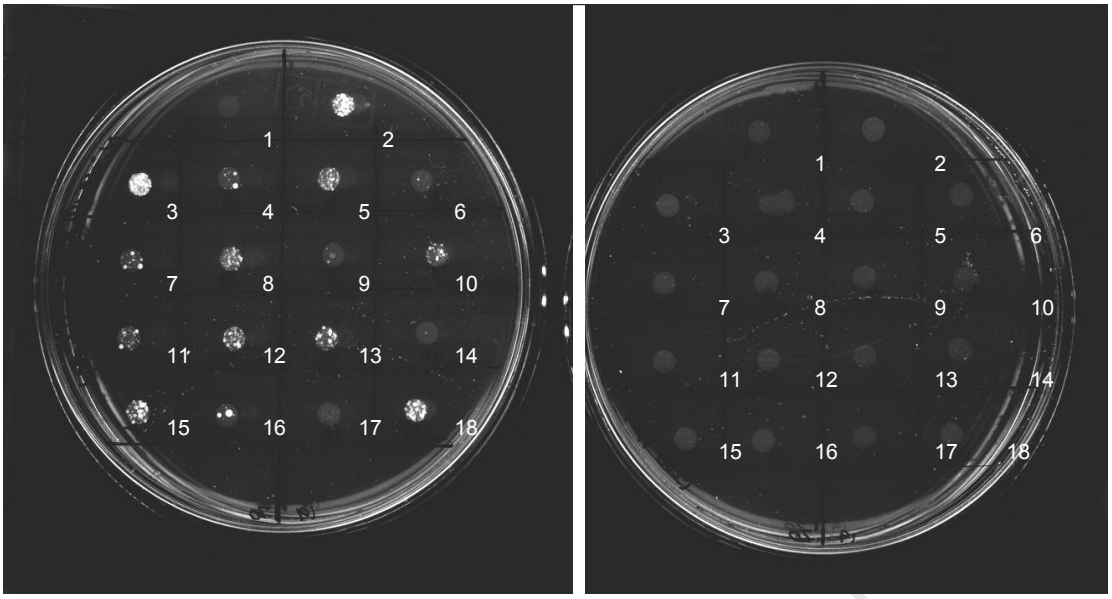


Figure 4: Graphical representation of pOne-1 vector (A) before insertion of DNA fragment and (B) after insertion of DNA fragment. ARS/CEN6, sequence for stable and low-copy number replication in yeast; TRP1, yeast selectable marker for tryptophan; MCS, multiple cloning site; Gal 1-10, Gal 1-10 minimal 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.

(A) **(B)**



(C)

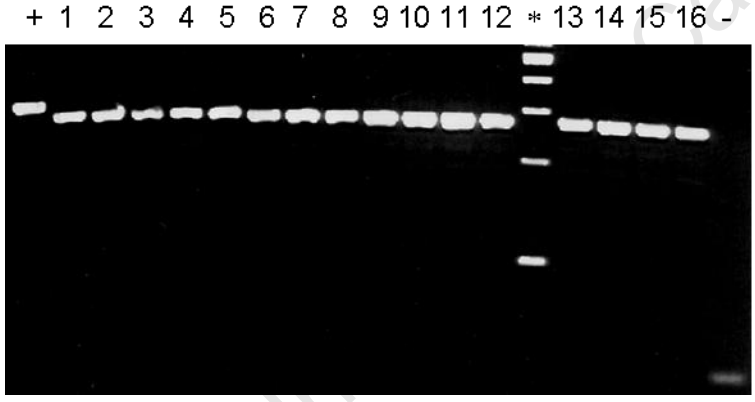


Figure 5: Yeast YM4271 cells harbouring the Wbox::pOne-1 construct. SD media without (A) tryptophan; (B) without tryptophan and histidine and supplemented with 1 mM 3-AT; (C) PCR confirming the presence of the Wbox::pOne-1 construct. + is the positive control and – is the negative control. * represents 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 *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 5×10^4 pfu were used as recommended by the manufacturer (Stratagene, La Jolla). The pAD-GAL4-2.1 phagemid vector excised *in vivo* from the HybriZAP-2.1 vector that had been transformed into *E.coli* DH5α 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.

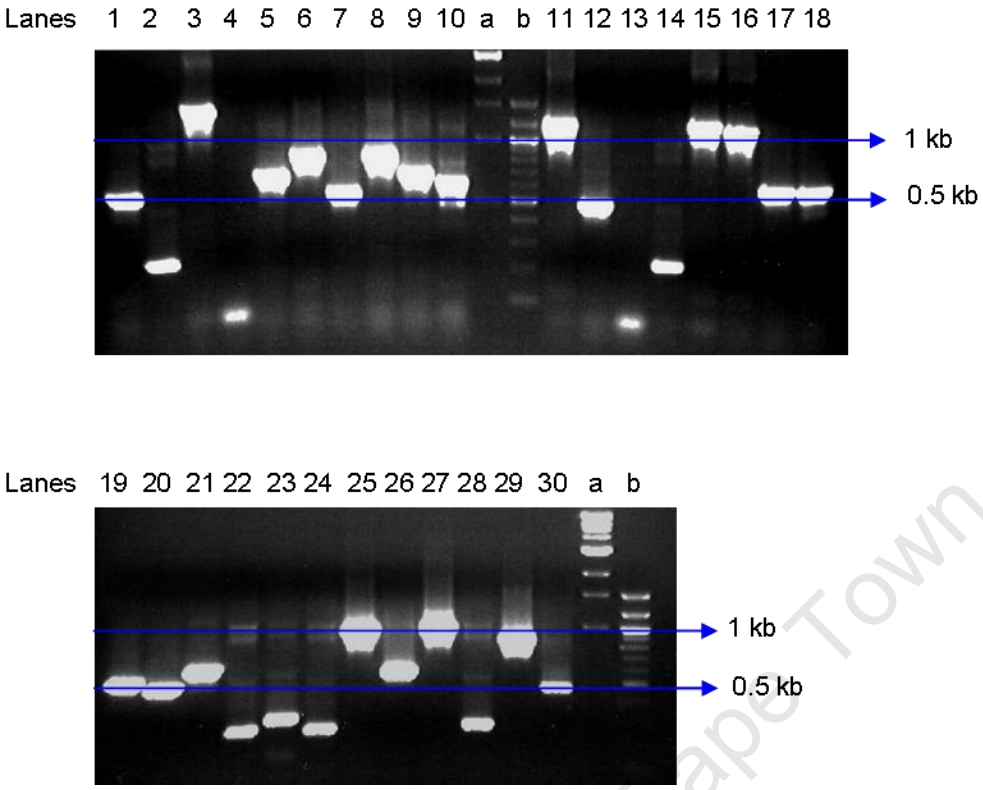


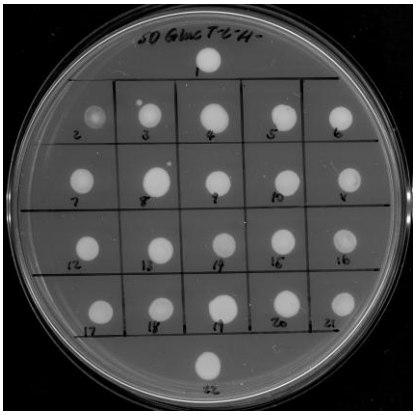
Figure 6: PCR analysis of single rescued colonies each containing a pAD-GAL4-2.1 phagemid vector with varying DNA inserts. Lanes 1 to 30 represent individual colonies, a is the 1 kb molecular weight marker and b is the 100 bp molecular weight marker. The blue lines are an indication of marker sizes.

4.2.3) Screening the yeast one-hybrid libraries

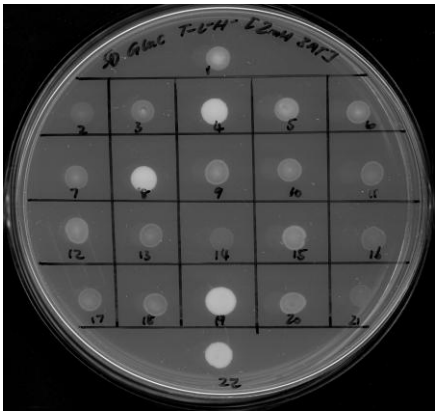
A total of 16 million clones were screened by transforming yeast YM4271 cells harbouring the Wbox::pOne-1 construct with the cDNA libraries. The transformed yeast cells were selected on SD media lacking tryptophan, leucine and histidine. Positive clones will be able to produce their own tryptophan as 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 *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 *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.

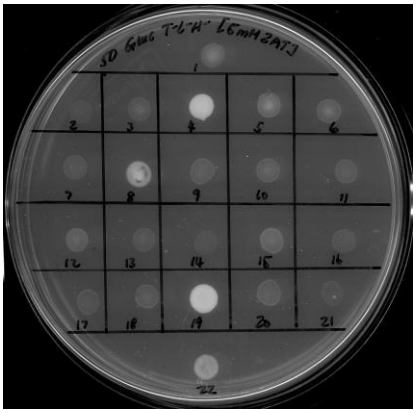
(A) 0 mM



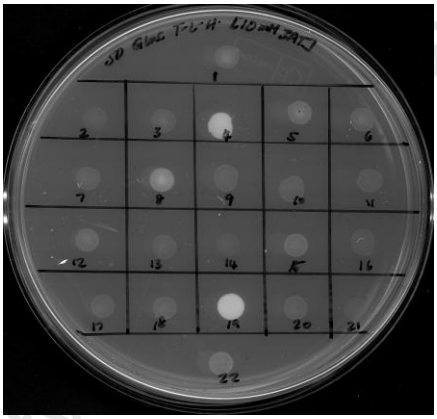
(B) 2 mM



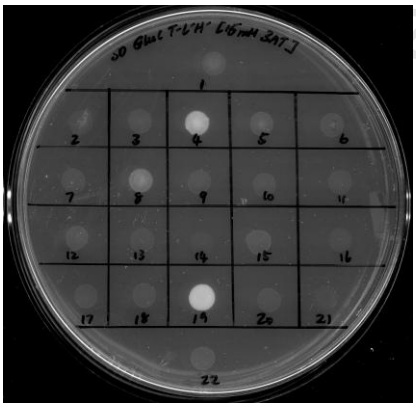
(C) 5 mM



(D) 10 mM



(E) 15 mM



(F) 30 mM

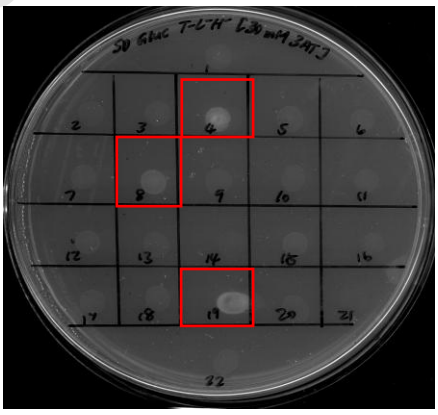


Figure 7: Screening for positive clones. (A) to (F), positive clones on SD media with increasing concentrations of 3-AT and lacking tryptophan, leucine and histidine. White spots and red boxes represent yeast growth while translucent spots represent no growth.

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 *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 *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 Peocke 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 *PI-II* 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). WRKY11 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.

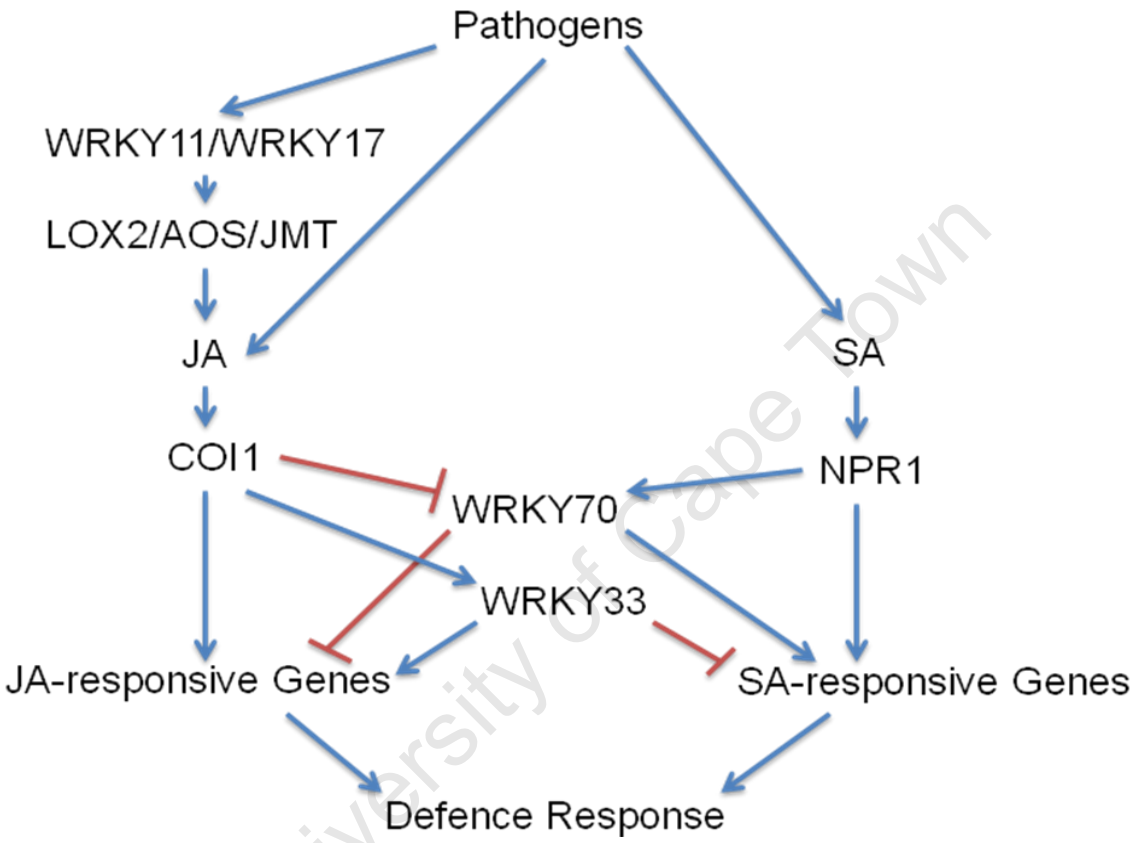


Figure 1: Working model for WRKY action in JA and SA signal crosstalk. Upon pathogen infection either JA or SA or both are synthesised. WRKY11 and WRKY17 positively regulate JA biosynthetic enzymes *LOX2*, *OAS* and *JMT* to activate JA-responsive genes through the JA signalling pathway. They may inhibit 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

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R.A., and Mengiste, T. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. Plant J 48, 28-44.

Andreasson, E., Jenkins, T., Brodersen, P., Thorgrimsen, S., Petersen, N.H., Zhu, S., Qiu, J.L., Micheelsen, P., Rocher, A., Petersen, M., Newman, M.A., Bjorn Nielsen, H., Hirt, H., Somssich, I., Mattsson, O., and Mundy, J. (2005). The MAP kinase substrate MKS1 is a regulator of plant defense responses. Embo J 24, 2579-2589.

Aravind, L., and Landsman, D. (1998). AT-hook motifs identified in a wide variety of DNA-binding proteins. Nucleic Acids Res 26, 4413-4421.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987). Current protocols in molecular biology. (New York: J. Wiley and Sons).

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S.P. (1997). Signaling in plant-microbe interactions. Science 276, 726-733.

Baldwin, I.T., and Schultz, J.C. (1983). Rapid Changes in Tree Leaf Chemistry Induced by Damage:Evidence for Communication Between Plants. Science 221, 277-279.

Baldwin, I.T., Kessler, A., and Halitschke, R. (2002). Volatile signaling in plant-plant-herbivore interactions: what is real? Curr Opin Plant Biol 5, 351-354.

Beaudoin, N., and Rothstein, S.J. (1997). Developmental regulation of two tomato lipoxygenase promoters in transgenic tobacco and tomato. Plant Mol Biol 33, 835-846.

Bell, E., Creelman, R.A., and Mullet, J.E. (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proc Natl Acad Sci U S A 92, 8675-8679.

Berger, S. (2002). Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling. Planta 214, 497-504.

Berger, S., Bell, E., and Mullet, J.E. (1996). Two Methyl Jasmonate-Insensitive Mutants Show Altered Expression of AtVsp in Response to Methyl Jasmonate and Wounding. Plant Physiol 111, 525-531.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J 29, 23-32.

Biesgen, C., and Weiler, E.W. (1999). Structure and regulation of OPR1 and OPR2, two closely related genes encoding 12-oxophytodienoic acid-10,11-reductases from Arabidopsis thaliana. Planta 208, 155-165.

Birkett, M.A., Campbell, C.A., Chamberlain, K., Guerrieri, E., Hick, A.J., Martin, J.L., Matthes, M., Napier, J.A., Pettersson, J., Pickett, J.A., Poppy, G.M., Pow, E.M., Pye, B.J., Smart, L.E., Wadhams, G.H., Wadhams, L.J., and Woodcock, C.M. (2000). New roles for cis-jasmone as an insect semiochemical and in plant defense. Proc Natl Acad Sci U S A 97, 9329-9334.

Blechert, S., Bockelmann, C., Füßlein, M., Schrader, T.V., Stelmach, B., Niesel, U., and Weiler, E.W. (1999). Structure-activity analyses reveal the existence of two separate groups of active octadecanoids in elicitation of the tendril-coiling response of *Bryonia dioica* Jacq. Planta 207, 470-479.

Boter, M., Ruiz-Rivero, O., Abdeen, A., and Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. Genes Dev 18, 1577-1591.

Brader, G., Tas, E., and Palva, E.T. (2001). Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen Erwinia carotovora. Plant Physiol 126, 849-860.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248-254.

Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M. (2003). A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of Arabidopsis. Plant Physiol 132, 1020-1032.

Bulow, L., Schindler, M., and Hehl, R. (2007). PathoPlant: a platform for microarray expression data to analyze co-regulated genes involved in plant defense responses. Nucleic Acids Res 35, D841-845.

Cabrera, H.M., Argandoña, V.H., Zúñiga, G.E., and Corcuera, L.J. (1995). Effect of infestation by aphids on the water status of barley and insect development Phytochemistry 40, 1083-108

Cantin, G.T., Stevens, J.L., and Berk, A.J. (2003). Activation domain-mediator interactions promote transcription preinitiation complex assembly on promoter DNA. Proc Natl Acad Sci U S A 100, 12003-12008.

Castillo, M.C., Martínez, C., Buchala, A., Métraux, J., and León, J. (2004). Gene-Specific Involvement of β-Oxidation in Wound-Activated Responses in Arabidopsis. Plant Physiology 135, 85-94.

Chassot, C., and Métraux, J.P. (2005). The cuticle as source of signals for plant defense. Plant Biosystems 139, 28-31.

Chatel, G., Montiel, G., Pre, M., Memelink, J., Thiersault, M., Saint-Pierre, B., Doireau, P., and Gantet, P. (2003). CrMYC1, a Catharanthus roseus elicitor- and jasmonate-responsive

bHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. J Exp Bot 54, 2587-2588.

Chen, H., Wilkerson, C.G., Kuchar, J.A., Phinney, B.S., and Howe, G.A. (2005). Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. Proc Natl Acad Sci U S A 102, 19237-19242.

Cheong, Y.H., Chang, H.S., Gupta, R., Wang, X., Zhu, T., and Luan, S. (2002). Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol 129, 661-677.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448, 666-671.

Ciolkowski, I., Wanke, D., Birkenbihl, R.P., and Somssich, I.E. (2008). Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. Plant Mol Biol 68, 81-92.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J. 16, 735-743.

Constabel, C.P., Bergey, D.R., and Ryan, C.A. (1995). Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci U S A 92, 407-411.

Cormack, R.S., Eulgem, T., Rushton, P.J., Kochner, P., Hahlbrock, K., and Somssich, I.E. (2002). Leucine zipper-containing WRKY proteins widen the spectrum of immediate early elicitor-induced WRKY transcription factors in parsley. Biochim Biophys Acta 1576, 92-100.

Creelman, R.A., Tierney, M.L., and Mullet, J.E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci U S A 89, 4938-4941.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature 411, 826-833.

Das, M.K., and Dai, H.K. (2007). A survey of DNA motif finding algorithms. BMC Bioinformatics 8 Suppl 7, S21.

Davies, P.J. (2004). Plant hormones:biosynthesis, signal transduction, action! (Dordrecht; Boston: Kluwer Academics).

de Pater, S., Katagiri, F., Kijne, J., and Chua, N.H. (1994). bZIP proteins bind to a palindromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. Plant J 6, 133-140.

de Ruijter, N.C.A., Verhees, J., van Leeuwen, W., and van der Krol, A.R. (2003). Evaluation and Comparison of the GUS, LUC, and GFP Reporter System for Gene Expression Studies in Plants. Plant Biol (Stuttg) 5, 103-115.

Delker, C., Zolman, B.K., Miersch, O., and Wasternack, C. (2007). Jasmonate biosynthesis in Arabidopsis thaliana requires peroxisomal beta-oxidation enzymes--additional proof by properties of pex6 and aim1. Phytochemistry 68, 1642-1650.

Denby, K.J., Jason, L.J., Murray, S.L., and Last, R.L. (2005). ups1, an Arabidopsis thaliana camalexin accumulation mutant defective in multiple defence signalling pathways. Plant J 41, 673-684.

Desveaux, D., Subramaniam, R., Despres, C., Mess, J.N., Levesque, C., Fobert, P.R., Dangl, J.L., and Brisson, N. (2004). A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Dev Cell 6, 229-240.

Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T., and Turner, J.G. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol 58, 497-513.

Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J 32, 457-466.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., Kobe, B., and Ellis, J.G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proc Natl Acad Sci U S A 103, 8888-8893.

Dong, J., Chen, C., and Chen, Z. (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol 51, 21-37.

Dong, X. (1998). SA, JA, ethylene, and disease resistance in plants. Curr. Opin. Plant Biol. 1, 316-323.

Dormann, P. (2005). Membrane lipids. In Plant lipids: biology, utilization and manipulation., D.J. Murphy, ed (Oxford: Blackwell Publishing Ltd), pp. 123-161.

Du, L., and Chen, Z. (2000). Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in Arabidopsis. Plant J 24, 837-847.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annu Rev Phytopathol 42, 185-209.

Ellis, C., and Turner, J.G. (2001). The Arabidopsis mutant cev1 has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell 13, 1025-1033.

Ellis, C., Karafyllidis, I., and Turner, J.G. (2002a). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to Erysiphe cichoracearum, Pseudomonas syringae, and Myzus persicae. Mol Plant Microbe Interact 15, 1025-1030.

Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G. (2002b). The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. Plant Cell 14, 1557-1566.

Ellis, J.G., Llewellyn, D.J., Walker, J.C., Dennis, E.S., and Peacock, W.J. (1987). The *ocs* element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer. EMBO J 6, 3203-3208.

Epple, P., Apel, K., and Bohlmann, H. (1995). An Arabidopsis thaliana thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol 109, 813-820.

Eulgem, T. (2005). Regulation of the Arabidopsis defense transcriptome. Trends Plant Sci 10, 71-78.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. Trends in Plant Science 5, 199-206.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K., and Somssich, I.E. (1999). Early nuclear events in plant defence signalling: Rapid activation by WRKY transcription factors. EMBO J. 18.

Falkenstein, E., Groth, B., Mithöfer, A., and Weiler, E.W. (1991). Methyljasmonate and α-linolenic acid are potent inducers of tendril coiling. Planta 185, 316-322.

Farmer, E.E. (2001). Surface-to-air signals. Nature 411, 854-856.

Farmer, E.E., and Ryan, C.A. (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci U S A 87, 7713-7716.

Farmer, E.E., Weber, H., and Vollenweider, S. (1998). Fatty acid signaling in Arabidopsis. Planta 206, 167-174.

Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W. (2003). The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. Plant Cell 15, 1083-1094.

Ferreira, R.B., Monteiro, S., Freitas, R., Santos, C.N., Chen, Z., Batista, L.M., Duarte, J., Borges, A., and Teixeira, A.R. (2007). The role of plant defence proteins in fungal pathogenesis. Molecular Plant Pathology 8, 677-700.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis Mutants Selected for Resistance to the Phytotoxin Coronatine Are Male Sterile, Insensitive to Methyl Jasmonate, and Resistant to a Bacterial Pathogen. Plant Cell 6, 751-759.

Flor, H.H. (1955). Host-parasite interactions in flax-its genetics and other implications. Phytopathol. 45, 680-685.

Forde, C.E., Gonzales, A.D., Smessaert, J.M., Murphy, G.A., Shields, S.J., Fitch, J.P., and McCutchen-Maloney, S.L. (2002). A rapid method to capture and screen for transcription factors by SELDI mass spectrometry. Biochem Biophys Res Commun 290, 1328-1335.

Froehlich, J.E., Itoh, A., and Howe, G.A. (2001). Tomato allene oxide synthase and fatty acid hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol 125, 306-317.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell 12, 393-404.

Fukuda, Y. (1997). Interaction of tobacco nuclear protein with an elicitor-responsive element in the promoter of a basic class I chitinase gene. Plant Mol Biol 34, 81-87.

Garcia-Hernandez, M., Berardini, T.Z., Chen, G., Crist, D., Doyle, A., Huala, E., Knee, E., Lambrecht, M., Miller, N., Mueller, L.A., Mundodi, S., Reiser, L., Rhee, S.Y., Scholl, R., Tacklind, J., Weems, D.C., Wu, Y., Xu, I., Yoo, D., Yoon, J., and Zhang, P. (2002). TAIR: a resource for integrated Arabidopsis data. Funct Integr Genomics 2, 239-253.

Garrett, R., and Grisham, C. (1995). Biochemistry. (Orlando: Harcourt Brace College Publishers).

Gidda, S.K., Miersch, O., Levitin, A., Schmidt, J., Wasternack, C., and Varin, L. (2003). Biochemical and molecular characterization of a hydroxyjasmonate sulfotransferase from Arabidopsis thaliana. J Biol Chem 278, 17895-17900.

Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P.A., and Cashmore, A.R. (1988). An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci U S A 85, 7089-7093.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43, 205-227.

Glazebrook, J., and Ausubel, F.M. (1994). Isolation of phytoalexin-deficient mutants of Arabidopsis thaliana and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci U S A 91, 8955-8959.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1997). Use of Arabidopsis for genetic dissection of plant defense responses. Annu. Rev. Genet. 31, 547-569.

Gleave, A.P. (1992). A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol 20, 1203-1207.

Gray, W.M. (2002). Plant defence: a new weapon in the arsenal. Curr Biol 12, R352-354.

Guerineau, F., Benjdia, M., and Zhou, D.X. (2003). A jasmonate-responsive element within the A. thaliana vsp1 promoter. J Exp Bot 54, 1153-1162.

Guo, A., He, K., Liu, D., Bai, S., Gu, X., Wei, L., and Luo, J. (2005). DATF: a database of Arabidopsis transcription factors. Bioinformatics 21, 2568-2569.

Hamberg, M., and Gardner, H.W. (1992). Oxylipin pathway to jasmonates: biochemistry and biological significance. Biochim Biophys Acta 1165, 1-18.

Hao, D., Ohme-Takagi, M., and Sarai, A. (1998). Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. J Biol Chem 273, 26857-26861.

Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., and Wasternack, C. (2000). Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J 24, 113-126.

Hayashi, Y., Yamada, K., Shimada, T., Matsushima, R., Nishizawa, N.K., Nishimura, M., and Hara-Nishimura, I. (2001). A proteinase-storing body that prepares for cell death or stresses in the epidermal cells of Arabidopsis. Plant Cell Physiol 42, 894-899.

He, Y., and Gan, S. (2002). A gene encoding an acyl hydrolase is involved in leaf senescence in Arabidopsis. Plant Cell 14, 805-815.

He, Y., Fukushige, H., Hildebrand, D.F., and Gan, S. (2002). Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol 128, 876-884.

He, Y., Tang, W., Swain, J.D., Green, A.L., Jack, T.P., and Gan, S. (2001). Networking senescence-regulating pathways by using Arabidopsis enhancer trap lines. Plant Physiol 126, 707-716.

Heath, M.C. (2000a). Hypersensitive response-related death. Plant Molec. Biol. 44, 321-334.

Heath, M.C. (2000b). Nonhost resistance and nonspecific plant defenses. Curr Opin Plant Biol 3, 315-319.

Hedden, P., and Thomas, S.G. (2006). Plant hormone signaling. (Oxford, UK; Ames, Iowa: Blackwell Publishing).

Heidenreich, B., Bieber, E., Sandermann, H., and Ernst, D. (2006). Identification of a new member of the WRKY family in tobacco. Involved in ozone-induced gene regulation? Acta Physiologiae Plantarum 28, 117-125.

Heil, M. (2008). Indirect defence via tritrophic interactions. New Phytol 178, 41-61.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27, 297-300.

Hilpert, B., Bohlmann, H., op den Camp, R.O., Przybyla, D., Miersch, O., Buchala, A., and Apel, K. (2001). Isolation and characterization of signal transduction mutants of Arabidopsis thaliana that constitutively activate the octadecanoid pathway and form necrotic microlesions. Plant J 26, 435-446.

Hoch, H.C., Staples, R.C., Whitehead, B., Comeau, J., and Wolf, E.D. (1987). Signaling for Growth Orientation and Cell Differentiation by Surface Topography in Uromyces. Science 235, 1659-1662.

Honda, I., Seto, H., Turuspekov, Y., Watanabe, Y., and Yoshida, S. (2006). Inhibitory effects of jasmonic acid and its analogues on barley (*Hordeum vulgare* L.) anther extrusion. Plant Growth Regulation 48, 201-206.

Howe, G.A. (2004). PLANT HORMONES: Biosynthesis, Signal Transduction, Action! (Dordrecht: Kluwer Academic Publishers).

Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K. (2001). The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell 13, 2191-2209.

Ishikawa, A., Yoshihara, T., and Nakamura, K. (1994). Jasmonate-inducible expression of a potato cathepsin D inhibitor-GUS gene fusion in tobacco cells. Plant Mol Biol 26, 403-414.

Izawa, T., Foster, R., and Chua, N.H. (1993). Plant bZIP protein DNA binding specificity. J Mol Biol 230, 1131-1144.

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., and Parcy, F. (2002). bZIP transcription factors in Arabidopsis. Trends Plant Sci 7, 106-111.

Jensen, A.B., Raventos, D., and Mundy, J. (2002). Fusion genetic analysis of jasmonate-signalling mutants in Arabidopsis. Plant J 29, 595-606.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 19, 4004-4014.

Journot-Catalino, N., Somssich, I.E., Roby, D., and Kroj, T. (2006). The transcription factors WRKY11 and WRKY17 act as negative regulators of basal resistance in Arabidopsis thaliana. Plant Cell 18, 3289-3302.

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., and Howe, G.A. (2008). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci U S A 105, 7100-7105.

Kawano, T. (2003). Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep 21, 829-837.

Kazan, K., and Manners, J.M. (2008). Jasmonate signaling: toward an integrated view. Plant Physiol 146, 1459-1468.

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53, 299-328.

Kim, S.-R., Kim, Y., Costa, M.A., and An, G. (1992a). Inhibition of sucrose enhancer effect of the potato proteinase inhibitor II promoter by Salicylic acid. Plant Physiol. 98, 1479-1483.

Kim, S.R., Kim, Y., and An, G. (1993). Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (nos) promoter. Plant Physiol 103, 97-103.

Kim, S.R., Choi, J.L., Costa, M.A., and An, G. (1992b). Identification of G-Box Sequence as an Essential Element for Methyl Jasmonate Response of Potato Proteinase Inhibitor II Promoter. Plant Physiol 99, 627-631.

Kim, S.Y., Chung, H.J., and Thomas, T.L. (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J 11, 1237-1251.

Kirsch, C., Logemann, E., Lippok, B., Schmelzer, E., and Hahlbrock, K. (2001). A highly specific pathogen-responsive promoter element from the immediate-early activated CMPG1 gene in Petroselinum crispum. Plant J 26, 217-227.

Kizis, D., and Pages, M. (2002). Maize DRE-binding proteins DBF1 and DBF2 are involved in rab17 regulation through the drought-responsive element in an ABA-dependent pathway. Plant J 30, 679-689.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. Plant J 44, 25-36.

Knoth, C., and Eulgem, T. (2008). The oomycete response gene LURP1 is required for defense against Hyaloperonospora parasitica in Arabidopsis thaliana. Plant J 55, 53-64.

Kobayashi, Y., Yamada, M., Kobayashi, I., and Kunoh, H. (1997). Actin Microfilaments are Required for the Expression of Nonhost Resistance in Higher Plants. Plant Cell Physiol 38, 725-733.

Kolomiets, M.V., Hannapel, D.J., Chen, H., Tymeson, M., and Gladon, R.J. (2001). Lipoxygenase is involved in the control of potato tuber development. Plant Cell 13, 613-626.

Kong, Z., Li, M., Yang, W., Xu, W., and Xue, Y. (2006). A novel nuclear-localized CCCH-type zinc finger protein, OsDOS, is involved in delaying leaf senescence in rice. Plant Physiol 141, 1376-1388.

Kramell, R., Atzorn, R., Schneider, G., Miersch, O., Brückner, C., Schmidt, J., Sembdner, G., and Parthier, B. (1995). Occurrence and identification of jasmonic acid and its amino acid conjugates induced by osmotic stress in barley leaf tissue. Journal of Plant Growth Regulation 14, 29-36.

Kroeger, K.M., and Abraham, L.J. (1997). Magnetic bead purification of specific transcription factors using mutant competitor oligonucleotides. Anal Biochem 250, 127-129.

Kubigsteltig, I., Laudert, D., and Weiler, E.W. (1999). Structure and regulation of the Arabidopsis thaliana allene oxide synthase gene. Planta 208, 463-471.

Laloi, C., Mestres-Ortega, D., Marco, Y., Meyer, Y., and Reichheld, J.P. (2004). The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiol 134, 1006-1016.

Lamb, C., and Dixon, R.A. (1997). The Oxidative Burst in Plant Disease Resistance. Annu Rev Plant Physiol Plant Mol Biol 48, 251-275.

Latchman, D.S. (1997). Transcription factors: an overview. Int J Biochem Cell Biol 29, 1305-1312.

Laudert, D., Pfannschmidt, U., Lottspeich, F., Hollander-Czytko, H., and Weiler, E.W. (1996). Cloning, molecular and functional characterization of Arabidopsis thaliana allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol 31, 323-335.

Li, C., Schilmiller, A.L., Liu, G., Lee, G.I., Jayanty, S., Sageman, C., Vrebalov, J., Giovannoni, J.J., Yagi, K., Kobayashi, Y., and Howe, G.A. (2005). Role of beta-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. Plant Cell 17, 971-986.

Li, J., Brader, G., and Palva, E.T. (2004a). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16, 319-331.

Li, J., Brader, G., Kariola, T., and Palva, E.T. (2006). WRKY70 modulates the selection of signaling pathways in plant defense. Plant J 46, 477-491.

Li, L., Li, C., Lee, G.I., and Howe, G.A. (2002). Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc Natl Acad Sci U S A 99, 6416-6421.

Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E., and Howe, G.A. (2004b). The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell 16, 126-143.

Lipka, U., Fuchs, R., and Lipka, V. (2008). Arabidopsis non-host resistance to powdery mildews. Curr Opin Plant Biol 11, 404-411.

Lisso, J., Steinhauser, D., Altmann, T., Kopka, J., and Mussig, C. (2005). Identification of brassinosteroid-related genes by means of transcript co-response analyses. Nucleic Acids Res 33, 2685-2696.

Liu, J., Wilson, T.E., and Milbrandt, J. (1993). Identifying DNA-Binding Sites and Analyzing DNA-Binding Domains Using a Yeast Selection System. METHODS: A Companion to Methods Enzymol 5, 125-137.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., and Solano, R. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15, 165-178.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16, 1938-1950.

Lu, M., Tang, X., and Zhou, J.-M. (2001). Arabidopsis *NHO1* is required for general resistance against *Pseudomonas* bacteria. Plant Cell 13, 437-447.

Mackey, D., Holt, B.F., 3rd, Wiig, A., and Dangl, J.L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell 108, 743-754.

Maleck, K., and Dietrich, R.A. (1999). Defense on multiple fronts: how do plants cope with diverse enemies? Trends Plant Sci 4, 215-219.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000). The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nat. Genet. 26, 403-410.

Manners, J.M., Penninckx, I.A., Vermaere, K., Kazan, K., Brown, R.L., Morgan, A., Maclean, D.J., Curtis, M.D., Cammue, B.P., and Broekaert, W.F. (1998). The promoter of the plant defensin gene PDF1.2 from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. Plant Mol Biol 38, 1071-1080.

Mao, P., Duan, M., Wei, C., and Li, Y. (2007). WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression. Plant Cell Physiol 48, 833-842.

Mason, H.S., DeWald, D.B., and Mullet, J.E. (1993). Identification of a methyl jasmonate-responsive domain in the soybean vspB promoter. Plant Cell 5, 241-251.

Matsushima, R., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2003). A novel ER-derived compartment, the ER body, selectively accumulates a beta-glucosidase with an ER-retention signal in Arabidopsis. Plant J 33, 493-502.

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.

McCaughern-Carucci, J. (1997). The NEVERFAIL northern blot.

McConn, M., and Browse, J. (1996). The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant. Plant Cell 8, 403-416.

Melotto, M., Mecey, C., Niu, Y., Chung, H.S., Katsir, L., Yao, J., Zeng, W., Thines, B., Staswick, P., Browse, J., Howe, G.A., and He, S.Y. (2008). A critical role of two positively charged amino acids in the Jas motif of Arabidopsis JAZ proteins in mediating coronatine- and jasmonoyl isoleucine-dependent interactions with the COI1 F-box protein. Plant J 55, 979-988.

Memelink, J., Verpoorte, R., and Kijne, J.W. (2001). ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. Trends Plant Sci 6, 212-219.

Menke, F.L., Champion, A., Kijne, J.W., and Memelink, J. (1999a). A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. Embo J 18, 4455-4463.

Menke, F.L., Parchmann, S., Mueller, M.J., Kijne, J.W., and Memelink, J. (1999b). Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in catharanthus roseus. Plant Physiol 119, 1289-1296.

Menkens, A.E., Schindler, U., and Cashmore, A.R. (1995). The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends Biochem Sci 20, 506-510.

Meshi, T., and Iwabuchi, M. (1995). Plant transcription factors. Plant Cell Physiol 36, 1405-1420.

Miao, Y., Laun, T., Zimmermann, P., and Zentgraf, U. (2004). Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol Biol 55, 853-867.

Miersch, O., Sembdner, G., and Schreiber, K. (1989). OCCURANCE OF JASMONIC ACID ANALOGUES IN *VICIA FABA*. Phytochemistry 28, 339-340.

Miersch, O., Kramell, R., Parthier, B., and Wasternack, C. (1999). Structure-activity relations of substituted, deleted or stereospecifically altered jasmonic acid in gene expression of barley leaves. Phytochemistry 50, 353-361.

Miersch, O., Neumerkel, J., Dippe, M., Stenzel, I., and Wasternack, C. (2008). Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. New Phytol 177, 114-127.

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A. (2003). Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. Plant Physiol 131, 298-308.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Murray, S.L., Thomson, C., Chini, A., Read, N.D., and Loake, G.J. (2002). Characterization of a novel, defense-related Arabidopsis mutant, cir1, isolated by luciferase imaging. Mol Plant Microbe Interact 15, 557-566.

Mysore, K.S., and Ryu, C.M. (2004). Nonhost resistance: how much do we know? Trends Plant Sci 9, 97-104.

Nagano, A.J., Matsushima, R., and Hara-Nishimura, I. (2005). Activation of an ER-body-localized beta-glucosidase via a cytosolic binding partner in damaged tissues of Arabidopsis thaliana. Plant Cell Physiol 46, 1140-1148.

Narvaez-Vasquez, J., Florin-Christensen, J., and Ryan, C.A. (1999). Positional specificity of a phospholipase A activity induced by wounding, systemin, and oligosaccharide elicitors in tomato leaves. Plant Cell 11, 2249-2260.

Nikolov, D.B., and Burley, S.K. (1997). RNA polymerase II transcription initiation: a structural view. Proc Natl Acad Sci U S A 94, 15-22.

Nishiuchi, T., Kodama, H., Yanagisawa, S., and Iba, K. (1999). Wound-induced expression of the FAD7 gene is mediated by different regulatory domains of its promoter in leaves/stems and roots. Plant Physiol 121, 1239-1246.

Nisius, A. (1988). The stromacentre in *Avena* plastids: an aggregation of β-glucosidase responsible for the activation of oat-leaf saponins. Planta 173, 474-481.

Nitz, I., Berkefeld, H., Puzio, P.S., and Grundler, F.M. (2001). Pyk10, a seedling and root specific gene and promoter from Arabidopsis thaliana. Plant Sci 161, 337-346.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell 7, 173-182.

Onate-Sanchez, L., and Singh, K.B. (2002). Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. Plant Physiol 128, 1313-1322.

Osbourn, A.E. (1996). Preformed Anitimicrobial Coumpounds and Plant Defense against Fungal Attack. The Plant Cell 8, 1821-1831.

Papadopoulou, K., Melton, R.E., Leggett, M., Daniels, M.J., and Osbourn, A.E. (1999). Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci U S A 96, 12923-12928.

Park, J.H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., and Feyereisen, R. (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J 31, 1-12.

Parthier, B. (1990). Jasmonates: Hormonal Regulators or Stress Factors in Leaf Senescence? Jounal of Plant Growth Regulation 9, 57-63.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell 10, 2103-2113.

Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell 8, 2309-2323.

Persson, S., Wei, H., Milne, J., Page, G.P., and Somerville, C.R. (2005). Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. Proc Natl Acad Sci U S A 102, 8633-8638.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johanson, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell 103, 1111-1120.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and van Loon, L.C. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10, 1571-1580.

Pre, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M., and Memelink, J. (2008). The AP2/ERF-domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol.

Preston, C.A., Betts, H., and Baldwi, I.T. (2002). Methyl jasmonate as an allelopathic agent: sagebrush inhibits germination of a neighboring tobacco, Nicotiana attenuata. J Chem Ecol 28, 2343-2369.

Raacke, I.C., Mueller, M.J., and Berger, S. (2006). Defects in Allene Oxide Synthase and 12-Oxa-Phytodienoic Acid Reductase Alter the Resistance to *Pseudomonas syringae* and *Botrytis cinerea*. J. Phytopathology 154, 740-744.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol 42, 93-113.

Reymond, P., Weber, H., Damond, M., and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell 12, 707-720.

Richmond, T.A., and Bleecker, A.B. (1999). A defect in beta-oxidation causes abnormal inflorescence development in Arabidopsis. Plant Cell 11, 1911-1924.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., and Yu, G. (2000). Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290, 2105-2110.

Rocher, A., Dumas, C., and Cock, J.M. (2005). A W-box is required for full expression of the SA-responsive gene SFR2. Gene 344, 181-192.

Rouster, J., Leah, R., Mundy, J., and Cameron-Mills, V. (1997). Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. Plant J 11, 513-523.

Ruiz-Rivero, O.J., and Prat, S. (1998). A -308 deletion of the tomato LAP promoters is able to direct flower-specific and MeJA-induced expression in transgenic plants. Plant Mol Biol 36, 639-648.

Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I.E. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. Embo J 15, 5690-5700.

Ryu, K.H., Kang, Y.H., Park, Y.H., Hwang, I., Schiefelbein, J., and Lee, M.M. (2005). The WEREWOLF MYB protein directly regulates CAPRICE transcription during cell fate specification in the Arabidopsis root epidermis. Development 132, 4765-4775.

Sagi, M., and Fluhr, R. (2001). Superoxide production by plant homologues of the gp1-phox NADPH oxidase. Modulation of activity by calcium and Tobacco Mosaic Virus.9. Plant Phsyiol. 126, 1281-1290.

Sanders, P.M., Lee, P.Y., Biesgen, C., Boone, J.D., Beals, T.P., Weiler, E.W., and Goldberg, R.B. (2000). The arabidopsis DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell 12, 1041-1061.

Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, K., Ohta, H., and Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res 8, 153-161.

Schaller, F., Biesgen, C., Mussig, C., Altmann, T., and Weiler, E.W. (2000). 12-Oxophytodienoate reductase 3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta 210, 979-984.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc. Natl. Acad. Sci., USA 97, 11655-11660.

Schilmiller, A.L., Koo, A.J., and Howe, G.A. (2007). Functional diversification of acyl-coenzyme A oxidases in jasmonic acid biosynthesis and action. Plant Physiol 143, 812-824.

Schindler, U., Beckmann, H., and Cashmore, A.R. (1992). TGA1 and G-box binding factors: two distinct classes of Arabidopsis leucine zipper proteins compete for the G-box-like element TGACGTGG. The Plant Cell 4, 1309-1319.

Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., Wasternack, C., Kombrink, E., and Stuible, H.P. (2005). A new type of peroxisomal acyl-coenzyme A synthetase from Arabidopsis thaliana has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. J Biol Chem 280, 13962-13972.

Sembdner, G., and Parthier, B. (1993). THE BIOCHEMISTRY AND THE PHYSIOLOGICAL AND MOLECULAR ACTIONS OF JASMONATES. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 569-589.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D. (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc Natl Acad Sci U S A 98, 4788-4793.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., and Innes, R.W. (2003). Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science 301, 1230-1233.

Sharma, V.K., Monostori, T., Gobel, C., Hansch, R., Bittner, F., Wasternack, C., Feussner, I., Mendel, R.R., Hause, B., and Schulze, J. (2006). Transgenic barley plants

overexpressing a 13-lipoxygenase to modify oxylipin signature. Phytochemistry 67, 264-276.

Shen, Y.G., Zhang, W.K., He, S.J., Zhang, J.S., Liu, Q., and Chen, S.Y. (2003). An EREBP/AP2-type protein in Triticum aestivum was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. Theor Appl Genet 106, 923-930.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M., and Oelmuller, R. (2008). PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between Arabidopsis thaliana and the endophytic fungus Piriformospora indica. Plant J 54, 428-439.

Siberil, Y., Doireau, P., and Gantet, P. (2001). Plant bZIP G-box binding factors. Modular structure and activation mechanisms. Eur J Biochem 268, 5655-5666.

Singh, K.B. (1998). Transcriptional regulation in plants: the importance of combinatorial control. Plant Physiol 118, 1111-1120.

Sobajima, H., Tani, T., Chujo, T., Okada, K., Suzuki, K., Mori, S., Minami, E., Nishiyama, M., Nojiri, H., and Yamane, H. (2007). Identification of a jasmonic acid-responsive region in the promoter of the rice 12-oxophytodienoic acid reductase 1 gene OsOPR1. Biosci Biotechnol Biochem 71, 3110-3115.

Somerville, C., and Browse, J. (1991). Plant Lipids: Metabolism, Mutants, and Membranes. Science 252, 80-87.

Stanley, D. (2006). Prostaglandins and other eicosanoids in insects: biological significance. Annu Rev Entomol 51, 25-44.

Staswick, P.E., and Tiryaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell 16, 2117-2127.

Staswick, P.E., Huang, J.F., and Rhee, Y. (1991). Nitrogen and Methyl Jasmonate Induction of Soybean Vegetative Storage Protein Genes. Plant Physiol 96, 130-136.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an Arabidopsis thaliana mutant. Proc Natl Acad Sci U S A 89, 6837-6840.

Staswick, P.E., Tiryaki, I., and Rowe, M.L. (2002). Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14, 1405-1415.

Stenzel, I., Hause, B., Miersch, O., Kurz, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., and Wasternack, C. (2003). Jasmonate biosynthesis and the allene oxide cyclase family of Arabidopsis thaliana. Plant Mol Biol 51, 895-911.

Stintzi, A., and Browse, J. (2000). The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci U S A 97, 10625-10630.

Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc Natl Acad Sci U S A 98, 12837-12842.

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., and Mitchell-Olds, T. (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. Plant Physiol 124, 1007-1018.

Strassner, J., Schaller, F., Frick, U.B., Howe, G.A., Weiler, E.W., Amrhein, N., Macheroux, P., and Schaller, A. (2002). Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J 32, 585-601.

Suza, W.P., and Staswick, P.E. (2008). The role of JAR1 in Jasmonoyl-L: -isoleucine production during Arabidopsis wound response. Planta 227, 1221-1232.

Swiatek, A., Van Dongen, W., Esmans, E.L., and Van Onckelen, H. (2004). Metabolic fate of jasmonates in tobacco bright yellow-2 cells. Plant Physiol 135, 161-172.

Tabor, S., and Richardson, C.C. (1987). DNA sequence analysis with a modified T7 DNA polymerase. Proc. NAtl. Acad. Sci., USA 84, 4767-4771.

Taipalensuu, J., Falk, A., Ek, B., and Rask, L. (1997). Myrosinase-binding proteins are derived from a large wound-inducible and repetitive transcript. Eur J Biochem 243, 605-611.

Taki, N., Sasaki-Sekimoto, Y., Obayashi, T., Kikuta, A., Kobayashi, K., Ainai, T., Yagi, K., Sakurai, N., Suzuki, H., Masuda, T., Takamiya, K., Shibata, D., Kobayashi, Y., and Ohta, H. (2005). 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. Plant Physiol 139, 1268-1283.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996). Physical interaction of avrPto and the Pto kinase defines a recognition event involved in plant disease resistance. Science 274, 2060-2063.

Thaler, J.S., Owen, B., and Higgins, V.J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. Plant Physiol 135, 530-538.

Thaler, J.S., Farag, M.A., Pare, P.W., and Dicke, M. (2002). Jasmonate-deficient plants have reduced direct and indirect defences against herbivores. Ecology Letters 5, 764-774.

Theodoulou, F.L., Job, K., Slocombe, S.P., Footitt, S., Holdsworth, M., Baker, A., Larson, T.R., and Graham, I.A. (2005). Jasmonic acid levels are reduced in COMATOSE ATP-binding

cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. Plant Physiol 137, 835-840.

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A., and Browse, J. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448, 661-665.

Thomma, B.P., Nelissen, I., Eggermont, K., and Broekaert, W.F. (1999a). Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. Plant J 19, 163-171.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.J., and Broekaert, W.F. (1999b). Requirement of functional *ethylene insensitive 2* gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea.* Plant Physiol. 121, 1093-1101.

Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.A. (2000). Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonate. Plant Phsyiol. Biochem 38, 421-427.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci., USA 95, 15107-15111.

Tiryaki, I., and Staswick, P.E. (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant axr1. Plant Physiol 130, 887-894.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005). The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. Plant J 43, 153-163.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. Proc Natl Acad Sci U S A 104, 1075-1080.

Turck, F., Zhou, A., and Somssich, I.E. (2004). Stimulus-dependent, promoter-specific binding of transcription factor WRKY1 to Its native promoter and the defense-related gene PcPR1-1 in Parsley. Plant Cell 16, 2573-2585.

Ueda, J., and Kato, J. (1980). Isolation and Identification of a Senescence-promoting Substance from Wormwood (Artemisia absinthium L.). Plant Physiol 66, 246-249.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E., and Ryals, J. (1993). Biological induction of systemic acquired resistance in *Arabidopsis*. Molec. Plant-Microbe Interact. 6, 692-698.

Vallad, G.E., and Goodman, R.M. (2004). Systemic Acquired Resistance and Induced Systemic Resistance in Conventional Agriculture. Crop Science 44, 1920-1934.

Van Damme, E.J., Zhang, W., and Peumans, W.J. (2004). Induction of cytoplasmic mannose-binding jacalin-related lectins is a common phenomenon in cereals treated with jasmonate methyl ester. Commun Agric Appl Biol Sci 69, 23-31.

van der Fits, L., and Memelink, J. (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289, 295-297.

van der Fits, L., and Memelink, J. (2001). The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25, 43-53.

van Loon, L.C., Bakker, P.A., and Pieterse, C.M. (1998). Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36, 453-483.

Van Poecke, R.M.P., and Dicke, M. (2002). Induced parasitoid attraction by Arabidopsis thaliana: involvement of the octadecanoid and the salicylic acid pathway. Journal of Experimental Botany 53, 1793-1799.

van Verk, M.C., Pappaioannou, D., Neeleman, L., Bol, J.F., and Linthorst, H.J. (2008). A Novel WRKY Transcription Factor Is Required for Induction of PR-1a Gene Expression by Salicylic Acid and Bacterial Elicitors. Plant Physiol 146, 1983-1995.

Vick, B.A., and Zimmerman, D.C. (1983). The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. Biochem Biophys Res Commun 111, 470-477.

Vijayan, P., Shockey, J., L慸esque, A., Cook, R.J., and Browse, J. (1998). A role for jasmonate in pathogen defense of Arabidopsis. Proc.Natl. Acad. Sci., USA 95, 7209-7214.

Walling, L.L. (2000). The Myriad Plant Responses to Herbivores. J Plant Growth Regul 19, 195-216.

Wallis, J.G., and Browse, J. (2002). Mutants of Arabidopsis reveal many roles for membrane lipids. Prog Lipid Res 41, 254-278.

Wasternack, C., and Hause, B. (2002). Jasmonates and octadecanoids: signals in plant stress responses and development. Prog Nucleic Acid Res Mol Biol 72, 165-221.

Weber, H., Vick, B.A., and Farmer, E.E. (1997). Dinor-oxo-phytodienoic acid: a new hexadecanoid signal in the jasmonate family. Proc Natl Acad Sci U S A 94, 10473-10478.

Wei, Y. (2002). Differential Display Identifies Jasmonate-Responsive Genes in Arabidopsis thaliana. In Molecular and Cell Biology (Cape Town: University of Cape Town), pp. 48.

Weigel, D., and Glazebrook, J. (2002). Arabidopsis. (Cold Spring Harbor: Cold Spring Harbor Laboratory Press).

Williams, M.E., Foster, R., and Chua, N.H. (1992). Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell 4, 485-496.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An "electronic fluorescent pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE 2, e718.

Woo, H.R., Chung, K.M., Park, J.H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K., and Nam, H.G. (2001). ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell 13, 1779-1790.

Wood, K.V. (1998). The Chemistry of Bioluminescent Reporter Assays. Promega Notes 65, 14.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G. (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280, 1091-1094.

Xu, B., and Timko, M. (2004). Methyl jasmonate induced expression of the tobacco putrescine N -methyltransferase genes requires both G-box and GCC-motif elements. Plant Mol Biol 55, 743-761.

Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell 14, 1919-1935.

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell 18, 1310-1326.

Xu, Y.H., Wang, J.W., Wang, S., Wang, J.Y., and Chen, X.Y. (2004). Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-delta-cadinene synthase-A. Plant Physiol 135, 507-515.

Yamane, H., Takagi, H., Abe, H., Yokota, T., and Takahashi, N. (1981). Identification of Jasmonic Acid in Three Species of Higher Plants and Its Biological Activities. Plant & Cell Physiol. 22, 689-697.

Yang, W., Devaiah, S.P., Pan, X., Isaac, G., Welti, R., and Wang, X. (2007). AtPLAI is an acyl hydrolase involved in basal jasmonic acid production and Arabidopsis resistance to Botrytis cinerea. J Biol Chem 282, 18116-18128.

Yoshihara, T., Omer, E.A., Koshino, H., Sakamura, S., Kikuta, Y., and Koda, Y. (1989). Structure of a Tuber-inducing Stimulus from Potato Leaves (*Solanum tuberosum* L.). Agric. Biol. Chem. 53, 2835-2837.

Yoshihara, T., Amanuma, M., Tsutsumi, T., Okumura, Y., Matsuura, H., and Ichihara, A. (1996). Metabolism and Transport of [2-^{14}C](±) Jasmonic Acid in the Potato Plant. Plant Cell Physiol 37, 586-590.

Yoshiharu, Y., Obokata, Y., and Obokata, J. (2007). ppdb: a plant promoter database. Nucleic Acids Res 36, 977-981.

Yu, D., Chen, C., and Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 13, 1527-1540.

Zheng, Z., Qamar, S.A., Chen, Z., and Mengiste, T. (2006). Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J 48, 592-605.

Zolman, B.K., and Bartel, B. (2004). An Arabidopsis indole-3-butyric acid-response mutant defective in PEROXIN6, an apparent ATPase implicated in peroxisomal function. Proc Natl Acad Sci U S A 101, 1786-1791.