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

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

University of Cape Town

Genetic and Phenotypic Characterisation of an *Arabidopsis thaliana* Jasmonic Acid Signalling Mutant, *jam2*.

Micheline Sanderson

University of Cape Town

Submitted in fulfillment of the requirements for the degree of Master of Science in the Department of Molecular and Cell Biology, University of Cape Town, South Africa.
November 2003

DECLARATION

I hereby declare that this thesis entitled:

Genetic and phenotypic Characterisation of an *Arabidopsis thaliana* Jasmonic Acid Signaling Mutant, *jam2*.

Is my own work and has not previously in its entirety or in part been submitted at any university for another degree.

signature removed

Signature

06/05/2004

Date

University of Cape Town

Abstract

Jasmonic acid (JA) is a plant hormone with diverse functions, ranging from development to stress responses. Moreover, a role for JA in mediating defence against pathogen attack has been established, seemingly specific against necrotrophic pathogens such as *Botrytis cinerea*. Despite these known roles of JA, it is not known exactly how JA activated downstream responses, such as induced gene expression. To further our understanding of JA signalling, this work aimed to identify new components involved in JA signal transduction. A novel screening method based on lack of anthocyanin accumulation after exogenous application of the methyl ester of JA, methyl jasmonate (MeJA), was employed. A recessive, monogenic mutant, *jasmonic acid modified2* (*jam2*), was isolated from T-DNA activation tagged lines and characterized genetically and phenotypically. *jam2* was found not to be T-DNA tagged as the T-DNA segregates independently of the mutation. *jam2* is unlikely to be an anthocyanin biosynthetic mutant but shows delayed anthocyanin accumulation after exogenous MeJA treatment. Resistance to MeJA root growth inhibition is a phenotype shared by all JA insensitive mutants. Contrary to this, *jam2*, like Col-0, exhibits stunted root growth on MeJA. The expression of the antifungal peptide, *PDF1.2* can be induced by exogenous MeJA treatment. To assess how *PDF1.2* expression was affected in *jam2*, plants were treated with external liquid and vaporous MeJA. Interestingly, the *PDF1.2* expression pattern after MeJA application (liquid or gaseous) was biphasic for Col-0, *jam2* and *jar1*. However, compared to Col-0 and *jar1*, *jam2* appeared to be affected in the first induction peak upon liquid MeJA treatment, whilst in the second after gaseous treatment. *PDF1.2* expression can also be seen as a marker for JA-mediated defence responses. Upon infection with *B. cinerea*, *jam2* and *jar1* showed intermediate resistance and faster *PDF1.2* expression, compared to Col-0 and *coi1-1*. These findings suggest that *jam2* is possibly involved in temporal regulation of anthocyanin accumulation and *PDF1.2* expression after MeJA application and *B. cinerea* infection. Therefore, *jam2* may define a novel component within the JA signalling pathway and further genetic and phenotypic characterisation could confirm this.

University of Cape Town

Acknowledgements

I would like to express my sincere gratitude to the following people or organisations:

- The Equity Development Program (EDP), the National Research Foundation (NRF) as well as the Department of Molecular and Cell Biology at the University of Cape Town for providing the necessary financial support for this project
- My supervisor, Dr. Katherine J. Denby, for her invaluable guidance and support
- Dr. Gary J. Loake for hosting me in his laboratory at the University of Edinburgh, Scotland
- My parents, brother, family and friends for their never-ending love, support and motivation
- The Denby and Loake lab members for always supporting me and being my friends

Lastly I would like to thank my Heavenly Father for providing me with the wisdom, knowledge, determination and perseverance through this trying and challenging time of my life.

University of Cape Town

Table of Contents

Page Number

Abstract
Acknowledgement
Abbreviations
List of Tables
List of Figures

Chapter 1 JA responses, biosynthesis and signalling in *Arabidopsis thaliana*
Introduction 1
The JA biosynthetic pathway 5
JA intermediates are biologically active 15
JA signalling 16
Mutants acting upstream of JA biosynthesis 17
JA downstream signalling 19
Regulation of JA-responsive genes 21
Cross talk with other signalling pathways 26
Concluding remarks 30

Chapter 2 Materials and Methods
Seed stock used 33
Plant growth 33
Chemical treatments of plants 34
Root growth analysis 35
Manual cross pollinations 35
Anthocyanin extraction 35
Infection with *Botrytis cinerea* 35
RNA analysis 36
RNA extraction, gel electrophoresis and transfer onto nitrocellulose membrane 36
RNA membrane hybridization 37
Radiolabelling of the PDF1.2 probe 37
Prehybridisation and Hybridisation of RNA membranes 37
Washing of hybridised membranes 38

Chapter 3 Genetic characterisation of *jam2*
Introduction 40
Results
Primary screen for new JA signalling mutants 44
Secondary screen of putative mutants 44
Heritability of the *jam2* mutation 45
jam2 is not allelic to *coi1-1* 46
Determination of the number of T-DNA insertions in *jam2* 47

University of Cape Town

	Page number
Linkage analysis between *jam2* and the T-DNA insertion	48
Discussion	49
Chapter 4 Phenotypic characterisation of *jam2*	
Introduction	52
Results	54
jam2 is not an anthocyanin biosynthetic mutant	54
jam2 has delayed anthocyanin accumulation	54
Root growth in *jam2* is inhibited by MeJA	57
PDF1.2 induction in *jam2* is similar to *jar1* after MeJA treatment	58
jam2 is more resistant to *Botrytis cinerea* than Col-0	60
Discussion	63
Chapter 5 Conclusion and Future Prospects	68
Chapter 6 References	72

University of Cape Town

List of Abbreviations

ABA	Abscisic acid
A. brassicicola	*Alternaria brassisicola*
Aco	*Acyl-CoA oxidase*
A. tumefaciens	*Agrobacterium tumefaciens*
AMP	Adenosine monophosphate
AP2	Apetalla2
ASK1	*Arabidopsis* Skp1-like protein1
ASK2	*Arabidopsis* Skp-like protein2
AtCUL1	*Arabidopsis Cullin1*
ATP	Adenosine triphosphate
AtRbx1	*Arabidopsis Rbx1*
AtVSP2	*Arabidopsis thaliana* vegetative storage protein2
B. cinerea	*Botrytis cinerea*
B. impatiens	*Bradysia impatiens*
CaMV	Cauliflower mosaic virus
CESA3	Cellulose synthase3
cM	Centimorgans
CHIB	Chitinase B
Col	Columbia
χ^2	Chi squared value
C. roseus	*Catharanthus roseus*
DES	*Divinyl ether synthase*
DNA	Deoxyribonucleic acid
E. carotovora	*Erwinia carotovora*
E. coli	*Escherichia coli*
EMS	Ethylmethane sulfonate
ER	Endoplasmic reticulum
ERF	Ethylene response factor
GUS	*Escherichia coli* β-glucuronidase
HEL	*Hevein-like*
HPL	Hydroperoxide lyase
i-PCR	Inverse polymerase chain reaction
JA	Jasmonic acid
JR1	Jasmonic acid responsive1
JR2	Jasmonic acid responsive2

University of Cape Town

JR3	Jasmonic acid responsive3
JERE	Jasmonate and elicitor response element
LRR	Leucine rich repeat
LUC	Luciferase
mRNA	Messenger Ribonucleic acid
NADPH	Nicotinamide-adenine dinucleotide phosphate
Nos	Nopaline synthase
ORCA	Octadecanoid-responsive *Catharanthus roseus Apetalla2* protein
ORCA2	Octadecanoid-responsive *Catharanthus roseus Apetalla2* protein2
ORCA3	Octadecanoid-responsive *Catharanthus roseus Apetalla2* protein3
PAL	Phenylalanine ammonia lyase
PDF1.2	Plant defensin1.2
PIN	Proteinase inhibitor
Pin2	Protein inhibitor2
P. syringae	*Pseudomonas syringae*
P. irregulare	*Pythium irregulare*
P. mastophorum	*Pythium mastophorum*
pv	pathovar
PR1	Pathogenesis related protein1
PR4	Pathogenesis related protein4
PR5	Pathogenesis related protein5
P. fluorescens	*Pythium mastophorum*
PCR	Polymerase chain reaction
RP	Regulatory protein
rcd1	*radical-induced cell death1*
Ri	Root-inducing
SA	Salicylic acid
Str	Strictosidine synthase
SAR	Systemic acquired resistance
Thi2.1	Thionin2.1
T-DNA	Transfer DNA
Ti	Tumor-inducing
TAIL-PCR	Thermal asymmetric interlaced PCR
UV-C	Ultraviolet-C
VSPs	Vegetative storage proteins
VSPB	Vegetative storage proteinB

University of Cape Town

X. campestris	*Xanthomonas campestris*

Arabidopsis mutants and transgenic lines

JA mutants and transgenic lines

aim1	Abnormal inflorescence meristem
aos	Allene oxide synthase mutant
cet	Constitutive expression of thionin2.1
cev1	Constitutive expression of VSP1
cex1	Constitutive expression1
coi1	Coronatine insensitive1
dad1	Delayed anther dehiscence1
dde1	Delayed dehiscence1
dde2	Delayed dehiscence2
jam2	Jasmonic acid modified2
jar1	Jasmonic acid resistant1
JMT-OE	Jasmonic acid methyltransferase overexpressing
jin1	Jasmonic acid insensitive1
jin4	Jasmonic acid insensitive4
joe1	Jasmonate overexpression1
joe2	Jasmonate overexpression2
jue2	Jasmonate underexpression2
jue1	Jasmonate underexpression1
opr3	12-oxo-phytodienoic acid reductase3

SA mutants

cpr1	Constitutive expression of PR proteins1
cpr6	Constitutive expression of PR proteins2
cir1	Constitutively induced resistance1
npr1	Nonexpression of PR1
nim1	non-inducible immunity1
sai1	Salicylic acid insensitive1
ssi2	Suppressor of SA insenitivity2

Ethylene mutants

etr1	Ethylene resistant1
ein2	Ethylene insensitive2

JA biosynthetic substrates, enzymes and products

α - LA	Alpha linolenic acid
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
AtLOX1	*Arabidopsis thaliana* lipoxygenase1

University of Cape Town

AtLOX2	*Arabidopsis thaliana* lipoxygenase2
CHS	Chalcone synthase
dnOPDA	dinor-12-oxo-phytodienoic acid
12, 13-EOT	12, 13- epoxy-octadecatrienoic acid
FAD	Fatty acid desaturase
FAD3	Fatty acid desaturase3
FAD7	Fatty acid desaturase7
FAD8	Fatty acid desaturase8
13(S)-HPOT	13-hydroperoxy-octadecatrienoic acid
JMT	Jasmonic acid methyltransferase
LOX	Lipoxygenase
MeJA	Methyl jasmonate
MeOPDA	Methyl 12-oxo-phytodienoic acid
OPC8:0	3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-oxo-phytodienoic acid
OPR	12-oxo-phytodienoid acid reductase
OPR1	12-oxo-phytodienoic reductase1
OPR2	12-oxo-phytodienoic reductase2
OPR3	12-oxo-phytodienoic reductase3
PLA1	Phospholipase A1
PLDα	Phospholipase Dα
A	Absorbance
°C	Centigrade Celsius
cps	counts per minute
g	gram/s
hr	Hour
µl	Microlitre
µM	Micromolar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
nm	nanometer
PN medium	Plant nutrient medium
v/v	Volume per volume
w/v	weight per volume

University of Cape Town

List of Tables

Table 1.1 JA biosynthetic mutants, gene products and localisation of gene products
Table 1.2 Insensitive JA signaling mutants
Table 1.3 Constitutive JA signaling mutants
Table 3.1 *jam1* segregates as a recessive, monogenic mutation
Table 3.2 *jam1* is not allelic to *coi1-1*
Table 3.3 *jam2* contains a single T-DNA insertion
Table 3.4 *jam2* is not genetically linked to BASTA resistance
Table 4.1 Anthocyanin levels of Col-0, *jam2* and *jar1* after treatment with 45 µM MeJA
Table 4.2 Disease indices of Col-0, *jam2* and *jar1* after infection with *B. cinerea*
Table 5.1 JA-mediated responses as elucidated by *Arabidopsis* JA signalling mutants

List of Figures

Figure 1.1 Structures of α-LA, arachidonic acid, OPDA, MeJA and Prostaglandin A_2
Figure 1.2 The JA biosynthetic pathway
Figure 1.3 The JA signalling pathway
Figure 1.4 Model of putative COI1 function in ubiquitin modulated protein degradation
Figure 1.5 Cross talk between the JA signalling pathway, the ethylene and the salicylic acid signaling pathways.
Figure 2.1 Activation tagging vector pSKI015
Figure 4.1 Anthocyanin production and accumulation of Col-0 and *jam2*
Figure 4.2 Visible anthocyanin production of Col-0, *jam2* and *jar1*
Figure 4.3 Average root lengths on 30, 45 and 100 µM MeJA of Col-0, *jam2* and *jar1*
Figure 4.4 Exogenous liquid MeJA treatment of Col-0, *jam2* and *jar1*
Figure 4.5 Gaseous MeJA treatment of Col-0, *jam2* and *jar1*
Figure 4.5 Assessment of disease development in Col-0, *jam2*, *jar1* and *coi1-1* (H)
Figure 4.7 *B.cinerea* infection of Col-0, *jam2* and *jar1*
Figure 5.1 The JA signalling pathway

University of Cape Town

Chapter 1

JA responses, biosynthesis and signalling in *Arabidopsis thaliana*

Literature Review

University of Cape Town

Introduction

Signal transduction pathways can be seen as channels of communication used by organisms to relay internal and external stimuli from the cell surface to the nucleus. This leads to changes in metabolism and gene expression culminating in the appropriate responses. Upon activation of signalling pathways, synthesis of specific signalling molecules (often hormones) is increased. These signalling molecules are able to activate signalling pathways, as well as act as effector molecules, governing various biological processes. In plants, phytohormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid (ABA) have established roles in regulating various aspects of growth and development (reviewed in Coenen and Lomax, 1997; Steptonova and Ecker, 2000; Sun, 2000; Kakimoto, 2003; Schaller, 2003; Vogler and Kuhlmeier, 2003). Ethylene and ABA also play a part in stress responses (Hildmann et al., 1992; Thaler; 1999; Steptonova and Ecker, 2000), as do signalling compounds such as brassinosteroids, salicylic acid (SA) and jasmonic acid (JA) (Reymond and Farmer, 1998; Bishop and Koncz, 2002; Schaller 2003). JA and brassinosteroids have also been shown to modulate growth activities (reviewed in Sembdner and Parthier, 1993; Creelman and Mullet, 1995; Bishop and Koncz, 2002; Schaller, 2003). Importantly, there is increasing evidence indicating that these molecules do not act independently, but act in concert in complex signalling networks to regulate constitutive and inducible plant responses (Hildman et al., 1992; Xu et al., 1994; Dammann et al., 1997; Norman-Setterblad et al., 2000).

University of Cape Town

Initial interest in JA stemmed from the isolation of its volatile methyl ester, methyl jasmonate (MeJA) (Fig.1.1), from jasmine (Sembdner and Parthier, 1993). MeJA is widely used in the perfume industry. Aside from this, JA is found in some fungi (Sembdner and Parthier, 1993) and even acts as a pheromone attracting natural pollinators of plants (Birkett et al., 2000; Farmer, 2001). However, the greatest interest in JA sprouts from its analogy to the animal defence compounds prostaglandins (Bergey et al., 1996). JA and prostaglandins are both derived from fatty acids: JA from the C18 fatty acid, α-linolenic acid (α-LA) and prostaglandins from the C20 fatty acid, arachidonic acid, yielding products with similar structures (Fig.1.1) (Bergey et al., 1996). In addition, substrates for both biosynthetic pathways are released from membranes through the actions of phospholipases (Bergey et al., 1996; Wang et al., 2000; Ishiguro et al., 2001). Moreover, prostaglandins act in anti-inflammatory and stress responses in animals whilst JA acts as regulator of plant stress responses (Bergey et al., 1996).

One other interesting feature of JA is that its' biosynthetic intermediates (with JA, collectively known as jasmonates) have different biological activities (Stintzi et al., 2001). For example, due to

MeJA's volatile nature, it has been implicated in interplant communication (Farmer and Ryan, 1990). Thus, JA is a remarkable biological compound, found in all plants (Creelman and Mullet, 1995) and has diverse biological functions. These characteristics make JA a molecule of general biological interest and the elucidation of how JA functions in plants can make a significant contribution to our understanding of signalling pathways in plants.

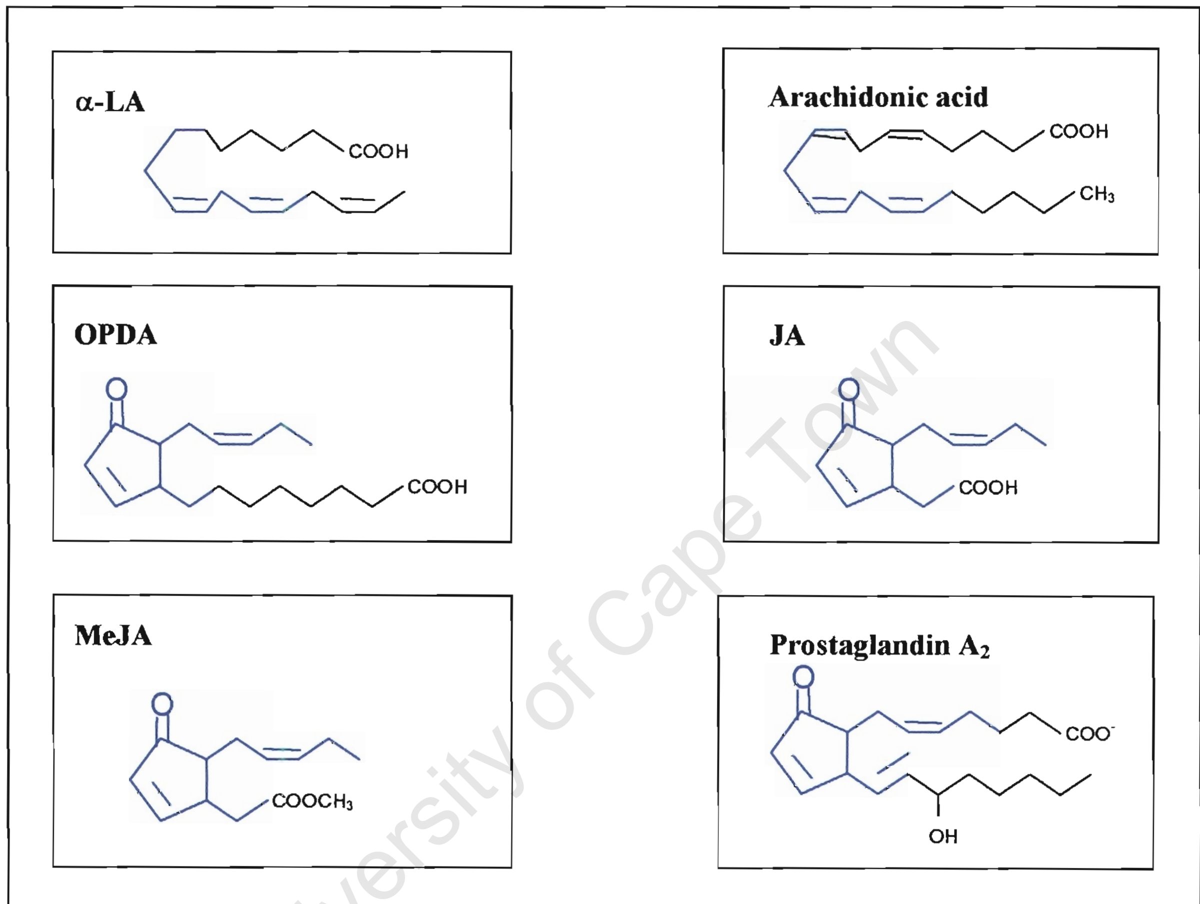


Figure 1.1: Structures of α LA linolenic acid; Arachidonic acid, OPDA 12-oxo-phytodienoic acid; JA jasmonic acid; MeJA methyl jasmonate and Prostaglandin A_2.

University of Cape Town

This introductory chapter will focus on the role that the phytohormone, JA, plays in plants, how JA is synthesised and how increases in JA lead to changes in gene expression and downstream responses. Most of this work has been done in tomato and the model plant *Arabidopsis thaliana*. Biochemical, physical and gene expression studies have been instrumental in elucidating different aspects of JA biology.

What responses are regulated by JA?

Processes regulated by JA were initially identified based on two observations: (1) the accumulation of endogenous JA under various conditions and upon various treatments, and (2) the effects exogenously applied JA and MeJA have on plants. These responses have been observed in a variety

of plant species, in seedlings and mature plants as well as soybean cell suspension cultures, demonstrating the ubiquity of JA in plants.

JA is thought to regulate numerous aspects of plant growth, development and physiology ranging from seed development and germination to photosynthesis and stomatal closure (reviewed in Sembdner and Parthier, 1993; Creelman and Mullet, 1997). An interesting developmental response involving JA and MeJA is tendril coiling in *Bryonia dioica*. Exogenous application of JA and MeJA induces tendrils to *coil*, mimicking the touch stimulus that normally induces such coiling (Falkenstein et al., 1991). Another developmental process thought to be regulated by JA is senescence. Senescence, a form of programmed cell death, is the final stage in leaf development. During senescence levels of JA were shown to increase concurrently with the induction of JA biosynthetic genes suggesting that JA is produced *de novo* during senescence (He et al., 2002). A role for JA in nitrogen storage has been suggested since endogenous levels of JA are high in young leaves, flowers and fruit of soybean plants and in developing organs including the hypocotyl hooks and plumule (Creelman and Mullet, 1995). Although JA levels are low in seeds, levels increased in developing soybean axes within 12 hours of germination (Creelman and Mullet, 1997). This rise in JA levels in vegetative sinks such as the soybean axes, plumule, hypocotyls hooks and in growing seedlings compared to older leaves, supports the suggestion that JA plays a role in vegetative storage (Creelman and Mullet, 1997). Other supporting evidence for the JA mediated regulation of nitrogen storage arose from the treatment of soybean cell cultures with exogenous MeJA that led to the accumulation of vegetative storage proteins (VSPs) (Creelman and Mullet, 1992). Interestingly, low-level atmospheric MeJA was also able to induce VSPs in soybean plants (Franceschi and Grimes, 1991).

University of Cape Town

Although JA plays a major part in regulating various aspects of plant development, it is also known for modulating stress responses such as wounding and pathogen defence responses. Initial studies to identify JA-regulated stress responses were based on the observation that JA levels increase upon chemical and physical treatments able to elicit defence responses in cell cultures and plants. Oligogalacturonides and chitosan oligosaccharides are accepted as elicitor molecules produced during wounding and pathogen attack, either by the pathogen or plant, and capable of activating plant defences. Upon treatment of cell cultures and plants with elicitors derived from yeast (Gundlach et al., 1992; Blechert et al., 1995) and fungi (Mueller et al., 1993; Menke et al., 1999), levels of jasmonates were shown to increase. The wounding response is well studied in tomato. Treatment of tomato leaves with elicitors such as systemin (wounding), oligogalacturonides (insects/wounding) and chitosan oligosaccharides (pathogens) resulted in an increase in endogenous

JA levels (Doares et al., 1995). Jasmonate levels were also shown to increase in wounded soybean hypocotyls (Creelman and Mullet, 1992; Bell et al., 1995), in local and systemic leaves of *Arabidopsis* challenged with *Alternaria brassicicola* (Penninckx et al., 1996), in barley leaves subjected to osmotic stress (Lehmann et al., 1995) and in *Arabidopsis* plants in response to ozone treatment (Rao et al., 2000).

Application of exogenous MeJA or JA can mimic stress responses by inducing transcription of defence related genes. The exogenous application of MeJA to *Arabidopsis* plants led to the induction of the wound-inducible JA biosynthetic lipoxygenase (*LOX*) genes (Melan et al., 1993; Bell and Mullet, 1993) and the antifungal peptides thionin 2.1 (*Thi2.1*) (Epple et al., 1995) and plant defensin (*PDF1.2*) (Penninckx et al., 1996). When exogenously applied to tomato plants, MeJA was found to induce the synthesis of wound-inducible proteinase inhibitor (PIN) proteins (Farmer and Ryan, 1990) and the JA biosynthetic gene allene oxide synthase (*AOS*) (Sivasankar et al., 2000). Addition of MeJA to soybean cell suspensions also led to an increase in mRNA transcripts of *VSP*, the phenylpropanoid enzyme, chalcone synthase (*CHS*), and proline-rich cell wall protein (Franceschi and Grimes, 1991; Creelman and Mullet, 1992). These genes are all associated with stress responses and can be induced by exogenous JA.

University of Cape Town

Defence responses against pathogens are accompanied by the release of low molecular weight antimicrobial secondary metabolites. Interestingly, a link between JA and secondary metabolites has been established. α-LA, MeJA and the JA intermediate, 12-oxo-phytodienoic acid (OPDA), are able to induce antimicrobial alkaloids in cell cultures and in *Catharanthus roseus* (Madagascan periwinkle) (Menke et al., 1999). Furthermore, MeJA was able to up-regulate the glucosinolate biosynthetic enzymes as well as induce the accumulation of glucosinolates in *Arabidopsis* (Brader et al., 2001; Mikkelson et al., 2003). Noteworthy is that different glucosinolate biosynthetic enzymes were induced upon MeJA and SA treatments, leading to the accumulation of specific glucosinolates upon these treatments. This indicates that different signalling pathways regulate the induction of specific glucosinolate biosynthetic enzymes, producing glucosinolates characteristic of those particular signalling pathways (Gundlach et al., 1992; Blechert et al., 1995 and Mikkelson et al., 2003).

A general role for JA has been established in development and stress responses. Additional evidence for the role of JA comes from the characterisation of JA biosynthetic mutants. JA biosynthetic mutants are disrupted in enzymes leading to the production of JA and contain altered JA levels. These plants with altered JA levels have been used to investigate the importance of JA in

plant responses, i.e. how does the absence of JA affect plant responses? The following section will focus on how JA is synthesised and how JA biosynthetic enzymes are regulated. What JA biosynthetic mutants have revealed about the importance of JA will then be discussed.

The JA biosynthetic pathway

JA is synthesized from the fatty acid-derived α-LA via the octadecanoid pathway (Fig.1.2). Through the sequential action of biosynthetic enzymes such as LOX, AOS, allene oxide cyclase (AOC) and 12-oxo-phytodienoc acid reductase (OPR), α-LA is oxygenated, dehydrated, cyclised and reduced to yield stereospecific products (Fig.1.2). The final biosynthetic step prior to JA formation involves three cycles of β-oxidation after which JA can be modified, for example through methylation and adenylation (Fig.1.2) (Seo et al., 2001; Staswick et al., 2002).

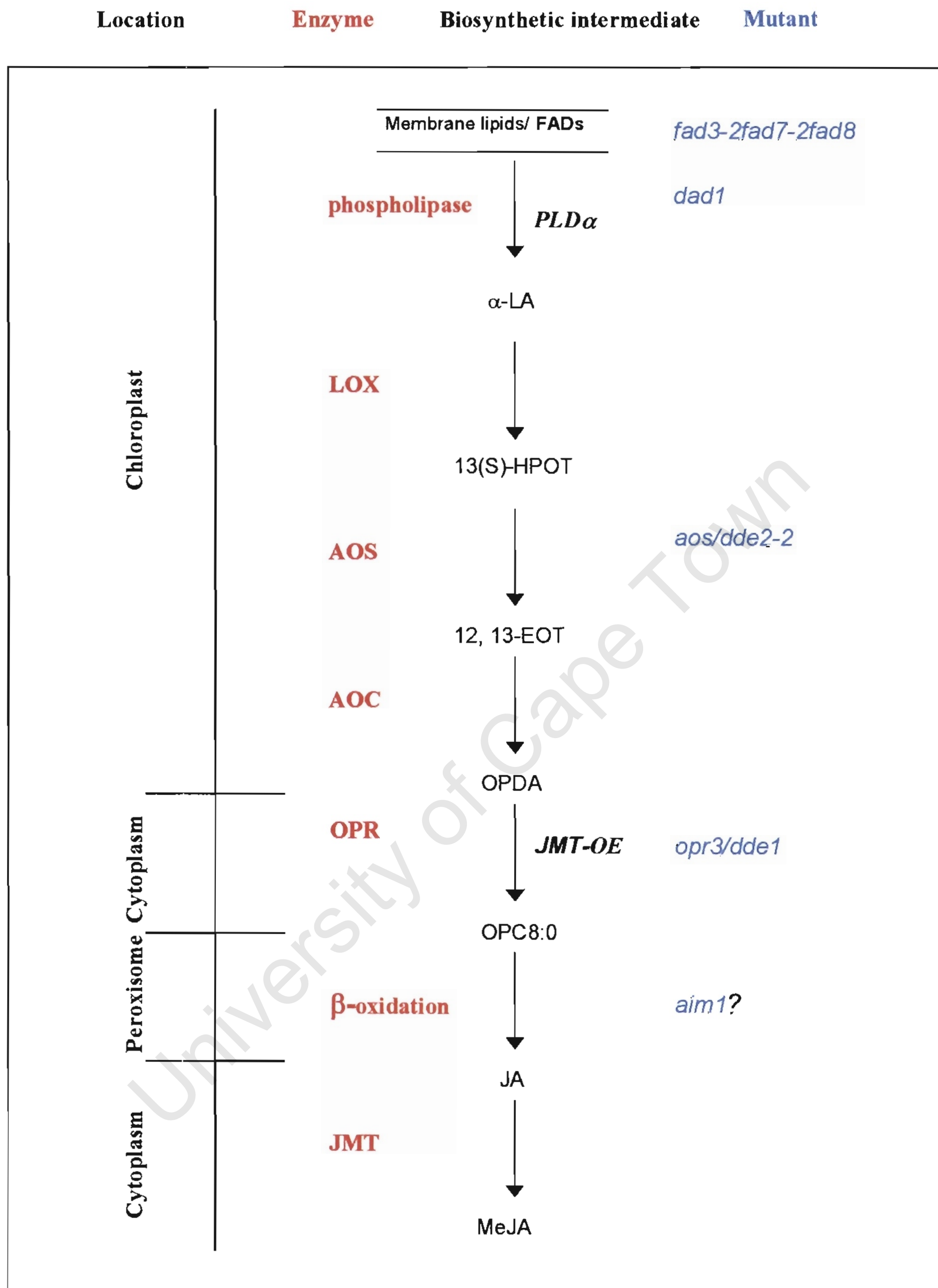
University of Cape Town

Vick and Zimmerman (1984), through biochemical analyses have elucidated all the enzymes involved in the JA biosynthetic pathway. The availability of JA biosynthetic mutants deficient in certain JA biosynthetic enzymes made a significant contribution to our understanding of how the flux through the pathway is controlled by identifying control points and rate limiting enzymes (Laudert and Weiler, 1998; Sanders et al., 2000; Ishiguro et al., 2001; Stintzi et al., 2001; Park et al., 2002; von Malek et al., 2002). The JA biosynthetic pathway, its biosynthetic enzymes and corresponding mutants are illustrated in Fig.1.2 and Table 1.1 and will be discussed.

<u>Release of α-LA by lipases/ phospholipases</u>

The octadecatrienoic fatty acid, α-LA, serves as substrate for JA biosynthesis (Fig.1.2) (Vick and Zimmerman, 1984). α-LA is a polyunsaturated fatty acid derived from linoleic acid through the action of fatty acid desaturases (FADs) and is a major constituent of chloroplast membranes (McConn et al., 1994; Creelman and Mulpuri, 2001). α-LA availability is important for the rate of JA biosynthesis as exogenous application of α-LA to tomato plants led to an increase in endogenous JA levels (Farmer and Ryan, 1992). Three genes encoding FADs, which insert double bonds at the ω-3 positions of hexadecadienoic (16:2) and octadecadienoic (18:2) acids, have been identified in *Arabidopsis* (Browse et al., 1986; Browse et al., 1993; McConn et al., 1994). The enzymes FAD7 and FAD8 are located in the chloroplast, whereas FAD3 is located in the endoplasmic reticulum (ER) (Browse et al., 1986; Kunst et al., 1989; Browse et al., 1993). These enzymes are responsible for providing most of the unsaturated trienoic fatty acids constituting leaf cell membranes. Notably, mutations in any of these genes lead to a decrease but not a total deficiency in trienoic acids (Browse et al., 1986; Browse et al., 1993; McConn et al., 1994). This

can be attributed to residual desaturase activity as well as transport of trienoic acids between the chloroplast and the ER (Browse et al., 1986; Browse et al., 1993).



University of Cape Town

Figure 1.2: The JA biosynthetic pathway. The JA biosynthetic enzymes are indicated on the left in red whilst the biosynthetic mutants are indicated in blue. Biosynthetic genes abbreviated as LOX lipoxygenase; AOS allene oxide synthase; AOC allene oxide cyclase; OPR OPDA reductase; JMT jasmonic acid carboxyl methyltransferase. JAs intermediates: α-LA α-linolenic acid; 13(S)-HPOT 13-hydroperoxy-octadecatrienoic acid; 12, 13-EOT 12, 13-epoxy-pctadecatrienoic acid; OPDA 12-oxo-phytodienoic acid; OPC8:0 3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid; JA jasmonic acid; MeJA methyl jasmonate. Biosynthetic mutants: *dad1* delayed anther dehiscence1; *fad3-2fad7-2fad8* fatty acid desaturase triple mutant; *dde2* delayed dehiscence2, *aos* allene oxide synthase; *dde1* delayed dehiscence1; *opr3* OPDA reductase3; *aim1* abnormal inflorescence meristem1; ? denotes the proposed position of *aim1* since it is not an established biosynthetic mutant yet. Transgenics are indicated in bold italics: PLDα suppressed line, AtLOX2 co-suppressed line; JMT-OE JMT overexpressing line

Table 1.1: JA biosynthetic mutants, gene products and localisation of gene products

Mutant	Abbreviation	Gene product	Gene product location	Reference
Defective in anther dehiscence1	*dad1*	Phospholipase A1	chloroplast	Ishiguro et al., 2001
fatty acid desaturase triple mutant	*fad3-2fad7-2fad8*	Fatty acid desaturase	FAD7 and FAD8 in chloroplast; FAD3 in endoplasmic reticulum	McConn and Browse, 1996
delayed dehiscence1	*dde1*	OPDA reductase1/-2	pistil, petals and stamen filaments	Sanders et al., 2000
OPDA reductase3	*opr3*	OPDA reductase3	anthers, flowers and leaves	Stintzi and Browse, 2000
delayed dehiscence2-2	*dde2-2*	Allene oxide synthase	n.d.#	von Malek et al., 2002
abnormal inflorescence meristem *	*aim1*	Multifunctional protein involved in β-oxidation	roots, rosette leaves, flowers, siliques and hypocotyls	Richmond and Bleecker, 1999

* The *aim1* mutant is proposed to be a jasmonate biosynthetic mutant based on phenotypic similarities with known jasmonate biosynthetic mutants. Although changes in fatty acid composition are observed, especially 18:1 and 18:2, the jasmonate levels of *aim1* still need to be determined. # not determined

University of Cape Town

The *Arabidopsis* triple mutant, *fad3-2fad7-1fad8* (Fig.1.2 and Table1.1), has negligible amounts of trienoic acids and high dienoic acid levels (McConn and Browse, 1996), confirming a lack of desaturase activity. This lack of trienoic acids in *fad3-2fad7-1fad8* leads to a lack of JA (McConn and Browse, 1996). This triple mutant was instrumental in demonstrating a role for JA in male fertility, nitrogen storage, wounding and defence against pathogens (McConn and Browse, 1996; Vijayan et al., 1998; Stintzi et al., 2000). *fad3-2fad7-1fad8* mutants are male sterile (McConn and Browse, 1996). Upon exogenous MeJA treatment, the male sterile phenotype of the *fad* triple mutants was rescued, demonstrating that JA is needed for male fertile plants (McConn and Browse, 1996). When *VSP* mRNA levels were studied in this triple mutant, no *VSP* transcripts were detected in untreated tissue. However, upon treatment with MeJA, *VSP* transcripts were detected, confirming that JA is required for *VSP* expression (McConn and Browse, 1996). f*ad3-2fad7-1fad8* mutants are unable to induce expression of *VSP* and *PAL* indicating the involvement of JA in wound responses (McConn and Browse, 1996).

A role for JA in defence against insect attack was also suggested since *fad3-2fad7-1fad8* mutants were highly susceptible to the fungus gnat *Bradysia impatiens* (McConn and Browse, 1996). Moreover, *fad3-2fad7-1fad8* was substantially protected against insect attack by exogenous MeJA treatment (McConn and Browse, 1996). *fad* triple mutants were also highly susceptible to root rot caused by *Pythium mastophorum* and again application of MeJA provided substantial protection (Vijayan et al., 1998). The *fad3-2fad7-1fad8* mutant was also susceptible to *A. brassicicola*, suggesting a role for JA in defence against fungal pathogens (Stintzi and Browse, 2000).

It has long been postulated that LA is released from membranes through the action of lipases or phospholipases (Farmer and Ryan, 1992). The involvement of two phospholipases, phospholipase Dα (PLDα) and phospholipase A1 (PLA1), in the release of LA has been suggested recently (Fig.1.2 and Table 1.1) (Wang et al., 2000; Ishiguro et al., 2001). A causal link between PLDα activity and wound-induced JA synthesis was established (Wang et al., 2000). In antisense *Arabidopsis* plants suppressing *PLD*α expression, and therefore lacking PLDα activity, JA production and expression of *VSP* and *AtLOX2* upon wounding were substantially reduced (Wang et al., 2000). The JA biosynthetic mutant, *defective in anther dehiscence1* (*dad1*), encodes a PLA1 (Fig.1.2 and Table 1.1) containing a putative N-terminal chloroplast localisation signal motif and a conserved lipase active site (Ishiguro et al., 2001). *dad1* mutants were male sterile and showed no anther dehiscence or pollen release upon flower opening (Ishiguro et al., 2001). Moreover, levels of JA and MeJA were decreased in *dad1* flower buds (Ishiguro et al., 2001). The male sterile phenotype of *dad1* mutants was rescued by application of JA or α-LA, indicating that *dad1* is affected in JA biosynthesis (Ishiguro et al., 2001). Although an increase in wildtype *DAD1* expression upon wounding was observed, the increase in JA levels after wounding was 100-fold for both the *dad1* mutants and wild type plants (Ishiguro et al., 2001). This suggests the presence of other lipolytic enzymes mediating JA accumulation upon wounding, presumably *PLDα. DAD1* seems to be required to mediate jasmonate accumulation during pollen and anther development and *DAD1* was specifically expressed in stamen filaments before flower opening as determined through GUS expression studies (Ishiguro et al., 2001). Therefore, it seems that the release of α-LA for JA biosynthesis during wounding and pollen development is probably provided via the action of two distinct lipolytic enzymes.

Lipoxygenases

After release of α-LA from the membrane, lipoxygenases (LOXs) oxygenate LA to form the stereospecificity isomer (9Z, 11E, 15Z, 13S-3-hydroperoxy-9, 11, 15-octadecatrienoic acid (13(S)-

University of Cape Town

HPOT) (Vick and Zimmerman, 1987; Creelman and Mullet, 1997). Lipoxygenases are non-haem iron-containing dioxygenases which catalyse the oxygenation of C_{18} fatty acids on either position 9 or 13 (Howe and Schilmiller, 2001; Schaller 2001). *Arabidopsis* has two different *LOX* genes, designated *AtLOX1* (Melan et al., 1993) and *AtLOX2* (Bell and Mullet, 1993). These two LOX isoforms are differentially expressed in different organs. *AtLOX1* is highly expressed in roots and young seedlings and is found at lower levels in leaves and inflorescences (Melan et al., 1993). In mature plants, *AtLOX1* is upregulated by MeJA and pathogens (Melan et al., 1993). After infection with an avirulent strain of *Pseudomonas syringae*, *AtLOX1* reached its maximum induction at 12 hr and maximum induction upon infection with the virulent strain was reached at 48 hr (Melan et al., 1993). On the other hand, *AtLOX2* is located in chloroplasts and is highly expressed in leaves and inflorescences, with lower expression in seeds, roots and stems (Bell and Mullet, 1993; Bell et al., 1995). *AtLOX2* mRNA increases upon treatment with MeJA and after wounding in both local and systemic leaves (Bell and Mullet, 1993).

University of Cape Town

Transgenic approaches aimed at the manipulation of jasmonate biosynthetic enzymes to alter endogenous jasmonate levels have helped in the identification of flux regulation through the JA biosynthetic pathway. Transgenic *Arabidopsis* plants exhibiting co-suppression of *LOX2* did not accumulate JA after wounding indicating that *LOX2* is required for the wound-inducible JA increase (Bell et al., 1995). One other line of evidence implicating a *LOX2*-dependent JA increase in the wound response is that the wound-induced increase in *VSP* mRNA was also reduced in transgenic leaves lacking *LOX2*. However, LOX2 is apparently not necessary to maintain basal levels of jasmonates because JA levels in unwounded wild type and unwounded *LOX2* transgenic plants were the same (Bell et al., 1995).

The hydroperoxide products of LOX serve as substrates for a variety of enzymes catalysing the synthesis of octadecanoid-derived metabolites. These enzymes include hydroperoxide lyase (HPL), divinyl ether synthase (DES), epoxy alcohol synthase, peroxygenase, alkyl hydroperoxide reductase and AOS (Howe and Schilmiller, 2002). However, only AOS will be discussed as it is the committing enzyme for JA biosynthesis.

Allene oxide synthase

Through the action of AOS, 13(S)-HPOT is dehydrated to form an unstable epoxide, 12, 13 (S)-epoxy-9 (Z), 11, 15 (Z)-octadecatrienoic acid (12, 13-EOT) (Vick and Zimmerman, 1981; Vick and Zimmerman, 1987). The AOS enzyme is the committing enzyme for JA biosynthesis and can also use 13(S)-hydroperoxylinoleic acid as a substrate (Laudert and Weiler, 1998). AOS is a cytochrome

P450 enzyme of the *CYP74* family (Laudert and Weiler, 1998; Schaller, 2001; Tijet and Brash, 2002). The activity of AOS is dependent on oxygen, nicotinamide-adenine dinucleotide phosphate (NADPH) and P450 reductases (Schaller, 2001).

Arabidopsis has a single *AOS* gene encoding a protein containing a chloroplast localisation signal (Laudert et al., 1996). *AOS* is highly expressed in leaves and flowers and at lower levels in seeds, roots, stems and siliques (Laudert et al., 1996). Wounding causes an increase in both *AOS* mRNA and protein expression with a concomitant increase in jasmonate levels (Laudert and Weiler, 1998; Kubigsteltig et al., 1999).

An attempt to increase endogenous JA levels by overexpressing *AOS* in *Arabidopsis*, did not lead to an increase in basal JA levels, although it did lead to higher JA accumulation in wounded transgenic plants (Laudert et al., 2000). In contrast to this, a Flax *AOS* gene overexpressed in potato led to increases of basal JA levels, although the wound-inducible *Pin2* gene was not constitutively activated (Harms et al., 1995). Noteworthy is that the basal levels of JA in these transgenic plants overexpressing *AOS* were similar to those obtained in wounded wild type plants (Harms et al., 1995). This suggests that previous stimulation to release α-LA is not required for JA synthesis in potato and implicates AOS as a rate-limiting enzyme in jasmonate biosynthesis. However, upon wounding and water stress, transgenic *AOS* potato plants accumulated even higher levels of JA followed by gene activation of wound and water stress related genes (Harms et al., 1995). The authors ascribe this discrepancy to either the existence of an alternative cytoplasmic jasmonate biosynthetic pathway that could be activated upon wounding and water stress or translocation of sequestered JA from the chloroplast or peroxisome to the cytoplasm. However, the mechanism by which sequestered JA would be relocated is unknown. The inconsistency between *Arabidopsis* and potato overexpressing *AOS* could mean that AOS-directed jasmonate synthesis is regulated differently in different plant species.

Recently, an *AOS* knockout mutant (*aos*), in *Arabidopsis* was found to be male sterile due to defects in pollen and anther development (Fig. 1.2 and Table 1.1) (Park et al., 2002). This knockout also had impaired wounding responses (Park et al., 2002). The *aos* male sterility phenotype was completely restored by complementation with *AOS* under a constitutive promoter (Park et al., 2002). Moreover, application of OPDA and JA, but not α-LA, led to the rescue of the male sterile phenotype (Park et al., 2002). Another mutant defective in the *AOS* gene, *delayed dehiscence2-2* (*dde2-2*) has also been identified (Fig. 1.2 and Table 1.1) (von Malek et al., 2002). *dde2-2* was also male sterile due to

University of Cape Town

defects in anther dehiscence and filament elongation (von Malek et al., 2002). Although the jasmonate levels were not determined in *dde2-2*, transcripts of *AOS* were detected in wild type plants but not in *dde2-2* mutants (von Malek et al., 2002). Furthermore, exogenous application of MeJA restored male fertility in *dde2-2* (von Malek et al., 2002). No increase in endogenous JA levels or induction of the wound-responsive genes *AtLOX2* and *AtVSP2* was recorded in *aos* mutants upon wounding. *AtLOX2* and *AtVSP2* remained at basal levels even after wounding in *aos* mutants (Park et al., 2002). However, in transgenic plants overexpressing *AOS*, the levels of JA remained unchanged in unwounded transgenic plants whereas the JA levels in wounded transgenic plants increased, even higher than that in wounded wild type plants (Park et al., 2002). These results confirm the vital role *AOS* plays in male fertility and that *AOS* is necessary for the wound-induced rise in JA levels leading to the induction of wound-responsive genes, *AtLOX2* and *AtVSP2*.

Allene oxide cyclase

The unstable epoxide, 12, 13-EOT, is cyclized by allene oxide cyclase (AOC) to yield a cyclopentanone derivative 12-oxo-10, 15 (Z)-phytodienoic acid (OPDA) (Vick and Zimmerman, 1984).

University of Cape Town

Four possible *Arabidopsis AOC* genes were identified in homology searches using the amino acid sequence of the tomato AOC protein (Creelman and Mulpuri, 2002). These four genes show very high homology to the tomato *AOC* and three of these putative *AOC* genes in *Arabidopsis* have motifs resembling plastid localisation signals (Creelman and Mulpuri, 2002). To date, no *Arabidopsis AOC* homolog has been functionally characterised. However, a single *AOC* gene from tomato, which is found on chromosome 2, was recently cloned (Ziegler et al., 2000). In tomato, *AOC* is highly expressed in roots, flower stalks and at low levels in stems, young leaves and flower pistils (Hause et al., 2000). The tomato AOC was shown to have a plastid localisation peptide, which was in accordance with the chloroplastic localisation of the AOC protein (Ziegler et al., 2002). In tomato, AOC was shown to specifically form *cis*-OPDA, which increases upon wounding and wounding led to an increase in *AOC* mRNA concurrently with increases in JA levels (Ziegler et al., 2002).

OPDA reductase

A NADPH-dependent OPDA reductase (OPR) reduces the 10, 11-double bond of OPDA to form 3-oxo-2 (2'-(Z)-pentanyl)-cyclopentane-1-octanoic acid (OPC 8:0) (Vick and Zimmerman, et al., 1984). *Arabidopsis* is believed to contain at least three *OPR* genes in its genome: *OPR1*, *OPR2* and

OPR3 (Schaller and Weiler, 1997; Schaller et al., 1998; Biesgen and Weiler, 1999; Schaller and Weiler, 2000). Interestingly, the isoenzymes OPR1 and OPR2 exhibit different substrate stereospecificity to OPR3. The OPR3 isoenzyme specifically converts (9S, 13S)-12-OPDA believed to be the natural isomer for JA biosynthesis, whereas OPR1 preferentially converts (9R, 13R)-12-OPDA and to a lower extent, (9S, 13R)-12-OPDA (Schaller et al., 1998; Schaller and Weiler, 2000). OPR2, on the other hand, is able to reduce all four OPDA isomers (Schaller et al., 1998). Therefore, OPR3 could be postulated to be the enzyme most pertinent to JA biosynthesis.

The *OPR* genes are differentially expressed in organs. Both *OPR1* and *OPR2* are highly expressed in roots, whilst *OPR1* is predominantly expressed in young seedlings and *OPR2* is highly expressed in pollen (Biesgen and Weiler, 1999). In leaves, shoots and flowers, *OPR1* and *OPR2* were expressed at 20% of the level of roots (Biesgen and Weiler, 1999). *OPR3*, on the other hand, is expressed throughout *Arabidopsis* plants, with highest expression in flowers and leaves (Sanders et al., 2000; Stintzi and Browse, 2000). *OPR1* and *OPR2* mRNA is transiently induced by wounding, UV-C light and cold. However, this increase is not reflected in protein levels nor enzyme activity (Biesgen and Weiler, 1999). On the other hand, *OPR3* is also induced under these conditions as well as by brassinosteroids, but the enzyme activity relative to mRNA levels has not yet been determined (Müssig et al., 2000; Schaller and Weiler, 2000).

University of Cape Town

Two mutants defective in *OPR* genes have recently been identified: *delayed dehiscence1* (*dde1*) and *OPDA reductase3* (*opr3*) (Sanders et al., 2000; Stintzi and Browse, 2000). *dde1* was shown to have 67% homology to both OPR1 and OPR2, suggesting that *DDE1* might be a different member of the *OPR* gene family (Sanders et al., 2000). Both *dde1* and *opr3* are male sterile, attributed to the inability of pollen to dehisce and poor filament elongation (Sanders et al., 2000; Stintzi and Browse, 2000). Exogenous application of JA rescued the male sterile phenotypes of both these mutants (Sanders et al., 2000; Stintzi and Browse, 2000). When OPDA was applied to *opr3*, male fertility was not restored, indicating that anther dehiscence and filament elongation require JA specifically and not OPDA (Stintzi and Browse, 2000). Endogenous JA levels were not measured in *opr3* and *dde1*. Transcript levels of *OPR3* were detected in wild type plants but not in *opr3* mutants which could be due to the rapid degradation of *OPR3* mRNA transcripts (Stintzi and Browse, 2000; von Malek et al., 2002).

Evidence that OPDA can confer disease resistance against insects and fungal infection in the absence of JA was obtained when *opr3* showed enhanced resistance to *B. impatiens* and *A.*

brassicicola infection, in contrast to the *fad* triple mutant (Stintzi et al., 2001). Moreover, wounded *opr3* plants expressed JA-responsive genes, indicating that OPDA is able to induce gene expression after wounding, a role previously ascribed to JA (Stintzi et al., 2001). Furthermore, *Arabidopsis* plants exogenously treated with OPDA were able to increase expression of a subset of JA-responsive genes (Stintzi et al., 2001). This suggests that expression of defence genes could be concomitantly regulated by JA and OPDA.

β-oxidation

OPC 8:0 then undergoes three cycles of β-oxidation catalysed by multiple enzymes (Vick and Zimmerman, 1984; Kindl et al., 1993). These enzymes are proposed to include a fatty acyl-CoA synthetase, acyl-CoA oxidase and possibly a multifunctional enzyme with enoyl-CoA-hydratase, β-hydroxy-acyl-CoA dehydrogenase and thiolase activity (Léon and Sánchez-Serrano, 1999). β-oxidation is the final step in JA biosynthesis and is thought to occur in the peroxisome (Fig. 1.2) (Gerhardt, 1983; Vick and Zimmerman, 1984; Kindl et al., 1993).

The β-oxidation step in JA biosynthesis is not well defined. However, differential displays identified an acyl-CoA oxidase (*Aco*) gene in *Arabidopsis* plants after wounding (Titarenko et al., 1997). Moreover, *Aco* mRNA transcripts accumulated after wounding and JA treatment, thus indicating that it is both wound and JA responsive (Titarenko et al., 1997). Also, the *abnormal inflorescence meristem1* (*aim1*) mutant is male sterile due to abnormal inflorescence and floral meristems (Fig.1.2 and Table 1.1) (Richmond and Bleecker, 1999). a*im1* has altered fatty acid compositions and the AIM1 amino acid sequence shows homology to a cucumber multifunctional protein involved in β-oxidation of fatty acids (Richmond and Bleecker, 1999). *AIM1* transcripts were highly expressed in cotyledons and at the junction between the root and hypocotyl. In mature plants, *AIM1* was highly expressed in rosette and cauline leaves, stems, inflorescence meristem and flowers (Richmond and Bleecker, 1999). Therefore, *AIM1* is highly expressed in flowers, known tissue where JA is important. Other *Arabidopsis* mutants lacking peroxisomal fatty acid β-oxidation have been isolated and further characterisation of these mutants should help elucidate the involvement of β-oxidation in JA biosynthesis (Hayashi et al., 1998).

Modification of JA

JA can be methylated by JMT

After release into the cytoplasm, JA can be enzymatically modified. However, the exact mechanism for JA transport to various compartments, as well as transport within and between cells, is not known. The methyl ester of JA is formed when the enzyme S-adenosyl-L-methionine: jasmonic acid

University of Cape Town

carboxyl methyltransferase (JMT) methylates JA, (Seo et al., 2001). JMT is expressed in rosette and cauline leaves and in developing flowers but not in young seedlings (Seo et al., 2001). Additionally, wounding and MeJA treatment induce the expression of JMT, both locally and systemically, indicating that JMT can respond to external stimuli (Seo et al., 2001). Interestingly, when JMT is overexpressed, transgenic plants showed increased internal MeJA levels, without any changes in JA levels suggesting JA homeostasis (Seo et al., 2001). Moreover, these JMT overexpressing transgenics exhibited constitutive expression of the JA-responsive genes *PDF1.2* and a *VSP*, as well as enhanced resistance to the necrotrophic fungus *Botrytis cinerea* (Seo et al., 2001), suggesting that JMT is involved in regulating developmental and defence responses possibly through MeJA.

JA can possibly be modified via adenylation by JAR1

The *JAR1* locus has recently been cloned (Staswick et al., 2002). The deduced amino acid sequence had homology to 19 *Arabidopsis* genes, putatively encoding proteins with 47-91% homology to each other (Staswick et al., 2002). JAR1 showed structural similarity, but low sequence homology, to the firefly luciferase family of adenylate-forming enzymes (Staswick et al., 2002). Reactions catalysed by these enzymes require magnesium and ATP to activate carboxyl groups with AMP, which then undergo biochemical modifications. JAR1 activity was shown to be specific for JA and was inactive on LA, OPDA and MeJA (Staswick et al., 2002). Requirements for JAR1 activity were JA, magnesium, ATP and a free carboxyl group (Staswick et al., 2002). The proposed role for adenylated JA is firstly the regulation of JA activity through either the initiation of conjugation to amino acids or sugars or through cellular location (Staswick et al., 2002). Secondly, adenylated JA may control JA turnover (Staswick et al., 2002).

University of Cape Town

Not much is known about the function of JAR1 in JA signalling, other than that it could positively regulate JA signalling (Staswick et al., 1998; Staswick et al., 2002). This brings about the question of the importance of adenylation to JA signalling. As previously stated, of the 19 related *Arabidopsis* genes tested, only JAR1 showed activity with JA, thereby implying that only one gene is responsible for JA adenylation (Staswick et al., 2002). When looking at the phenotype of *jar1*, it can be speculated that adenylation is not needed for male fertility since *jar1* is not male sterile but is necessary for root growth (Staswick et al., 1998; Staswick et al., 2002). *jar1* was isolated for its resistance to MeJA root growth inhibition and showed the same level of insensitivity to JA (Staswick et al., 1998). Due to the similar resistance to MeJA and free JA exhibited by *jar1*, it can be suggested that MeJA is demethylated before adenylation since JAR1 is only active on JA (Staswick et al., 2002). *jar1* is affected in defence responses against necrotrophic pathogens and thus a putative role for adenylation in response to necrotrophic pathogen infection can be speculated. It

seems as if adenylation might be involved in the regulation of JA stress responses and not in general development since *jar1* roots grow normally without addition of JA (Staswick et al., 2002).

JA intermediates are biologically active

An interesting characteristic of the JA biosynthetic pathway is that some of its intermediates are themselves biologically active. When levels of endogenous jasmonates were measured, *Arabidopsis* and soybean leaves displayed low levels of JA and its intermediate, dinor-OPDA (dnOPDA) and surprisingly high levels of the biologically active JA intermediate, OPDA (Weber et al., 1997). Contrary to this, however, in potato leaves OPDA and JA were the most abundant jasmonates found in healthy mature leaves (Weber et al., 1997). This indicates that different plant species have different relative levels of different jasmonates. Weber et al. (1997) termed these profiles "oxylipin signatures". Furthermore, the timing and location of increased endogenous jasmonates, seems to be important for jasmonate regulated developmental processes. Increases in JA levels were found to be biphasic in both locally wounded and systemic leaves of *Arabidopsis* plants, although lower in systemic leaves (Laudert and Weiler, 1998). OPDA, on the other hand, did not increase biphasically in wounded leaves and had no change in systemic leaves.

University of Cape Town

It was found that MeJA, methyl OPDA (MeOPDA) and the *P. syringae* OPDA analogue, coronatine induced expression of AOS mRNA, all with different kinetics (Laudert and Weiler, 1998). Notably, MeOPDA and coronatine showed the fastest induction whilst MeJA the slowest (Laudert and Weiler, 1998). The levels of JA and OPDA showed no significant increase upon treatment with coronatine. Coronatine, however, induced *AOS* mRNA directly (Laudert and Weiler, 1998). Other JA biosynthetic intermediates can also induce gene expression. Exogenous JA precursors: LA, 13(S)-HPOT and OPDA were able to induce PIN protein synthesis (Farmer and Ryan, 1992).OPDA also induced *AOS* expression in tomato (Sivasankar et al., 2000). A volatile compound related to MeJA, *cis*-jasmone produced by an extra β-oxidation step, plays a role in insect predation by attracting natural predators of insects (Birkett et al., 2000).

The above section dealt with the biosynthesis and modification of JA. In summary, JA biogenesis occurs in three compartments: the chloroplast (LA to OPDA), cytoplasm (OPDA to OPC8:0) and the peroxisome (β-oxidation). JA is present at low levels in healthy, mature *Arabidopsis* plants and increases upon wounding (Bell et al., 1995; McConn et al., 1997). Therefore, the JA biosynthetic enzymes are constitutively active and can be induced upon wounding. Moreover, JA and its intermediates are able to induce the expression of some of these biosynthetic enzymes (*DAD1*,

LOX1 and *LOX2*, *AOS*, *OPR3* and *JMT*) (Melan et al., 1995; Bell and Mullet, 1993; Laudert and Weiler, 1998; Müssig et al., 2000; Ishiguro et al., 2001), indicating that JA biosynthesis is regulated through positive feedback. This was confirmed by microarray analysis, in which 5 out of 41 genes influenced by JA were JA biosynthesis genes (Sasaki et al., 2001).

All JA biosynthetic mutants, with the exception of *jar1*, share a common characteristic: they are all male sterile. Although pollen development proceeds to the normal trinucleate stage: 1 vegetative and 2 generative nuclei, these mutants do not release pollen after flower opening (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; von Malek et al., 2002). Moreover, exogenous application of JA or its intermediate below the mutated step in the biosynthetic pathway completely rescued the male phenotypes of JA biosynthetic mutants (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; von Malek et al., 2002).

University of Cape Town

Understanding how JA is synthesised, perceived and transduced as a systemic signal to elicit developmental and stress responses, would aid in the elucidation of the regulatory role of JA in these responses. The following section will focus on JA signalling. The role of JA signalling mutants in the identification and regulation of JA-mediated responses will be highlighted.

JA signalling

It is widely speculated that JA interacts with receptors to activate downstream responses. However, to date no JA receptors have been identified. The screens employed to isolate JA insensitive signalling mutants failed to identify any genes encoding putative JA receptor/s. Therefore, it is possible that multiple, genetically redundant, JA receptors exist, emphasising the need for the isolation of more JA signalling mutants utilising different screening strategies as before. The isolation of JA signalling mutants has helped elucidate the jasmonate signal transduction cascade downstream of JA biosynthesis. JA signalling mutants are impaired in the perception of jasmonates or transduction of this signal, resulting in the abrogation of JA-mediated responses. Mutational analysis could lead to the identification of jasmonate receptor/s as well as both positive and negative regulators of JA signalling. To date *Arabidopsis* JA signalling mutants have been identified that were either insensitive to JA or have constitutive JA responses. Most of these mutants are affected in more than one response and to differing degrees. All the JA insensitive mutants isolated to date are recessive, loss-of-function mutants and it is speculated that their gene products encode positive regulators of JA signalling (Tables 1.2 and 1.3) (Staswick et al., 1992;

Feys et al., 1994; Berger et al., 1996). Recently, mutants with constant JA responses were isolated (listed in Table 1.3), which could be helpful to isolate possible redundant JA signalling components such as receptors. Besides this, JA constitutive mutants are postulated to encode negative regulators of JA signalling. These JA signalling mutants were used to investigate how disrupted JA signalling would affect plant responses as well as how signal transduction was occurring. The JA signalling mutants isolated to date are listed in Tables 1.2 and 1.3 and Figure 1.3.

Mutants acting upstream of JA biosynthesis

One of the earliest acting mutants is thought to be the constitutive JA signalling mutant, *constitutive expression of VSP1* (*cev1*) (Table 1.3) (Ellis and Turner, 2001). *cev1* exhibits stunted growth, shorter roots, constitutive JA and ethylene production and constitutive expression of JA-responsive genes (Ellis and Turner, 2001; Turner et al., 2002). Another interesting *cev1* phenotype is anthocyanin accumulation without application of MeJA (Ellis and Turner, 2001). Moreover, analysis of double mutants of *cev1* with the JA-insensitive mutant *coi1* and the *ethylene resistant* mutant, *etr1* reveals that *cev1* is only partially suppressed in these backgrounds, indicating that *cev1* requires both ethylene and JA signalling (Ellis and Turner, 2001).

Furthermore, in the triple mutant *cev1coi1etr1* the mutant phenotype reverted to wild type (Ellis et al., 2002). Taken together, these results suggest that *cev1* is involved in JA and ethylene biosynthesis (Ellis and Turner, 2001). The *CEV1* gene has been cloned and was shown to encode the cellulose synthetase gene *CESA3* (Ellis et al., 2002). In accordance with this, the *cev1* mutant exhibits reduced levels of cellulose especially in the roots, and treatment of wild type plants with cellulose synthase inhibitors reproduced the *cev1* phenotype (Ellis et al., 2002). It has been established that cell wall-derived elicitors can induce wound and pathogen defence responses (Gundlach et al., 1992; Mueller et al., 1993; Blechert et al., 1995; Doares et al., 1995; Menke et al., 1999), but this is the first account directly linking JA biosynthesis and cell wall activities. To establish how defence responses are affected in *cev1*, mutant plants have been challenged with assorted powdery mildew species and found to be resistant to these pathogens (Fig.1.3) (Ellis and Turner, 2001). These findings suggest that the wild type product of *cev1* encodes a negative regulator governing resistance to powdery mildew.

University of Cape Town

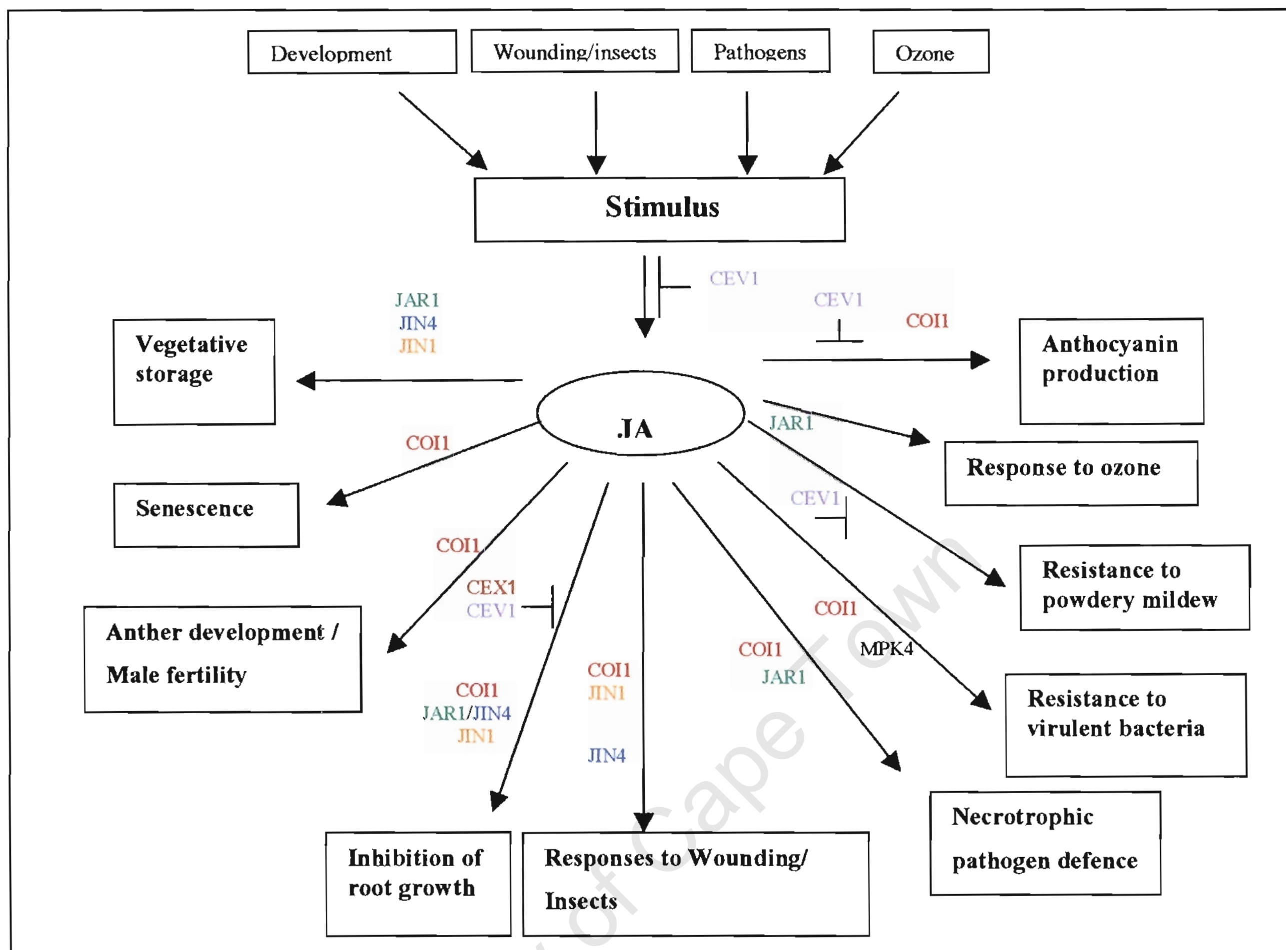


Figure 1.3: The JA signalling pathway. External or internal stimuli are perceived with consequent JA biosynthesis. After perception of JA, JA-responsive genes are expressed leading to the appropriate responses. JA insensitive mutants are defective in signalling components, which positively regulate JA responses (indicated by ⟶), whilst JA constitutive mutants define negative regulators of JA responses (indicated by ⊥).

Table 1.2: Insensitive JA signalling mutants

Mutant	Abbreviation	D/R	Phenotypes	Gene product	Reference
coronatine insensitive1	*coi1*	R	Male sterile, reduced expression of JA-responsive genes, highly susceptible to necrotrophic fungi and insects, resistant to virulent bacteria, increased sensitivity to ozone	F-box protein containing LRR	Feys et al, 1994
jasmonic acid responsive1	*jar1*	R	Reduced expression of JA-responsive genes, highly susceptible to necrotrophic fungi, increased sensitivity to ozone	Acyl adenylate forming enzyme	Staswick et al., 1992
jasmonic acid insensitive 1	*jin1*	R	Reduced expression of JA-responsive genes	#n.d.	Berger et al., 1996
jasmonic acid insensitive 4	*jin4*	R	Reduced expression of JA-responsive genes	#n.d.	Berger et al., 1996

University of Cape Town

Table 1.3: Constitutive JA signalling mutants

Mutant	Abbreviation	D/R	Phenotypes	Gene product	Reference
constitutive expression of thionin2.1 1 to 9	*cet1-cet9*	R, D	Increased expression of JA-responsive genes,	#nd	Hilpert et al., 2001
constitutive expression of VSP1	*cev1*	R	Increased expression of JA-responsive genes	cellulose synthetase	Ellis and Turner, 2001
constitutive expression1	*cex1*	R	Increased expression of JA-responsive genes	#nd	Xu et al., 2001
jasmonate overexpression 1 and -2	*joe1, joe2**	R	Increased expression of JA-responsive genes	#nd	Jensen et al., 2002

*joe1, joe2** are mutants exhibiting overexpression of JA-responsive genes
R = recessive mutation
D = dominant mutation
# nd = not determine

University of Cape Town

JA downstream signalling

As mentioned earlier, several JA signalling mutants have been isolated, insensitive as well as constitutive. The *jasmonic acid resistant1* (*jar1*) and the *jasmonic acid insensitive1* and *4* (*jin1* and *jin4*) mutants were isolated for their resistance to MeJA inhibition of root growth whilst *coronatine insensitive1* (*coi1*) was isolated for its resistance to coronatine root growth inhibition (Table 1.2 and Fig. 1.3) (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996). Nine alleles, representing five different loci, of *constitutive expression of thionin 2.1* (*cet*) mutants have been isolated, some of which are recessive and some which are dominant (Table 1.3) (Hilpert et al., 2001). The *cet* mutants also exhibit spontaneous necrotic lesions (Table 1.3) (Hilpert et al., 2001). Like *cev1*, *constitutive expression1* (*cex1*) exhibits stunted growth and roots phenotype and constitutively induces the *AtVSP* gene (Fig.1.3 and Table 1.3) (Ellis and Turner, 2001; Xu et al., 2001). These constitutive mutants exhibit constant expression of the JA-responsive genes, *Thi2*.1 and *PDF1.2* without MeJA treatment (Fig.1.3 and Table 1.3) (Ellis and Turner, 2001; Hilpert et al., 2001; Xu et al., 2001). *Thi2.1* was also upregulated in *cet* mutants inoculated with the *Fusarium oxysporum* (Table 1.3) (Hilpert et al., 2001). Recently, two recessive mutants, *jasmonic acid overexpressing1* and *2* (*joe1* and *joe2*), which overexpressed the *LOX2* promoter fused to the firefly luciferase (*LUC*) reporter

gene, after MeJA treatment were isolated (Jensen et al., 2002). After MeJA treatment, *joe1* also exhibited increased anthocyanin accumulation, whilst *joe2* showed resistance to root growth inhibition (Jensen et al., 2002).

These abovementioned signalling mutants reinforce the role of JA as initially seen from JA accumulation during various developmental stages and stress responses. The difference between responses affected in these mutants can help indicate how the wild type gene products act in the JA signalling pathway. For example, the well-characterised JA insensitive mutant, *coi1*, is affected in all JA responses, whilst *jin1* is only affected in root growth and the induction of the wound and JA responsive gene, *AtVSP* (Figure 1.3) (Feys et al., 1994, Benedetti et al., 1995; Berger et al., 1996). *coi1* is severely affected in response to wounding and pathogen attack, thus playing an instrumental role in unravelling JA regulation of these responses. Upon mechanical wounding, *coi1* fails to induce *AtVSP* and two other wound-inducible transcripts *JA responsive (JR) 1* and *2* (Titarenko et al., 1997). Moreover, *coi1* mutants were also extremely sensitive to chewing insects (Xie et al., 1998). These results indicate that COI1 is required for the wound response. Similar to the jasmonate biosynthetic mutants, the jasmonate insensitive mutants are highly susceptible to a range of pathogens, especially necrotrophic fungi. *jar1* is susceptible to *P. irregulare* (Staswick et al., 1998) and *coi1* is susceptible to *P. mastophorum*, *A. brassisicola*, *Botrytis cinerea* and the gram-negative bacterium, *Erwinia carotovora* (Penninckx et al., 1996; Thomma et al., 1998; Vijayan et al., 1998; Thomma et al., 1999; Norman-Setterblad, 2000; Stintzi et al., 2001; Zimmerli et al., 2001). The *coi1* mutant is also susceptible to virulent pathogens such as *Erisyphe cichoracearum* and cauliflower mosaic virus (Kloek et al., 2001). *coi1*, however, is resistant to *P. syringae* pv *tomato* and *P. syringae maculicola* (Kloek et al., 2001) and to the biotrophic fungus *Peronospora parasitica* (Thomma et al., 1998), suggesting that resistance to these pathogens is independent of COI1. Furthermore, this also indicates that JA signalling is not only involved in the resistance against these mentioned pathogens, but that signalling is pathogen specific. Another pathogen elicited response, the expression of *PDF1.2* is not induced in *coi1* mutants upon MeJA treatment, indicating that *PDF1.2* induction is COI1 dependent (Penninckx et al., 1996; Penninckx et al., 1998). These findings indicate that the COI1 gene product is an essential component of JA signalling, placing *coi1* upstream from the other JA mutants in the JA signalling network.

Not much work has been done on most of the constitutive mutants and thus they cannot yet be positioned in the JA signalling pathway. However, the constitutive expression of *AtVSP* in *cev1* was shown to be dependent on COI1, although its stunted root phenotype was shown to be independent of COI as revealed through double mutant analysis and thus places *cev1* upstream of COI1 in the JA

University of Cape Town

signalling pathway (Ellis and Turner, 2001). Moreover, *joe1* and *joe2* were also placed upstream of COI1 since *coi1/joe1* and *coi1/joe2* double mutants lacked increased expression of *LOX2*-LUC/GUS reporters after MeJA treatment. Allelism tests still need to be performed to establish whether *cev1* and *cex1* are allelic since they have common phenotypes. However, putative positions were assigned to *cev1* and *cex1* (Fig.1.3). *cev1* and *cex1* have been implicated in negatively regulating root growth, whereas *cev1* has also been suggested to negatively regulate anthocyanin production and resistance to powdery mildew (Fig.1.3) (Ellis and Turner, 2001; Xu et al., 2001).

Regulation of JA-responsive genes

The regulation of gene expression is governed by transcription factors interacting with corresponding target *cis*-elements within promoter regions or other transcription regulators. Transcription factors that bind DNA directly are called activators or repressors. The regulatory role transcription factors play in gene expression can either be through gene activation or repression. Other transcription factors that do not bind DNA directly, but instead interact with other transcription factors to affect gene regulation are called corepressors or coactivators. Not much is known about transcriptional regulation of JA responses.

As previously mentioned, JA is capable of inducing various genes involved in processes ranging from development, metabolism to stress responses. Hence, the promoters of these genes must contain JA-responsive *cis*-elements. The core sequences of these *cis*-acting elements are palindromic DNA sequences that function as recognition sites for transcription factors. Thus far three types of *cis*- elements have been found in studied JA-responsive promoters. These are (1) G-box motifs found in the promoters of the *Arabidopsis* chalcone synthase (*CHS*) gene, the soybean *VSPB* and the potato *PIN II* genes, (2) the C-box motif found in the promoters of barley *LOX1* and the *Agrobacterium* nopaline synthase (*Nos*) and (3) the GCC-box-like motif found in the *C. roseus* strictosidine synthase (*Str*) promoter (Kim et al., 1992; Kim et al., 1993; Mason et al., 1993; Rouster et al., 1997; Hartmann et al., 1998; Menke et al., 1999). Moreover, GCC-box elements were found in the promoters of the JA-responsive *PDF1.2*, *Thi2.1* and *PR4* promoters (Zhou et al., 1997 and Manners et al., 1998). Recently, the GCC-box in *PDF1.2* was found to be crucial for its JA responsiveness (Brown et al., 2003). These DNA motifs have been analysed and found to have the following consensus sequences or derivatives thereof: (1) the G-box is a hexameric sequence consisting of the consensus sequences CACGTG, (2) the C-box motif contains the sequence TGACG, whilst the GCC-box-like motif comprises a variation of the sequence AGCCGCC (Menkens et al., 1995; Cheong et al., 1998; Riechman and Meyerowitz, 1998; Pasquali et al., 1999).

University of Cape Town

A JA- and elicitor -responsive *cis*-element was found in the well characterised *C. roseus* strictosidine synthase (*Str)* gene called JERE (jasmonate and elicitor response elements) (Menke et al., 1999). Strictosidine synthase catalyses the condensation of tryptamine and secologanin, yielding strictosidine (Memelink et al., 2001). This terpenoid indole alkaloid serves as precursor for a variety of alkaloids (Memelink et al., 2001). JERE sequences were shown to be responsive specifically to jasmonic acid and elicitors (Menke et al., 1999). This could be seen in expression studies where tetramers of JERE were fused to minimal promoters, leading to transcriptional activation upon JA and elicitor treatments (Menke et al., 1999). Mutations in JERE regions caused the *Str* promoter to be unresponsive to MeJA and elicitor treatments (Menke et al., 1999). This finding, that responses to JA and elicitors are similarly affected by mutations in JERE regions, corroborates a role for JA in elicitor–induced signal transduction (Menke et al., 1999).

University of Cape Town

Transcription factors that interact with JERE have also been identified in *C. roseus* (Menke et al., 1999; van der Fits and Memelink, 2000; van der Fits and Memelink, 2001). To date, two of these transcription factors designated octadecanoid-responsive *C. roseus* AP2-domain proteins (ORCA1 and ORCA2) have been shown to interact with JEREs (Menke et al., 1999; van der Fits and Memelink, 2000; van der Fits and Memelink, 2001). These ORCAs belong to the AP2/ERF family of transcription factors that are unique to plants (Memelink et al., 2001). The AP2/ERF transcription factors are so designated because both the Apetalla 2 (AP2) gene and ethylene response factors (ERFs) contain a similar DNA binding domain namely the AP2 DNA-binding domain (Riechmann and Meyerowitz, 1998). AP2/ERF proteins contain a single copy of the AP2 DNA binding domain and comprise a subfamily of AP2-domain proteins (Riechmann and Meyerowitz, 1998). The AP2/ERF domains for both ORCA2 and ORCA3 are located at the C-terminal regions of these proteins whereas acidic regions can be found near the N-terminals (Menke et al., 1999; van der Fits and Memelink, 2001). Additionally, ORCA3 also has a serine–rich region in the C-terminus as well as a putative bipartite nuclear localisation signal in its AP2/ERF domain (van der Fits and Memelink, 2001). These serine-rich clusters are found to be in different family members of the AP2/ERF proteins and are proposed to be involved in transcriptional activation (Riechmann and Meyerowitz, 1998; van der Fitz and Memelink, 2001). However, deletion analysis of serine residues from the ORCA3 C-terminus caused an increase in trans-activation (van der Fitz and Memelink, 2001). Three possible reasons for this finding are (1) the ORCA3 protein or mRNA is more stable, (2) the ORCA3 C-terminal could possess a repressor binding site which would lead to decreased activation and (3) these serine residues could act as phosphorylation sites and could therefore be subjected to regulation by other factors (van der Fitz and Memelink, 2001). These ORCA proteins also induce some of the same genes as well as different genes (Memelink et al., 2001; van der Fits

and Memelink, 2001). This serves as preliminary evidence that ORCA2 and ORCA3 have different functions and that both are required for JA-induced gene expression (Memelink et al., 2001; van der Fits and Memelink, 2001). These ORCAs specifically bind to the JERE and transactivate the periwinkle *Str* gene (Menke et al., 1999; van der Fits and Memelink, 2001). More importantly, MeJA induced both *Orca2* and *Orca3* gene expression rapidly and transiently (Menke et al., 1999; van der Fits and Memelink, 2001). *Orca2* expression was induced by elicitor treatment as well (Menke et al., 1999). The expression of *Orca3* upon elicitor treatment has not been tested.

The induction kinetics of these *Orca* genes after MeJA treatment was biphasic (Menke et al., 1999; van der Fits and Memelink, 2001). Biphasic gene expression is suggested to be due to transient activation of gene expression, very unstable mRNA or due to transcription factors possessing auto-regulatory function. Interestingly, *Orca3* mRNA also accumulated after treatment with the JA intermediates α-LA and OPDA (van der Fits and Memelink, 2001). However, it is unclear whether these intermediates have a direct effect on *Orca* gene expression since they can easily be converted to JA. *Orca3* is proposed to be an early JA responsive gene. This is based on the finding that MeJA induction of *Orca3* does not require *de novo* protein synthesis because the protein synthesis inhibitor, cyclohexamide, itself induced *Orca3* expression and caused superinduction combined with MeJA (van der Fits and Memelink, 2001).

The JA and ethylene signalling pathways positively regulate responses to wounding/insects and pathogen infection and are required in concert for some responses (Xu et al., 1994; O'Donnell et al., 1996; Penninckx et al., 1996; Penninckx et al., 1998; Pieterse et al., 1998; Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998; Thomma et al., 1999; Norman-Setterblad et al., 2000; Thomma et al., 2000). It was recently shown that JA and ethylene signal transduction pathways are needed for the activation of the pathogen-induced ethylene transcription factor, ERF1 (Lorenzo et al., 2003). Exogenous treatment with MeJA, ethylene or a combination of MeJA/ethylene caused ERF1 activation (Lorenzo et al., 2003). *coi1* and *etylene insensitive2* (*ein2*) were also analysed with these exogenous hormone treatments and induction of ERF1 and the JA/ethylene responsive gene chitinase B (*CHIB*) was blocked in both mutants with all hormone treatments, indicating that activation of ERF1 requires both JA and ethylene (Lorenzo et al., 2003). More importantly, complementation studies shows that overexpression of ERF1 can rescue both *coi1* and *ein2* phenotypes associated with pathogen defence but not responses associated with development, indicating that ERF1 mediates responses downstream of *coi1* and *ein2* (Solano et al., 1998; Lorenzo et al., 2003). Another ERF, *AtERF2* was also found to be responsive to JA and pathogens (Brown et

University of Cape Town

al., 2003). Overexpression of *AtERF2* in *Arabidopsis* led to the expression of *PDF1.2*, *Thi2.1* and *PR4* (Brown et al., 2003). Interestingly, these are all GCC-box containing genes (Zhou et al., 1997 and Manners et al., 1998). Therefore, it can be speculated that the effects of this ERF on JA and pathogen responses could be mediated via interaction with the GCC-box *cis*-element (Brown et al., 2003).

Identified transcription factors acting downstream in the JA and ethylene signal transduction cascade could not only be used to unravel the molecular events occurring upon activation of defence and other stress responses, but they could also be utilised to dissect how different signalling pathways communicate with each other on a molecular level to regulate these responses. Recently, gene expression profiles revealed the activation or repression of transcription factors in *Arabidopsis* during different developmental stages and upon different stress treatments (Chen et al., 2002). A total of 402 genes were examined and 72 of these genes, responding to bacterial pathogens, were either reduced in expression or not expressed in *coi1*, *ein2* and *nonexpression of PR1* (*npr1*), indicating that JA, ethylene and SA are involved in the regulation of these 72 genes (Chen et al., 2002). Not only was cross communication between signalling pathways revealed by this work, but also cross talk between different stress responses was identified (Chen et al., 2002). Senescence induced the expression of 43 transcription factors and 28 of these genes were also induced by other stress treatments. Moreover, a highly conserved motif was found in promoters of genes responding to a variety of pathogens (Chen et al., 2002).

University of Cape Town

JA responses are post-translationally regulated via the ubiquitin protein degradation pathway

The wild type gene encoded by *coi1* has been cloned (Xie et al., 1998). The deduced amino acid sequence of *COI1* contains two conserved motifs: an F-box and leucine rich repeats (LRR) postulated to be involved in protein-protein interactions (Kobe and Deisenhofer, 1994; Xie et al., 1998). Homology searches revealed that COI1 was related to previously isolated F-Box proteins: (1) the *Arabidopsis* TIR1 involved in auxin signalling and (2) the cell cycle regulatory proteins, human Skp2 and the yeast Grr1. TIR1, Skp2 and Grr1 are all involved in recruiting proteins for degradation via the ubiquitin protein degradation pathway (Xie et al., 1998). Xie et al. (1998) postulated that COI1 is an F-box protein that targets negative regulators of pollen development and defence responses for modification by ubiquitin. Ubiquitin is transferred to target proteins in three enzymatic steps before the target protein is degraded by the 26S proteosome: an E1 enzyme which activates ubiquitin, an E2 conjugating and E3 ligase enzymes which together conjugates the target protein to ubiquitin (Fig.1.4) (Skowyra et al., 1997). Recently, COI1 interacting proteins were

identified in a yeast-two-hybrid screen and *in planta* association of these components was demonstrated through coimmunoprecipitation (Xu et al., 2002). These interacting components were identified as: ASK1 and ASK2 (*Arabidopsis* Skp1- like proteins), AtRbx1 (an *Arabidopsis* RING

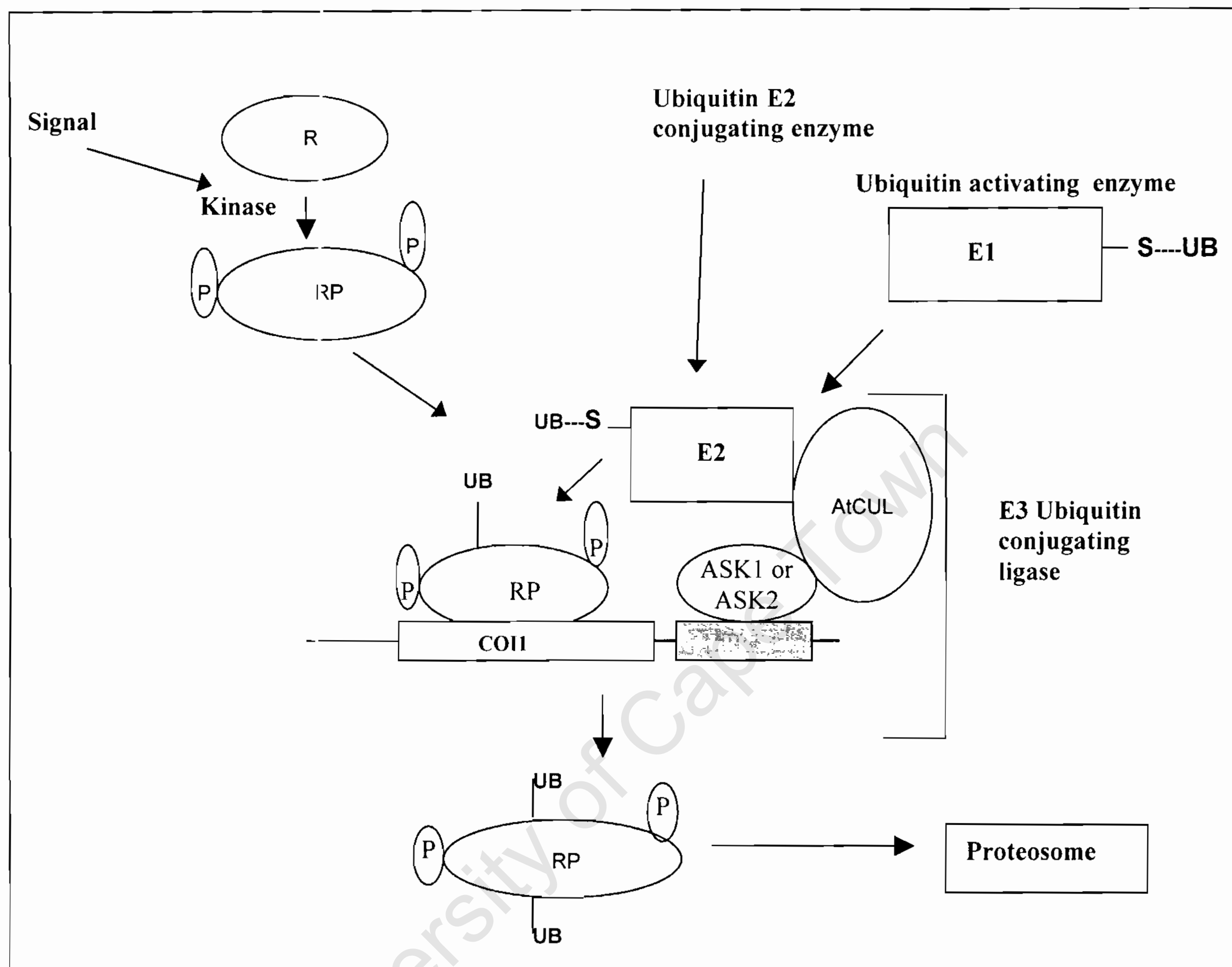


Figure 1.4: Model of putative COI1 function in ubiquitin modulated protein degradation. Abbreviations: AtCUL *Arabidopsis* Cullin, ASK1/ASK2 *Arabidopsis* Skp1-like proteins, COI1 wild type gene product of *coi1*, E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme, E3 ubiquitin conjugating ligase, LRR leucine rich repeat, RP repressor protein, UB ubiquitin.

University of Cape Town

finger protein) and AtCUL1 (*Arabidopsis* Cullin), demonstrating that an intact SCF^{COI1} complex can be formed *in planta* (Fig.1.4) (Xu et al., 2002). Upon perception of an internal or external signal, it is speculated that a kinase is activated catalysing the phosphorylation of the regulatory protein (RP) (Fig. 1.4). The involvement of other phosphorylation events in JA signalling has been reported previously (Rojo et al., 1998). A protein kinase and phosphatase has been identified in the JA-regulated wound response (Rojo et al., 1998). The serine-threonine kinase inhibitor, staurosporine, could induce the same genes as JA (Rojo et al., 1998). The serine-threonine phosphataseI inhibitor, okadaic acid, inhibited JA induction of *JR1*, *JR2*, *JR3 VSP*, *HPL* and *CHS* (Rojo et al., 1998). These results indicate that a protein kinase negatively regulates JA signalling whereas a protein phosphatase positively influences JA-regulated gene expression. The

phosphorylated RP then binds to COI1. Following this, E1 transfers an ubiquitin molecule to E2. The SCF complex then acts as an ubiquitin ligase and transfers the ubiquitin to the RP. The polyubiquitinated RP is then degraded in the proteosome (Xu et al., 2002). The SCF complex comprising of these components was designated SCF^{COI1} (Xu et al., 2002). SCF complexes have been identified in mammals (SCF^{SKP2}) (Michel and Xiong, 1998; Lisztwan et al., 1998; Lyapina et al., 1998; Yu et al., 1998), yeast (SCF^{GRR1}) (Bai et al., 1996; Skowyra et al., 1997; Skowyra et al., 1999) and in plants (SCF^{TIR}) (reviewed in Dharmasiri and Estelle, 2002; Gray et al., 1999; Gray et al., 2001). It was also found that COI1 forms two separate complexes with ASK1 and ASK2, however the functional significance of this is not clear (Xu et al., 2002). More importantly, the disruption of any SCF^{COI1} subunits was found to abolish JA responses, not only indicating *in planta* functionality but also the importance of this complex to the regulation of JAs signalling. One other interesting result was the discovery of AtCUL1 modification through AXR1 (the locus encoded by the auxin insensitive mutant *axr1*) and the importance of this modification to the SCF^{COI1} complex (Xu et al., 2002). The auxin insensitive mutant, *axr1*, was found to be allelic to a MeJA insensitive mutant *axr1-24*, again confirming the involvement of *axr1* in JAs responses (Tiryaki and Staswick, 2002).

University of Cape Town

Cross talk with other signalling pathways

JA regulates plant defence responses to insects (McConn and Browse, 1996; Xie et al., 1998; Stintzi et al., 2001) and pathogens (Xu et al., 1994; Penninckx et al., 1996; Penninckx et al., 1998; Pieterse et al., 1998; Staswick et al., 1998; Thomma et al., 1998, Vijayan et al., 1998; Thomma et al., 1999; Norman-Setterblad et al., 2000; Thomma et al., 2000). Upon pathogen attack levels of JA increase, leading to the expression of specific defence-related genes and then local and systemic resistance (Gundlach et al., 1992; Mueller et al., 1993; Blechert et al., 1995; Penninckx et al., 1996; Menke et al., 1999). More specifically, JA is implicated in resistance to insects and especially necrotrophic pathogens (Penninckx et al., 1998; Staswick et al., 1998; Thomma et al., 1998, Vijayan et al., 1998; Feys and Parker, 2000; Norman-Setterblad et al., 2000; Stintzi et al., 2001). In addition to a role in pathogen defence, JA is well known for its roles in regulating insect- induced wound responses (McConn and Browse, 1996; Stintzi et al., 2001).

Plant defence response regulation is proving to be very complex. The complexity of signalling networks regulating plant responses to pathogens and insects is exacerbated by cross communication between different signal transduction cascades. The nature of the relationship

between these different signalling pathways, whether antagonistic or synergistic, in conjunction with the type of pathogen/insect, govern which specific defence response is launched by the plant.

The signalling relationship between JA and ethylene was found to be synergistic (Fig. 1.5) (Penninckx et al., 1996; Norman-Setterblad, 2000). Both ethylene and JA are required for the induction of the antifungal peptide *PDF1.2* upon infection with *A. brassicicola* and *E. carotovora* in *Arabidopsis* (Fig.1.5) (Penninckx et al., 1996; Norman-Setterblad et al., 2000). *PDF1.2* and PR proteins are typically expressed after pathogen infection in the JA and SA defence pathways respectively and can thus be seen as markers of resistance against pathogens. These markers can be used to follow the progress of a resistance response. One other antifungal peptide that can be seen as an indication of JA-mediated defence responses is *THI2.1*, which is specific for JA and does not require ethylene (Epple et al., 1995). Ethylene is also needed for the induction of other JA-inducible genes such as hevein-like protein (HEL) and chitinase-B (*CHIB)* against *E. carotovora* in tobacco (Norman-Setterblad, 2000). When MeJA and ethylene are added in combination exogenously, increased expression of *Pdf1.2*, *HEL*, *PR1b* and *PR5* (thaumatin) is observed in *Arabidopsis* (Penninckx et al., 1998; Norman-Setterblad et al., 2000) and tobacco (Xu et al., 1994) compared to MeJA and ethylene treatment alone.

In addition to helping elucidate the role JA plays in plant responses, mutant analysis also revealed that cross talk between signalling networks exist. As with the JA-signalling mutants, the ethylene insensitive mutant (*ein2*) is highly susceptible to *B. cinerea* and *E. carotovora* (Thomma et al., 1999; Norman-Setterblad et al., 2000). Moreover, just like *coi1*, *ein2* is resistant to the virulent bacterial pathogens *P. syringae* and *X. campestris* (Bent et al., 1992). Also, in tobacco the expression of JA-responsive genes *PDF1.2*, *Thi2.1*, *HEL* and *CHIB* requires *EIN2* (Xu et al., 1994). To add to the synergistic relationship between JA and ethylene, the establishment of induced systemic resistance (ISR) against *P. fluorescens*, requires both JA and ethylene (Pieterse and van Loon, 1999). Most of the reported interactions between JA and ethylene have been synergistic. However, an antagonistic relationship between JA and ethylene has been reported in ozone-induced cell death (Overmeyer et al., 2000). In the *Arabidopsis* ozone-sensitive mutant *rcd1* (*radical-induced cell death1*), ethylene is required for the lesion propagation stage of cell death whilst JA is required for the lesion containment stage (Overmeyer et al., 2000).

The relationship between the JA and SA defence signalling pathways has been found to be antagonistic. Systemic acquired resistance (SAR) is a SA-dependent defence response activated upon pathogen infection. Treatment of wild type plants with SA and MeJA induced the expression

University of Cape Town

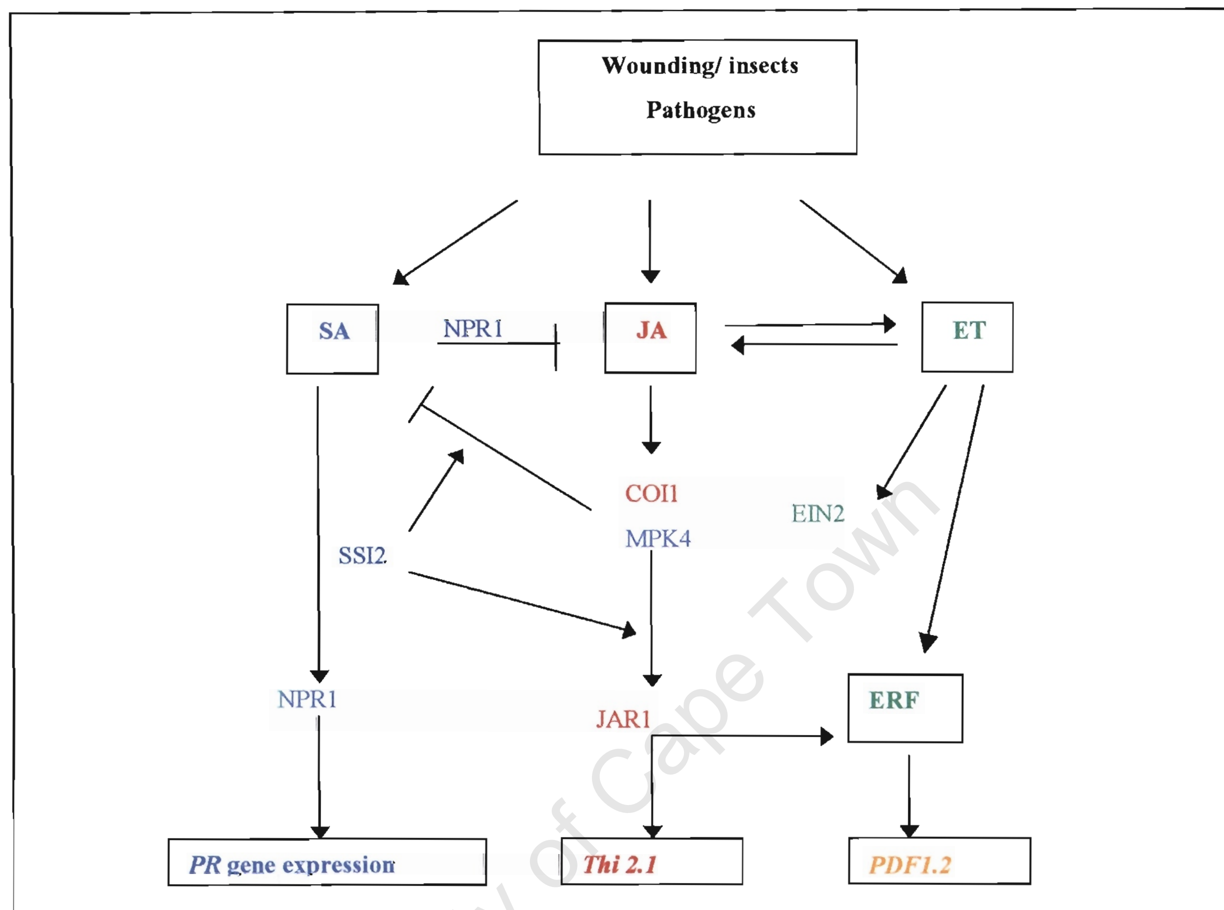


Figure 1.5: Cross talk between the JA signalling pathway and the ethylene and salicylic acid signalling pathway. Abbreviations: EIN2 ethylene insensitive 2, ERF ethylene response factor, ET ethylene, JA jasmonic acid, JAR1 JA responsive 1, SA salicylic acid, MPK4 map kinase 4, NPR1 non-expression of PR1, PDF1.2 plant defensin 1.2, PR proteins pathogenesis related proteins, SSI suppressor of SA insensitivity 2, *Thi2.1* thionin 2.1. SA signalling mutants and responsive genes are indicated in blue, JA signalling mutants and responsive genes are indicated in red whilst ethylene mutants and responsive genes are indicated in green, with exception of *PDF1.2* which is regulated by both JA and ethylene, indicated in orange. Positive regulation is indicated with and negative regulation is indicated with

University of Cape Town

of *PR1* and the JA-responsive genes: *LOX2*, *VSP* and *Pdf1.2*, respectively (Spoel et al., 2003). However, when wild type plants were treated with a combination of SA and MeJA, PR1 genes were still expressed whilst SA suppressed the expression of the JA-responsive genes, verifying the negative relationship between SA and JA signalling pathways (Spoel et al., 2003). The SA signalling mutant, *npr1* (also known as *non-inducible immunity1*; *nim1* or *salicylic acid insensitive1*; *sai1*) (Cao et al., 1994; Delaney et al., 1994; Shah et al., 1997), does not launch SAR and its accompanied expression of *PR* genes, nor does it accumulate SA after pathogen infection (O'Donnell et al., 1996). Whilst treatment of *npr1* mutants with MeJA lead to the expression of *LOX2*, *VSP* and *PDF1.2*, combined SA and MeJA treatment did not cause inhibition of JA-

responsive genes (Spoel et al., 2003). This result confirms that SA is needed to suppress JA-inducible gene expression. In addition to this, one other systemic defence response, ISR, is governed by JA and ethylene, but interestingly also requires NPR1 (Pieterse et al., 1998). NPR1 is suggested to be a modular switch used by the SAR and ISR defence pathways in response to different pathogens by selectively recruiting regulators of the different signalling pathways (Spoel et al., 2003). Thus NPR1 can function in a SA-dependent as well as SA-independent defence responses. This role could be fulfilled through protein-protein interactions via the ankyrin repeat found in the NPR1 sequence (Cao et al., 1997; Ryals et al., 1997). Further, after pathogen infection, NPR1 is transported to the nucleus where it interacts with transcription factors of the basic leucine zipper subclass, thus activating the SA-dependent PR genes (Kinkema et al., 2000). Recently, it was found that NPR1 antagonistically regulates SA-independent but JA-dependent defence signalling in the cytosol, instead of the nucleus (Spoel et al., 2003). Moreover, internal SA suppressed JA biosynthesis, possibly by inhibiting the expression of LOX2 by SA (Spoel et al., 2003).

Even though the relationship between JA and SA is mostly antagonistic, some synergism between the two pathways has also been reported. The SA-inducible PR protein, PR1b, was significantly induced by SA but combined treatment of SA and MeJA had a greater effect on PR1 expression (Xu et al., 1994). Furthermore, activation of both the SA-and JA-dependent defence pathways heightened ISR, adding to the suggestion of a synergistic relationship between JA and SA (van Wees et al., 2000).

A constitutive SA signalling mutant, *suppressor of SA insensitivity 2* (*ssi2*) exhibits constitutive PR gene expression, has elevated SA levels and is resistant to *Pseudomonas* infection (Shah et al., 1999). More interestingly, *ssi2* constitutively expresses the JA/ethylene-dependent *PDF1.2* gene (Shah et al., 1999). Furthermore, *ssi2* suppresses the *npr1* mutation including *PDF 1.2* expression, indicating that *ssi2* acts independently of *npr1* in expression of PR genes and SAR and that *PDF1.2* induction requires SA (Shah et al., 1999). However, this is not the only report in which the JA-dependent *PDF1.2* and SA-dependent *PR* genes are induced concomitantly. The *cpr5*, *cpr6* and *cir1* (*constitutively induced resistance*) express both *PDF1.2* and PR genes upon (Bowling et al., 1997; Clarke et al., 1998; Murray et al., 2002).

Microarray analysis of gene expression is a powerful tool. Recently, cross communication between JA, ethylene and SA has been identified in such gene expression studies in response to MeJA treatment, upon pathogen attack, wounding and insect feeding (Schenk et al., 2000 and Sasaki et al., 2001). In a cDNA microarray analysis concomitantly investigating the expression profiles of 2 375

University of Cape Town

Arabidopsis genes upon treatment with pathogen or defence-related hormones, expression profiles of 705 known genes were changed (Schenk et al., 2000). Expression of 168 genes increased upon pathogen infection whereas 39 were reduced. Interestingly, upon JA treatment, expression patterns of 221 genes were increased compared to the 192 and 55 genes induced by treatment with SA and ethylene, respectively (Schenk et al., 2000). Moreover, a large number of genes were co-regulated by SA and MeJA and 50% of ethylene-induced genes, were also induced by MeJA. This indicates that different signalling networks work in concert to regulate plant defence responses. Upon treatment of *Arabidopsis* plants with MeJA, 41 JA-responsive genes with a fold induction greater than 3 were identified out of 2880 cDNA screened (Sasaki et al., 2001). Of these 41 JA-responsive genes upregulated by MeJA, a small number of genes were involved in other pathways, including the SA and ethylene signalling pathways (Sasaki et al., 2001). Although these microarray results differ, they suggest that JA and SA might co-regulate a group of genes.

University of Cape Town

Concluding remarks

JA has diverse functions in plants ranging from development to responses to environmental stress factors including wounding and pathogen attack (reviewed in Sembdner and Parthier, 1993; Creelman and Mullet, 1995). In *Arabidopsis* especially, JA is involved in regulating developmental processes such as vegetative storage, senescence, production of viable pollen and root growth (Staswick et al., 1992; Feys et al., 1994; Benedetti et al., 1995; Berger et al., 1996; McConn and Browse, 1996; Xie et al., 1998; Richmond and Bleecker, 1999; Sanders et al., 2000; Stintzi and Browse 2000; Ellis and Turner, 2001; Ishiguro et al., 2001; Xu et al., 2001; He et al., 2002, von Malek et al., 2002). These functions of JA have been elucidated through chemical, physiological and genetic studies, including the use of mutants defective in JA biosynthesis. JA also plays an integral role in regulating responses to abiotic stress like ozone damage and mechanical wounding as well as biotic stress such as defence against pathogens and wounding caused by insects (Farmer and Ryan, 1990; Farmer and Ryan, 1992; Sharma and Davis, 1994; Berger et al., 1996; McConn and Browse, 1996; Staswick et al., 1998; Vijayan et al., 1998; Xie et al., 1998; Overmeyer et al., 2000; Rao et al., 2000; Stintzi et al., 2000; Seo et al., 2001; Stintzi et al., 2001; Park et al., 2002 and Stotz et al., 2002). The JA-dependent systemic resistance pathway is specifically implicated in the modulation of defence responses against necrotrophic fungi such as *B. cinerea* (Penninckx et al., 1996; Penninckx et al., 1998; Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998; Thomma et al., 1999; Thomma et al., 2000; Kloek et al., 2001).

Upon these developmental and stress stimuli, JA levels increase and this increase coincides with increased expression of JA-responsive genes, the effectors of JA-regulated responses. JA is derived

from α-LA via the octadecanoic pathway. Genes encoding JA biosynthetic enzymes are constitutively active, responsible for maintaining basal levels of JA in plants. However, the enzymes catalysing JA synthesis can be induced upon developmental cues and stress agents such as wounding/insects and pathogens and transcription of these genes occur at the site of JA biosynthesis (Turner et al., 2002). This indicates that JA can be synthesised locally in response to these external and internal factors. JA biosynthetic enzymes are differentially located in different organelles and tissues, indicating a possible temporal and spatial differentiating regulation mechanism of JA biosynthesis. Moreover, feedback regulation of JA biosynthesis has been specifically suggested since jasmonates themselves are able to induce JA biosynthetic enzymes. AOS has been identified as a major regulating point of JA biosynthesis in potato and *Arabidopsis* (Harms et al., 1995; Laudert and Weiler, 1998). The AOS enzyme is needed for the wound-induced increase in JA levels as well as the induction of *AtLOX2* as demonstrated by transgenic potato plants overexpressing AOS (Harms et al., 1995). Interestingly, JA intermediates such as OPDA are biologically active, adding to the complexity of JA signalling (Farmer and Ryan, 1992; Weber et al., 1997; Laudert and Weiler, 1998; Sivasankar et al., 2000; Stintzi et al., 2001). In addition, the *JAR1* locus encodes an *Arabidopsis* homologue of the firefly luciferase adenylating family, which possibly modifies JA through adenylation to control JA activity (Staswick et al., 2002). However, some questions pertaining to JA biosynthesis still remain. The most important of which is how do jasmonates move in and between cells and tissues to reach their sites of action. Another important question is what are the *in vivo* concentrations of jasmonates needed to elicit JA-regulated responses, since the concentration used in studies were at non-physiological concentrations (Creelman and Mullet, 1995). Answers to these questions could better our understanding of the mode of jasmonate action.

University of Cape Town

After perception of the JA signalling molecule and transduction of the signal from the cell surface to the nucleus, transcription factors recognising specific *cis*-acting elements are activated or repressed to accordingly regulate gene expression governed by JA. In regard to signalling events occurring after JA perception, signalling mutants impaired in sensing JA as well as downstream responses are not well understood. The *coi1* mutant is disrupted in F-box containing protein involved in the ubiquitin protein degradation pathway through which repressors of development and stress responses can be removed (Xie et al, 1998; Xu et al., 2002). Thus far, only ORCAs have been identified as JA responsive transcription factors (Menke et al., 1999; van der Fits and Memelink, 2000; van der Fits and Memelink, 2001). Moreover, only three *cis*-acting elements have been identified in promoters of JA-responsive genes (Kim et al., 1992; Kim et al., 1993; Mason et al., 1993; Rouster et al., 1997; Zhou et al., 1997; Hartman et al., 1998; Manners et al., 1998; Menke et al., 1999; Brown et al., 2003).

The complex JA signal transduction pathway is slowly being elucidated. However, many questions still remain unanswered. The precise mechanism of how JA regulates these responses is an ongoing investigation. An even more pressing question is whether there is a JA receptor/s in plants since extensive investigations have failed to deliver putative JA receptor/s. Also, how these receptor/s would distinguish between the different biologically active jasmonates is an interesting question. In this regard, an interesting hypothesis involving jasmonates acting as molecular sensors have been proposed by Weber et al. (1997). The identification of more *cis*-elements in JA-responsive genes and transcription factors recognising these sequences as well as other interacting factors is crucial to the unravelling of JA signalling. Answers to these questions can be obtained through the identification of more JA signalling mutants. In addition, since cross communication is a vital part of JA signalling and the regulation of JA-responses, it is often difficult to distinguish whether the phenotypes exhibited by JA mutants are solely due to disruption of JA signalling or an affect caused by a defect in another signalling pathway. The analysis of double, even triple mutants affected in more that one pathway would help provide answers to the affect of other signalling pathways on JA signalling. In addition to this, transgenic approaches manipulating the biosynthetic pathways of JA, ethylene and SA would also make an invaluable contribution to see how these interacting pathways affect one another. The contribution of gene expression studies to our understanding of how gene expression is affected during JA signalling cannot go unmentioned. Microarray analysis is a powerful technique, which could be used to identify candidate genes playing an important role in JA signalling. The elucidation of these various aspects of the JA signalling pathway could eventually be applied to agricultural use, improving crop yields through manipulation of this pathway.

University of Cape Town

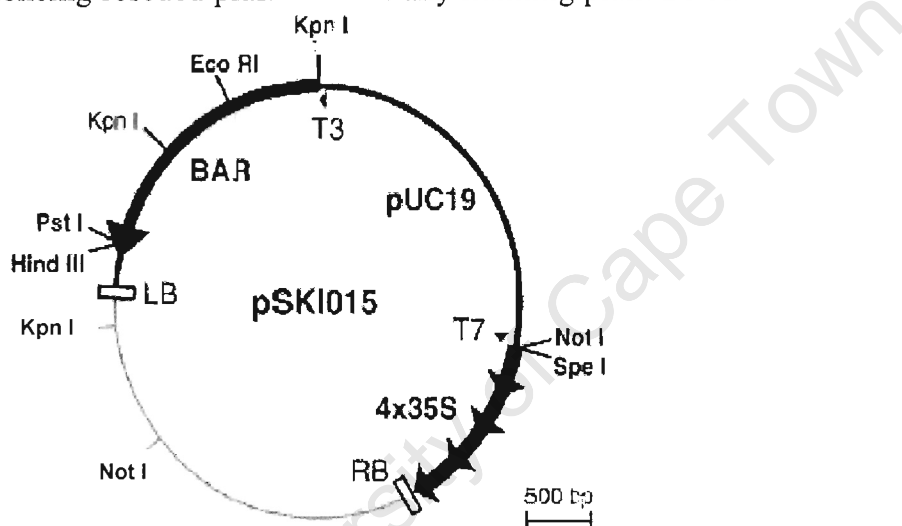
Chapter 2

Materials and Methods

University of Cape Town

Seed stock used

The T-DNA transformed seed pools used in this study were obtained from the Arabidopsis Biological Resource Centre (ABRC-www.arabidopsis.org), stock number: CS21995. The map of the engineered T-DNA binary vector, pSKI015, used to transform Col-0 seed (Weigel et al., 2000) is given in Figure 2.1. pSKI015 contains the tetramerised CaMV 35S enhancer, the glufosinate resistance gene, *bar,* pUC19 sequences including an *ori*V bacterial origin of replication and ampicillin resistance gene and T-DNA left and right borders containing specific restriction sites. The latter two features of pSKI015 could be used for plasmid rescue of T-DNA and flanking plant sequences from transformed plants. pSKI015 has been engineered to contain specific primer sequences as well as the T3 and T7 universal primers for sequencing rescued plasmids and any flanking plant DNA.



University of Cape Town

Figure 2.1: Activation tagging vector pSKI015 (figure taken from Weigel et al., 2000).

coi1-1, *jar1*, *jin1* and *jin4* seeds used in this study were kindly provided by Drs John Turner, John Browse and Suzanne Berger respectively.

Plant growth

Axenic growth conditions

Seeds were surface sterilised by initially shaking in 1 ml of 70% (v/v) ethanol for 7 min. The ethanol was removed and seeds were shaken in 1 ml of 10% bleach/ 0.02% (v/v) Triton X-100 solution, shaking for 15 min. After removing the 10 % bleach/ 0.02% (v/v) Triton X-100 solution, seeds were washed 3 times with sterile water, resuspended in 1 ml of 0.1% (w/v) sterile agar and stratified for 1 week at 4 °C before plating. All seedlings were grown on 25 ml of sterile plant nutrient (PN) medium (Haughn and Somerville, 1986) for 2 weeks under

axenic conditions. Plants were grown at a light intensity of 50 –52 μmoles photons.m^{-2}. s^{-1} at 22 °C under continuous light.

Plant growth in soil

For growing in soil or jiffy- 7 discs (AS Jiffy products Ltd., Norway) seeds were resuspended in 0.1% (w/v) agar and stratified at 4 °C for 1 week before sowing. Soil (Cornell mix, Landry et al., 1995) was placed in plant pots, watered 3 times and left to stand overnight for optimal hydration. Jiffy-7 discs were re-hydrated with water for up to 3 hr to ensure complete rehydration. After seeds were sown, pots and jiffy discs were covered with cling wrap for one week to promote germination after which the covers were removed. Seedlings were treated with Kompel fertiliser (Starke Ayres, South Africa) and treated with VectoBac 12 AS (Abbott Laboratories, South Africa (Pty) Ltd.) one week thereafter and subsequently every fortnight. Plants were grown under light intensity of 80-100 μmoles photons. m^{-2}.s^{-1}, humidity of 50% and light cycle of 16 hr light and 8 hr dark at 24 °C.

Chemical treatments of plants

Liquid treatments

MeJA (Sigma) stock solutions were dissolved in absolute ethanol to the appropriate concentrations. Stock concentrations were diluted with sterile Mili-Q water or added to either PN medium (Haughn and Somerville, 1986) or melted petroleum jelly to obtain working concentrations. Axenically grown seedlings were treated with 2.5 ml of 450μM MeJA/0.1% (v/v) solution to a final concentration of 45μM/ 0.01% (v/v) ethanol (1:10 final dilution). As control treatment, seedlings were treated with 0.01% (v/v) ethanol. Samples were harvested into liquid nitrogen and stored at – 70 °C until subsequent analysis.

Gaseous MeJA treatment

To treat 4-week-old plants with MeJA exogenously, 1 ml of 5 and 15 mM MeJA stock solutions were added to 18 ml (equivalent weight of 15 g) of melted unscented petroleum jelly (Johnson and Johnson, South Africa), respectively. As a control treatment, 1 ml of ethanol was added to 18 ml of melted petroleum jelly. Prior to adding the MeJA stock solutions or ethanol, melted petroleum jelly was cooled down to 50 °C. These suspensions were applied to the corners and mid sections of propagation tray lids to ensure even distribution of MeJA or ethanol vapours. Lids were immediately placed on the trays housing plants on jiffy-7 discs, to minimise evaporation of MeJA or ethanol. Samples were harvested into liquid nitrogen and stored at – 70 °C until subsequent analysis.

University of Cape Town

BASTA treatment

Soil grown plants were sprayed with 10 mg/ml of a BASTA derivative called Challenge (Aventis). Plants were assessed 1 week after treatment. Axenically grown seedlings were propagated on PN plates containing 5 μg/ml BASTA for one week before subsequent analysis.

Root growth analysis

For root growth analysis, MeJA stock concentrations 30 mM, 45 mM and 100 mM were diluted 1:1000 by direct addition to the PN medium, thus yielding final concentrations of 30 μM, 45 μM and 100 μM in 0.1% (v/v) ethanol. Seeds were germinated on 25 ml of MeJA containing PN medium and grown in an upright position for 1 week to allow gravitrophic growth of roots. Root lengths were measured in millimetres.

Selection of homozygous coi1-1 mutants

Selection of homozygous *coi1-1* mutants was performed on PN plates containing 30 μM MeJA/0.1% (v/v) ethanol and grown in an upright position.

University of Cape Town

Manual cross pollinations

In the case of Col-0 x *jam2*, Col-0 was emasculated and pollinated with *jam2* pollen 2 days thereafter whereas *coi1-1* was used as the female parent for the *coi1-1* x *jam2* cross.

Anthocyanin extraction

Approximately 100 mg of tissue per sample was ground in 300 μl of acidic (1% HCl, w/v) methanol and left shaking at 30 °C overnight (method adapted from Rabino and Mancinelli, 1986; Feinbaum and Ausubel, 1988 and Kubasek et al., 1992). Tissue debris was spun down in a bench top centrifuge for 2 min and removed. Pigments were extracted twice in 300 μl chloroform and 600 μl of water was added to each methanol phase. After the addition of water, a white precipitate forms which was pelleted using a bench top centrifuge. The absorbance (A) of the clarified extracts was then measured at 537 and 657 nm. To compensate for the presence of possible chlorophyll and its degradation products, A_{657} was subtracted from A_{537}.

Infection with *Botrytis cinerea*

The *B. cinerea* strain used in this study was isolated from *Capsicum annuum* (courtesy of Dr. G.J. Loake, University of Edinburgh, Scotland). *B. cinerea* spores were sub-cultured at 28 °C for 2 weeks on apricots (Natural Lite, South Africa) to maintain virulence. Spores were harvested by adding 5-10 ml of sterile Mili-Q water to plates then gently scraping off spores with a sterile glass rod. To remove mycelia, the spore suspension was passed through a

syringe plugged with sterile glasswool. The spore suspension was placed in sterile SS34 centrifuge tubes and spun at 2000 x g for 5 min to pellet spores. After removing the water fraction, spores were resuspended in 200 μl of 2.4% (w/v) potato dextrose broth (Sigma). To determine the spore concentration, a 1:100 dilution was prepared in potato dextrose broth and 5 μL of this spore suspension was placed on a haemocytometer (Neubauer) and counted using light microscope (Zeiss Standard RA Microscope, West Germany) (1/10 magnification).

Leaves of 4-week-old plants were inoculated with a spore suspension of $1x10^3$ spores/ ml 2.4% (w/v) potato dextrose broth (Sigma). Leave tissue was harvested into liquid nitrogen and stored at – 70 °C until further analysis.

For the detached leaf assay, leaves were placed on plates containing 25 ml of sterile 0.8% (w/v) agar and a 5 μl droplet of a $1x10^3$ spore/ml suspension was placed along the mid-vein of leaves. Leaves were assessed for lesion development 4 and 5 days post infection. Lesion size was measured in millimetres.

University of Cape Town

RNA Analysis

RNA extraction, gel electrophoresis and transfer onto nitrocellulose membrane

Total RNA was extracted from 100-200 mg of leaf tissue according to the method of Chomczynski and Sacchi (1987). Tissue was homogenised in liquid nitrogen using a mortar and pestle. Homogenised tissue was placed in microcentrifuge tubes containing 500 μl of Solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 70 μl β-mercaptoethanol) prior to adding 500 μl of water-saturated phenol (pH 4), 50 μl of 3 M sodium acetate (pH 4) and 150 μl of chloroform:isoamylalcohol (49:1). Samples were incubated on ice for 15 min before centrifugation at 9700 x g for 20 min at 4 °C. Supernatant fractions were removed and placed in clean microcentrifuge tubes before addition of 500 μl of isopropanol. Samples were incubated on ice for 10 min before centrifugation at 9700 x g for 20 min at 4 °C. After removal of the supernatant fraction, RNA pellets were washed with 500μl of 75% (v/v) ethanol and centrifuged at 9700 x g for 10 min at 4 °C. Supernatant fractions were removed and pellets were air dried for 5 min before resuspension into 70 μl of 0.1% (v/v) DEPC treated water.

Equal volumes of RNA sample application buffer (300 μl of 10x MOPS (0.2 M MOPS, 0.05 M sodium acetate. 0.001 M EDTA), 80 μl of 37% formaldehyde, 900 μl of deionised formamide, 2 μl of 10mg/ml ethidium bromide) were added to 10-20 μg of total RNA and electrophoresed

on a 1% (w/v) agarose/formaldehyde gel in 1x MOPS buffer. Gels were visualised and recorded on a GeneGenius Bioimaging system (Syngene).

Electrophoresed RNA samples were reverse transferred to Hybond N^+ nitrocellulose membrane (Amersham) according to the method of Ingelbrecht et al. (1998) for 5-6 hr. After transfer, RNA was crosslinked to the nitrocellulose membrane using a UV crosslinker (Amersham, Life Sciences, RPN 2500/2501) at a UV exposure setting of 70 000 μjoules.cm^{-2} at 254 nm short wave and washed in 2x SSC to lower salt concentrations. To visualise transferred total RNA samples, membranes were stained with methylene blue and photographed under visual light using a GeneGenius Bioimaging system (Biorad).

RNA membrane hybridisation

RNA nitrocellulose membranes were prehybridised for 4 hr and hybridised for 16 hr using the Denhardt's method in Sambrook et al. (1989).

Radiolabelling of the PDF1.2 probe

^{32} P-labelled PDF1.2 (100ng) probes were prepared using the Megaprime DNA labelling system (Amersham) according to manufacturer's instructions. A short Pasteur pipette was used as a column to remove unincorporated nucleotides via gravity flow. The column was prepared by creating a plug of sterile glass wool at the narrow end and packed with Sephadex G-50 in 1x STE (3% (w/v) NaCl, 500 mM EDTA, 5 M Tris(pH8)) until the column bed was 1.5 cm away from the top of the pipette. The column was equilibrated with 1x STE.

To follow the progress of the labelled probe through the column, 8 μl of dye (3% (w/v) Dextran Blue, 1% (w/v) Orange G in 15mM NaCl) was added to the probe mixture. A total volume of 63μl of the radioactive probe mixture was applied to the column and fractions of 4 drops were collected in microcentrifuge tubes. Using a Geiger counter, the fractions with the highest counts per second (cps) were identified after which these fractions were pooled. To determine the % incorporation, 2μl aliquots were taken before and after the removal of unincorporated nucleotides.

Prehybridisation and Hybridisation of RNA membranes

RNA membranes were prehybridised (6 x SSPE/ 0.25 x Denhardt's solution (2% (w/v) Ficoll/2% (w/v) Polyvinylpyrroldine/ 2% (w/v) BSA)/ 0.05% (v/v) SDS/ 80 μg/μl denatured sheared salmon sperm) for 4 hr at 65 °C. PDF 1.2 probes were denatured at 65 °C, added to the hybridisation buffer (6 x SSPE/ 0.25 x Denhardt's solution/ 0.05% (v/v) SDS/ 80 μg/μl denatured sheared salmon sperm) at $1x10^6$ counts per ml and hybridised at 65 °C overnight.

University of Cape Town

Washing of hybridised RNA membranes

Membranes were washed twice at 65°C for 30 min in 6x SSPE/ 0.1% (v/v) SDS and once in 2x SSPE/ 0.1% (v/v) SDS. Thereafter, membranes were washed once for 30 min at 65°C in 1x SSPE/ 0.1% (v/v) SDS and again in 0.2% SSPE/ 0.1% (v/v) SDS. Membranes were washed until counts of 2-5 cps were recorded with a Geiger counter, then sealed in plastic bags and placed in an autoradiograph cassette. To ensure that no buffer leaked from the plastic bags, paper was placed on top of the membranes for 30 min before removal. Images were visualised via phosphoimager (Molecular Imager FX, Biorad) analysis. The intensity of detected transcripts was calculated using Quantity One Quantitation Software V4 Windows® and Macintosh® (Biorad).

University of Cape Town

Chapter 3

Genetic characterisation of *jam2*

University of Cape Town

Introduction

The model plant *Arabidopsis thaliana* was used as experimental system in this study. This cruciferous plant offers many advantages, which can be exploited in experimental studies investigating diverse aspects of plant biology. It is small in size with a short life cycle, lasting between 6-8 weeks (from seed to seed), making it easy to grow and work with in a laboratory setting. Another attractive characteristic of *Arabidopsis* is that it propagates via self-fertilisation, producing large amounts of seed per plant. Thus, in a short time span, large numbers of individual plants can be generated and examined. In addition, it has a small genome (125 Mb) consisting of 5 chromosomes harbouring an estimated 25 000 genes (The *Arabidopsis* Genome Initiative, 2000). These characteristics make *Arabidopsis* an excellent system for molecular and genetic studies.

This study aimed to isolate new *Arabidopsis* JA signalling mutants using a novel screening method. The following chapters will describe how one mutant, *jasmonic acid modified2* (*jam2*), was isolated and genetically and phenotypically characterised. This chapter will focus on the genetic characterisation of *jam2*, which was isolated from transfer DNA (T-DNA) activation tagged seed lines.

A genetic approach was adopted to isolate components of the JA signal transduction pathway. Various aspects of signal transduction have previously been studied using physical, molecular and genetic techniques. Physical and molecular studies, such as gel shift assays and yeast-two-hybrid studies, are mainly conducted *in vitro* and thus do not truly reflect *in vivo* events. Genetic studies, on the other hand, are mainly based on mutant analysis to dissect a variety of signalling pathways, hence providing an *in vivo* system of study. Mutants defective in genes encoding specific proteins can be identified using a genetic approach. Moreover, mutants defective in regulatory elements such as transcription factors, DNA binding sites and other interacting regulators could also be identified via a genetic approach. This approach has been extensively used to study the model plant system *Arabidopsis,* culminating in the isolation of numerous mutants. These mutants are defective in various aspects of signalling ranging from development, physiology, morphology and metabolism. Thus, there are many *Arabidopsis* signalling mutants available that could be genetically studied to help elucidate these affected signalling pathways.

Mutant analysis is a powerful genetic tool to investigate many aspects of plant biology. The analysis of *Arabidopsis* mutants has been a valuable tool that has led to the identification, cloning and sequencing of many genes. Therefore, another attractive feature of *Arabidopsis* is that it can easily be genetically

University of Cape Town

manipulated to create mutants. Three mutagenesis approaches have mainly been used to generate *Arabidopsis* mutants: (a) chemical mutagens such as ethylmethane sulfonate (EMS), (b) gamma irradiation and (c) biologica mutagens such as insertions of T-DNA mediated by *Agrobacterium tumefaciens* transformation.

EMS mutagenesis mainly introduces point mutations, whereas gamma irradiation more likely introduces deletions (Rédei and Koncz, 1992; Anderson and Wilson, 2000). T-DNA insertion relies on the DNA transfer system of *A. tumefaciens*. A segment of the *A. tumefaciens* tumour inducing (Ti) or root inducing (Ri) plasmids is transferred into plant cells (Koncz and Schell, 1992; Aarts et al., 2000). These segments are flanked by 25 bp border repeats (Koncz and Schell, 1992; Aarts et al., 2000). The transferred DNA is transported to the nuclei of infected cells, where it is stably integrated into their genomes (Koncz and Schell, 1992; Aarts et al., 2000). This transfer process is regulated by Ti or Ri plasmid virulence genes that are expressed in the bacteria (Koncz and Schell, 1992; Aarts et al., 2000). These virulence factors have to be provided in *cis* or *trans* to facilitate the transfer of foreign DNA utilising T-DNA based vectors (Koncz and Schell, 1992; Aarts et al., 2000).

University of Cape Town

One advantage of chemical mutagenesis is that the mutation rate is high compared to that of T-DNA insertional mutagenesis. Therefore, one can obtain a higher number of M_1 individuals to obtain mutational saturation of the *Arabidopsis* genome with chemical mutagenesis than with T-DNA mutagenesis. However, T-DNA mutagenesis offers several advantages above those of chemical and physical mutagenesis. A major advantage of T-DNA mutagenised lines is that T-DNA can facilitate easy gene cloning if a mutation is tightly linked to the T-DNA insertion, since the T-DNA left and right border sequences are known. T-DNA vectors are often engineered to contain specific restriction enzyme sites, selectable markers for easy screening and even plasmid origins of replication that could be used in plasmid rescue attempts to recover flanking DNA. This technique entails the digestion of mutant plant DNA with an appropriate enzyme and ligation of digested fragments. The ligated fragments are then transformed into *Escherichia coli* because T-DNA segments contain a plasmid origin of replication, thus allowing replication. Transformants are selected based on antibiotic resistance. Plant flanking regions can then be identified through sequencing of transformants, using the known T-DNA borders as primers. Polymerase chain reaction (PCR) techniques such as inverse PCR (i-PCR) or thermal asymmetric interlaced (TAIL) PCR could also be employed to directly amplify plant flanking DNA. This thus avoids time-consuming and labour intensive map-based cloning that has to be employed in the cloning of genes disrupted in mutants created by chemical or physical

mutagenesis. Other advantages of using T-DNA insertional mutagenesis is that (1) single copies are normally inserted at a locus (2) due to their large size, they are more or less guaranteed to cause gene aberrations when inserted into introns, untranslated regions as well as coding regions and (3) insertions are generally stable (Koncz and Schell, 1992; Aarts et al., 2000). Another insertional mutagen widely used is transposon tagging. This approach offers the same advantages as T-DNA mutagenesis, except that they do not integrate stably into genomes (Coupland, 1992; Aarts et al., 2000). However, stable transposon mutagenised lines can be obtained through selection for excision or separation of an inactivated transposable element from the transposase gene (Coupland, 1992; Aarts et al., 2000). Hence the advantages of T-DNA mutagenesis outweigh those of other conventional mutagenic approaches such as using chemical and physical mutagens. In this study, T-DNA mutagenised seed lines were analysed.

University of Cape Town

Reporter gene constructs have recently been exploited in fusion genetic analysis used to screen for constitutive mutants of JA signalling. In these experiments known JA-inducible promoters have been fused to different reporter constructs. This approach led to the identification of constitutive JA signalling mutants (Ellis and Turner, 2001; Hilpert et al., 2001, Jensen et al., 2002). Multiple *cet* mutants were isolated in a screen using the JA-inducible *Thi2.1* promoter fused to the dominant marker gene *bar* (Hilpert et al., 2001). The *cev1* mutant was isolated in a screen where the JA-responsive *Vsp* promoter was fused to the firefly luciferase reporter gene (Ellis and Turner, 2001) for its constitutive expression of *Vsp1*. The *jue1*, *-2* and *–3* (*jasmonate underexpressing*) and *joe1* and-2 (*jasmonate overexpressing*) were isolated upon treatment with MeJA in a screen which also employed the firefly luciferase reporter gene and the *E. coli* β-glucuronidase reporter fused to the promoter of the JA-inducible *LOX2* gene (Jensen et al., 2002). This approach relies on the fact that a disruption in JA signalling will alter JA-induced gene expression.

Mutants can be studied using a forward or a reverse genetic approach. In reverse genetics, a gene of interest is cloned based on its sequence and is molecularly characterised. Following this, the wild type gene is silenced and the resulting phenotypes observed. A drawback of this method is that a visible phenotype is not guaranteed, especially in the case of redundant genes. For example, if two genes have the same function, the loss of one would have minimal or no effect on phenotype. When using a forward genetic approach, a mutant is identified based on defective response/s (i.e. mutated gene is not known), its function is again inferred from phenotypic analysis and the wild type gene is cloned. If for example, chemical mutagenesis was employed, the wild type gene has to be cloned using a map-based

approach. In this study a forward genetic approach was adopted. A disadvantage of forward genetics is that it entails time-consuming, laborious screening for mutants. Also, in forward genetics lethal mutations are often lost. An advantage of forward genetics is that abrogation of gene function can lead to inference of gene function, meaning that the observed mutant phenotype can be an indication of the type of gene that was disrupted. This study was conducted to find new genes involved in JA signalling, warranting the utilisation of new screening methods. Different types of screens have led to the isolation of various mutants, hence a different type of screen method was adopted in this study. A new screening method and one that can yield dominant mutations is that of activation tagging. Multimers of enhancer elements such as the constitutive enhancers of the cauliflower mosaic virus (CaMV) 35S gene were inserted into near the borders of T-DNA vectors (Weigel et al., 2000). Insertion of these T-DNA segments containing multimerised enhancers into genomes can cause transcriptional activation of adjacent genes. Activated genes are thus associated with the T-DNA insert, hence the term activation tagging. Activation tagging could be useful as a means of analysing functionally redundant genes or genes essential to the survival of an organism that is difficult to identify through loss-of –function screens.

Although the abovementioned mutants have shed some light on JA signalling, our knowledge of JA perception, downstream signalling and molecular events leading to JA end responses is far from complete. Only through the identification and analysis of more and novel mutants affected in JA perception and signalling using diverse screening methods may we advance our understanding of JA signal transduction.

University of Cape Town

Results

Mutant screen

Primary screen for new JA signalling mutants

Exogenous application of MeJA or the OPDA structural analogue, coronatine, stimulates anthocyanin accumulation in wild type *Arabidopsis* plants but not in the JA signalling mutant *coi1* (Feys et al., 1994). Based on this observation, a simple screen using plate grown plants was devised. Lack of anthocyanin accumulation after exogenous MeJA treatment is a visual phenotype that could easily be tracked in subsequent generations and characterisation studies. Visible phenotypes can simplify attempts to map mutations onto the *Arabidopsis* genome in order to clone wild type genes.

University of Cape Town

One hundred two-week-old seedlings of 86 pools of T_2 generation T-DNA transformed Arabidopsis were screened for lack of visual anthocyanin accumulation after exogenous application of 45 µM MeJA. In initial experiments, a number of plants had a water-soaked appearance after treatment with 45 µM MeJA treatment. These water-soaked plants did not accumulate anthocyanins after MeJA treatment. To avoid water-soaking of plants in subsequent experiments, plates were left covered in a flow hood overnight before treatment to remove any condensation on plate lids and water-soaked plants were excluded from the primary screen. 13 putative mutants from 5 pools were identified that did not show visible anthocyanin accumulation 48 hr after treatment. Col-0 control seedlings on the same plates exhibited anthocyanin accumulation. These 13 putative mutants were transplanted into soil and 7 plants from 4 different pools survived the transplant and produced T_3 seeds, which were used in subsequent analyses. These plants are likely to be non-siblings because they were isolated from different pools.

Secondary screen of putative mutants

To assess the heritability of the lack of anthocyanin phenotype in the isolated putative mutants, T_3 seeds were used in a secondary screen. In the secondary screen, 50 T_3 seeds from each plant were analysed as before. Of the 7 putative mutants screened, 1 line lacked visible anthocyanin accumulation whilst the other 6 showed very reduced anthocyanin accumulation 48 hrs after treatment compared to Col-0. To correlate the visual lack of anthocyanin phenotype with the actual levels of anthocyanins accumulated, anthocyanins were extracted and quantified from the 7 putative mutants after treatment. Only 1 line produced levels of anthocyanins significantly lower than Col-0 48 and 72 hr after treatment with 45µM MeJA (data not shown). This mutant was designated *jam2* (*jasmonic acid modified2*). All

50 *jam2* seedlings tested showed no visible anthocyanin, thus indicating that the mutation was homozygous and not segregating.

Heritability of the *jam2* mutation

Genetic analysis in *Arabidopsis* is normally performed in the F_2 generation (Koornneef and Stam, 2000). To obtain *Arabidopsis* F_2 progeny, a homozygous mutant is crossed to its parental line to segregate out other possible mutations and to obtain homozygous mutants of interest. This cross delivers the F_1 generation. F_1 plants are used to assess the dominant or recessive nature of a mutation. If a mutation is dominant, all F_1 plants would display the mutant phenotype. On the other hand, for a recessive mutation, the F_1 plants would all have wild type phenotypes. The F_1 plants are allowed to self-fertilise to generate the F_2 generation. A recessive monogenic mutation would have a Mendelian segregation ratio of 3:1 (wt: mutant) in the F_2 generation. Monogenic ratios could deviate from the 3:1 segregation ratio. This could be due to an intermediate phenotype of heterozygotes, which would have a segregation ratio of 1:2:1. Other factors contributing to deviation from the Mendelian 3: 1 ratio could be due to genotypes causing poor propagation, lethality or certation (reduced transmission of certain alleles through the gamete). The segregation ratios are determined statistically using the Chi square test. The Chi square test, as used in this work, assesses whether observed numbers deviate from theoretical expected numbers within certain probability limits.

University of Cape Town

To determine whether the *jam2* mutation is recessive or dominant to the wild type allele, and whether the *jam2* mutant phenotype is conferred by a single gene, a backcross to the Col-0 parent was performed. *jam2* was used as the pollinator and F_1 progeny were treated with BASTA to confirm the success of the cross. BASTA resistance was used to determine the success of the cross to Col-0 since the T-DNA seed lines used contained the dominant *bar* gene which confers resistance to the BASTA herbicide. Thus the *bar* gene is located on the T-DNA and is not present elsewhere in the genome. The BASTA resistance of two-week old F_1 seedlings was assessed on plates containing 5 µg/ml BASTA (data not shown) because at this concentration Col-0 plants were found to be BASTA sensitive. BASTA resistant F_1 plants were selected and transferred to soil. F_2 seeds were harvested from 20 resistant plants representing 4 F_2 lines. The segregation of the mutant and wild type phenotype was analysed in the F_2 generation of 1 F_2 line.

It was found that the *jam2* lack of anthocyanin phenotype segregates in a classical Mendelian ratio of 3:1 (wt: mutant) indicating that it is a recessive, monogenic mutation (Table 3.1).

Table 3.1. : *jam2* segregates as a recessive, monogenic mutation. *jam2* was used as the pollen donor and Col-0 as the pollen recipient.

	Hypothesis	Number tested	A^+		A^-		χ^2 value	P=5%
F_2			O	E	O	E		
jam2 x Col-0	3:1[a]	179	143	135.25	36	44.75	2.155	≥ 3.841

The χ^2 value was calculated and compared to the critical value at the 5% confidence level (Ennos, 2000).
[a] The hypothesis was that if *jam2* was a recessive monogenic mutation, a segregation ratio of 3(A^+) :1 (A^-) would be obtained.
A^+: plants displaying visible anthocyanin accumulation 48 hr after treatment with 45µM MeJA.
A^-: plants not displaying visible anthocyanin accumulation 48 hr after treatment with 45µM MeJA.
O: Observed
E: Expected

jam2* is not allelic to *coi1-1

Allelism or complementation tests are carried out to determine whether monogenic, recessive mutants with identical or similar phenotypes are due to mutations in the same genes (allelic) or different genes (non-allelic). Two homozygous recessive mutants are crossed and the resulting F_1 progeny is analysed. If the two mutants are allelic, all the progeny would have mutant phenotypes. In the case of two non-allelic mutants, the F_1 generation would have wild type phenotypes, indicating complementation of the two mutants. The F_2 generation could also be used to conduct allelism tests, although the F_1 generation is standard. In the F_2 generation, if two monogenic recessive mutants are allelic, all plants would have the mutant phenotype. If two monogenic recessive mutants are non-allelic, a segregation ratio of 9: 7 (wt: mutant) would be expected in the F_2 generation.

coi1-1 is defective in most JA-dependent responses including anthocyanin accumulation and comprises an integral component of the JA signalling cascade (Feys et al, 1994; Xu et al, 2002). Due to the high frequency of *coi1* alleles identified and the fact that *coi1* and *jam2* share a lack of anthocyanin accumulation phenotype after MeJA treatment, an allelism test between these 2 mutants was conducted. F_1 progeny resulting from a cross between homozygous *coi1-1* (female parent) and a homozygous *jam2* (pollen donor) were screened for BASTA resistance, indicating a successful cross and were grown to maturity to generate F_2 seeds. As both *coi1-1* and *jam2* are recessive mutations, a phenotypic segregation ratio of 9 (wt): 3 (*coi1-1*): 3 (*jam2*): 1 (*coi1-1 jam2*) would be expected in the F_2 generation if *jam2* and *coi1-1* are not allelic and unlinked. This would lead to an anthocyanin phenotype ratio of 9 (anthocyanin$^+$) (A^+): 7 (anthocyanin$^-$) (A^-). As seen in Table 3.2, the predicted 9 (A^+): 7 (A^-) phenotypic ratio was obtained, indicating that *jam2* and *coi1-1* are not allelic.

University of Cape Town

Table 3.2. : *jam2* is not allelic to *coi1-1*.

Generation	Hypothesis	Total number of plants scored	A^+		A^-		χ^2 value	P=5%
F_2			O	E	O	E		
jam2 x coi1-1	9 : 7[a]	307	161	172.69	146	134.31	1.808	≥ 3.841

The χ^2 value was calculated and compared to the critical value at the 5% confidence level (Ennos, 2000).
[a] Hypothesis: If *jam2* is not allelic to *coi1-1*, the F_2 generation of a cross between homozygous *coi1-1* and *jam2* would segregate 9 (A^+): 7(A^-).
A^+: plants displaying visible anthocyanin accumulation 48 hr after treatment with 45μM MeJA.
A^-: plants not displaying visible anthocyanin accumulation 48 hr after treatment with 45μM MeJA.
O: Observed
E: Expected

Determination of the number of T-DNA insertions in *jam2*

It is essential to determine the number of T-DNA insertions into the genome. This is to ensure that only one T-DNA insert is integrated into the genome that may be the cause of the mutant phenotype. T-DNA vectors are normally engineered to carry dominant selectable markers such as antibiotic (often kanamycin) or herbicide resistance genes (often glufosinate) (Aarts et al., 2000). The segregation of this dominant gene can be used to determine the number of T-DNA insertion events. If a single insertion occurred, the segregation ratio resistant: sensitive would 3:1 in the F_2 population. The activation tagged seed lines used in this study were transformed with T-DNA vectors containing the dominant glufosinate (*bar)* resistance gene that confers resistance to the BASTA herbicide.

University of Cape Town

To determine the number of T-DNA inserts present in *jam2*, F_2 progeny from *jam2* x Col-0 were tested for BASTA resistance. Col-0 and T_5 *jam2* were included as positive and negative controls respectively. All Col-0 were found to be sensitive to BASTA whereas all T_5 *jam2* were found to be resistant. A segregation ratio of BASTA resistance: sensitivity of 3:1 was obtained in the F_2 progeny indicating that *jam2* contains one or multiple copies of T-DNA at a single locus (Table 3.3).

Table 3.3. : *jam2* contains a single T-DNA insertion.

Generation	Hypothesis	Total number of plants scored	BASTA R$^+$		BASTA R$^-$		χ^2 value	P=5%
F_2			O	E	O	E		
jam2 x Col-0	3: 1[a]	125	97	93.75	28	31.25	0.451	≥ 3.841

The χ^2 value was calculated and compared to the critical value at the 5% confidence level (Ennos, 2000).
[a]The hypothesis was that if *jam2* has only 1 T-DNA insertion, a segregation ratio of 3(R$^+$): 1 (R$^-$) would be obtained.
R$^+$: plants resistant to BASTA
R$^-$: plants sensitive to BASTA
O: Observed
E: Expected

Linkage analysis between *jam2* and the T-DNA insertion

The ultimate aim of genetic analysis is to clone the wild type allele of the mutated gene. Before T-DNA mutants can be used for cloning, genetic linkage between the mutant phenotype and T-DNA insertion (through the selectable marker) needs to be established because untagged mutations may occur. The separation of untagged mutations from possible tagged ones requires rather large F_2 or F_3 populations in genetic linkage studies, due to the dominance of the selectable marker (Aarts et al., 2000). Aarts et al. (2000) states that in a mutant population of 1000, if no untagged mutant is found, linkage within approximately 3.2 cM can be deduced.

If the *jam2* mutation is tagged by a T-DNA insert, cloning of *JAM2* would be simplified by using techniques such as plasmid rescue or inverse PCR. In order to establish genetic linkage between the *jam2* mutant phenotype and BASTA resistance conferred by the dominant marker gene *BAR*, F_2 progeny from a cross between Col-0 and *jam2* where pollen from *jam2* was used to pollinate Col-0, were selected for the lack of anthocyanin mutant phenotype 48 hr after treatment with 45 μM MeJA. Plants displaying the mutant phenotype, as well as those with the wild type phenotype, were transplanted into soil and grown for 1 week before treatment with BASTA. If the mutant phenotype is caused by the T-DNA insertion, all the F_2 *jam2* x Col-0 progeny displaying the mutant phenotype should be BASTA resistant. 149 mutants were assessed (Table 3.4). Of these 102 were found to be resistant to BASTA and 47 were BASTA sensitive (Table 3.4). Since *jam2* selected mutants were found that were not resistant to BASTA treatment, it is an indication that the lack of anthocyanin is not linked to the T-DNA insert. Moreover, this confirms that *jam2* contains a single T-DNA insertion locus and that the T-DNA is unlinked and independent to the mutation.

University of Cape Town

Table 3.4. : *jam2* is not genetically linked to BASTA resistance.

Generation	Total number of plants scored	R^+	R^-
F_2		Observed	Observed
jam2	149	102	47
Col-0	20	0	20
jam2 (T_5)	19	19	0

R^+: plants resistant to BASTA

R^-: plants sensitive to BASTA

Discussion

To isolate components of JA signalling, a novel screening method to identify mutants defective in JA signalling was used. Using a visual phenotype, which could be easily tracked in subsequent generations, was an important consideration in this work. This serves to aid possible later mapping attempts. The selection criteria used was lack of anthocyanin accumulation after exogenous MeJA treatment. This phenotype is also exhibited in the JA insensitive mutant, *coi1*, in which most JA responses are attenuated (Feys et al., 1994; Xie et al. 1998).

This study was conducted on the premise that plants unable to accumulate anthocyanin upon MeJA treatment would either be (1) defective in JA perception or signal transduction or (2) affected in anthocyanin biosynthesis. Out of 86 T_2 T-DNA transformed pools screened in the primary screen, 13 possible mutants were identified representing 5 pools. Col-0 was used as positive control in all experiments since it is the parental line of T-DNA transformants used. Col-0 accumulated visible amounts of anthocyanin at 48 hr after 45 μM MeJA treatment and this time point was used to identify possible mutants. During the primary screen it was observed that water-soaked seedlings, wild type or mutagenised pools, did not produce any visible anthocyanins. This observation suggests that MeJA must be sensed as a gas, due to water filling the interstitial spaces. All water-soaked plants were therefore excluded from the primary screen.

To check whether the lack of anthocyanin phenotype was heritable, a secondary screen was performed using the T_3 generation. The lack of anthocyanin phenotype was only heritable in one mutant. This mutant was designated *jam2* and used in subsequent experiments. Moreover, all *jam2* plantlets tested

University of Cape Town

exhibited the lack of anthocyanin phenotype after MeJA treatment. This shows that *jam2* is a homozygous mutant and that it does not segregate.

The F_2 generation of a cross between homozygous *jam2* (pollen donor) and Col-0 (pollen acceptor) was used to carry out genetic studies on *jam2*. Due to the small size of *Arabidopsis* flowers, it was difficult to obtain successful crosses. Therefore, to confirm whether crosses were successful and thus ruling out self or cross fertilisation as aided through insect vectors, all F_1 progeny were treated for BASTA resistance. F_2 progeny were harvested and used in subsequent genetic studies.

Genetic analysis revealed that *jam2* was inherited as a recessive mutation at a single locus (Table 3.1). Although the *jam2* mutation was caused by one T-DNA inserted at a single locus (Table 3.2), further investigation showed that *jam2* was independent and unlinked to the T-DNA insert (Table 3.3). Moreover, this result confirmed that only one T-DNA insertion site was present in *jam2* since a segregation ratio of 3 (BASTA resistant): 1 (BASTA sensitive) (Table 3.3) was obtained. Furthermore, this study showed that *jam2* was not allelic to *coi1*. The lack of anthocyanin phenotype is shared only between *jam2* and *coi1* (Feys et al., 1994) and not by the other JA insensitive mutants *jar1*, *jin1* and *jin4* (Staswick et al., 1992 and Berger et al., 1996). It is thus unlikely that *jam2* would be allelic to these latter mutants and would therefore define a novel JA mutant. Another postulation would be that *jam2* might define a stronger allele of *jar1*, *jin1* or *jin4*, since *jam2* does not exhibit anthocyanin accumulation after MeJA treatment. This however still needs to be investigated through further characterisation studies to find possible common phenotypes between these mutants and *jam2*.

It is not uncommon that observable mutations do not co-segregate with T-DNA inserts. This observation extends to both seed and tissue culture-derived T-DNA transformants. Of 115 mutants derived from seed transformations and extensively characterised by Castle et al. (1993), 64% were not tagged by T-DNA inserts. Also, analysis of tissue culture derived T-DNA transformants by Koncz et al. (1992), revealed that only 10-30% of mutants displaying visible phenotypes were tagged by T-DNA. The mutation causing the *jam2* phenotype could possibly arise from a segment of T-DNA inserted into the genome or a random mutation. Since the *jam2* mutation is not T-DNA tagged, conventional mapping techniques would have to be employed to clone *JAM2*. This would entail creating mapping populations by crossing homozygous *jam2* to, for example, the Landsberg ecotype, since it contains polymorphisms, compared to Col, for various known markers.

University of Cape Town

This study identified a novel, recessive monogenic *Arabidopsis* mutant, *jam2*, disrupted in a gene required for anthocyanin accumulation. The next chapter will deal with identifying other JA related phenotypes in which *jam2* could be affected.

University of Cape Town

Chapter 4

Phenotypic Characterisation of *jam2*

University of Cape Town

Introduction

In order to establish how JA signalling is affected in *jam2*, a phenotypic characterisation study was conducted. The disruption of JA signalling can result in multiple responses being affected, culminating in pleiotropic mutant phenotypes. A good example is the JA insensitive mutant, *coi1*, which seems to be affected in all JA responses (as outlined in Chapter 1) (Feys et al., 1994, Benedetti et al., 1995 and Titarenko et al., 1997). Although several other JA insensitive signalling mutants have been isolated, all of which exhibit different phenotypes, they are not as severely affected as *coi1* (Staswick et al., 1992, Berger et al., 1996). Since abrogation of JA signalling can lead to numerous responses being influenced, it is imperative to perform phenotypic characterisation studies on newly isolated mutants. Through establishing the full range of affected responses, one can hypothesize the function of the corresponding wild type gene and its role in the JA signalling pathway.

University of Cape Town

Since anthocyanin production is induced by MeJA (Franceschi and Grimes, 1991; Feys et al., 1994) and is a JA-regulated response, the ability of *jam2* to produce and accumulate anthocyanins compared to Col-0 was assessed. In this work, the JA mutant, *jam2*, was isolated for its lack of anthocyanin accumulation after exogenous application of MeJA. However, to assess the overall anthocyanin production capacity in *jam2,* seedlings were subjected to nutrient stress by growing plantlets on high sucrose and no nitrogen (Lam et al., 2003). Accumulated anthocyanins in *jam2* compared to Col-0 and *jar1* were also quantified after MeJA treatment. This visual phenotype displayed by *jam2,* as previously mentioned, would be beneficial in subsequent mapping attempts to clone *JAM2*.

To determine how JA signalling was affected in *jam2*, further characterisation studies were performed. MeJA induced root growth inhibition was used as selection criteria in screens to isolate JA insensitive mutants (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996). All JA insensitive mutants isolated to date, displayed different degrees of resistance to MeJA root inhibition (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996). MeJA is able to induce the expression of *PDF1.2* in *Arabidopsis* (Penninckx et al., 1996, Penninckx et al., 1998). This antifungal peptide can be seen as a marker for JA mediated defense responses since it is induced by a variety of fungal and bacterial pathogens (Feys et al., 1994; Penninckx et al., 1996; Penninckx et al., 1998; Manners et al, 1998; Norman-Setterblad et al., 2000; Govrin and Levine, 2002). These are all well-known JA-mediated responses, used to investigate JA signalling in *jam2*. Moreover, this study was directed at investigating the signalling role of JA in response to pathogen attack. JA is specifically implicated in defense against necrotrophic pathogens

(Penninckx et al., 1996; Penninckx et al., 1998, Staswick et al., 1998; Thomma et al., 1998; Vijayan et al., 1998; Thomma et al., 1999, Thomma et al., 2000 and Kloek et al., 2001), therefore the fungus *Botrytis cinerea* was used.

The following sections wil focus on the phenotypic analysis of *jam2*.

University of Cape Town

Results

jam2 is not an anthocyanin biosynthetic mutant

Since *jam2* does not display any visible anthocyanin after MeJA treatment, the possibility exists that it might be an anthocyanin biosynthetic mutant. This means that the locus defined by *jam2* could encode an anthocyanin biosynthetic enzyme, which when mutated, leads to attenuated anthocyanin accumulation. This would not be ideal, since this work is interested in JA signalling and not anthocyanin production per se. Therefore, before any other phenotypic characterisation was performed, the ability of *jam2* to produce anthocyanin was assessed.

Plants produce and accumulate purple pigments called anthocyanins under stress conditions such as high light conditions, drought, ozone damage, nutrient deficiency and pathogen infection (reviewed in Dixon and Paiva, 1995; Holton and Cornish, 1995; Weisshaar and Jenkins, 1998; Winkel-Shirley, 2002). To determine whether *jam2* is able to produce and accumulate anthocyanins, *jam2* seedlings were subjected to severe nutrient deficiency by germination on medium containing no nitrogen and high sucrose (Lam et al., 2003). Seedlings were planted on the same plate as Col-0. Both Col-0 and *jam2* one-week-old seedlings produced visible purple anthocyanins (Fig.1). The anthocyanins produced after nutrient stress, were the same colour as those produced after MeJA treatment of Col-0, as could be seen from the extracts' colour. This indicates that *jam2* can produce anthocyanins in response to nutrient deficiency and therefore the anthocyanin biosynthetic enzymes appear to be intact.

University of Cape Town

jam2 has delayed anthocyanin accumulation

Despite the fact that *jam2* is able to produce anthocyanins upon nutrient deficiency, it accumulates reduced levels of visible anthocyanins after MeJA treatment. *jam2* produced lower levels of visible anthocyanins compared to Col-0 and *jar1* after treatment with 45 μM MeJA. Despite the obvious visible difference between Col-0, *jar1* and *jam2*, to determine the actual differences, anthocyanins were extracted and quantified spectrophotometrically. The findings of two independent experiments are illustrated in Table 4.1. In both experiments, Col-0 and *jar1* produced significant levels of visible anthocyanins 48 hr after MeJA treatment (Table 4.1, Figure 4.2), whereas *jam2* did not. Even though visible anthocyanins accumulated in *jam2* 120 hr after treatment, the levels produced were still much lower than that of Col-0 and *jar1*. Therefore, in comparison to Col-0 and *jar1*, *jam2* shows reduced and delayed anthocyanin production and accumulation upon treatment with 45 μM MeJA.

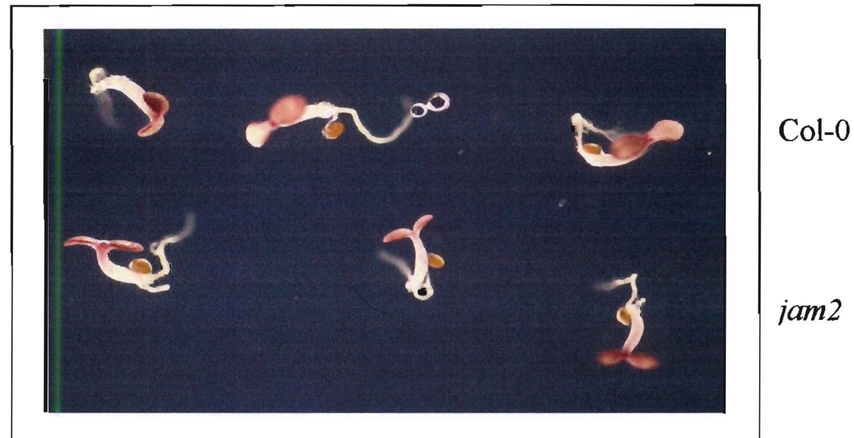


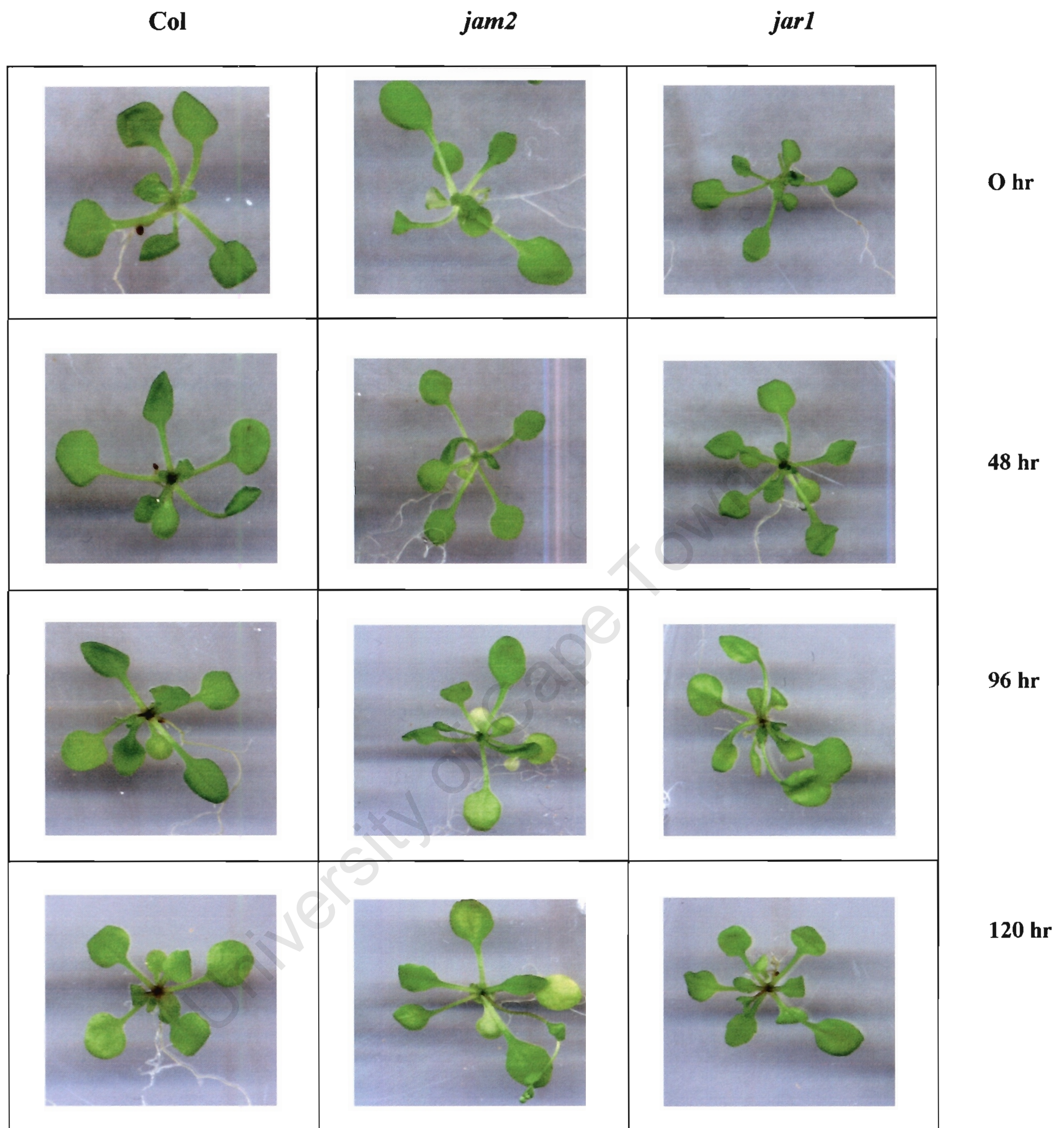
Figure 4.1: Anthocyanin production and accumulation of Col-0 and *jam2*. Seedlings were germinated on PN medium containing no nitrogen and high sucrose and grown for one week.

University of Cape Town

Table 4.1: Anthocyanin levels of Col-0, *jam2* and *jar1* after treatment with 45µM MJA.

Mean anthocyanin levels of Col-0, *jam2* and *jar1*. Two-week-old seedlings were treated with 45 µM MeJA. Anthocyanins were extracted at 0, 48 and 120 hr and the absorbances at 537 and 657 nm were measured. Results are expressed as percentages of the highest Col-0 anthocyanin production at 120 hr.

	% Anthocyanin levels					
	Col-0		***jam2***		***jar1***	
hr	**Exp1**	**Exp2**	**Exp1**	**Exp2**	**Exp1**	**Exp2**
0	15.5	21.0	0.5	0.8	9.5	16.4
48	17.1	55.4	3.0	6.8	15.2	59.4
120	100	100	9.1	18.4	65.5	76.7



University of Cape Town

Figure 4.2: Visible anthocyanin accumulation in Col-0, *jam2* and *jar1*. Two-week old seedlings were treated with 45 µM MeJA and assessed at 0, 48, 96 and 120 hr.

Root growth in *jam2* is inhibited by MeJA

When wild type *Arabidopsis* plants are grown on MeJA or coronatine containing plates, their root growth is inhibited (Staswick et al., 1992; Feys et al., 1994 and Berger et al., 1996). This characteristic was exploited in mutant screens for JA insensitive and constitutive mutants and can be seen as a typical JA mediated response (Staswick et al., 1992; Feys et al., 1994 and Berger et al., 1996; Xu et al., 2001).

To investigate how MeJA affects *jam2* root growth, seedlings were germinated on 30, 45 and 100 µM MeJA. Col-0 and *jam2* showed stunted root growth on all MeJA concentrations tested, indicating that JA-mediated root growth was not affected in *jam2* (Fig. 4.3). The JA signalling mutants, *jin1*, *jin4* and *jar1* was used as positive controls and as expected, displayed reduced sensitivity to MeJA-induced root inhibition (Fig. 4.3). Interestingly, the average root length of *jin1* increased with increasing concentrations of MeJA. In contrast to the JA-insensitive mutants isolated to date, *jam2* is sensitive to MeJA-induced root elongation, suggesting that root growth is independent of *jam2* and that *jam2* might define a novel JA-signalling mutant.

University of Cape Town

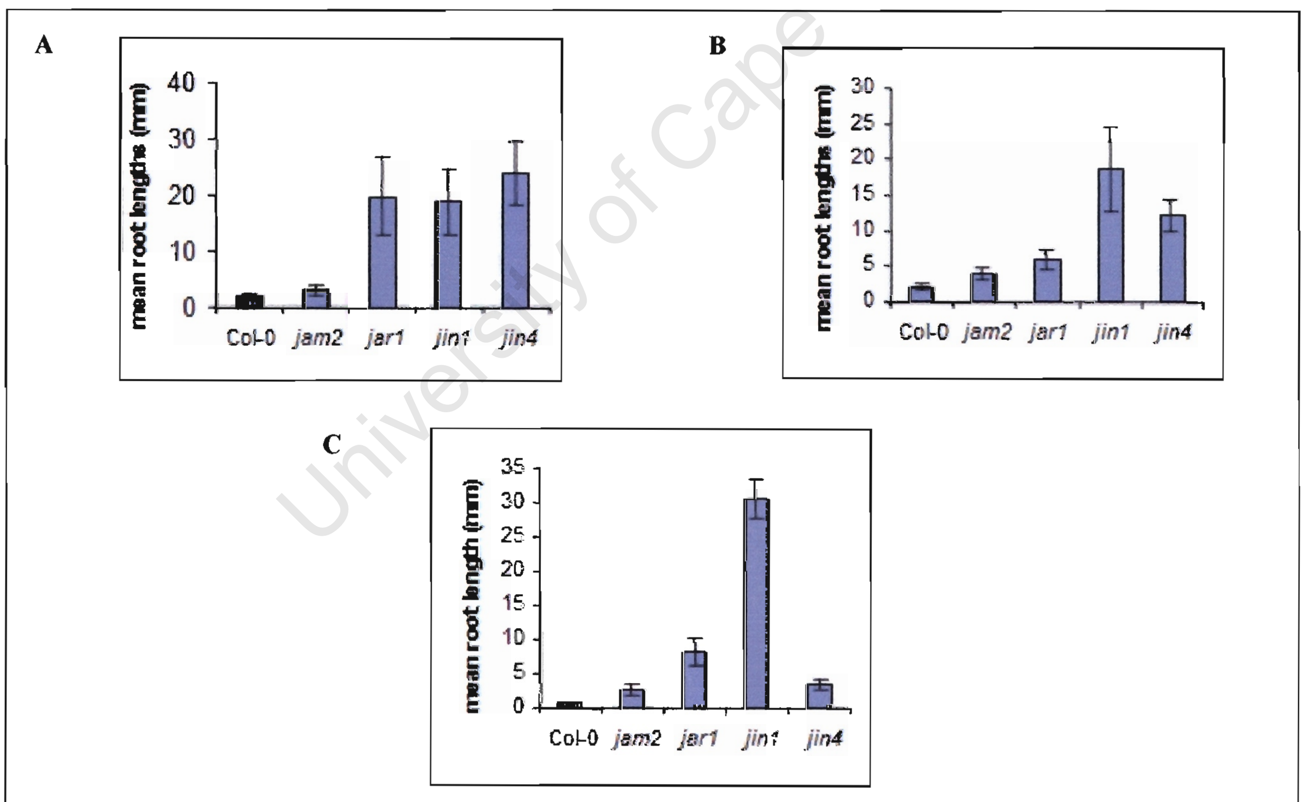


Figure 4.3: Effect of MeJA treatment on root lengths of Col-0, *jam2*, *jar1*, jin1 and *jin1*. Average root lengths were determined on 30 (**A**), 45 (**B**) and 100µM (**C**) MeJA. Root lengths were measured in mm after 1 week. Error bars represents standard errors, n < 10 plants. All Col-0 roots were 2 mm on 100 µM MeJA, hence no standard error is reported.

***PDF1.2* induction in *jam2* is similar to *jar1* after MeJA treatment**

Another JA-mediated response investigated in *jam2* was *PDF1.2* expression. Expression of this plant defensin can be induced by exogenous application of MeJA (Penninckx et al., 1996; Penninckx et al., 1998; Manners et al., 1998). Moreover, the JA signalling mutants *coi1* and *jar1* are unable to induce expression of *PDF1.2* after MeJA treatment (Penninckx et al., 1996; Penninckx et al., 1998 and Kachroo et al., 2000). For this reason, *PDF1.2* expression was used as a measure to check how the JA signalling pathway was affected in *jam2*.

In previous reported studies, MeJA was applied as droplets to leaves of soil- and axenically-grown plants and the concentrations used ranged from 5 μM to 50 μM, with 45 μM being used predominantly (Penninckx et al., 1996; Penninckx et al., 1998; Manners et al., 1998). As already mentioned, MeJA is the volatile methyl ester of JA and low atmospheric levels induced *Vsp* expression in soybean hypocotyls (Fransceschi and Grimes; 1991). Moreover, MeJA was shown to be involved in interplant communication (Farmer and Ryan; 1990). This suggests that MeJA functions as a plant volatile, which is able to elicit plant responses including gene expression (Farmer and Ryan, 1990; Fransceschi and Grimes; 1991). In this study the effect of different MeJA concentrations and application methods on *PDF1.2* expression in *jam2* was investigated (Fig. 4.4 A and B). MeJA was applied as a solution on axenically grown plants. Soil grown plants were treated with MeJA vapours. Concentrations used in this work were 45 and 100 μM MeJA on plates and 15 mM for soil grown plants.

At a MeJA concentration of 45 μM, *PDF1.2* expression was highly induced at 24 and 72 hr (Fig.4.4 A). At 48 hr after treatment with 45 μM MeJA, however, *PDF1.2* levels were reduced (Fig.4.4 A). This biphasic induction pattern was observed in Col-0, *jam2* and *jar1* (Fig.4.4 A). In comparison with Col-0, *PDF1.2* levels in *jam2* and *jar1* were reduced at 48 and 72 hr (Fig.4.4 A). These lower levels of *PDF1.2* at 72 hr suggest that these mutants are affected in the induction of the second *PDF1.2* expression peak. Interestingly, this biphasic induction pattern of *PDF1.2* expression was not previously reported as studies were performed only at 48 hr after treatment with 45 or 50 μM MeJA (Penninckx et al., 1996; Penninckx et al., 1998 and Kachroo et al., 2001). Therefore, the biphasic *PDF1.2* expression pattern after 45 μM MeJA treatment reported here is novel.

At 100 μM MeJA, the induction pattern of *PDF1.2* expression was also biphasic (Fig.4.4 B). However, Col-0 and *jam2* showed delayed *PDF1.2* induction, peaking at 72 hr compared to *jar1* (Fig.4.4 B). For *jam2*, this result is contrary to the result obtained with 45 μM MeJA (Fig.4.4 A and B).

University of Cape Town

This slower induction could be a result of the higher MeJA concentration used. In the case of *jar1*, *PDF1.2* induction followed the same pattern obtained with 45 µM MeJA, although the second peak was reduced (Fig.4.4 A and B). It therefore appears that at a concentration of 100 µM MeJA, there is no difference in *PDF1.2* induction in Col-0 and *jam2*. In addition, overall expression levels were higher at higher MeJA concentrations.

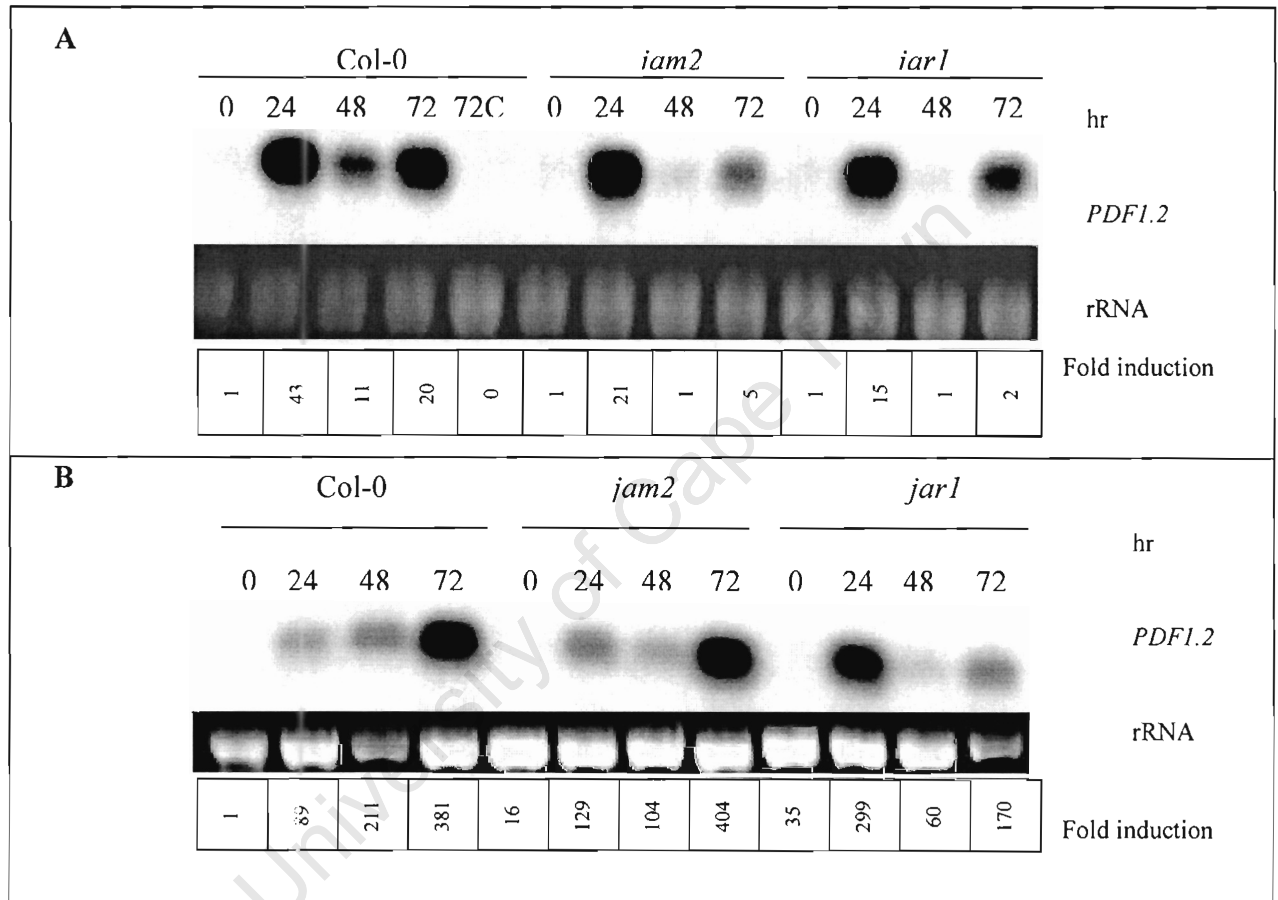


Figure 4.4: Effect of exogenous MeJA treatment on expression of *PDF1.2* by *jam2*. Two-week-old seedlings were treated with (**A**) 45 and (**B**) 100 µM MeJA. Tissue was harvested at 0, 24, 48 and 72 hr. 20 µg of total RNA was extracted and electrophoresed on a 1.2% Agarose/Formaldehyde gel. Total RNA was transferred on a Hybond N^+ membrane and probed with α-^{32}P labelled *PDF1.2* PCR product. Similar trends were seen for repeat experiments. Fold induction is the ratio of sample value (*PDF1.2* intensity/rRNA intensity) to Col-0 time 0 value (*PDF1.2* intensity/rRNA intensity).

Surprisingly, the biphasic induction pattern of *PDF1.2* obtained for liquid MeJA treatment was mirrored by treatment with MeJA vapours (Fig.4.4 and Fig. 4.5). In *jam2*, the highest *PDF1.2* levels were obtained at 72 hr, whilst Col-0 and *jar1* levels peaked at both 24 and 72 hr (Fig.4.5). Therefore it seems that *jam2* is affected the first induction peaked upon gaseous MeJA treatment, a response also obtained for 100 µM MeJA, as compared to Col. The phenotype of *jam2* is therefore dependent on the

concentration and perhaps method of delivery of MeJA. These experiments were performed thrice and similar trends were observed. The *PDF1.2* expression pattern obtained for *jar1* is unexpected since published data showed that *jar1* did not exhibit *PDF1.2* expression 48 hr after liquid MeJA treatment (Kachroo et al., 2001).

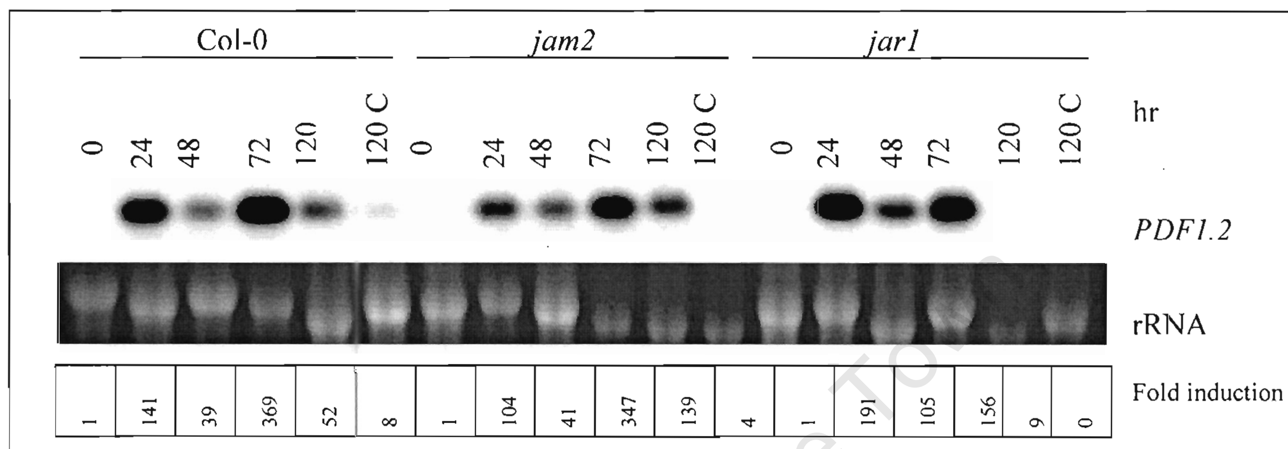


Figure 4.5: Effect of gaseous MeJA treatment on *PDF1.2* expression by *jam2*. Four week-old plants were treated with 15 mM MeJA and Ethanol as controls. Tissue was harvested at 0, 24, 48, 72 and 120 hr. 20μg of total RNA was extracted and electrophoresed on a 1.2% Agarose/Formaldehyde gel. Total RNA was transferred on a Hybond N^+ membrane and probed with α-^{32}P labelled *PDF1.2* PCR product. This experiment was repeated twice with similar results. Fold induction is the ratio of sample value (*PDF1.2* intensity/rRNA intensity) to Col-0 time 0 value (*PDF1.2* intensity/rRNA intensity).

jam2 is more resistant to _Botrytis cinerea_ than Col-0

JA is implicated in defense specifically against necrotrophic pathogens such as *Botrytis cinerea*. Disease symptoms caused by *B. cinerea* infection are manifested by the development of necrotic lesions (Thomma et al., 1998; Thomma et al., 1999; Govrin and Levine, 2002). Disease development in *jam2* upon infection with *B. cinerea* was assessed using 2 methods (1) inoculation of entire leaves and (2) drop-inoculation by placing a 5μl drop of a suspension of 1 x 10^3 spores/ml on the midvein of leaves. To analyse resistance of Col-0, *jam2* and *jar1*, a disease index was calculated for leaves inoculated with *B. cinerea*. To assay disease development in detached leaves, lesion diameters were measured in mm for drop-inoculated leaves.

The calculated disease indices for resistance against *B. cinerea* in 2 independent experiments were: Col-0 3.64 and 2.68, *jam2* 2.76 and 1.44 (Figure 4.6 A and B). *jar1* had disease indices of 1.76 and 2.2. In comparison with Col-0, the disease indices obtained for *jam2* were slightly lower, indicating that *jam2* was more resistant to *B. cinerea* infection than Col-0. *jar1* exhibited similar resistance trends as

University of Cape Town

jam2 when compared to Col-0. When comparing the disease indices for *jam2* and *jar1* no significant difference was found.

Lesion size was the second method used to study disease development in *jam2*. The mean lesion diameters of Col-0 were higher than those of *jam2* and *jar1*, indicating that Col-0 is more susceptible to *B. cinerea* than *jam2* and *jar1* (Fig. 4.6 C). When comparing lesion diameters for *jam2* and *jar1*, *jam2* no significant difference was observed (Fig.4.6 C). Although *coi1-1* homozygotes displayed the largest average lesion growth, lesion sizes was not significantly different from Col-0. This finding is surprising since published data indicates that *coi1-1* is more susceptible to *B. cinerea* infection than Col-0 (Thomma et al., 1998; Thomma et al., 1999; Zimmerli et al., 2001).

As mentioned previously, *PDF1.2* expression can be used as a marker to follow JA-mediated disease resistance responses. To assess how JA-regulated defense responses against necrotrophic fungi are affected in *jam2*, *PDF1.2* expression after infection with *B. cinerea* was studied. The *PDF1.2* expression patterns in *jam2* and *jar1* differed from that of Col-0 (Fig.4.7). In Col-0, initial *PDF1.2* levels were low, increasing at 48 hr after infection (fig.4.7). In contrast, *jam2* and *jar1* expressed *PDF1.2* at high levels at 24 hr, with a reduction at 48 hr (Fig.4.7). Interestingly, *jam2* and *jar1* displayed similar *PDF1.2* expression patterns (Fig.4.7). These results therefore indicate that *jam2* and *jar1* exhibit faster induction of *PDF1.2* expression than Col-0. Interestingly, no *PDF1.2* expression was observed in Col-0, *jam2* and *jar1* at 72 hr after infection.

University of Cape Town

Table 4.2: Disease indices after infection with *B. cinerea*.

Plants were infected with 1 x 10^3 spores/ml and analysed 5d post infection. Data represents the DI of 5 plants. Two independent experiments were performed.

			Exp1					**Exp2**		
Plant	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Col-0	3.2	3.8	4.0	3.6	3.6	2.4	2.6	2.2	3.2	3.0
jam2	2.8	2.8	3.2	2.8	2.2	1.2	2.4	0.6	0.8	2.2
jar1	1.8	3.0	1.2	1.8	1.0	1.6	1.8	1.6	3.0	2.6

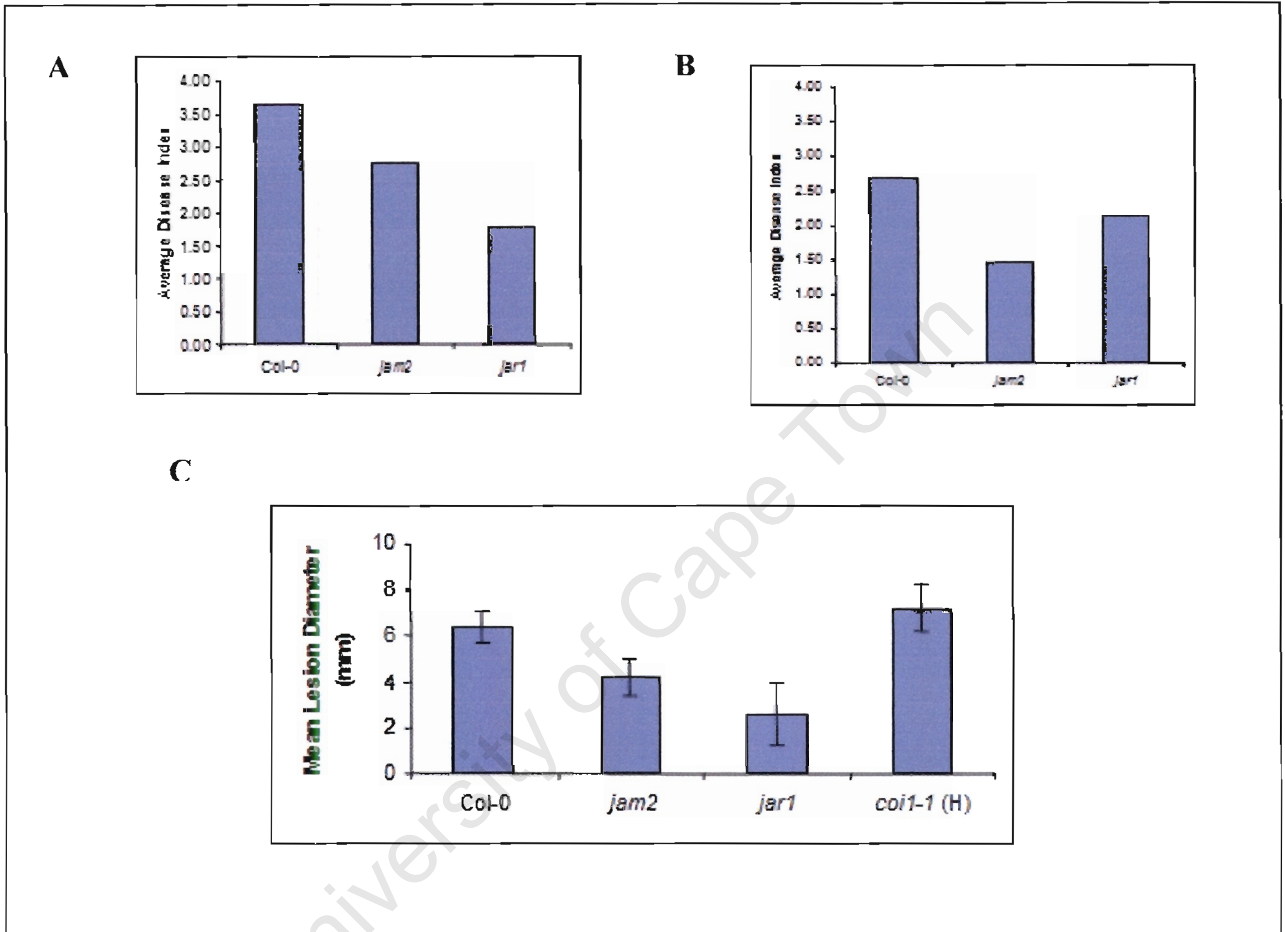


Figure 4.6: Assessment of disease development in Col-0, *jam2*, *jar1* and *coi1-1* (Homozygotes). The *B. cinerea* spore density used for all 3 experiments was 1 x 10^3 spores/ml. **A** and **B**: bars represent disease indices (DI)* for 2 independent experiments where n=5. **C**: bars represent the mean diameter of lesions measured in mm. Lesions of not less than 10 plants were assessed. Error bars represents standard errors. * DI=Σ (i x j/n) where i = infection class, j = the amount of plants scored in infection class, n = the total amount of plants in the replicate. Infection classes were scored as 0 = no infection, 1 = 50% of infected leave area showing faint chlorosis, 2 = ≥50% of infected leave area showing strong chlorosis, 3 = ≤50 % of infected leave area showing necrosis, 4 = ≥50% of infected leaf area showing necrosis of all infected leaves. DI calculation was adapted from Epple et al. (1997).

University of Cape Town

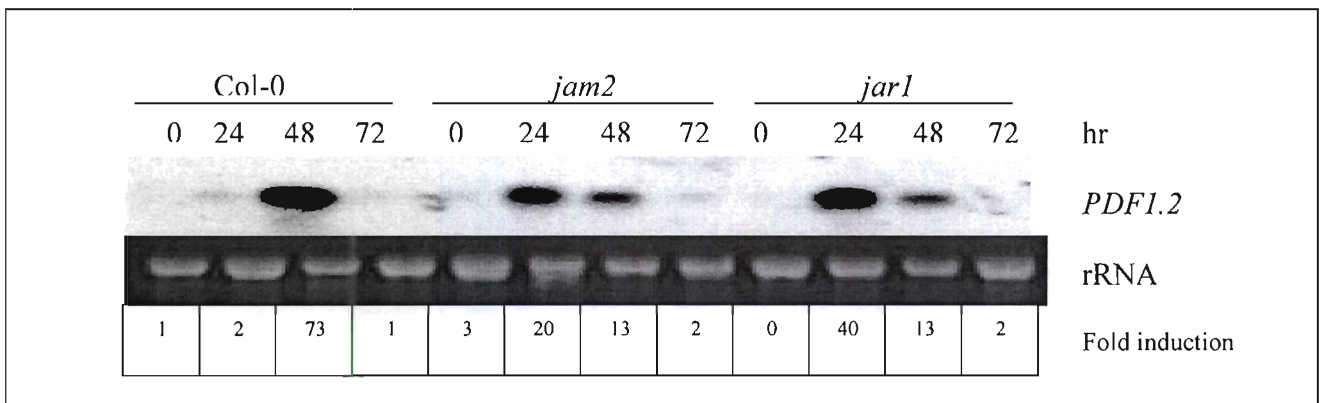


Figure 4.7: Effect of infection by *B. cinerea* on the expression of *PDF1.2* by *jam2*. Four-week-old plants were infected with 1 x 10^3 spores/ml. Tissue was harvested at 0, 24, 48 and 72 hr. Total RNA was extracted and 20μg were electrophoresed on a 1.2% Agarose/Formaldehyde gel. Total RNA was transferred onto Hybond N^+ membrane and probed with α-^{32}P labelled *PDF1.2* PCR product. Image was visualised using phosphoimaging. This experiment was repeated once with similar results.

University of Cape Town

Discussion

The aim of this study was to investigate JA signalling via a forward genetic approach. The isolation of novel JA signalling mutants would help identify components involved in JA signalling, thus aiding in the elucidation of this pathway. This was achieved through the isolation of a JA signalling mutant using a novel screening method.

The screening criterion used in this study was lack of anthocyanin accumulation upon MeJA treatment. Anthocyanins are blue, purple and red flavonoid-derived pigments, produced by plants in vegetative tissue at specific developmental stages and upon various stress conditions (Chapple et al., 1994). These stress conditions include high light, nutrient stress, cold, wounding and pathogens (reviewed in Mol et al., Lam et al., 2003). Exogenous MeJA is able to induce anthocyanin accumulation in soybean and *Arabidopsis* (Fransceschi and Grimes, 1991; Creelman and Mullet, 1992 and Feys et al., 1994). This work was based on the premise that lack of anthocyanin accumulation after MeJA application could be indicative of a disrupted JA signalling pathway. In this study, it was shown that *jam2* could produce and accumulate visible anthocyanins at the same rate as Col-0 upon lack of nutrients. Therefore, *jam2* is not affected in general anthocyanin production and accumulation. This result therefore suggests that the anthocyanin biosynthetic enzymes are not disrupted in *jam2* and that *jam2* represents a genuine JA signalling mutant. Another aim of this work was to identify other possible JA-mediated responses that could be affected in *jam2* to help determine the role of the wildtype *JAM2* gene and its possible position in a signalling pathway.

Of the four well-known JA-controlled responses analysed in this work, *jam2* was affected in only two. It was found that *jam2* displays delayed anthocyanin accumulation after treatment with MeJA. To determine the actual difference in accumulated anthocyanin levels upon MeJA treatment between Col-0, *jam2* and *jar1*, visible anthocyanins were extracted and quantified. The levels of accumulated anthocyanins in *jam2* were significantly less that in Col-0 and *jar1*, even at 120hr. Another JA signalling mutant, *coi1*, also does not produce visible anthocyanins on MeJA or coronatine (Feys et al., 1994). Similar to *jam2*, *coi1* is not an anthocyanin biosynthetic mutant because it produces anthocyanins when subjected to continuous white light or water stress (Feys et al., 1994). *COI1* is speculated to be a positively regulator of JA responses through the removal of negative regulators via the ubiquitin protein degradation pathway (Xie et al., 1998; Xu et al., 2002). It can thus be speculated that like *COI1*, *JAM2* plays a regulatory function in anthocyanin production and accumulation. What this possible regulatory role of *JAM2* in anthocyanin biosynthesis is still needs to be elucidated. However, it could be suggested that *JAM2* is involved in the temporal regulation of anthocyanin accumulation since *jam2* showed reduced and delayed accumulation upon MeJA treatment. High light conditions have been shown to induce the expression of CHS, the committing enzyme to anthocyanin biosynthesis (Feinbaum and Ausubel, 1998). More importantly, increased expression of CHS was correlated with increased enzyme activity as well as the accumulation of anthocyanin (Feinbaum and Ausubel, 1998). This is an indication that flavonoid and thus anthocyanin accumulation is regulated on a transcriptional level. MeJA induces *CHS* expression (Franscechi and Grimes, 1991; Creelman and Mullet, 1992). Moreover, no other flavonoid biosynthetic enzyme was shown to be induced by MeJA. Thus the possibility exists that *JAM2* might be involved in the regulation of *CHS*. Another possibility is that a lesion in the *CHS* promoter occurred in *jam2* mutants, thus reducing JA-responsiveness. This would be in accordance with the delayed anthocyanin accumulation phenotype of *jam2* after MeJA treatment. However, such a mutation would not account for the gene expression phenotype or pathogen resistance phenotype of *jam2*.

In contrast to the other JA insensitive mutants which are all resistant to MeJA root growth inhibition (Staswick et al., 1992, Feys et al., 1994; Berger et al., 1996), *jam2* displays stunted root growth upon MeJA treatment. Three different MeJA concentrations were tested and *jam2* exhibited stunted roots at all concentrations, similar to Col-0. The result obtained thus indicates that root growth is independent of *JAM2*. This is a surprising finding because normal root growth was found to be dependent on *COI1*, *JAR1*, *JIN1* AND *JIN4* (Staswick et al., 1992, Feys et al., 1994; Berger et al., 1996). In other words, root growth seems to be a common and general JA response affected in these JA signalling mutants.

University of Cape Town

The JA insensitive mutants used as positive controls in this study, all showed reduced root inhibition to MeJA. An interesting observation was that *jin1* roots increased concomitantly with MeJA concentrations. This means that the reduction of root growth inhibition in *jin1* is greater at higher MeJA concentrations, suggesting that *jin1* might be a strong allele for this phenotype.

PDF1.2 expression was another JA-mediated response used to assess JA signalling in *jam2*. In contrast to *coi1*, *jam2* is able to express *PDF1*.2 upon treatment with MeJA (Penninckx et al., 1996; Penninckx et al., 1998). However, *PDF1.2* expression was also induced in *jar1*, a characterised JA signalling mutant. This finding is contrary to previous reports that found no *PDF1*.2 expression in *jar1* after liquid MeJA treatment at 48 hr (Kachroo et al., 2000). It was decided to use 45 μM because this was the predominant concentration used to study *PDF1.2* expression. In this study, *PDF1*.2 induction at higher MeJA concentrations on plates and in soil was also assessed The concentration of MeJA needed to induce *PDF1.2* expression in soil grown plants was determined through titration of MeJA concentrations as adjusted from plates. The volume of air in covered plates were extrapolated to the dimensions of propagation trays used, thus taking into account diffusion of MeJA vapours to equilibrium levels. The concentrations used in the MeJA titration experiment were 5, 15, 20 and 25mM. All concentrations were able to induce *PDF1.2* (data not shown). It was decided to use 15mM MeJA because this was close to the calculated concentration. An interesting observation was the *PDF1.2* temporal expression pattern, which appeared to be biphasic for all concentrations tested. Another interesting finding was that the high concentrations of MeJA appeared to rescue the delayed second *PDF1*.2 induction peak in *jam2 and jar1*. Thus the concentration of MeJA applied to plants could have an effect on the induction patterns of JA-inducible genes, especially considering that concentrations used in experiments are higher than physiological levels. Moreover, the method of application does not seem to play a role since a biphasic induction pattern of *PDF1.2* was observed for liquid treatments of plates and for the gaseous treatment of soil grown plants. However, the *PDF1.2* induction peaks in *jam2* appeared to be differentially affected. *jam2* seems to be defective in the second peak upon liquid treatment and in the first peak upon gaseous treatment. These experiments were repeated with similar trends, although *PDF1.2* was not induced to the same intensity. This deviation could be ascribed to variation between treatments.

This biphasic phenomenon was observed for liquid and gaseous MeJA as well as after infection with *B. cinerea*. A possible explanation for this biphasic kinetics could be that MeJA induced stomatal closure (reviewed in Creelman and Mullet, 1992) and thus perception of MeJA was interrupted, leading to a

University of Cape Town

reduction in *PDF1.2* expression at 48 hr. The biphasic induction pattern of *PDF1.2* could also account for a lack of detectable *PDF1.2* in *jar1* after MeJA treatment, since *PDF1.2* expression was studied at only 48 hr (Kachroo et al., 2000). Interestingly, studies looking at P*DF1*.2 expression in *coi1* after MeJA were also conducted at only 48 hr (Penninckx et al., 1996 and 1998). Therefore this is an exciting finding, which warrants a more complete investigation of *PDF1.2* expression patterns after MeJA, including earlier time points and lower concentrations. Preliminary results showed that *PDF1.2* is induced at a MeJA concentration of 20 μM, therefore indicating that *PDF1.2* induction can occur at lower MeJA concentrations (data not shown). In addition, *PDF1.2* expression was induced at 6 and 12 hr in Col-0 and *jar1* but only at 12 hr in *jam2* at both 20 and 45 μM MeJA (data not shown), suggesting a difference in the early temporal induction between *jam2, jar1* and Col-0. However, these are preliminary results that can be followed up. Other plausible reasons for the two-phased *PDF1.2* induction pattern could be that *PDF1.2* is transiently expressed, *PDF1.2* mRNA is unstable or that *PDF1.2* expression is controlled by self-regulating transcription factors. Interestingly, *Orca* transcription factors were expressed in a biphasic manner after MeJA treatment (Menke et al., 1999; van der Fits and Memelink, 2001). Another JA-inducible gene, the *Str* gene of *C. roseus*, was also found to have a two-stage induction pattern (Menke et al., 1999). Furthermore, JA-and elicitor-responsive elements were found in the promoter of the *Str* gene to which *Orcas* bind (Menke et al., 1999). This could provide a link between the expression patterns of transcription patterns and downstream genes and thus a possible transcriptional control mechanism. Moreover, JA was found to increase in two phases after wounding (Laudert and Weiler, 1998). Whether there is a direct link between the biphasic increase of JA and the two-phase expression patterns of some JA-responsive genes is not clear.

University of Cape Town

As mentioned previously, JA specifically mediates resistance against necrotrophic pathogens. The necrotrophic fungus, *B.* cinerea was used to assess JA regulated disease resistance in *jam2*. *jam2* and *jar1* showed increased resistance to *B. cinerea* infection than Col-0. This experiment was first performed at the University of Edinburgh where similar trends were observed (data not shown). Not only does this indicate that the experiment was reproducible but it also indicates that the *B. cinerea* strain used retained its virulence in two different climates. In accordance with the slight increase in resistance in *jam2* and *jar1*, lesion growth in these mutants was slightly smaller compared to Col-0. *coi1* was used as positive control in these lesion growth experiments. Contrary to previous reports, *coi1-1* did not show significantly larger lesions than Col-0, *jam2* and *jar1* (Thomma et al., 1998; Thomma et al., 1999; Zimmerli et al., 2001). Interestingly, *jam2* and *jar1* exhibited the same *PDF1.2*

expression upon infection. These mutants displayed a faster and longer induction of *PDF1.2* compared to Col-0. Moreover, this is in accordance to the intermediate resistance shown in this work for *jam2* and *jar1*. Therefore, these results suggest that *PDF1.2* expression upon *B. cinerea* infection is either negatively regulated by *JAM2* and *JAR1* or independent of *JAM2* and *JAR1*.

These findings thus suggest that the newly isolated *jam2* mutant might be involved in the temporal regulation of anthocyanin biosynthesis and *PDF1.2* expression upon MeJA treatment and *B. cinerea* infection. Also, unlike *coi1*, *jam2* seems to be more resistant to this necrotrophic fungus. An exciting finding, however, is that *jar1* and *jam2* share these phenotypes. Therefore the possibility exists that these two mutants could be allelic. Although the *JAR1* locus has been cloned, not much characterisation of *jar1* has been performed. *JAR1* was found to encode a protein with structural homology to the firefly luciferase adenylating enzyme family (Staswick et al., 2002). Adenylated JA was proposed as a possible mechanism to control JA activity (Staswick et al., 2002). It would thus be interesting to see whether *jam2* is allelic to *jar1*. However, *jam2* and *jar1* exhibit different root growth phenotypes on MeJA, suggesting that *jam2* might define a novel locus. Therefore, further characterisation of these mutants could contribute significantly to our understanding of JA signalling.

The following chapter will focus on a possible model for *JAM2* function as well as future work

University of Cape Town

Chapter 5

Conclusion and Future Prospects

University of Cape Town

Chapter 5 Conclusion and Future Prospects

Although much work has been done to elucidate JA signal transduction, our knowledge thereof is far from complete. Various *Arabidopsis* mutants with altered JA and intermediate levels of JA have been instrumental in identifying processes requiring JA. In *Arabidopsis*, various signalling mutants have also been isolated (Table 5.1) Characterisation of these signalling mutants led to the identification of additional responses regulated by JA in *Arabidopsis* (Table 5.1). These responses range from developmental processes to various environmental stresses (Table 5.1). Moreover, the JA signalling pathway is proving to be very complex. This complexity is exacerbated by cross talk with other signalling pathways such as the ethylene and SA response pathways, particularly in defense responses. Through employing novel and unique screening methods, additional JA signalling mutants could be isolated and characterized, aiding in the elucidation of JA signalling

In this study, a novel recessive JA signalling mutant, *jam2*, was isolated using a new screening method. Like the previously isolated JA signalling mutants, with the exception of *coi1*, *jam2* is not defective in most JA-mediated responses. *jam2* is not affected in root growth, contrary to the JA insensitive mutants. This mutant, however, displays delayed anthocyanin accumulation after MeJA treatment. Moreover, *jam2* appears to be affected in MeJA induced *PDF1.2* expression. Furthermore, *jam2* exhibits slightly increased resistance to *B. cinerea* infection, which is in contrast to other JA insensitive mutants. In this study, lesion size was used to assess disease development and therefore pathogen growth. However, whether lesion size is correlated to pathogen growth was not established. Upon infection with *B. cinerea*, *jam2* exhibits faster *PDF1.2* induction, possibly leading to its increased resistance. Whether there is a correlation between *PDF1.2* expression and resistance to *B. cinerea* in *jam2* needs to be established since previous reports demonstrate that resistance to *B. cinerea* and *PDF1.2* expression is not correlated (Mengiste et al., 2003). Our working hypothesis is that *JAM2* plays a regulatory role in the accumulation of anthocyanins, *PDF1.2* expression and resistance to *B. cinerea*. The fact that *jam2* does not show strong phenotypes related to disrupted JA signalling, indicates that it is possibly not a null mutant. Such mutants are often still informative, as is the case with *jar1*. *jar1* is postulated to modify JA itself via adenylation, possibly controlling JA activity (Staswick et al., 2002). A possible model for the proposed function of *JAM2* is portrayed in Fig.5.1.

University of Cape Town

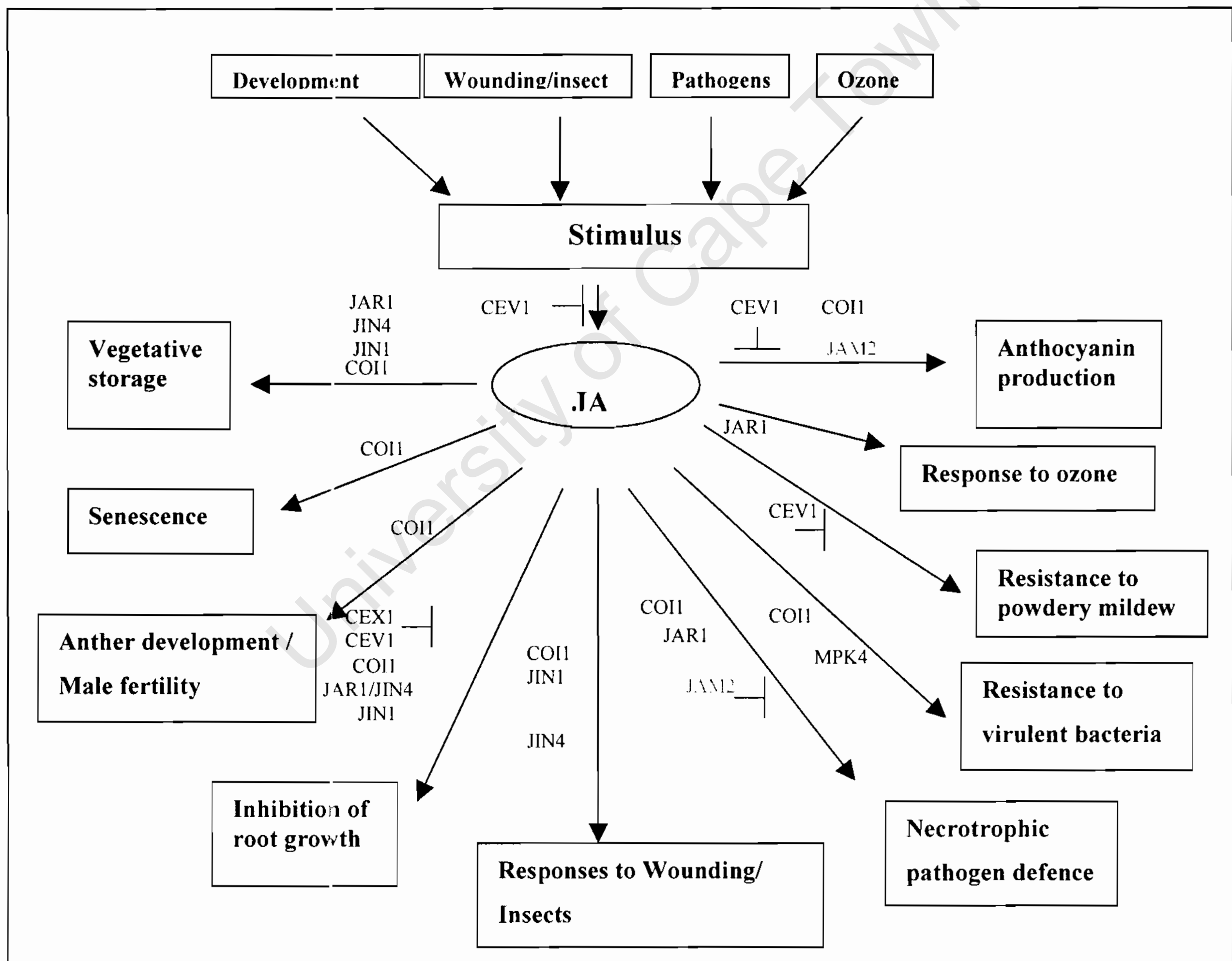
To clearly understand the function of *jam2* in JA signalling, further genetic and phenotypic analyses would have to be conducted. Only the heritability of the lack of anthocyanin accumulation phenotype displayed by *jam2* has thus far been assessed. To conclusively assess whether all the phenotypes displayed by *jam2* are due to the same recessive mutation, the segregation of anthocyanin accumulation, *PDF1.2* expression and *B. cinerea* resistance must be analysed in a backcrossed F_2 population. The possibility exists that *jam2* and *jar1* could be allelic due to their similar *PDF1.2* expression patterns and resistance profiles to *B. cinerea*. Therefore, it needs to be established whether *jam2* and *jar1* are allelic or not. The allelism test between *jam2* and *jar1* could be based on the *PDF1.2* expression pattern after MeJA treatment or infection with *B. cinerea*.

Table 5.1: JA-mediated responses as elucidated by *Arabidopsis* JA signalling mutants.

Response	JA Signalling Mutant	References
Development		
Male Fertility	*coi1*	Feys et al., 1994
Nitrogen Storage	*coi1*	Benedetti et al., 1995
	jar1	Staswick et al., 1992
	jin1 and *jin4*	Berger et al., 1996
	cev1	Ellis and Turner, 2001
	cex1	Xu et al., 2001
Senescence	*coi1*	He et al., 2001
Root Growth	*coi1*	Feys et al., 1994
	jar1	Staswick et al., 1992
	jin1 and *jin4*	Berger et al., 1996
	cev1	Ellis and Turner, 2001
	cex1	Xu et al., 2001
	joe2	Jensen et al., 2002
Cellulose synthesis	*cev1*	Ellis et al., 2002
Environmental Stress		
Wounding/Insect Herbivory	*coi1*	Titarenko et al., 1997; Stotz et al., 2002
	jin1 and *jin4*	Berger et al., 1996
Pathogen	*coi1*	Feys et al., 1994
	jar1	Staswick et al., 1998
	cev1	Ellis and Turner, 2001
	cex1	Xu et al., 2001
	cet mutants	Hilpert et al., 2001
Anthocyanin	*coi1*	Feys et al., 1994
	cev1	Ellis and Turner, 2001
	joe1	Jensen et al., 2002
Ozone	*jar1*	Overmeyer et al., 2000; Rao et al., 2000

University of Cape Town

To analyse whether *JAM2* function is dependent on any other known JA signalling component, double or triple mutants can be created with existing JA mutants, especially *coi1* and *jar1*. Through studying possible additive effects in these double or triple mutants, the position of *JAM2* in the JA signal transduction pathway could be elucidated. Since there are numerous JA responses affected in these mutants, it could prove very difficult to position *JAM2* in the JA signalling cascade. One possible aspect that could be studied to determine whether *JAM2* function is dependent on *COI1*, is MeJA root growth inhibition, since root growth in *coi1* and *jam2* is differently affected. Similarly, *jam2* and *jar1* exhibit different anthocyanin accumulation patterns after MeJA therefore the dependence of *JAM2* on *JAR1* could be established



University of Cape Town

Figure 5.1: The JA signalling pathway. External or internal stimuli are perceived with consequent JA biosynthesis. After perception of JA, JA-responsive genes are expressed leading to the appropriate responses. JA insensitive mutants are defective in signalling components, which positively regulate JA responses (indicated by →), whilst JA constitutive mutants define negative regulators of JA responses (indicated by ⊥).

As outlined in Chapter 1, JA positively regulates its own biosynthesis, therefore JA levels may be altered in mutants defective in JA signalling. Considering the different *PDF1.2* expression patterns obtained in *jam2* and *jar1* after MeJA application and *B. cinerea* infection, it therefore would be interesting to measure the JA levels present after these treatments in these genotypes. The potentiating effect of ethylene on *PDF1.2* expression and ethylene's synergistic relationship with the JA pathway cannot be discounted, thus it would also be interesting to measure ethylene levels in these mutants as well. JA and ethylene levels could be measured using gas chromatography. To further explore crosstalk between the JA and ethylene signalling pathways the analysis of double mutants between *jam2* and ethylene signalling mutants could be informative. It would also be interesting to see how *jam2* responds to wounding and insect herbivory, additional well known JA responses.

The most exciting future prospect would be to map *jam2* onto the *Arabidopsis* genome and to clone the wild type gene. The cloning of *JAM2* would be simplified due to the visible mutant phenotype exhibited by *jam2*. Following the map-based cloning of *JAM2*, complementation studies would have to be conducted to determine if the wild type gene would complement the mutant phenotypes, thereby indicating that the correct gene was cloned. The cloning of *JAM2* would open up numerous avenues to explore, including promoter analysis through deletion constructs and the isolation of possible interacting partners through techniques such as yeast-two-hybrid. Further characterisation of this newly isolated JA signalling mutant, will hopefully provide valuable answers pertaining to the function of *JAM2* in JA signalling.

University of Cape Town

Chapter 6

References

University of Cape Town

Chapter 6 References

Aarts M.G.M., Koncz C. and Pereira A. (2000). Transposon and T-DNA mutagenesis. In Wilson Z.A., *eds*, *Arabidopsis* a practical approach. Oxford University Press, Oxford, pp 143-169
Anderson M. and Wilson F. (2000). Growth, maintenance and use of *Arabidopsis* genetic resources. In Wilson Z.A., *eds*, *Arabidopsis* a practical approach. Oxford University Press, Oxford, pp **1-28**
Bai C., Sen P., Hofmann K., Ma L., Goebl M., Harper J.and Elledge S. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86**: 263-274.
Bell E. and Mullet J. E. (1993) Characterisation of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol. **103**: 1133-1137
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. USA **92**: 8675-8679
Benedetti C. E., Xie D. and Turner J. G. (1995). *COI1*-dependent expression of an *Arabidopsis* vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiol. **109**: 567-572
Bent F., Innes R., Ecker J., Staskawicz B. (1992). Disease development in ethylene-insensitive Arabidopsis thaliana infected with virulent and avirulent Pseudomonas and Xanthomonas pathogens. Mol Plant Microbe Interact. **5**: 372-378
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
Bergey D., Howe G. and Ryan C. (1996). Polypeptide signaling for plant defensive genes exhibits analogies with defense signaling in animals. PNAS **93**: 12053-12058
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. M., 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. USA **97**: 9329-9334
Bishop G. and Koncz C. (2002). Brassinosteroids and plant steroid hormone signaling.Plant Cell **14 Suppl: S97-110**
Blechert S., Brodschelm W., Hölder S., Kammerer L., Kutchan T. M., Mueller M. J., Xia Z. and Zenk M. H. (1995). The octadecanoid pathway: Signal molecules for the regulation of secondary pathways. Proc. Natl. Acad. USA **92**: 4099-4105
Bowling S., Clarke J., Liu Y., Klessig D. and Dong X. (1997). The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell **9**: 1573-1584
Brader G., Tas E. and Palva E. (2001). Jasmonate-dependent induction of indole glucosinolates in *Arabidopsis* by culture filtrates of the nonspec fic pathogen *Erwinia carotovora*. Plant Phys. **126**: 849-860
Brown R., Kazan K., McGrath K., Maclean D. and Manners J. (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene in *Arabidopsis*. Plant Physiol. **132**: 1-13
Browse J., McConn M., James D. and Miquel M. (1993). Mutants in *Arabidopsis* deficient in the synthesis of α-linolenate. J. Biol. Chem. **268**: 16345-16351
Browse J., McCourt P. and Somerville P. (1986). A mutant of *Arabidopsis* deficient in $C_{18:3}$ and $C_{16:3}$ leaf lipids. Plant Physiol. **81**: 859-964
Cao H., Bowling s., Gordon S and Dong X. (1994). Characterisation of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6**: 1583-1592
Chen W., Provart N., Glazebrook J., Katagiri F., Chang H., Eulgem T., Mauch F., Luan S., Zou G., Whitham S., Budworth P., Tao Y., Xie Z. Chen X., Lam S., Kreps J., Harper J., Si-Ammour A., Mauch-Mani B., Heinien M., Kobayashi K., Hohn T., Dangl J., Wang X. and Zhu T. (2002). Expression profile matrix if *Arabidopsis* transcription factor genes suggests putative functions in response to environmental stresses. Plant Cell **14**: 559-574
Cheong Y., Yoo C., Park J., Ryu G., Goekjian V., Nagao R., Key J., Cho M. and Hong J. (1998). STF1 is a novel TGACG-binding factor with a zinc-finger motif and a bZIP domain which heterodimerizes with GBF proteins. Plant Journal **15**: 199-209
Chomczynski P. and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**: 156-159
Coenen C. and Lomax T. (1997). Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends in Plant Sci. **2**: 351-356
Coupling G. Transposon tagg ng in *Arabidopsis*. In Koncz C., Chua N-H. and Schell J., *eds*, Methods in *Arabidopsis* research. World Scientific Publishing Co, Pte. Ltd., London, pp 290-309
Creelman R. A. and Mullet J. E. (1995). Jasmonic acid ditribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. USA **92**: 4114-4119
Creelman R. A. and Mullet J E. (1997). Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48**: 355-381
Creelman R. A. and Mulpuri R., Jasmonate (09/30/02), The *Arabidopsis* Book, *eds*. Somerville C. R. and Meyerowitz E. M., American Society of Plant Biologists, Rockville, MD, doi/10,1199/tab.0012, http://www.aspb.org/publications/arabidopsis/

University of Cape Town

Creelman R. A., Tierney M. L. and Mullet J. E. (1992). Jasmonic acid/ methyl jsmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA **89**: 4938-4941
Dammann C., Rojo E. and Sánchez-Serrano J. (1997). Abscisic acid and jasmonic acid activate wound-inducible genes in potato through separate, organ-specific signal transduction pathways. Plant Journal **11**: 773-782
Delaney T. P., Uknes S., Vernooij B., Friedrich L., Weymann K., Negrotto D., Gaffney T., Gut-Rella M., Kessmann H., Ward E. and Ryals J. (1994). Science **266**: 1247-1250
Dharmasiri S, Estelle M. (2002). The role of regulated protein degradation in auxin response. Plant Mol. Biol. **49**: 401-409
Doares S. H., Syrovets T., Weiler E. W. and Ryan C. A. (1995). Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc. Natl. Acad. Sci. USA **92**: 4095-4098
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. and Turner J. G. (2002). A conditionally fertile *coi1* allele indicates cross-talk between plant hormone signalling pathways in *Arabidopsis thaliana* seeds and seedlings. Planta **215**: 549-556
Ellis C., Karafyllidis I, Wasternack C. and Turner J. (2002). The *Arabidopsis* mutant *cev1* links cell wall signalling to jasmonate and ethylene responses. Plant Cell **14**: 1557-1566
Ennos R. (2000). Statistical and data handling skills in biology. Pearson education limited, Essex.
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**: 8136-820
Falkenstein E., Groth B., Mithöfer and Weiler E. (1991). Methyljasmonate and α-linolenic acid are potent inducers of tendril coiling. Planta **185**: 316-322
Farmer 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. USA **87**: 7713-7716
Farmer E. E. and Ryan C. A. (1992). Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinas inhibitors. Plant Cell **4**: 129-134
Feinbaum R.L. and F.M. Ausubel. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. And Cell. Biol. **8**: 1985-1992
Feys B. J. F., 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
Feys B.J. and Parker J.E. (2000). Interplay of signalling pathways in plant disease resistance. Trends in Genet. **16**: 449-455
Franceschi V. R. and Grimes H. D (1991). Induction of soybean vegetative storage proteins and anthocyanins by low-level atmospheric methyl jasmonate. Proc. Natl. Acad. Sci. USA **88**: 6745-6749
Gerhardt B. (1983). Localisation of β-oxidation enzymes in peroxisomes isolated from non-fatty plant tissues. Planta **159**: 238-246
Govrin E.M. and Levine A. (2000). Infection of *Arabidopsis* with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). Plant Mol. Biol. **48**: 267-276
Gray W., del Pozo J., Walker L., Hobbie L., Risseeuw E., Banks T., Crosby W., Yang M., Ma H., Estelle M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. Genes Dev. **13**: 1678-1991
Gray W., Kepinski S., Rouse D., Leyser O. and Estelle M. (2001). Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature **414**: 271-276
Gundlach H., Müller M. J., Kutchan T. M. and Zenk M. H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA **89**: 2389-2393
Harms K., Atzorn R., Brash A., Kühn H., Wasternack C., Willmitzer L. and Peña-Cortés H. (1995). Expression of a flax allene oxide synthase cDNA leads to increased endogenous jasmonic acid (JA) levels in transgenic potato plants but not to a corresponding activation of JA-responding genes. Plant Cell **7**: 1645-1654
Hartmann U., Valentine W.J., Christie J.M., Hays J., Jenkins G.I. and Weisshaar B. (1998). Identification of UV/blue-light response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. Plant Mol. Biol. **36**: 741-754
Haughn G.W. and Somerville C. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Genetics **204**: 430-434
Hause B., Hause G., Kutter C., Miersch O., Wasternack C. (2000). Enzymes of jasmonate biosynthesis occur in tomato sieve elements. Plant Cell Physiol. **44**: 643-648
Hayashi M., Toriyama K., Kondo M., Nishimura M. (1998). 2,4-Dichlorophenoxybutyric acid-resistant mutants of Arabidopsis have defects in glyoxysomal fatty acid beta-oxidation. Plant Cell **10**:183-195.
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
Hildmann T., Ebneth M., Peña-Cortés H., Sánchez-Serrano J., Willmitzer L. and Prat S. (1992). General roles of abscisic acid and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4**: 1157-1170

University of Cape Town

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

Holton T.A. and Cornish E.C. (1995). Genetics and Biochemistry of anthocyanin biosynthesis. Plant Cell **7**: 1071-1083

Howe G. A. and Schilmiller A. L. (2001). Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. **5**: 230-236

Ingelbrecht I. L., Mandelbaun C. I. And Mirkov T. E. (1998). Highly sensitive northern hybridisation using a rapid protocol for downward alkaline blotting of RNA. Biotechniques **25**: 420-425

Ishiguro S., Kawai-Oda A., Ueda J., Nishida I. and Okada K. (2001). The *defective in anther dehiscence1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence and flowering in *Arabidopsis*. Plant Cell **13**: 2191-2209

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

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

Kachroo P., Shanklin J., Shah J., Whittle E.J. and Klessig D.F. (2001). A fatty acid desaturase modulates the activation of defense signalling pathways in plants. Proc. Natl. Acad. Sci. USA **98**: 9448-9453

Kakimoto T. (2003). Perception and signal transduction of cytokinins. Annu Rev Plant Biol. **54**: 605-627.

Kim S., Choi J., Costa M. and An G. (1992). Identification of a G-box sequence as an essential element for methyl jasmonate response of tomato proteinase inhibitor II promoter. Plant Physiol. **99**: 627-631

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

Kinkema M., Fan W., Dong X. (2000). Nuclear localization of NPR1 is required for activation of PR gene expression. Plant Cell **12**: 2339-2350.

Kloek A. P., Verbsky M. L., Sharma S. B., Schoelz J. E., Vogel J., Klessig D. F. and Kunkel B. N. (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanism. Plant Journal **26**: 509-522

Kobe B., Deisenhofer J. (1994). The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci. **19**: 415-421

Koornneef M. and Stam P. (2000). Genetic analysis. In Koncz C., Chua N-H. and schell J., *eds*, Methods in *Arabidopsis* research. World Scientific Publishing Co, Pte. Ltd., London, pp 83-99

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

Kunst L., Browse J. and Somerville C. (1989). A mutant of *Arabidopsis* deficient on desaturation of palmitic acid in leaf lipids. Plant Physiol. **90**: 943-947

Landry L.G., Chapple C.C.S and Last R.L. (1995). Arabidopsis mutants lacking phenolic sunscreens exhibits enhanced UV-B injury and oxidative damage. Plant Physiol. **109**: 1159-1166

Laudert D. and Weiler E. W. (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant Journal **15**: 675-684

Laudert D., Pfannschmidt U., Lottspeich F., Holländer-Czytko H. 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

Laudert D., Schaller F. and Weiler E. (2000). Transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* plants overexpressing allene oxide synthase. Planta **211**: 163-165

Lehmann J., Atzorn R., Brückner C., Reinbothe S., Leopold J., Wasternack C. and Parthier B. (1995). Accumulation of jasmonate, abscisic acid, specific transcripts and proteins in osmotically stressed barley leaf segments. Planta **197**: 156-162

Léon J. and Sánchez-Serrano J. J. (1999). Molecular biology of jasmonic acid biosynthesis in plants. Plant Physiol. Biochem. **37**: 373-380

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

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

Manners J.M, Penninckx I.A.M.A, Vermaere K., Kazan K., Brown R.I., Morgan A., Maclean D.J., Curtis M.D., Cammue B.P.A. 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

Mason H. and Mullet J. (1990). Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding and jasmonic acid. Plant Cell **2**: 569-579

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

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

McConn M., Creelman R., Bell E., Mullet J. and Browse J. (1997). Jasmonate is essential for insect defense in *Arabidopsis*. Plant Biol. **94**: 5473-5477

University of Cape Town

McConn M., Hugly S., Browse J. and Somerville C. (1994). A mutation at the *fad8* locus of *Arabidopsis* identifies a second chloroplast ω-3 desaturase. Plant Physiol. **106**: 1609-1614
Melan M. A., Dong X., Endara M. E., Davis K. R., Ausubel F. M. and Peterman T. K. (1993). An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid and methyl jasmonate. Plant Physiol. **101**: 441-450
Memelink J., Verpoorte R. and Kijne J. W. (2001). ORCAnisation of jasmonate responsive gene expression in alkaloid metabolism. Trends Plant Sci. 6: 212-219
Menke F., Champion A., Kijne J. and Memelink J. (1999). 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 Journal **18**: 4455-4463
Menkens A., Schindler U., Cashmore A. (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
Mengiste T., Chen X, Salmeron J. and Dietrich R. (2003). The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell **15**: 2551-2565
Mikkelson M. D., Petersen B. L., Glawischnig E., Jensen A. B., Andreasson E. and Halkier B. A. (2003). Modulation of the *CYP79* genes and glucosinolate profiles in *Arabidopsis* by defense signalling pathways. Plant Physiol. **131**: 298-308
Mikkelson M., Petersen B., Glawischnig E., Jensen A., Andreasson E. and Halkier B. (2003). Plant Phys. **131**: 298-308
Mol J., Stuitje A., Gerats A., van der Kol A. and Jorgensen R. (1989). Trends in Biotech. 7: 148-153
Moran P.J. and Thompson G.A. (2001). Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiol. **125**: 1074-1085
Mueller M. J., Brodschelm W, Spannagl E. and Zenk M. H. (1993). Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc. Natl. Acad. Sci. USA **90**: 7490-7494
Murray S. Thomson C., Chini A., Read N. and Loake G. (2002). Characterization of a novel, defense-related *Arabidopsis* mutant, *cir1*, isolated by luciferase imaging. Mol. Plant Microbe-Interaction **15**: 557-566
Müssig C., Biesgen C., Lisso J., Uwer u., Weiler E.W., Altmann T. (2000). A novel stress-inducible 12-oxophytodienoate reductase from *Arabidopsis thaliana* provides a potential link between brassinosteroids action and jasmonic acid synthesis. J. of Plant Physiol.
Nawrath C. and Metraux J-P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11**: 1393-404
Norman-Setterblad C., Vidal S. and Palva E. (2000). Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia* caraotovora. MPMI **13**: 430-438
Norman-Setterblad C., Vidal S. and Palva T. (1994). Interacting signal pathways control defense gene expressing in *Arabidopsis* in response to cell wall-degrading in enzymes from *Erwinia carotovora*. MPMI **13**: 430-438
O'Donnell P., Calvert C., Atzorn R., Wasternack C., Leyser H., Bowles D. (1996). Ethylene as a Signal Mediating the Wound Response of Tomato Plants. Science **274**: 1914-1917
Overmeyer K., Tuominen H., Kettunen R., Betz C., Langebartels C., Sandermann, Jr. H. and Kangasjärvi J. (2000). Ozone-sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signalling pathways in regulating superoxide-dependent cell death. Plant Cell **12**: 1849-1862
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 Journal **31**: 1-12
Pasquali G., Erven A., Ouwerkerk P., Menke F., Memelink J. (1999). The promoter of the strictosidine synthase gene from periwinkle confers elicitor-inducible expression in transgenic tobacco and binds nuclear factors GT-1 and GBF. Plant Mol. Biol. **39**:1299-1310
Penninckx I. A. M. A., Eggermont K., Terras F. R. G., Thomma B. P. H. J., De Samblanx G. W., Buchala A., Métraux J., 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
Penninckx I.A.M.A., Thomma B.P.H.J., Buchala A., Métraux J-P. and Broekaert W.F (1998). Concomittant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell **10**: 2103-2113
Pieterse C., van Loon L. (1999). Salicylic acid-independent plant defence pathways. Trends Plant Sci. **4**: 52-58.
Pieterse C., van Wees S., van Pelt J., Knoester M., Laan R., Gerrits H., Weisbeek P., van Loon L. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. (1998). Plant cell **10**: 1571-1580
Rao M. V., Lee H-L., Creelman R. A., Mullet J. E. and Davis K. R. (2000). Jasmonic acid signalling modulates ozone-induced hypersensitive cell death. Plant Cell **12**: 1633-1646
Rédei G.P. and Koncz C. (1992). Classical mutagenesis. In Koncz C., Chua N-H. and schell J., *eds*, Methods in *Arabidopsis* research. World Scientific Publishing Co, Pte. Ltd., London, pp 16-82
Reymond P. and Farmer E. (1998). Jasmonate and salicylate as global signals for defense gene expression. Curr. Opinin. Plant Biol. **1**: 404-411
Richmond T. A. and Bleecker A. B. (1999). A defect in β-oxidation causes abnormal inflorescence development in *Arabidopsis*. Plant Cell **11**: 1911-1923

University of Cape Town

Rojo E., Titarenko E., León J., Berger S., Vancanneyt G. and Sánchez-Serrano J. (1998). Reversible protein phosphorylation regulates jasmonic acid-dependent and –independent wound signal transduction pathways in *Arabidopsis thaliana*. Plant Journal **13**: 153-165
Rouster J., Leah R., Mundy J. and Cameron-Mills V. Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase1 gene expressed in barley grain. Plant Journal **11**: 513-523
Sambrook J., Fritsch E.F. and Maniatis T. (1989). Molecular Cloning: A laboratory manual, second edition. e*ds* Nolan C. Cold Spring Harbour Laboratory Press, New York pp. **9.49, B.15**
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 research **8**: 153-161
Sharma Y. K. and Davis K. R. (1994). Ozone-induced expression of stress-related gene sin *Arabidopsis thaliana*. Plant Physiol. **105**: 1089-1096
Schaller F. (2001). Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. J. Exp. Bot. **52**: 11-23
Schaller F. and Weiler E. W. Molecular cloning and characterisation of 12-oxophytodienoate reductase, an enzyme of the octadecanoid signalling pathway from *Arabisopsis thaliana*. J. Biol. Chem. **272**: 28066-28072
Schaller F., Biesgen C., Müssig C., Altmann T. and Weiler E. (2000). 12-oxophytodienoate reductase3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta **210**: 979-984
Schaller F., Hennig P. and Weiler E. W. (1998). 12-oxophytodienoate-10, 11-reductase: Occurrence of two isozymes of different specificity against stereoisomers of 12-oxophytodienoic acid. Plant Physiol. **118**: 1345-1351
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
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 transferase: A key enzyme for jasmonic acid regulated plant responses. Proc. Natl. Acad. Sci. USA **98**: 4788-4793
Shah J., Kachroo P., Klessig D. (1999). The Arabidopsis *ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. Plant Cell **11**:191-206
Shah J., Tsui F. and Klessig D. (1997). Characterization of a Salicylic Acid-Insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. MPMI **10**: 69-78
Sivasankar S., Sheldrick B. and Rothstien S. J. (2000). Expression of allene oxide synthase determines defense gene activation in tomato. Plant Physiol. **122**: 1335-1342
Skowyra D., Craig K.L., Tyers M., Elledge S.J. and Harper J. W. (1997). F-Box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell **91**: 209-219
Skowyra et al.(1999) Reconstitution of the G_1 cyclin ubiquitination with complexes containing SCF^{GRR1} and RBX1. Science **284**:662-665
Solano R., Ecker J. (1998). Ethylene gas: perception, signaling and response. Curr Opin. Plant Biol. **1**: 393-398
Solano R., Stepanova A., Chao Q., Ecker J. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **1**: 3703-3714
Spoel S., Koornneef A., Claessens S., Korzelius J., van Pelt J., Mueller M., Buchala A., Métraux J., Brown R., Kazan K., van Loon L., Dong X. and Pieterse C. (2003). NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15**: 760-770
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. USA **89**: 6837-6840
Staswick P. E., Yen G. Y. and Lehman C. C. (1998). Jasmonate signalling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant Journal **15**: 747-754
Staswick P., Tiryaki I. and Rowe M. (2002). Jasmonate response locus *JAR1* and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity in jasmonic, salicylic acid and indole-3-acetic acids in an assay for adenylation. Plant Cell **14**: 1405-1415
Stepanova A. and Ecker J. (2000). Ethylene signaling: from mutants to molecules. Curr. Opin. Plant Biol. **3**: 353-360
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. USA **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 cyclopentanones. Proc. Natl. Acad. Sci. USA **98**: 12837-12842
Stotz H. U., Koch T., Biedermann A., Weniger K., Boland W. and Mitchell-Olds T. (2002). Evidence for regulation of resistance in *Arabidopsis* to Egyptian cotton worm by salicylic and jasmonic acid signalling pathways. Planta **214**: 648-652
Sun T. (2000). Gibberellin signal transduction. Curr. Opin. Plant Biol. **3**: 374-380

University of Cape Town

Thaler J. (1999). Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature **399**: 686-688
The *Arabidopsis* Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**: 796-815
Thomma B. P. H. J., Eggermont K., Broekeaert W. F. and Cammue B. P. A. (2000). Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. Plant Physiol. 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
Thomma B. P. H. J., Eggermont K., Tierens K. F. M-J. and Broekaert W. F. (1999). Requirement of functional *Ethylene-Insensitive2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. **121**: 1093-1101
Tijet N. and Brash A. R. (2002). Allene oxide synthases and allene oxides. Prostaglandins and other Lipid mediators **68-69**: 423-431
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
Titarenko E., Rojo E., León J and Sánchez-Serrano J. J. (1997). Jasmonic acid –dependent and –independent signalling pathways contolr wound-induced gene activationin *Arabidopsis thaliana*. Plant Physiol. **115**: 817-826
Turner J., Ellis C. and Devoto A. (2002). The jasmonate signal pathway. Plant Cell **S153-S164**
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 Journal **25**: 43-53
van der Fits L., Zhang H., Menke F., Deneka M. and Memelink J. (2000). A *Catharanthus roseus* BPF-1 homologue interacts with an elicitor-responsive region of the secondary metabolite biosynthetic gene *Str* and is induced by elicitor via a JA-idependent signal transduction pathway. Plant Mol. Biol. **44**: 675-685
van Wees S., de Swart E., van Pelt J., van Loon C. and Pieterse C. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate-and jasmonate –dependent defense pathways in *Arabidopsis thaliana*. PNAS **97**: 8711-8716
Vick B. and Zimmerman D. (1981). Lipoxygenase, hydroperoxide isomerase and hydroperoxide cyclase in young cotton seedlings. Plant Phys. **67**: 92-97
Vick B. and Zimmerman D. (1984). Biosynthesis of jasmonic acid be several plant species. Plant Physiol. **75**: 458-461
Vick B. and Zimmerman D. (1987). Pathways of fatty acid hydroperoxide metabolism in spinach leaf chloroplast. Plant Physiology **85**: 1073-1078
Vijayan P., Shockey J., Lévesque C. A., Cook R. J. and Browse J. (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Plant Biol. **95**: 7209-7214
Vogler H. and Kuhlemeier C. (2003). Simple hormones but complex signalling. Curr. Opin. Plant Biol. **6**: 51-6.
von Malek B., van der Graaff E., Schneitz K. and Keller B. (2002). The *Arabidopsis* male-sterile mutant *dde2-2* is defective in the allene oxide synthase gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216**: 187-192
Wang C., Zien C. A., Afitlhile M., Welti R., Hildebrand D. F. and Wang X. (2000). Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in *Arabidopsis*. Plant Cell **12**: 2237-2246
Weber H., Vick B. and Farmer E. (1997). Dinor-oxo-phytodienoic acid: A new hexadecanoid signal in the jasmonate family. PNAS **94**: 10473-10478
Weigel D., Ahn J.H, Blásques M.A., Borevitz J.O., Christensen S.K., Fankhauser C., Ferrándiz C., Kardailsky I., Malancharuvil E.J., Neff M.M., Nguyen J.T., Sato S., Wang Z-Y., Xia Y., Dixon R.A., Harrison M.J., Lamb C.J., Yanofsky M.F. and Chory J. (2000). Activation tagging in *Arabidopsis*. Plant Phys.**122**: 1003-1013
Weisshaar B. and Jenkins G.I. (1998). Phenylpropanoid biosynthesis and its regulation. Curr. Opin. Plant Biol. **1**: 251-257
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 L., Liu F., Lechner E., Genscik P., Crosby W.L., Ma H., Peng W., Huang D. and Xie D. (2002). The SCF^{COI} ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell **14**: 1919-1935
Xu L., Liu F., Wang Z., Peng W., Huang R., Huang D. and Xie D. (2001). An *Arabidopsis* mutant *cex1* exhibits constant accumulation of jasmonate-regulated *AtVsp*, *Thi2.1* and *PDF1.2*. FEBBS Letters **494**: 161-164
Xu Y., Chang P., Narasimhan M., Raghothama K., Hasegawa P. and Bressan R. (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6**: 1077-1085
Zhou J., Tang X. and Martin G. (1997). The *Pto* kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. Embo J. **16**: 3207-3218
Ziegler J., Stenzel I., Hause B., Maucher H., Hamberg M., Grimm R., Ganal M. and Wasternack C. (2000). Molecular cloning of allene oxide cyclase. Journal of Biological Chemistry **275**: 19132-19138
Zimmerli L., Métraux J-P. and Mauch-Mani B. (2001). β-Aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol. **126**: 517-523

University of Cape Town