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UNCOVERING THE ROLE OF S-NITROSYLATION IN JASMONIC ACID SIGNALLING DURING THE PLANT IMMUNE RESPONSE

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Abstract

Plants have evolved a plethora of effective mechanisms to protect themselves from biotic stresses. Jasmonates (JAs) are employed as vital defence signals against both insect and pathogen attack. Jasmonic acid (JA) signalling plays a central role in plant defence and development.

S-nitrosylation, a redox-based post-translational modification plays an important role in plant disease resistance. S-nitrosoglutathione (GSNO) is formed by the reaction of antioxidant glutathione (GSH) and nitric oxide (NO) and acts as a mobile reservoir of NO bioactivity. The *Arabidopsis thaliana S-NITROSOGLUTATHIONE REDUCTASE* (*AtGSNOR1*) controls multiple modes of disease resistance via S-nitrosylation. In this context, the *Arabidopsis* loss-of-function mutant *atgsnor1-3* exhibits higher susceptibility to *Botrytis cinerea* a necrotrophic pathogens and *Pieris rapae* insect attack. Accumulation of JA was reduced in *atgsnor1-3* after mechanical wounding. JA marker genes were also downregulated in *atgsnor1-3* compared to Col-0 after Methyl Jasmonate (Me-JA) treatment.

The relative gene expression of *Vegetative Storage Protein* (*VSP*) was reduced in *atgsnor1-3* compared to wild type. Further, protein-protein interaction experiments in yeast two hybrid assays revealed an inhibition of Coronatine-insensitive 1 (COI1) and Jasmonate ZIM domain (JAZ1) interactions upon NO donor application. Interestingly it was also shown that Nitric oxide donor may inhibited the degradation of JAZ1-β-glucoronidase (GUS) fusion protein driven by a CaMV*35s*:: *JAZ1*-GUS transgene in GUS histochemical analysis but not in flurometric assay.

A biotin switch assay of recombinant JAZ1-Maltose-binding protein (MBP) has shown that JAZ1-MBP was S-nitrosylated and mass spectrometry suggested Cysteine229 (Cys229) was the site of this modification. Further, CaMV35S::*JAZ1*-Flag transgene expressed in either a wild-type or *atgsnor1-3* genetic background, suggested that JAZ1 was S-nitrosylated *in vivo*.

Collectively, our data imply that JA-signalling engaged in response to either insect predation or attempted *B. cinerea* infection is under redox control as high SNO in *atgsnor1-3* has disrupted the JA signalling pathway. Furthermore, our data suggest that S-nitrosylation of Cys-229 of JAZ1 may control JA-mediated signalling by blocking the interaction of this protein with COI1, thus reducing the turnover of JAZ1 by the 26S proteasome and consequently enabling continued JAZ1-mediated repression of JA-dependent gene expression in the presence of Me-JA. Thus our findings highlight the importance of NO and associated S-nitrosylation in JA signalling during plant immune response.

Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

Priya Vijay Ayyar

Remark- Data shown in Chapter 4th Fig.4.1 & 4.2 were generated by Steven Charlesworth
Data shown in Chapter 6th Fig.6.1 was generated by Dr. Byung wook Yun

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Contents

Abbreviations

µg: Microgram

µl: Microlitre

35S: Cauliflower mosaic virus 35S promoter

ABA: Abscisic acid

ACS : (acyl-CoA synthetase),

ACX : (acyl-CoA oxidase)

α-linolenic acid (18:3) : (alpha linolenic),

AOS: (allene oxide synthase)

AOC: (allene oxide cyclase)

ASK: (Arabidopsis-SKP1-like (ASK) protein)

At: *Arabidopsis thaliana*

BLAST: Basic Local Alignment Search Tool

CaMV: Cauliflower Mosaic Virus

cev1:(constitutive expression of *VSP)*

COI1: (Coronatine insenstitive1)

Col-0: *Arabidopsis* ecotype Columbia

CUL1: (Cullin1)

cis-(+)-OPDA: (12-oxophytodienoic acid)

CysNO: S-nitrosocysteine

CTS: (peroxisomal ABC transporter protein COMATOSE)

DAD1: (*DEFECTIVE IN ANTHER DEHISCENCE1)*

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E. coli: *Escherichia coli*

EGL3: (Enhancer of Glabra3)

fad3/7/8: (f*atty acid desaturase 3/7/8)*

fou2: (*fatty acid oxygenation up-regulated 2)*

GL3: (Glabra3)

GM: Genetically Modified

GSH: Glutathione

GSNO: S-nitrosoglutathione

GSNOR: S-nitrosoglutathione Reductase

GSSG: Glutathione Disulphide

GST: Glutathione-S-Transferase

GUS: β-glucuronidase

HR: Hypersensitive response

IPTG: Isopropyl- β -thio Galactopyranoside

ICS: Isochorismate Synthase

JA: Jasmonic Acid

Jasmonoyl isoleucine: (JA-Ile)

Jasmonate ZIM Domain: (JAZ)

JAR1: (JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE1)

JA-CoA: (jasmonoyl CoA)

JMT:(Carboxyl methyltransferase)

(+)–7-*iso*-JA-lle: ((+)-7-iso-jasmonoyl isoleucine)

KAT: (3-ketoacyl-CoA thiolase)

kDa: Kilodalton

LB: Luria Bertani medium

LRR: Leucine-rich Repeat

lox3/4: (*lipoxygenase3/4*)

MAMP: Microbe-associated Molecular Pattern

MAPK: Mitogen-activated Protein Kinase

Me-JA: Methyl Jasmonate

MFP: (multifunctional protein)

MMTS: S-methylmethanethiosulfonate

MS: Murashige and Skoog medium

NahG: Bacterial transgene encoding Salicylcate hydroxylase

NADH: Nicotinamide Adenine Dinucleotide

NADP(H): Nicotine adenine dinucleotide phosphate (reduced form)

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NPR1: NONEXPRESSOR OF PR GENES, also known as NIM1

OD: Optical density

OPR: (12-oxophytodienoate reductase)

OPC8:0: (3-oxo-2(2'[*Z*]-pentenyl)-cyclopentane-1-octanoic acid)

opr3: (*12-oxophytodienoate reductase*)

OPC6:0: (3-oxo-2-(2'-pentenyl) cyclopentanehexanoic acid)

PAGE: Polyacrylamide Gel Electrophoresis

PAMP: Pathogen-associated Molecular Pattern

PBS: Phosphate Buffered Saline

PCD: Programmed Cell Death

PCR: Polymerase Chain Reaction

PIs: (Proteinase inhibitors)

PLA2: *(Phospholipase A2)*

PMT: (Putrescine *N-Methyltransferase)*

PR: Pathogen Related protein

R: Resistance gene

RBX: (RING box protein1)

REDOX: Reduction oxidation

RNA: Ribonucleic Acid

ROS: Reactive oxygen species

RT: Reverse transcription

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

SNO: S-nitrosothiol

spr8:(*suppressor of prosystemin-mediated responses8*)

TAIR: The *Arabidopsis* Information Resource

T-DNA: Transfer DNA

TT8: (Transparent Testa8)

Ub: Ubiquitin

TE: (thioesterase)

WT: Wild type

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Chapter 1

1. Introduction

1.1 General Introduction

1.1.1 Global Food Security

The world's population is expected to increase from 6.7 in 2012 to 9 billion by the year 2050 (kazanRonald, 2011). In respect to the Food and Agriculture Organization of the United Nations (FAO., 2009), 70% more food must be produced to nourish a human population. Agriculture plays a vital role in providing food and income for the majority of the population (Diao et al., 2007). Plant diseases can dramatically reduce the crop yield. Enormous losses of crops can occur at different stages of plant growth and development and from the time of seed sowing till the process of harvesting and storage. Historical evidence of plant disease epidemics are the Irish Famine due to late blight of potato (Ireland, 1845), Bengal famine due to the brown spot of rice (India, 1942) and coffee rust (Sri Lanka, 1967). Such epidemics left their effect on the economy of the affected countries. Losses in crop yield due to infections caused by pathogens range between 20-40% (Savary et al., 2012). Wheat and cotton losses are around 50-80%, while maize, barley, rice and soybean losses are 12%, and groundnuts and potatoes display a 24% yield loss due to pathogen infection (Oerke, 2006). At present, the record in 2015/16 in the European Union (EU) has demonstrated that the corn production has gone down to 23% from the last year's record (FAS, 2015). Modern science offers humankind a powerful instrument to assure food security for all. Pesticides and fungicides have provided effective protection under the regime of the green revolution, but their application compromised the soil texture, water quality and also promoted the emergence of resistant pathogen strains (Adenle, 2011). Chemical controls are usually beyond the means of farmers in developing nations (McDowell and Woffenden, 2003). Crop biotechnology offers some possible solutions to these pressing problems (Gressel, 2010). Genetically modified (GM) crops started initially in 1996 were first deployed in the USA and China.. A genetically engineered line Bt cotton contains genes from *Bacillus thuringiensis* (Bt), a soil bacterium which enables the plant to produce toxins that are harmful to the pests like *Helicoverpa armigera* and Spodoptera insects (Patil et al., 2013). Later there was a steady growth of

cultivation of GM crops in developing countries (Redenbaugh et al., 1992). Bt cotton hybrids were first introduced in 2002 in India and 7 million farmers have adopted Bt cotton on 26 million acres, covering 90% of the total Indian cotton area (James, 2011) despite the anti-GM movements. Countries like China and Pakistan have also utilised Bt transgenic approaches in cotton, tobacco, pepper, rice, tomato and cucurbits. The implementation of GM crops has decreased not only the production cost but also the use of toxic chemicals (Pearce, 2002). However, the risk assessment is set up by the European Food Safety Authority (EFSA) for GM crops where molecular characterisation, nutritional potential, agronomic value, potential toxicity and environmental impact are all taken into account (van Haver et al., 2008).

Thus, understanding the molecular mechanisms involved in plant signalling and plant pathogen interactions may help guide the future breeding or design of crops with increased disease resistance against multiple plant pathogens.

1.1.2 Plant Stress Response

Plants are living in a complex environment in which they closely interact with various potential microbial pathogens (Musser et al., 2002; Schoonhoven et al., 2005). Plants are constantly challenged by various environmental cues like water logging, unfavourable temperatures, drought, and too much or too little light, salinity and UV-B radiation. Biotic and abiotic stresses can have a huge impact on world agriculture that has reduced average yields to>50% for most major crop plants (Wang et al., 2003). Plants should be capable of adjusting multiple stress conditions, as in many cases individual stress would induce an opposing reaction. For example, plants are opening stomata in order to cool the leaves when they are under heat stress as heat stress is accompanied by enhanced respiration and stomata opening but this would be a disadvantage under drought conditions as more water would be lost (Rizhsky et al., 2004). Similarly under heat stress, increased transpiration may cause an enhanced uptake of heavy metals and salts, which could cause plant damage (Mittler and Blumwald, 2010). Plants have to efficiently balance between growth and defence against multiple stresses (Bechtold et al., 2010; Herms and Mattson 1992; Smith and Stitt, 2007; Tian et al., 2003). Plants exposed to stresses can reduce plant fitness in terms of growth and yield (Bolton, 2009; Massad et al., 2012) for example; insect herbivorous reduces plant fitness (Crawley, 1983; Marquis, 1982). Stress responsive transcription factors (TFs) play a key role in plant stress adaptation by controlling the expression of a plethora of gene products that collectively help ameliorate the impact of environmental stresses (AbuQamar et al., 2009;

Dubos et al., 2010; Mengiste et al., 2003; Narusaka et al., 2004; Vannini et al., 2006; Zhang et al., 2006). TFs may also get post- translationally modified.

1.2 Induction of Plant Immunity

1.2.1 PAMP-triggered immunity

In contrast to animals, plants do not have a circulating system, but have developed an inducible defence mechanism to defend themselves against microbial pathogens. The chemical and physical barriers present on the surface of plants hinder the invasion and colonisation of microbial pathogens. Sometimes the pathogen is able to enter inside through injury or openings like stomata. Thus, a sophisticated defence mechanism is activated upon attempted pathogen infection that is known as plant innate immunity ((Jones and Takemoto, 2004; Jones and Dangl, 2006). Pathogen-associated molecular patterns (PAMPs) are associated with a group of pathogens and microbial-associated molecular patterns (MAMPs) like lipopolysaccharides, flagellin are recognised by pathogen recognition receptors (PRRs). PAMPs are also referred to as MAMPS (Zipfel, 2008, 2009). Another class of elicitors of immune responses are damage-associated molecular patterns (DAMPs) that are the result of damaged host cells (Rubartelli and Lotze, 2007).

PRRs are predominantly located in the plasma membrane but also can be localised in the cytoplasm (Nurnberger et al., 2004). Plants recognise microbes via receptor-like kinases (RLKs) and receptor-like proteins (RLPs). These are the two types of PRRs found in plants (Fritz-Laylin et al., 2005).

PAMP-triggered immunity (PTI) is the first inducible response upon the PAMP perception. Bacterial flagellin (flg) is an elicitor of PTI that triggers the defence response in different plants (Gomez-Gomez and Boller, 2002). The flg22 is a compound of 22 amino acid from the N-terminal domain of bacterial flagellin ((Boller and Felix, 2009). Elongation factor (EF-Tu) is the most abundant bacterial protein that acts as a PAMP in *Arabidopsis* and the Brassicaceae family. The two peptides elf18/elf26 are an acetylated N-terminal portion of EF-Tu (Kunze et al., 2004). From the studies of signal transduction and PRR activation, a second RLK is identified involved in flagellin signalling termed as BAK1 (BRI1 associated receptor kinase 1) (Chinchilla et al., 2007; Heese et al., 2007). Recently BAK1 was identified as a positive regulator of PRRs (Heese et al., 2007). Silencing *BAK1* expression results in plants susceptibility against necrotrophic fungal infection in *Arabidopsis*

(Kemmerling et al., 2007). Mitogen-activated protein kinases (MAP Kinase) signalling cascades (MEKK1, MKK4/MKK5 and MPK3/MPK6) are activated by the FLS2-BAK1 (flagellin sensing 2) that results in the induction of TFs such as WRKY22 and WRKY29 that functions downstream of FLS2. Thus, the activation of MAPK cascades confer increased resistance in plants against fungal as well as bacterial infection (Asai et al., 2002).

1.2.2 PTI induction and Suppression

Pathogenic bacteria have acquired a collection of virulence factor termed as effectors that can be injected into the host cells to suppress the PTI using a type III secretion system (TTSS) (Jones and Dangl, 2006). *Pseudomonas syringae* (*P. syringae*), a bacterial strain is capable of secreting almost 20-30 effectors during the plant immune responses (Chang et al., 2005). This effector interfere the components of PTI resulting into effector-triggered susceptibility (ETS) (Schulze-Lefert and Panstruga, 2003). Similarly, oomycetes and pathogenic fungi are capable of delivering the effectors into the host cells by haustoria and extra-haustorial matrix (Birch et al., 2008). Resistance (R) protein, a plants intracellular immune receptor helps in keeping the cell under surveillance of any pathogen effectors (Spoel and Dong, 2012).

Various effectors have been recognised and characterised like AvrPto1, AvrE1, HopM1, AvrRpm1 and AvrRpt2, these effectors alter the basal defence by suppressing the papilla formation. Effectors like AvrB1 and Hop change hormonal responses while AvRpm1, AvrRpt2, AvrB2 and Hop suppress the cell death (Grant et al., 2006). *P. syringae* effectors like HopM target *Arabidopsis* protein families like *Arabidopsis* adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) manipulating vesicle transport of host cells (Nomura et al., 2006). The *Pseudomonas* avirulence effector perceived by tomato R protein Pto (AvrPtoB), is a bipartite protein. The amino terminus of AvrPtoB contributes to virulence while the effectors C- terminus is capable of blocking the cell death (Abramovitch et al., 2003). Further studies have shown the domain of the C-terminus folds into an active functional E3 ligase that involves host protein degradation (Janjusevic et al., 2006). The AvrPtoB E3 ligase ubiquitinated the Fen protein and promoted its degradation in a proteosome degradation manner (Rosebrock et al., 2007).

1.2.3 *R* gene mediated resistance

PTI is suppressed by effector interference that can be recognised in some cases by the resistance (*R*) gene product that triggers the second immune system like effective-triggered immunity (ETI). The recognised effectors are termed as avirulence (Avr) protein. Some R proteins have been identified and almost 150 are found in *Arabidopsis* (Meyers et al., 2003) and 600 in *Oryza sativa* (Goff et al., 2002). The understanding of *R* gene-mediated resistance was discovered a long time ago by the studies of flax (*Linum usitatissimum*) and fungal pathogen *Melampsora lini* in the 1940s by Flor (Campbell et al., 2002). Nucleotide-binding site-leucine rich repeat (NBS-LRR) has a domain structure similarity to the animal nucleotide-binding oligomerization domain (NOD) and LRR-containing protein (NLR) protein (Ting and Davis, 2005). The structure of NB-LRR defines as NBS domain in the centre. The C-terminus consists of LRR region while the amino terminus has the toll-interleukin-1 receptor (TIR) and the coiled-coil (CC) domain (Tameling and Joosten, 2007). NB-LRR is the largest class of proteins that is utilised by ETI (Dangl and Jones, 2001). However, there is an indirect recognition used by NB-LRR to detect effectors which is known as guard hypothesis. The NB-LRR proteins are capable of altering the effector targets to reduce the effectors interference (Dangl and Jones, 2001). In the guard hypothesis, the R protein is guards and effector targets which are referred as 'guardees' (Dangl and Jones, 2001; Marathe and Dinesh-Kumar, 2003; Van der Biezen and Jones, 1998) . One of the best-studied examples is the RPM1-Interacting protein 4(RIN4). The RIN4 are the 'guardees' of R proteins and are targeted and modified by the effectors of *P. syringae* like AvrB, AvrRpm1 and AvrRpt2 (Kim et al., 2005; Mackey et al., 2002). After the recognition of modified RIN4 by R protein RPM1 and RPS2 this leads to the hypersensitive response (HR) and plants develop resistance (Kim et al., 2005; Ong and Innes, 2006). However, some *P. syringae* have an Avr protein like AvrRpt2 used to overcome this phenomenon. AvrRpt2 is a cysteine protease that cleaves RIN4 and thus, plants are susceptible. In some plants, an R protein like RPS2 recognises AvrRpt2 cleavage of RIN4 and triggers plant defence (Axtell and Staskawicz, 2003; Mackey et al., 2003). For the function of various R proteins, post-translational modification are sometimes required like phosphorylation, protein degradation or localisation in host cells (Martin et al., 2003).

1.2.4 Hypersensitive response

The Hypersensitive response (HR) is a defence mechanism utilised by the plants against invading pathogens. It is the result of an interaction between products of the *Avr* gene of the pathogen and the *R* gene of plants (Dodds et al., 2006). HR is the result of an incompatible reaction in which the *Avr* gene matches the *R* gene of plants while the compatible reaction is when the *Avr* gene does not match with the plants *R* gene resulting in growth of the pathogen in plants and disease (Moffett, 2009). Based on some similarities in animal programmed cell death like apoptosis, HR is suggested to be a kind of programme cell death (PCD). PCD is associated with cell death at the site of infection as it inhibits the growth of invading pathogens by killing the cell (Heath, 2000; Jacobson et al., 1997).

The dying cells accumulate toxic compounds like phytoalexins (Dangl et al., 1996). HR also generates signals in plants which cause local and systemic changes in plants. The most rapid plant defence response following the plant recognition is the oxidative burst. After the elicitation of pathogen attack, the oxidative burst producing reactive oxygen species (ROS) includes the superoxide anion (O_2^-). The apoplastic superoxide dismutase (SOD) catalyses the dismutation of (O_2) rapidly to produces hydrogen peroxide (H_2O_2) (Grant and Loake, 2000; Lamb and Dixon, 1997). ROS plays a significant role in the plant disease resistance. Studies have also shown the plants NADPH oxidase catalyses the involvement of NADPH oxidase activity in the production of H_2O_2 as the reduction of dioxygen to O2 from the oxidation of NADPH. It has been revealed that ROS derived from the oxidative burst is generated by the plasma membrane NADPH oxidase. A gp91-phox is a protein involved in the respiratory oxidative burst in mammals during infection. The NADPH oxidase corresponds to the subunit gp91-phox protein (Torres et al., 1998). Ten forms of NADPH oxidase designated as RBOHA to RBOHF are encoded in *Arabidopsis* genome (Sagi and Fluhr, 2006; Torres et al., 2002). The two forms RBOHD and RBOHF are involved significantly in the pathogen-induced HR. After pathogen infection, RBOHD is the major source of extracellular ROS, while RBOHF functions to control the HR (Torres et al., 2002). Plant antioxidants are utilised to counteract any risk possessed by ROS, for example, glutathione (GSH) and ascorbate and enzymes like glutathione peroxidase (GPX) and glutathione-S-transferase (GST) uses GSH as an electron donor (Wojtaszek, 1997). RBOHD limits large scale cellular damage by obstructing the death of the adjacent cells by suppressing the salicylic acid (SA) and ethylene (ET) signalling. It has also shown the nitric oxide (NO) produced within the same timeframe

as H_2O_2 and these two molecules regulate synergistically to modulate the HR (Delledonne et al., 2001b). Interestingly the *Arabidopsis* mutant S-nitrosoglutathione reductase *gsnor1-3* has shown an elevation in NO and protein-S-nitrosothiols (SNO) levels and this correlates to reduced ROS mediated cell death (Chen et al., 2009; Feechan et al., 2005). Recently it has been shown that the Cysteine-890 present in the C-terminal of RBOHD is S-nitrosylated which in turn regulates the NADPH oxidase activity and ROS production limiting cell death (Yun et al., 2011b). SA, RBOHD and GSNOR balance NO and ROS production hence are the positive regulators of cell death in early stages of plant infection (Yun et al., 2011b).

1. 3 Plant Hormones

Hormones are small molecules that are important regulators of plants growth, development, and environmental interactions. Abscisic acid (ABA), indole-3-acetic acid (IAA or auxin), cytokinins (CK), ethylene (ET) gibberellic acid (GA), jasmonic acid (JA) and salicylic acid (SA) are all key phytohormones (Buchanan et al., 2000). Collectively each hormone has a different aspect in plant physiology (Jun et al., 2008).

Auxin is synthesised from actively growing tissues like shoot meristems, leaf primordia and expanding young leaves, developing seeds, fruits and pollens. Auxin regulates a numerous biological processes like cell division and its expansion, ion fluxes, root initiation, phototropism, geotropism, apical dominance, fruit development and parthenocarpy, and also abscission and sex expression. Meanwhile, auxin also causes hyperpolarization of the plasma membrane (Arteca, 1996; Kepinski and Leyser, 2002; Kulaeva and Prokoptseva, 2004). Auxin Response Factors (ARFs) and the interacting Aux/IAA proteins function as transcription regulators in the auxin signalling pathway (Kepinski and Leyser, 2002; Kulaeva and Prokoptseva, 2004; Santner and Estelle, 2009). Auxin is perceived by transport inhibitor response 1(TIR1) and triggers the degradation of AUX/IAA proteins by 26S proteasome degradation (Teale et al., 2006).

Ethylene (ET) is a volatile and gaseous molecule and regulates seed germination, fruit ripening and plant senescence (Van Der Straeten and Van Montagu, 1991; Yang and Hoffman, 1984). ET has well-characterised receptors categorised into two subsets like ETR1 and ERS1 that has three transmembrane domains and a C-terminus histidine kinase domain while the other receptors family is ETR2, EIN4 and ERS2 which contains four transmembrane regions and a C-terminus serine-threonine kinase domain (Kendrick and

Chang, 2008). Noteworthy EIN3 binds with the ERF1 and is the target of EIN3 driving the expression of ERF1 in turn results in the activation of ethylene-induced genes that consist of GCC box in the promoter (Ohme-Takagi et al., 2000). ET is also known to inhibit cell division (Stanley, 1973).

Gibberellin (GA) is dihydroxylated tetracyclic diterpene acid that regulates seed germination, stem elongation, flowering time and fruit patterning (Arnaud et al., 2010; Richards et al., 2001; Yamaguchi, 2008). The DELLAs REPRESSOR OF GA1-3-LIKE 1(RGL1-like) RGL1, RGL2 and RGL3 are repressors of GA signalling and GA-insensitive dwarf 1 (GID1) is the GA receptor in the GA signalling pathway (Schwechheimer, 2011). In response to a bioactive GA signal, DELLA proteins bind to GID1 forming a GID1-DELLA complex. The (SCFSLY1/GID2) recruits DELLA for polyubiquitination and degradation by the E3 ubiquitin and 26S proteasome protein degradation pathway (Itoh et al., 2003; Santner et al., 2009; Schwechheimer, 2008). Upon the perception of GA plants exhibit an increase in the intracellular Ca^{2+}/calmodulin, and a decrease in intracellular pH and elevates the cGMP a second messenger cGMP (Richards, 2001).

Salicylic acid (SA) is known to regulate flowering and thermogenesis. They are also known to regulate processes like photosynthesis and transpiration (Hayat et al., 2010). It is a phenol hormone known as critical in plant innate immunity (Arteca, 1996), as it activates both the local and systemic resistance. It promotes the pathogenesis-related proteins like peroxidase, catalase and hydrolase (Klessig et al., 2000; Kumar and Klessig, 2003). They are also known to regulate the host cell death and systemic acquired resistance (SAR). SA is identified in plant leaves and reproductive organs (Arteca, 1996). The transformed plants constitutively expressing the bacterial *NahG* gene encodes salicylate hydroxylase, that converts SA into inactive catechol has concluded the role of SA in plant defence (Delaney et al., 1994; Kachroo et al., 2000) as *nahG* are hypersusceptible to infection by biotrophs. JA hormone is discussed in details in later sections.

1.4 *Arabidopsis thaliana*

Arabidopsis thaliana is considered an excellent biological model to study various aspects of plant biology. *A. thaliana* belongs to the mustard family (Cruciferae or Brassicaceae). Its

distribution is throughout Asia, Europe and North America. *A. thaliana* has a fully sequenced genome. It has one of the smallest genomes (~157 megabase pairs) among flowering plants (Johnston et al., 2005). Characteristics like self-pollination, production of thousands of seeds from an individual and easy transformation, offer many advantages in plant research. It also completes its life cycle within six weeks. *A. thaliana* is well established as a powerful model plant for genetic and developmental studies. Knowledge and information achieved from the study of *A. thaliana* have been applied to increase crop yield and to enhance the resistance of plants to multiple stress responses (Somerville and Koornneef, 2002).

1.5 Jasmonates: A Defence Hormone

Jasmonic acid (JA) and related molecules like methyl-jasmonate (Me-JA), jasmonoyl-isoleucine (JA-Ile), cis-jasmone are derived from octadecanoids and are responsible for regulating diverse plant defence and developmental processes. JA is synthesised in response to multiple stresses such as herbivory or pathogen attack (Song et al., 2013). Plants produce a volatile derivative of JA such as Me-JAand also conjugate JA with isoleucine to form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile) (Creelman and Mulpuri, 2002), which together with other related molecules are termed jasmonates (JAs).

In the plant kingdom, JAs are widely distributed and play a vital role in plant-herbivory, plant-pathogen interactions and are also integral to a number of plant developmental processes (Cheong and Choi, 2003) (Figure 1.1). Conversion of JA to JA-Ile is mediated by (JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE1) (*JAR1*) (Fonseca et al., 2009; Suza et al., 2010; Wasternack and Kombrink, 2010). *Jar1* exhibits a male fertile phenotype (Staswick et al., 2002). Analysis of a *jar1* mutant which fails to convert JA to JA-Ile indicates JA-Ile is the primary signal for the defence response and this molecule is thought to be the primary bioactive form of JAs (Fonseca et al., 2009).

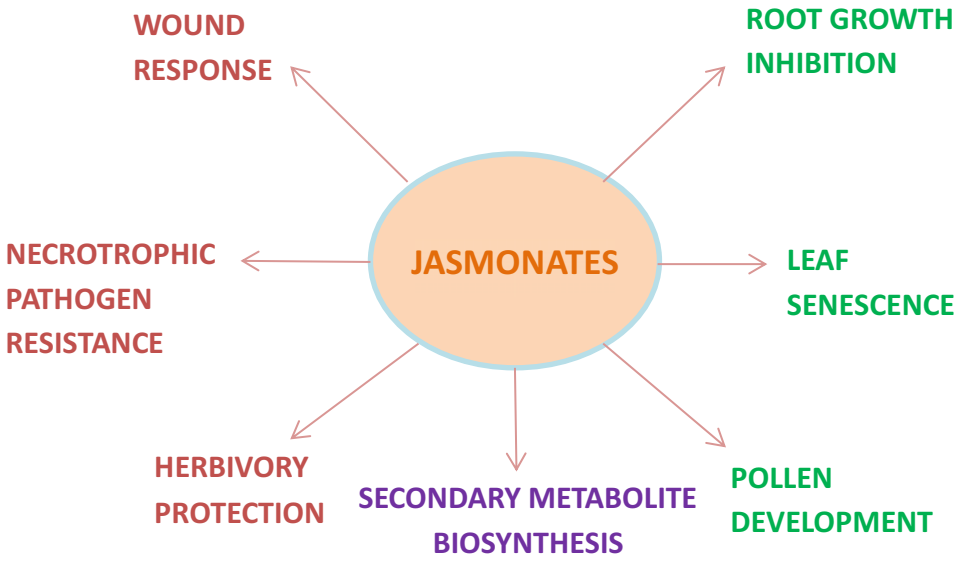


Figure 1.1 Functions of jasmonates in plant biology (Wasternack, 2014).

1.5.1 JA Biosynthesis

The process of JA synthesis occurs in two cellular compartments: the chloroplast and the peroxisome (Figure 1.2). The initial step of JA biosynthesis starts from free linolenic acid (LA) (18:3), which is released from the chloroplast membrane by phospholipase A_2 (PLA_2) (Wasternack, 2007). *Defective in anther dehiscence1* (*DAD1*) encodes the phospholipase enzyme which is thought to initiate JA biosynthesis in *Arabidopsis*. LA is then oxidised by molecular oxygen at carbon atom 13 yielding a hydroperoxy fatty acid (13*S*-hydroperoxy-(9*Z*,11*E*,15)-octadecatrienoic acid) (13(S) -HPOT) with the action of lipoxygenase (LOX). LOX enzymes are iron-containing dioxygenases which are widely distributed in plant and animal species (Shin et al., 2008). Allene oxide synthase (AOS) catalyses the first step in the biosynthesis of JA from LOX-derived hydroperoxides of free fatty acids (Sivasankar et al.,

2000). *AOS* genes have been cloned from flaxseed, rubber and *Arabidopsis* among others (Song et al., 1993). *Arabidopsis* allene oxide cyclase (AOC) is highly active and are chloroplast localised (Stenzel et al., 2003; Ziegler et al., 2000).In the chloroplast AOS and (AOC subsequently catalyse the conversion of linear allene oxide (13-HPOT) into 12-oxo-phytodienoic acid (cis-(+)-12- OPDA) (Devoto and Turner, 2003; Turner et al., 2002; Wasternack, 2007). LOX, AOS and AOC together catalyse hexadecatrienonic acid (C16:3) to form dinor-OPDA (dn-OPDA). The peroxisomal ABC transporter protein COMATOSE (CTS) is thought to transport OPDA into the peroxisome, where OPDA reductase 3 (OPR3) reduces the OPDA and dn-OPDA to form 12-oxophytoenic acid (OPC8) and 12-oxophytoenic acid (OPC6) respectively. OPDA is abundant in vegetative tissues of *Arabidopsis* and functions in the elongation of anther filaments. OPR3 is thought to be located in the plastids and then transported to the peroxisomes (Stintzi and Browse, 2000) OPDA, dn-OPDA, OPC8 and OPC6 are activated by acyl-coenzyme A synthetases to form CoA esters, so that the carboxylic acid side chains can be shortened by the three rounds of β-oxidation by acyl-CoA oxidase (ACX), a multifunctional protein (MFP) and L-3-ketoacyl CoA thiolase (KAT). Jasmonoyl-CoA, the final product of the β-oxidation reactions, is then cleaved by thioesterase (TE) to form cis-7-iso-jasmonic acid ((+)-7-iso-JA), which converts spontaneously to the stable form of (-)-JA. This molecule is conjugated to form (+)-7-*iso*-jasmonoyl-isoleucine by Jasmonate Resistant 1 (JAR1) (Schaller, 2001). Alternatively, Me-JA is produced by JA methylation mediated by a JA carboxyl methyltransferase (JMT) (Seo et al., 2001).

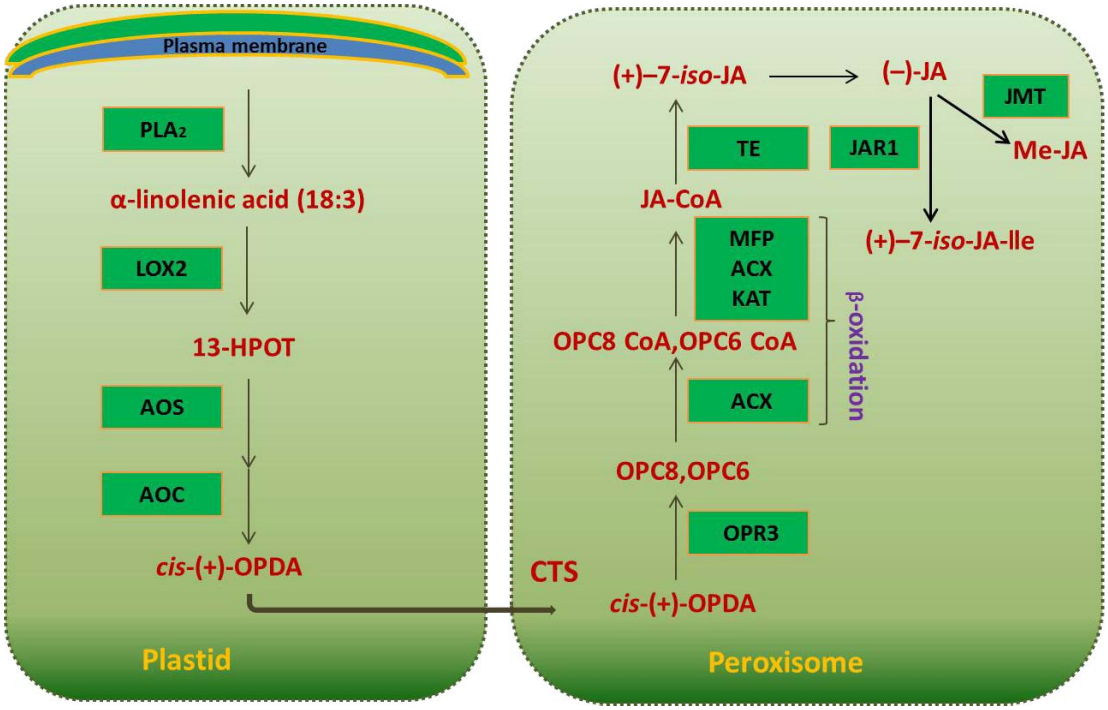


Figure 1.2 Jasmonate biosynthesis

Abbreviation: *PLA2 (Phospholipase A2), DAD1* (*DEFECTIVE IN ANTHER DEHISCENCE1),* α-linolenic acid (18:3) (alpha linolenic), AOS (allene oxide synthase), AOC (allene oxide cyclase), *cis*-(+)-OPDA (12-oxophytodienoic acid), CTS (peroxisomal ABC transporter protein COMATOSE), OPR (12-oxophytodienoate reductase), OPC8:0 (3-oxo-2(2'[*Z*]-pentenyl)-cyclopentane-1-octanoic acid), OPC6:0 (3-oxo-2-(2'-pentenyl) cyclopentanehexanoic acid), ACS (acyl-CoA synthetase), ACX (acyl-CoA oxidase), MFP (multifunctional protein), KAT (3-ketoacyl-CoA thiolase), TE (thioesterase), JAR1 (JASMONOYL ISOLEUCINE CONJUGATE SYNTHASE1), JA-CoA (jasmonoyl CoA) ,(+)–7-*iso*-JA-lle (+)-7-iso-jasmonoyl isoleucine), JMT (Carboxyl methyltransferase) and Me-JA (methyl jasmonate) (Figure adapted from (Wasternack, 2014).

1.5.2 Metabolites of JA

(+)-7-iso-JA epimerize to more stable trans configuration form, (-)-JA, generally known as JA. This occurs through a keto-end tautomerization, which involves the C6 ketone and C7 proton to form the corresponding diastereomers (Creelman and Mullet, 1997). Therefore, JA undergoes epimerization and various JA metabolites are induced in different tissues upon various stress responses (Gobel and Feussner, 2009; Koo and Howe, 2012; Wasternack, 2007). Me-JA is a methyl ester of JA and has been ascribed numerous signalling roles including the induction of wound response genes and the engagement of numerous

biochemical pathways responsible for the synthesis of an array of secondary metabolites. However, the amino acid conjugate of JA, (JA-Ile), is thought to be the bioactive hormone (Fonseca et al., 2009). Interestingly, it has been suggested that the pH changes promote conversion of (+)-7-iso-JA-L-Ile to the inactive (-)-JA-L-Ile form. This may therefore provide a simple mechanism that can regulate the activity of this hormone through epimerization (Fonseca et al., 2009).

Zea mays and *Glycine max* tissues also accumulate a sulphated form of JA, 12-hydroxyjasmonic acid sulfate (12-HSO4-JA), in addition to 12-hydroxyjasmonic acid (12-OH-JA) and a glycosylated derivative, 12-O-β-D-glucopyranosyljasmonic acid (12-*O*-glucosyl-JA) (Wasternack and Kombrink, 2010). Higher levels of 12-OH-JA, 12-HSO4-JA and 12-O-Glc-JA are associated with sex determination in the tassels of *Zea mays* (Wasternack and Hause, 2013). Especially noteworthy is the production of the phytotoxin, coronatine, which is produced by several strains of *P. syringae*. It is thought that coronatine acts as a molecular mimic of JA-Ile (Katsir et al., 2008). This is especially significant because JA-Ile suppresses the function of the immune activator, SA, required for the activation of plant defences against biotrophic and hemi-biotrophic pathogens, such as *P. syringae* species. Thus, the production of coronatine by these bacterial pathogens reduces the activation of resistance mechanisms leading to increased microbial susceptibility.

1.6 JA Signalling

1.6.1 The JA-ILE Receptor and the Mechanism Underpinning JA-ILE Signalling

CORONATINE INSENSITIVE 1 (COI1) contain Leucine-rich repeats (LRRs) and a degenerate F-box motif (Xie, 1998). The F-box is a protein structural motif of almost 50 amino acids that mediates protein-protein interactions (Kipreos and Pagano, 2000). C-terminal of the F-box is a horseshoe-formed solenoid, a structure which has 18 LRRs, of these four loops (loop-2, loop-12, loop-14 and loop-C) bind to JA-Ile (Fonseca et al., 2009). F-box proteins are one of the components of Skp1/Cullin/F-box (SCF) complex. S-phase kinase-associated protein 1A (Skp1A) is also known as *Arabidopsis-SKP1-like* (*ASK*). It is a small protein of approximately 160 amino acids which is essential for the recognition and binding of the F-box. Cullin 1 (CUL1) functions as a rigid scaffold that organises the SKP1-F-box and RING-box 1 (RBX1) proteins. The SCF complex leads a functional role as an E3 ubiquitin ligase, these enzymes target specific protein substrates for ubiquitination and leads

to subsequent degradation by the proteasome (Morgan, 2006). Upon the perception of JA-Ile (Sheard et al., 2010; Yan et al., 2009) the F-box of COI1 recognises a family of JASMONATE ZIM-domain (JAZ) proteins including JAZ1, JAZ2, JAZ3, JAZ6, JAZ9 and JAZ10. The JAZ has a degron sequence that contains a conserved helix for COI1 docking (Chini et al., 2009c; Chung and Howe, 2009; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2009). Significantly, this recognition promotes the ubiquitination of these proteins by the SCF^{COI} complex leading to their subsequent degradation by the 26S proteasome. The complex structure of COI1-ASK1 with the coronatine and JAZ1 degron is shown in (Figure1.3) (Sheard et al., 2010). JAZ proteins interact with the transcriptional activator, MYC2, a positive regulator of JA-dependent genes, suppressing activator function. Thus, following JA-Ile accumulation and the subsequent degradation of JAZ proteins, MYC2 is released from JAZ-mediated repression enabling the MYC2-dependent expression of JA-regulated genes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) (Figure 1.4) adapted from (Yan et al., 2013a). Thus, this mechanism facilitates the induction of a large series of genes following JA-Ile accrual.

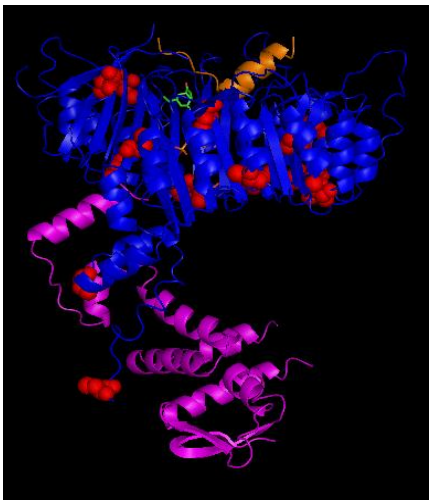


Figure 1.3 A ribbon structure of COI1, JA-Ile, JAZ1 degron and JA-Ile binding pocket. Blue represents COI1, pink represents SKP1 and the orange represents the JAZ degron. Red circular spots represent the cysteine residues in COI1.Structure obtained from NCBI (Sheard et al., 2010).

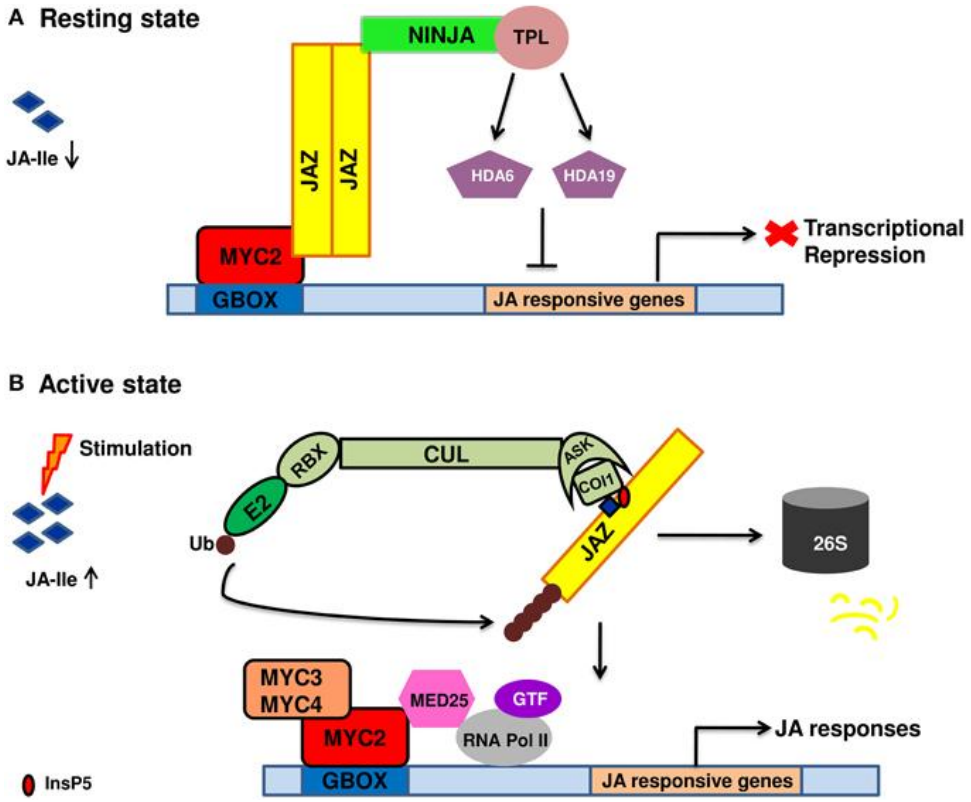


Figure 1.4 Mechanism of Jasmonoyl isoleucine (JA-Ile) regulated gene expression. **a**. Jasmonate ZIM Domain (JAZ) binds MYC2 in absence of JA-Ile inhibiting JA-Ile regulated gene expression JAZ proteins recruit TPL and adaptor NINJA and form an active transcriptional repressor complex in turn inhibiting jasmonate responses by converting an open complex to a closed complex by recruiting HDA6,HDA19. **b**. In active state JA is rapidly synthesised and readily epimerizes to JA-Ile. Then it binds to COI1-JAZ-$InsP_5$ co- receptor complex causing ubiquitination and proteasomal degradation of JAZ. MYC2 and its homologs from repression then binds to G-box element present downstream of JA-responsive genes upon homo/heterodimerization. This is followed by the recruitment of MED_{25} that in turn bring RNAPol II and general transcription factor hence, causing diverse jasmonate responses.

Abbreviation: COI1 (Coronatine insenstitive1), RBX (RING box protein1), CUL1 (Cullin1) and ASK (*Arabidopsis-SKP1-like* (*ASK*) protein), InsP5 (inositol pentakisphosphate), GTF (general transcription factor), HDA6, HDA19 (histone deacetylase 6, 19), MED25 ($medicator_{25}$), RNAPol II (RNA polymerase II) Diagram adapted from (Sharma and Laxmi, 2015) .

1.6.2 JAZ Proteins: Repressor of JA Signalling

JAZ proteins, the targets of the SCF^{COI} complex, are induced upon wounding or JA treatment (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009). *Arabidopsis* possesses 12 JAZ proteins that exhibits significant sequence variability. All JAZ proteins consist of three domains which are conserved and comprise their distinguishing characteristics. The N-terminal (NT) region consists of weakly conserved domain that is involved in a small set of protein-protein interactions. However, this domain remains poorly characterised (Hou et al., 2010). The ZINC-FINGER EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) is a putative transcription factor involved in inflorescence and flower development. The ZIM domain within this protein is found in a variety of other plant TFs that contain a GATA domain which is the GATA zinc-finger ($CX_2CX_{20}CX_2C$, where X present any amino acid (Nishii et al., 2000; Vanholme et al., 2007). The ZIM domain consists of 30 amino acids and forms the central portion of the JAZ peptide sequence and contains a highly conserved TIFY motif (TIF[F/Y]XG) (Vanholme et al., 2007). This motif is required for the formation of homo or heterodimers and also for the interaction of JAZ proteins with the Novel Interactor of JAZ (NINJA) adaptor protein. NINJA contains an ETHYLENE RESPONSIVE FACTOR–associated amphiphilic repression (EAR) motif in the N-terminal domain that mediates interaction with the corepressor TOPLESS (TPL) and TPL-related proteins (TPRs). Through the interaction with NINJA, JAZ recruits the TPL and TPRs proteins that repress transcription from JAZ targeted genes (Chini et al., 2009c; Chung et al., 2009; Pauwels et al., 2010).

The Jas domain, in the C-terminal region, is conserved across JAZ family members. Further, 12 of 29 amino acid residues in this domain are similar across all 12 JAZ family members with conservative substitutions across all *Arabidopsis* 12 JAZ proteins (Chini et al., 2007). The Jas domain is characterized by a S-L-X(2)-F-X(2)-K-R-X(2)-R core (Thines et al., 2007; Yan et al., 2007). The Jas domain is involved in a broad range of protein–protein interactions (Chini et al., 2009c; Hou et al., 2010; Song et al., 2011b; Zhu et al., 2011a), including the interaction with MYC2 (Chini et al., 2007; Melotto et al., 2008; Thines et al., 2007). The C-terminal region is also essential and adequate for interaction with COI1 (Katsir et al., 2008). Plants containing JAZ proteins deleted for the Jas domain display a diminished JA response (Chini et al., 2007).

1.6.3 MYC2

MYC proteins belong to the basic helix–loop–helix (bHLH) domain-containing family of TFs (Lorenzo et al., 2004). A small clade of *Arabidopsis* bHLH TFs (bHLH003, bHLH013 and bHLH017) are phylogenetically closely related to MYC2, MYC3 and MYC4 and all these TFs can interact with JAZ proteins *in vivo* (Fernandez-Calvo et al., 2011) However, MYC2 is thought to be the most prominent and a master regulator in the control of the JA-Ile-mediated defence and developmental responses. MYC TFs have two important domains a JAZ interaction domain (JID) adjacent to the N-terminus and a conserved TAC-like domain at the C-terminus. The TAC-like domain is essential for homo- and hetero-dimerization of MYCs (Cheng et al., 2011; Fernandez-Calvo et al., 2011). *JASMONATE-INSENSITIVE1* (*JAI1*/*JIN1*) encodes MYC2 and *jin1* mutants display a decreased sensitivity to JA showing a reduction in root-growth inhibition and anthocyanin accumulation demonstrating that MYC2 is required for JA responses (Dombrecht et al., 2007).
MYC2 is capable of interacting with all the 12 JAZ proteins (Browse, 2009) while MYC3 showed strong interactions with eight JAZ proteins (JAZ1, JAZ2, JAZ5, JAZ6, JAZ8, JAZ9, JAZ10, and JAZ11) in yeast (Cheng et al., 2011). MYC3 and MYC4 are known to interact with JAZ repressor and forms homo-heterodimers among all MYC TFs. MYC3 and MYC4 are also a nuclear protein that binds DNA with the specific sequence like MYC2. Mutants of these TFs are impaired in JA responses and also enhance in-sensitivity among *myc2* mutant (Kazan and Manners, 2013).

1.7 JA Regulation of Secondary Metabolites

At least 25% of all drugs can be traced to plant-derived molecules. Further, 73% of all cancer drugs developed since the 1940s are either natural products or derivatives thereof. *Catharanthus roseus* is exploited for the production of the vinca alkaloid, vinblastine. This blockbuster anticancer drug binds tubulin, thereby inhibiting the assembly of microtubules and thereby disrupting cell division (Dorr and Von-Hoff, 1994). Significantly, exogenous addition of Me-JA to cultured *C. roseus* cells strongly induces the production of vinblastine. However, JAs have been shown to have broad-spectrum utility to induce the biosynthesis of a large variety of plant secondary metabolites including nicotine, paclitaxel and camalexin glucosinolates (De Geyter et al., 2012). In tobacco, Putrescine *N- Methyltransferase* (*PMT*) is

a key structural gene involved in nicotine biosynthesis. The APETALA 2/ethylene responsive element binding factor (AP2/ERF) domain family TFs *NtORC1* and *NtJAP1* are required to up-regulate *PMT* in response to JA accumulation (Goossens et al., 2003) ORC1 and JAP1 are also referred as ERF222 and ERF10 that belongs to *NIC2* AP2/ERFs group IX of ERF gene (Rushton et al., 2008; Shoji et al., 2010). Interestingly, *NtORC1* is a homologue of the *C. roseus* AP2/ERF domain transcription factor octadecanoid-derivative response *Catharanthus* AP2 domain3 (ORCA3), which is pivotal for the JA-induction of genes integral to vinblastine biosynthesis (Shoji and Hashimoto, 2011; Shoji et al., 2008). A gene inherited from a common ancestor in two different species is a homolog, and this gene can have an identical sequence (Encyclopaedia Britannica online).

1.8 Role of JA in Plant Defence

Plants are closely associated with a vast variety of organisms including bacteria, fungi, oomycetes as well as herbivory pests. Plant-pathogens are characterised into one of two broad classes, biotrophs or necrotrophs depending on their lifestyle (Farmer and Ryan, 1990). Biotrophic pathogens parasitise living plant cells and derives their nutrients without extensive cell damage. In contrast, necrotrophs destroy plant cells by the production of phytotoxins and cell-degrading enzymes during the infection process. Hemibiotrophs exhibit both lifestyles during the life cycle of the pathogen (Glazebrook, 2005a).

JA biosynthesis is activated in response to attempted microbial infection, insect herbivory and mechanical wounding, leading to extensive changes in local plant gene expression often resulting in host resistance (Ballare, 2014; Howe and Jander, 2008). Further, PAL, peroxidase, and LOX-like genes are also expressed in systemic, unchallenged tissues resulting in enhanced resistance against anticipated infection attempts (Sticher et al., 1997). JA biosynthesis or perception mutants are compromised in their resistance against *Botrytis cinerea*, a necrotrophic fungus (Diaz et al., 2002). JA-deficient *Arabidopsis* are also highly susceptible to two additional necrotrophic pathogens, *Alternaria brassicicola* and *Pythium irregulare* (Staswick et al., 1998) and the bacterial pathogen *Erwinia carotovora* (Brooks et al., 2005; Norman-Setterblad et al., 2000). Further, the JA-related mutants *fad3-2, fad7-2, fad8*, *opr3, aos*, *jar1,* and *coi1* are highly susceptible to an array of insect pests including *Lepidoptera*, *Coleoptera* and *Homoptera* species (Howe and Jander, 2008). Conversely, mutations which result in the overexpression of the JA-pathway such as *fatty acid oxygenation up-regulated 2* (*fou2*), constitutive expression of *VSP* (*cev1*) and *cex1* are highly

resistant to insect herbivores and also necrotrophic fungal pathogens; (Bonaventure et al., 2007; Ellis and Turner, 2001a; Xu et al., 2001).

1.8.1 Role of JA in Systemic Signalling

Proteinase inhibitors (PIs) are proteins that inhibit the activity of proteases. Chewing insects have long been known to induce the accumulation of plant PIs which have been shown to inhibit the activity of insect gut proteases, reducing insect grazing on host plant leaves (Koiwa et al., 1998; Pannetier et al., 1997). Further, transferring a trypsin inhibitor gene from *Vigna unguiculata* to tobacco conferred resistance to a wide range of insect pests (Hilder et al., 1987). Thus, PIs are central to insect resistance in plants. A detailed study was carried out on wound-inducible PIs in tomato by Green and Ryan. Specific signals are generated at the wound-site that travel through the plants. These signals activate the expression of PIs and other defence-related genes in both local and systemic leaves (Green and Ryan, 1972). In this scenario, insect attack is thought to promote the rapid cleavage of systemin, an 18-amino-acid peptide signal, from the precursor protein, prosystemin (PS). Binding of systemin to its proposed receptor on the cell surface might then activate *PI* and associated gene expression by increasing the accumulation of JA and related molecules (Farmer and Ryan, 1990). Further, a central role for JAs in intercellular signalling is suggested by findings showing the exogenous application of Me-JA to a tomato leaf induces *PI* gene expression in distal untreated leaves (Farmer and Ryan, 1990).

Grafting experiments have also demonstrated that the graft-transmissible wound signal generated by plants expressing the CaMV*35S::PS* transgene can be readily recognised by *spr2* plants (a line insensitive to systemin). However, this signal is not perceived by *jai-1* plants (insensitive to JA). These findings imply that the *35S::PS* generated wound signal is through JA and not systemin (Li et al., 2002). This data is therefore at odds with the well-established view that systemin is the long-distance mobile signal for wound-induced *PI* gene expression (Stratmann, 2003) , Me-JA is volatile and it has been shown that air-bourne transmission of this molecule can activate *PI* expression in distant plants. Thus, Me-JA might also provide a plant-to-plant early warning system (Farmer and Ryan, 1990).

Recently, a genetic screen to identify mutations that suppress constitutive wound signalling due to the expression of a CaMV*35S::PS* transgene in tomato identified novel features of

JA/systemin signalling (Lee and Howe 2003; Li et al., 2003). For example, the *suppressor of prosystemin-mediated responses8* (*spr8*) mutant in tomato, which is defective in wound-induced defence gene expression, has been found to be more susceptible to insect attack and *B. cinerea* infection. It has recently been demonstrated that *spr8* encodes the tomato lipoxygenase D (*TomLoxD)* gene, a chloroplast-localized 13-lipoxygenase involved in the wound-induced JA biosynthesis. Significantly, overexpression of *TomLoxD* has been shown to increase the resistance against both insect herbivory and necrotrophic pathogens by enhancing the expression of a suite of wound-responsive genes including PIs (Yan et al., 2013b). These findings highlight the potential of TomLoxD and potentially other genes integral to JA/systemin biosynthesis/signalling for the crop improvement.

1. 9 Plant hormonal crosstalk

1.9.1 JA and Ethylene

In *Arabidopsis thaliana*, an intact JA-ET signalling pathway is thought to be necessary for resistance to necrotrophic pathogens, such as *Erwinia carotovora* and *Botrytis cinerea*. JA and ET signalling are required for the expression of the defence-related gene *PDF1.2* in response to infection by *A. brassicicola* and are regulated by the ethylene responsive transcription factor Octadecanoid-Responsive *Arabidopsis* AP2/ERF 59 (ORA59) (Leon-Reyes et al., 2010).

JA and ET signalling pathways are also both required for the induction of induced systemic resistance (ISR), a form of systemic resistance that is triggered by the root-colonizing bacterium *P. fluorescens*. JA and ET are also responsible for activation of defence related genes like encoding a hevein-like protein (HEL) (Potter et al., 1993), encoding a basic chitinase (CHIB) in *Arabidopsis* (Samac et al., 1990) and osmotin and *PR1* in tobacco against *B. cinerea* and *A. brassicicola* (Xu et al., 1994). Microarray analysis has suggested that JA-ET regulates coordinately various defence genes(Schenk et al., 2000).

1.9.2 JA and Salicylic Acid

Initially, the JA- SA crosstalk was observed in the wound response of tomato (Harms et al., 1998) but many *Arabidopsis* accession also showed JA-SA cross talk (Koornneef and

Pieterse, 2008). SA is involved in the response to biotrophic pathogens, whereas in response to necrotrophic pathogens and herbivorous insects JA is the central regulator, with the key components of JA signalling like COI1/JAZ/MYC2/NINJA and TPL (Pieterse et al., 2012b; Vlot et al., 2009). A defence mechanism that is activated locally or systemically and confers a long lasting protection in response to a broad spectrum of pathogens in plants is known as systemic acquired resistance (SAR) (Ryals et al., 1996; Sticher et al., 1997). SAR requires a signalling molecule SA and is associated with the accumulation of pathogenesis-related PR proteins (van Loon and van Kammen, 1970).

SA biosynthesis occurs via two parallel pathways, the PAL and ISOCHORISMATE SYNTHASE ICS/SID2 mediated pathways (Garcion and Métraux, 2006). The central regulator in SA signalling is NONEXPRESSOR OF PR GENES1 (NPR1) that, in the presence of SA, is a transcriptional co-activator for many defence genes. *Arabidopsis coi1*-1 is a JA perception mutant that shows an elevated expression of *PR1*, a marker gene of SA signalling while *npr1* an SA mutant exhibits an increase in the expression levels of antifungal defensin *PDF1.2*, a JA-ET responsive marker gene (Mur et al., 2006; Spoel et al., 2003). NPR1 is localised in the cytosol and controls the SA-induced suppression of JA signalling pathway (Koornneef and Pieterse, 2008; Spoel et al., 2003). Some TFs associated with NPR1 activity such as WRKY62, thought to function downstream of NPR1, are involved in JA-responsive gene suppression (Mao et al., 2007). SA activated NPR1 in the cytoplasm may bind a positive regulator of the JA signalling pathway, thus preventing its transportation to the nucleus. It may also be possible that the activity of negative regulator of JA pathway requires NPR1 (Dong, 2004).

Glutaredoxins (GRXs) and thioredoxins (TRXs) are redox regulators that maintain the redox state of proteins, where JA decreases and SA increases the glutathione pool (Spoel and Loake, 2011). Another important transcription factor involved in the JA-SA crosstalk is WRKY70 which activates SA-induced defence response by controlling AtMYB44 and represses the JA pathway (Shim et al., 2013).

1.10 Production of Nitric Oxide in Plants and Animals

Nitric oxide (NO) is a highly reactive gas that plays an important role in various biological processes like immune defence, regulation of cell death apoptosis and neurotransmission

(Kam and Govender, 1994). The initiative to uncover various roles of NO in plants is a recent one while its importance in animal biology like in respiration, gene expression, cell motility and blood flow is well characterised. NO was recognised as 'Molecule of the year' in 1992 by the journal Science (Guyer and Koshland 1989). NO has an unpaired electron and its simple chemical structure like a Stokes radius and neutral charge facilitates its rapid membrane diffusion and makes it an ideal signalling molecule within species from every biological kingdom (Lamattina et al., 2003).NO has recently emerged as an important signalling molecule in plant disease resistance (Arasimowicz and Floryszak-Wieczorek, 2007; Hong et al., 2008). In plants, NO is involved in physiological processes like defence response, signalling, cellular detoxification, flowering and lignin biosynthesis (Besson-Bard et al., 2008b; He et al., 2004). In animals nitric oxide synthase (NOS) is responsible for the production of NO (Wojtaszek, 2000). The NADPH-dependent deamination of arginine by nitric oxide synthase (NOS) to form citrulline and NO is an important mechanism for the generation of NO in animals (Cueto et al., 1996; Stuehr et al., 2004). In contrast, plants show a complex mechanism for the production of NO. Nitrate reductase (NR) enzymes may be responsible for the production of NO in plants (Rockel et al., 2002). Arginine pathway may also contribute to the production of NO. NR enzymes convert nitrate into nitrite (Crawford, 2006) and also nitrite into NO (Rockel et al., 2002) (Figure 1.5) explains the routes of NO production in plants. NO is produced in response to abiotic stress (San et al., 2008), during developmental process of flowering (Seligman et al., 2008) and at the time of stomatal closure (Bright, 2006; Neill et al., 2008). NO is also known to generate while lateral root induction (Kolbert et al., 2008) and in response to defence elicitors (Wu et al., 2009). Therefore, it has shown that through the cytosolic action of NR, NO is produced (Sang et al., 2008b; Wu et al., 2009). Lines silenced for *NR* or knockout mutants fail to accumulate NO, supporting the fact regarding the role of NR in NO production (Sang et al., 2008b; Wu et al., 2009). The *Arabidopsis* genome has two NR isoforms NIA1 and NIA2, where NIA1 is functional NO producing enzyme involved in ABA-induced stomatal closure, while NIA2 is responsible for the NR activity. NR inhibitors like sodium azide and potassium cyanide reduce the production of NO in plants (Farmer and Ryan, 1990; Sang et al., 2008a). Mutants like *nia1* or *nia2* may be further impaired in NO production after treating with NR inhibitors. The production of NO by NR depends upon factors like low O_2 and high NO_2^- concentration (Rockel et al., 2002).

NOS are associated with neuronal, smooth muscle relaxation, where they are classified as $Ca^{2+/}$calmodulin activated brain NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Forstermann and Sessa, 2012). nNOS and eNOS are considered as the constitutive forms of NOS which show fast and transient activation. The iNOS is the most stable, sustains longer and produces adequate NO, in turn exerting antimicrobial effects on the immune system (Beck et al., 1999). A NOS-like activity has been detected in the extracts of plant tissues and organelles like peroxisomes and mitochondria (Besson-Bard et al., 2008a). However, plant NOS activity failed to show any evidence of L-citrulline production *in vitro* nor has any protein or gene with sequence similarity to animal NOS proteins been identified in higher plants.

A search for an enzyme that triggers the pathogen-induced NO production in *Arabidopsis* led to the generation of AtNOS1. In *Arabidopsis* AtNOS1 protein was identified which showed sequence similarity with a snail protein that encodes the NOS-like activity (Crawford, 2006) and cross reacted with the mammalian NOS antibodies (Huang et al., 1997). The *Atnos1* mutant shows a depletion of NO production. *Atnos1* indicates an increased susceptibility against *P. syringae* pv. *tomato* ((*Pst*) DC3000, suggesting that *AtNOS1* is involved in pathogen-induced NOS activity (Zeidler et al., 2004). Several studies have shown that AtNOS1may not be a NOS but associated with NOS activity (Crawford, 2006). However, this protein has been shown to function as a GTPase (Moreau et al., 2008). AtNOS1 has been renamed as *Arabidopsis* nitric oxide associated (*AtNOA1*). Under stress conditions, *Atnoa1* exhibits low NO levels (Bright, 2006; He et al., 2004; Zeidler et al., 2004). Interestingly, studies have also shown that polyamines like spermine and spermidine can induce NO production in plants (Besson-Bard et al., 2008a; Tun et al., 2006).

Surprisingly, a marine single-celled green algae *Ostreococcus tauri* has been claimed to exhibit NOS activity which displayed a similarity to different isoforms of NOS. *O. tauri* NOS also possess similar properties to animal NOS in terms of Km of L-arginine and NADPH oxidation rate (Foresi et al., 2010).

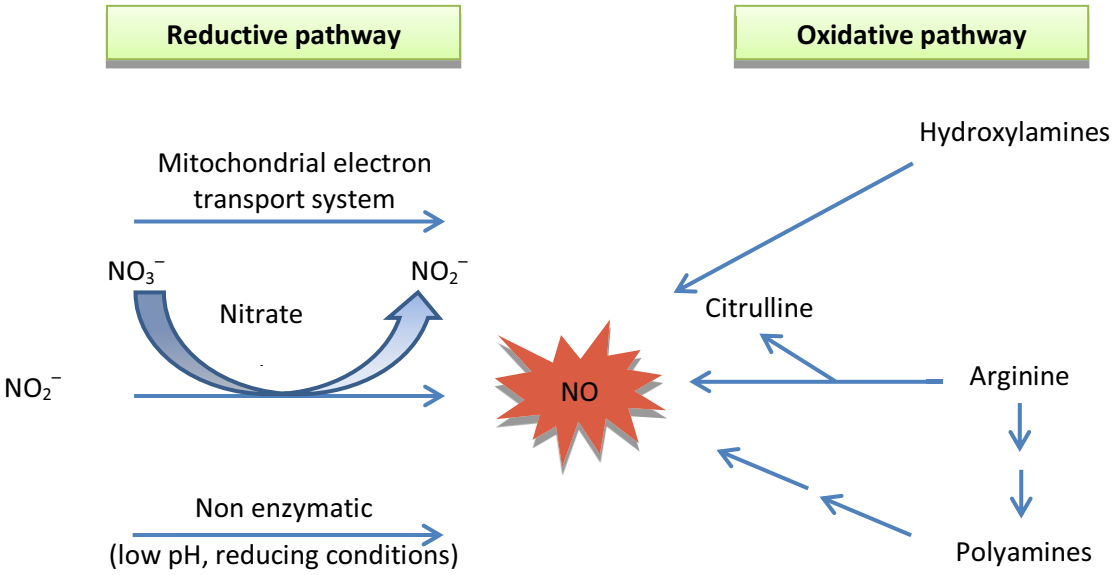


Figure 1.5 Two possible routes of NO formation in plants (Moreau et al., 2010).

1.10.1 S-nitrosylation in Plant Disease Resistance

NO is free radical that can react with the intra and extracellular targets and also acts as an inhibitor or an activator in ion channels, TFs, enzymes where in this way it regulates the plant development while pathogen interactions or abiotic and biotic stresses (Stamler, 1994). NO signalling attributes to other NO derivatives like reactive nitrogen species (RNS), that not only comprises of NO radical, its nitroxyl NO and nitrosonium NO^+ ions but also the peroxynitrite ($ONOO^-$), S-nitrosothiols, the higher nitrogen oxides and dinitrosyl iron (Leitner et al., 2009). RNS and NO exert their biological functions by modifying targets through chemical modification and also reacting with various amino acids. NO acts to bind the transition metal of metalloproteins (metal nitrosylation), tyrosine-3-nitration and the covalent modification of cysteine S-nitrosylation. Altogether these processes are categorised as post-translational modifications of proteins (Stamler et al., 2001). The covalent attachment of an NO group to the sulfhydryl side chain of a reactive cysteine thiol is known as S-nitrosylation It is a redox based post-translational modification process which is important in signal transduction (Hess et al., 2005). Reversible modification of cysteine thiols also

includes S-sulphenation, the formation of disulphide bridges, glutathionylation, a covalent attachment of glutathione and further oxidation of sulphenic groups to sulphinic and sulphonic forms (Figure.1.6) (Spadaro et al., 2010; Spoel and van Ooijen, 2013).S-nitrosylation can modify protein activity, protein-protein interactions and also protein localisation. S-nitrosylation a potential new paradigm in signal transduction has unique features such as formation and degradation that solely depends upon the chemical reaction (Martinez-Ruiz and Lamas, 2007). The $ONOO^-$, nitrosonium cation (NO^+) and higher oxides like dinitrogen trioxide (N_2O_3) are the effective nitrosylating agents (Nedospasov et al., 2000). The thiyl $RS^{\cdot}$ and $NO^{\cdot}$ radical a combination reaction is more efficient and faster compared to the reaction between NO and the thiol groups (Lane et al., 2001; Lindermayr and Durner, 2009). NO reacts rapidly with the antioxidant GSH and results in the formation of GSNO. GSNO acts as a mobile reservoir of NO bioactivity. S-nitrosylation of proteins is mainly regulated by the presence of NO availability and NO donor. SNO levels in the cells depend upon the balance between SNO synthesis and decomposition i.e. S-nitrosylation and de-nitrosylation under normal conditions. GSH is important in tolerance to various environmental stresses. Techniques like saville griess, mass spectrometry, biotin switch assay and gas-phase chemiluminescence are used to characterise nitrosylated proteins (Bari and Jones, 2009; Hao et al., 2006; Mannick, 2007; Mannick and Schonhoff, 2006; Palmer et al., 2008).

There are some common features of redox based signalling pathways like electrostatic interactions capable of controlling the thiols pKa, allosteric regulators like $Ca^{2+,}$ Mg^{2+} and O_2 and hydrophobic compartmentalisation. These features facilitate the thiol accessibility, reactivity and interaction in between the NO and the target protein (Hess et al., 2005). The motifs that are likely to be S-nitrosylated are known as SNO-motif. A cysteine (Cys) residue is embedded in an acid-base motif, for example is β-Cys93 of haemoglobin is an example of a Cys residue embedded in an acid-base motif. An acid-base motif is a Cys flanked by an amino acid (Asp, Glu) and a basic amino acid (Arg, His, Lys) in a protein that may contribute to changes in the target thiol pKa, rendering it susceptible for S-nitrosylation (Greco et al., 2006b; Hao et al., 2006; Marino and Gladyshev, 2010; Perez-Mato et al., 1999). S-nitrosylation of S-adenosylmethionine synthetase1, metacaspase9 and the SA binding protein3 (AtSABP3) in *Arabidopsis* have shown that the cysteine residues are surrounded by the acid-base amino acid motif (Lindermayr, 2006). Cys3635 of the ryanodine receptor of the skeletal muscle (RyR1) is S–nitrosylated which is intercalated in the hydrophobic region of

the calmodulin binding domain (Jourd'heuil et al., 2003).Proteomic approaches have helped to identify more than 100 proteins which were potential targets of S-nitrosylation (Lindermayr et al., 2005). NPR1 has been shown to be S-nitrosylated at Cys156 by GSNO, and this promotes oligomer formation (Tada et al., 2008). Methionine adenosyltransferase1 (MAT1) catalyses the synthesis of ethylene precursor S-adenosylmethionine. MAT1 inhibits this enzyme after S-nitrosylation at Cys114 by exogenous NO application (Lindermayr et al., 2006). Recently TIR1 is known to be S-nitrosylated and is associated with auxin signalling (Terrile et al., 2012b). *In vivo* S-nitrosylation of NADPH oxidase AtRBOHD at Cys890 facilitates the regulation of the hypersensitive cell death response (Yun et al., 2011b) .
NPR1 is thought to be a master regulator of plant immune response and functions as a co-activator (Fu Z. Q. and Dong, 2013). NPR1 has many cysteine residues. This complex is mediated by the formation of disulphide bonds by cysteines residues (Mou et al., 2003). In non-challenged plants, NPR1 resides in cytoplasm as an oligomer complex. Upon pathogen attack, disulphide bonds are reduced into monomeric NPR1 that translocate into the nucleus where it functions as a co-activator and drives SA dependent gene expression (Mou et al., 2003).

Although some proteins can transfer the NO group onto another protein (i.e. trans-nitrosylation), a specific enzyme to catalyse the S-nitrosylation of proteins has not yet been described.

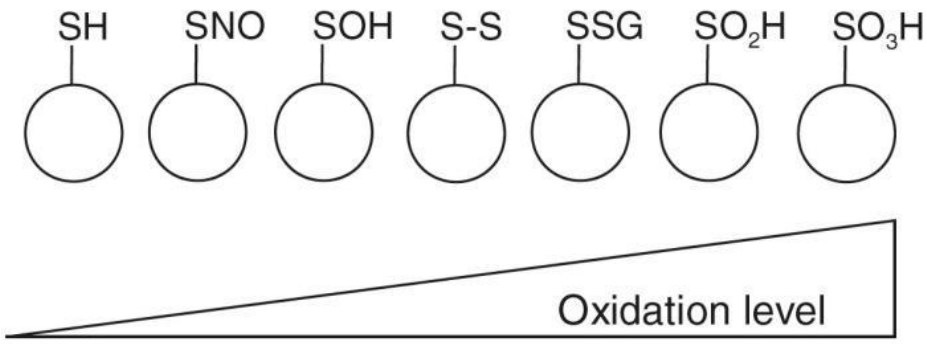


Figure 1.6 Schematic overview of cysteine thiol (SH) redox-based modifications: Cysteine thiol (SH) is reversibly modified by covalent attachment of NO to form S-nitrosothiol (SNO), thiol hydroxylation (sulphenic acid (SOH)), disulphide (S–S), S-glutathionylation (SSG), Further oxidation of sulphenic acid –SOH to

sulphinic acid (SO_2H) and irreversible sulphonic acid formation (SO_3H). This modification occurs from the reduced (left) to the higher oxidized level (right) diagram from (Spadaro et al., 2010).

1.10.2 Denitrosylation

The balance in between nitrosylation and denitrosylation determines the level of S–nitrosylation (Hogg, 2002; Stamler and Toone, 2002). The denitrosylation process can be carried out by the Thioredoxins (Trx). Trx have a conserved Cys-Gly-Pro-Cys redox active site and are ubiquitous which are essential for their function as oxidoreductase (Lillig and Holmgren, 2007). Oxidised cysteine is reduced upon the activation of plant immunity. Pathogen infection induces the expression of various Trx proteins. SA treatment also is known to induce the Trx protein (Laloi et al., 2004; Tada et al., 2008). Recently the Trx proteins were reported as denitrosylases in animals (Benhar et al., 2009), however plant Trx are documented to have denitrosylase activity in vitro, yet its activity in *planta* is still unknown (Spoel and Loake, 2011). Trx has caspase-3-denitrosylase activity *in vivo* that is NADPH- dependent (Benhar et al., 2009). Thioredoxin reductase (TrxR) catalyses NADPH to NADP+ that recycle the oxidised thioredoxin disulphide (Trx-S_2) to reduced thioredoxin (Trx-$(SH)_2$) (Lillig and Holmgren, 2007) therefore activates the Trx denitrosylase activity (Benhar et al., 2009). Redox enzymes like xanthine oxide and protein disulphide isomerase can reduce the GSNO to low molecular SNOs (Nikitovic and Holmgren, 1996; Sliskovic et al., 2005; Trujillo et al., 1998). Denitrosylase activity of GSNOR1 controls the SNO-proteins indirectly by turning over GSNO during the plant immune response (Spoel and Loake, 2011).

1. 10.3 GSNO/GSNOR in plant disease resistance

An enzyme, formaldehyde dehydrogenase (GS-FDH) purified from *Escherichia coli*, exhibits effective GSNO turnover (Liu et al., 2001). GS-FDH was renamed as S-nitrosoglutathione reductase (GSNOR). *Arabidopsis GSNOR* fully complement the GSNO hypersensitive phenotype in the yeast *sfa1* mutant (Sakamoto et al., 2002). GS-FDH is essential to protect the plants against nitrosative stress from GSNO (Liu et al., 2001). Deletion of SFA1 in yeast leads to an 11-fold increase in SNO. In plants, an enzyme, S-nitrosoglutathione reductase GSNOR, regulates global S-nitrosylation and acts as a key player in plant disease resistance.

GSNOR metabolises GSNO with NADH as an electron donor (Wilson et al., 2008a) and is associated with the removal of NO through GSNO metabolism. GSNOR is encoded by a single gene and is predicted to be localised in the cytosol (Xu et al., 2013).

GSNOR is conserved in almost all the living systems including plants (Diaz et al., 2003; Liu et al., 2001; Sakamoto et al., 2002). A T-DNA knock out mutant (*atgsnor1-3*) resulted in an elevated cellular level of S-nitrosothiols (SNOs) while a mutant overexpressing *GSNOR* (*atgsnor1-1*) exhibited reduced SNO levels. This implies that AtGSNOR1 regulates total cellular SNO levels in plants (Feechan et al., 2005). GSNOR also regulates thermotolerance and pathogen-triggered cell death (Lee et al., 2008b; Yun et al., 2011b). GSNOR has received increasing attention for its role in plant stress responses. Several factors regulate the intracellular stability of GSNO. These factors are chemically driven degradation reaction, such as thiol and metal mediated decomposition, while the enzymatic reaction so far is the reduction of GSNO by GSNOR. GSNOR reduces GSNO into glutathione disulphide (GSSG) and ammonia, in an NADPH-dependent manner (Figure 1.7).

$$GSNO + NADH + H^+ \rightarrow GSNHOH + NAD^+$$

$$GSNHOH + NADH^+ H+ \rightarrow GSNH_2 + NAD^+ + H_2O$$

$$GSNH_2 + GSH \rightarrow GSSG + NH_3$$

Figure 1.7- In *E. coli* GSNOR, ammonia (NH3) and glutathione disulphide (GSSG) identified as the main products of GSNOR enzyme activity described by **(Liu et al., 2001).**

GSNO acts as an NO donor and can modify proteins by transferring an NO group which is functionally important in plants. In *Arabidopsis*, loss of GSNOR function increases cellular SNO levels and leads to a loss of apical dominance. These mutants are also compromised in plant disease resistance (Feechan et al., 2005). Plants challenged with avirulent *Pst* DC3000, showed low levels of SA accumulation that resulted in a compromised disease resistance (Feechan et al., 2005; Yun et al., 2011b). A possible role for GSNOR in plant-insect interactions was uncovered recently: silencing GSNOR enhanced susceptibility to *Manduca sexta* in tobacco (*Nicotiana attenuata*) plants. This transgenic line exhibited low accumulation of JA and ET (Wunsche et al., 2011). This implies GSNOR might have a role

in herbivore defence systems. An avirulent isolate of *P. syringae* pv tomato induces a strong nitrosative burst leading to S-nitrosylation of SABP3 at Cys280 ((Wang et al., 2009). Carbonic anhydrase (CA) provides resistance against bacterial infection in SABP3. S-nitrosylation suppresses the CA activity as well as the binding of SA resulting in modulation of the plant disease resistance (Spadaro et al., 2010; Wang et al., 2009). GSNOR function is required for a normal development of fertility under optimal growth conditions (Lee et al., 2008a). By using the Bio-Array resources GSNOR (At5g43940) is expressed in all organs in *Arabidopsis,* an exception is the mature pollens. GSNOR is differentially expressed as it is higher in roots and leaf from the first stage of development (Espunya et al., 2006; Leterrier et al., 2011). *HOT5* is an *Arabidopsis* mutant that has shown to be sensitive to high temperature. It is affected in GSNOR, thus uncovering the role of GSNOR required for thermotolerance (Lee et al., 2008a). Screening of a mutant by using paraquat allowed the identification of knock-out mutant *par2* which exhibits resistance against this herbicide (Chen et al., 2009). *par2* correspond to *GSNOR* gene previously known as *HOT5* . Overexpression of *HOT5/PAR2/GSNOR1* exhibits a low level of NO while the knockouts *hot5/par2/gsnor1* displayed an increased NO level. This indicates GSNOR activity is involved in NO homeostasis (Leterrier et al., 2011).

1.10.4 Nitric oxide, ROS and JA signalling

During the certain developmental processes and stress conditions, ROS are produced. This formation of ROS changes the redox status of the cellular environment. The level of ROS can be exploited to redox signals which are beneficial for the organism to respond to various stresses. ROS damages the DNA, RNA and protein, therefore, needs to be detoxified.
GSH (γ-glutamyl-L-cyteinly-glycine) is a low molecular thiol and is the most abundant in cellular redox system which is used for detoxification of ROS. Glutathione-ascorbate cycle detoxifies ROS leading to the transient change in the cellular glutathione redox potential. Glutaredoxins (GRXs) are important as they are capable of sensing the redox potential shift of GSH. GRXs transfer the electron in the glutathione redox buffers and the thiol group of proteins. These proteins may be the TFs that might alter the expression of stress-related genes and also some metabolic enzymes. Recent studies suggest the ratio of reduced/oxidised small molecule redox couple might play an important signalling role in stress response. These is reduced/oxidised small molecules are NAD(P), GSH and ascorbate protect the cell from ROS

and RNS (Noctor et al., 2006). The enzymatic production of ROS and RNS depends on the availability of NAD(P)H in the cellular redox enzyme. In comparison to NAD(P)H, GSH and ascorbate molecules are more reduced, in turn, establishes a redox flux from NAD(P)H to GSH and ascorbate. For the regeneration of GRX/GSH reducing system, NADPH modifies the cellular ratio of reduced/oxidised GSH/GSSG (Noctor et al., 2006). GSH plays a vital role in redox signalling during a plant stress response (Marty et al., 2009) ROS is produced upon infection in the plants and leads to changes in a cellular redox potential. Application of a pharmacological defence related hormones like SA and JA also changes the GSH/GSSG ratio (Koornneef and Pieterse, 2008; Mou et al., 2003; Vanacker et al., 2000). Changes in the ratio in between the oxidised/reduced redox couple is determined by the reactive cysteines of target proteins (Spoel and Loake, 2011).

ROS and NO control the HR process, and the balance in both is the key determinants for HR (Delledonne et al., 2001a). Upon pathogen infection, NO is accumulated followed by ROS accumulation at the site of infection.

1.10.5 Limited information related to S-nitrosylation and JA signalling pathway

H_2O_2 and NO are important signalling molecules in plant stress response. Reactive form of ROS is H_2O_2 is generated via the superoxidation during photosynthesis (Alvarez et al., 1998). Emerging studies have shown, wounding or the application of JA defence hormone induces NADPH oxidase activity as well as ROS accumulation in tomato, however, the activity could be blocked using the pre-application of NADPH oxidase inhibitors (Hu et al., 2009).

Recent studies have shown the activation of ROS is dependent on the octadecanoid pathway.

Other molecules that are associated with the plant-herbivory interactions are MAPK, NO production, elicitation of phytohormones and ion influx (Wu and Baldwin, 2009). Wounding induces NO production in *Arabidopsis* (Huang et al., 2004). NO is capable of blocking the wound-induced proteinase inhibitor elicited by systemin, JA application and wounding. This blockage could be reversed by NO scavengers (Hu et al., 2009). NO is also capable of blocking H_2O_2 (Orozco-Cardenas and Ryan, 2002). JA acts downstream of NO generation and also the octadecanoid pathway is regulated by NO (Xu et al., 2005). Thus, very limited information is available regarding S-nitrosylation and JA signalling pathway.

1.11 Hypothesis

It is now a well-established fact that GSNOR plays a vital role in regulating the cellular SNO level. The *Atgsnor1-3* mutant exhibits compromised growth and disease resistant (Feechan et al., 2005). While JA signalling is known to regulate various aspects like development and responses towards stress (Turner et al., 2002). We hypothesised that post-translational modification may occur in the proteins involved in the JA signalling cascade, in turn attenuating the transcript of certain JA regulated signalling genes. By extension, changes in SNO levels in *atgsnor1-3* mutants may alter the disease resistance response to necrotrophic fungal pathogens and possibly also herbivory.

1.12 Objective

Changes in cellular redox status are a common feature during immune function across eukaryotes. GSNOR is a key regulator of cellular SNO levels and has been shown to control multiple modes of plant disease resistance. Therefore, this project aims to investigate a possible role for NO and associated S-nitrosylation in JA signalling during the plant defence response. The objectives of the research work are:

Aims

1. To compare the disease phenotype of study the phenotypic observation in *atgsnor1-3* and wild-type Col-0 plants in response to wounding, pathogen infection and insect feeding. To access any difference in gene expression of JA marker gene in *atgsnor1-3* compared to wild-type plants.

2. To uncover the dynamic nature of proteins involved in JA signalling during redox regulation (to explore the impact of S-nitrosylation on protein-protein interactions and protein degradation).

3. To identify the S-nitrosylation status of key proteins integral to JA signalling pathway.

This thesis document consists of seven chapters:-

Chapter 1 describes the background of plant immunity, the defence hormone JA and its various roles in immunity and development. Further, this chapter provides an overview of S-nitrosylation in a plant disease resistance.

Chapter 2 explains the techniques used to perform all experiments described in this thesis.

Chapter3 describes the phenotypic study conducted on *atgsnor1-3* plants stating the role of S-nitrosylation on JA mediated immunity. In this study, we have shown that due to increased accumulation of SNO, the JA signalling pathway is attenuated. We describe the phenotypic observations of *atgsnor1-3* plants in response to pathogen infection and herbivory insect feeding. For this purpose, we have used *Botrytis cinerea* and *Pieris rapae*. Wounding in *Arabidopsis* is known to induce JA levels. Therefore, we also studied the JA content in wild-type and *atgsnor1-3* plants. Also we explored the expression levels of JA marker genes in *atgnsor1-3* plants compared with a wild-type line after the exogenous application of Me-JA

Chapter 4 describes about the effects of S-nitrosylation on the proteins-protein interactions involved in JA, signalling including COI1-JAZ1 and JAZ1-MYC2. It also shows the blockage of JAZ1 protein degradation due to the exogenous application of NO donors.

Chapter 5 outlines the S-nitrosylation of JAZ1-MBP detected using the biotin switch assay and site identification.. Further, the site of JAZ1-SNO formation is described.

Chapter 6 describes the methodology of generation of transgenic plants to assess the S-nitrosylation of JAZ1 *in vivo* and its biological consequences.

Chapter 7 discusses the implications of the results described in the thesis and also will state a comprehensive analysis of future building on findings from this work.

Chapter-2

Methods and materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Co. Ltd., UK).

2.1 *Arabidopsis* Seeds and Growth Conditions

Arabidopsis thaliana (*Arabidopsis*) ecotype Columbia (Col-0) was used. Seeds were soaked in water for 2 days at 4^{o}C and were transferred on potting medium consisting of peat moss, vermiculite and sand (4:1:1) and then were placed in a growth room. Seeds were incubated under 16-h/8-h light/dark cycle conditions with light intensity 110 μmol m^{-2} s^{-1} at 20^{o}C. For aseptic conditions seeds were sterilised with commercial bleach 10% (v/v) with a drop of Triton X-100 for 5 min and then washed with distilled water for about 5-10 times and plated on Murashige and Skoog MS medium (1/2 MS salt, 1% sucrose, 0.4% phytoagar, pH 5.8) containing Me-JA (Sigma Aldrich, UK) when indicated.

Table 2.1 lists the *Arabidopsis* transgenic lines and mutants with their phenotypic characteristics and the reference sources.

Table 2.1 *Arabidopsis* transgenic lines and mutant strains.

Strains	Phenotype	Reference Source
Col-0	wild-type	NASC
atgsnor1-3	Loss of apical dominance	Gabi-Kat
35S::JAZ1	No phenotype studied	This study
coi1-1	Male sterile plant.well-developed roots on MS media supplemented with Me-JA	John Turner's lab
35S::*JAZ1 GUS*	Gus activity	John Browse's lab
35S::GSNOR1	Enhanced NR activity	Michael Skelly and Loake
jaz1	Homozygous	NASC T-DNA insertion

2.2 *Coi1*-1 seedlings sterilisation and selection

Coi1-1 and wild type seeds were sterilized using commercial bleach 10% with a drop of TritonX-100 for 5 min and then washed with 5-10 changes of distilled water. Seeds were sown on MS media supplemented with 10 µM Me-JA. Wild-type *Arabidopsis* had shown anthocyanin accumulation in cotyledons while *coi1*-1 is insensitive towards Me-JA treatment (Feys et al., 1994). *coi1*-1 are also male sterile plants.

2.3 Genomic DNA Extraction from *Arabidopsis*

Arabidopsis plant leaf was ground in 300 µL of Cetyltrimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCL, pH 8, 20 mM EDTA and 0.2 % 2-mercaptoethanol) in a 1.5 ml eppendorf tube. Incubation was carried at 65^{o}C for 20 mins. 300 µL of chloroform was added was added into the tube and vortexed for 10 sec before been centrifuged at 15,000 rpm for 5 min. The upper aqueous layer was transferred to a new eppendorf tube with 300 µl of isopropanol followed by centrifugation at 15,000 rpm for 5 min. Supernatant was discarded and the pellet was washed by 1 ml of 70% (v/v) cold ethanol. The tube was again centrifuged and residual ethanol was taken out by using a pipette. Pellet was air-dried and later dissolved in 50 µl of distilled water.

2.4 RNA Extraction and Reverse-Transcription (RT)

Four-week-old plants were used for total RNA extraction using TRI reagent (Sigma Aldrich, UK) according to the manufacturer's protocol and concentration (ng/ul) was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, UK). cDNA synthesis was carried out by Omniscript RT kit (Qiagen)..1 µg of total RNA made upto 5 µl with DEPC treated dH_2O was denatured at 65 ^{o}C for 5 min.5 µl of mixture (1x reaction buffer,0.5 mM dNTP, 2.5 µl of oligo(dt) primer, 4 U RNase inhibitor and 2 U of Omniscript RT was added. Sample was incubated for 1 hr at 37^{o}C followed by 65^{o}C for 10 min. cDNA was stored at -20 ^{o}C. PCR reaction was set up using crimson *Taq* buffer (NEB) in thin-walled 0.2 ml PCR tubes (Axygen Scientific) with gene specific primers set mentioned in (Table-2.2).

2.5 Genotyping PCR

Genotyping PCR was carried out by using 1 µl genomic DNA, dNTP mix 200 µM forward and reverse primer 1µM each, Crimson *Taq* buffer 1.25 U (NEB) at cycle 95^{o}C (30 sec), 52^{o}C (30 sec) and 72^{o}C (2 min) for 35 cycles. PCR product (5 µl) was separated on taken out to analyze in 1.2% agarose gel.

Table 2.2 Primers used in genotyping.

S. No	Gene/ID	Sequence (5′-3′)	Size of amplicon (bp)
1	BASTA (*BAR*)	F-ATGCCGCGGGGGGCATATCCGA R-TTCGAACTTCCTTCCTAGATC	1368
2	*Atgsnro1-3* T-DNA insertion	F- AAGGGAAGGTGTATGCAAGT R- CAGGTGGGACTACATAGCTC	500
3	*35S* promoter	F- TGAGACTTTTCAACAAAGGAT R-CTCCAAATGAAATGAACTTCCTTA	1219
4	*JAZ1*-T-DNA insertion	LP -----AGGTAAATGCGGAGAGAGAGG RP ------AGGCACCGCTAATAGCTTAGC	900-1100
5	*JAZ1*-T-DNA insertion	RP ------AGGCACCGCTAATAGCTTAGC LbB1.3----ATTTTGCCGATTTCGGAAC	1172

2.6 Insect feeding by *Pieris rapae*

Insect feeding causes high induction of the JA pathway in *Arabidopsis*(Elzinga et al., 2014). To investigate the role of GSNOR in resistance against insect attack, *Pieris rapae* larvae were allowed to feed on Me-JA treated and without Me-JA treated Col-0, *coi1*-1 and *atgsnor1-3* plants. The lepidopteron *P. rapae* is a common and economically important pest and feeds on

a number of plant species in the Brassicaceae. *P. rapae* caterpillars and butterflies were field collected at the Oxenford castle garden (Edinburgh, UK). The butterflies were reared on cabbage plants and fed with 20% of sucrose solution. Newly hatched larvae were carefully collected and allowed to feed on *Arabidopsis* plants grown in pots and covered with plastic cups cut at the bottom, to avoid escape of the larvae. The larvae were allowed to feed for 10 days.. The experiment was repeated at least with three independent biological replicates. The weight of the larvae was measured on 4^{th}, 7^{th} and 10^{th} day of feeding. Data was analysed by plotting a graph by using microsoft office excel 2010 results were statistically analysed by Student *t*-test.

2.7 Pathogen tests By *Botrytis cinerea*

Botrytis cinerea is a necrotrophic pathogen, and causes post infection induction of JA pathway. Four-week- old Col-0 and *atgsnor1-3* plants were spot inoculated with 5µl (5×10^5 /ml) of *B. cinerea* spore suspension. The necrotrophic fungus was grown on potato dextrose agar medium and the spores were collected in potato dextrose broth as suspension. Plants were covered with transparent lids after inoculation and grown under (16 h/8 h light/dark)) conditions for three days. Plants were analysed for symptoms development. Average size of lesions was measured in (mm) and leaves were stained with Trypan Blue. Trypan blue stain is selectively used to stain the dead cells blue (Louis and Siegel, 2011). Experiment was repeated with twice independent biological replicates.

2.8 Trypan Blue staining

Trypan blue stain was prepared by mixing 10 g phenol crystals 10 ml glycerol,10 ml lactic acid and 10 ml of water with 0.02 g of trypan blue. Working solution was prepared by diluting in 2 volumes of 96% ethanol. Infected leaves were boiled in trypan blue stain for 1 min and incubated at room temperature overnight. Leaves were de-stained next day in chloral hydrate. De-stained leaves were mounted on slides with a drop of glycerol for microscopic observation.

2.9 Quantification of jasmonic acid after mechanical wounding

Wild type Col-0, *atgsnor1-3* and *coi1*-1 mutant plants were grown for 4 weeks at 22°C. The plant leaves were wounded by crushing each leaf three times with a sharp scalpel.

Immediately after wounding samples were collected at 15 min, 30 min, and 1 hr time points. Samples of wounded and unwounded (control) plants were harvested, weighed, and immediately frozen in liquid N_2. Plants were stored at −80°C and jasmonate levels were measured by gas chromatography/mass spectrometry Gas chromatography and mass spectrometry were performed as described by Engelberth et al, 2003 and Lee et al, 2004 using dihrdojasmonic acid as an internal standards. Plant material was ground in LN_2 to a fine powder. Extraction was carried out using citric acid 50 mM in (H_2O/acetone, (30/70) (v/v) and internal standard dihydro JA 500 ng were dissolved in extraction buffer). Sonication was carried out for 15-20 min at RT followed by centrifugation at 4000 g at RT for 5 min. Diethyl ether was used to extract the remaining citric acid by vortexing. The extracts were loaded on the solid phase extraction cartridge (Germany). The cartridge contains 500 mg of aminopropyl sorbent and the cartridge was washed by 10 ml of trichoromethane:2-propanol (2:1, v/v). Internal standards and bound JA were eluted by using 10 ml of diethyl ether: acetic acid (98:2, v/v). Methanolysis helps in evaporating the solvent and volatilized compounds were trapped in SQ filter material. Elution was carried out using 200 µl of dichloromethane. Quantification of JA was performed by Prof. Peter Morris at Herriot-Watt University, UK Experiment was repeated with two independent biological replicates.

2.10 *Arabidopsis* transformation by floral dip method

Agrobacterium transformation in Arabidopsis plants was performed by using floral-dip method (Clough and Bent, 1998). *Agrobacterium* cells were grown at 28°C in 5ml LB broth (with antibiotics) overnight. Seed culture was added to 500 ml LB broth (with antibiotics) to bulk up and grown at 28°C overnight. Pellet was formed by centrifugation at 5000 rpm once the OD_{600} reached 0.8. Pellet was re-suspended by re-suspension buffer. *Arabidopsis* plants were dipped into re-suspended bacterial solution for 30-60 sec. Plant trays were covered with transparent plant lid to maintain high humidity and were transferred in growth room and kept in dark for 24 h. After 24 h the lids were removed and plants were allowed to grow upright.

Re-suspension solution (1L)

Chemicals	**1L**
MS salt 0.5x	2.151 g
B5 vitamins 1x solution is sterilized, use LFU	1 ml
Sucrose5%	50 g
Silvet L-770.02%	200 µl

2.11 Molecular biology techniques

2.11.1 RT-PCR

RNA was extracted from Col-0 and *atgsnor1-3* plants using RNeasy® Plant Mini Kit (Qiagen) according to manufacturer's instructions after spray application of 50 µM Me-JA to four-week- old plants. Plant samples were collected at 30 min, 2 hr, 4 hr and 6 hr time points after application and immediately stored in liquid N_2. Plants treated by 0.1% ethanol were served as control. Control leaves were collected immediately after spraying. Reverse transcription was conducted using Omniscript® RT kit (Qiagen) according to manufacturer's instructions, and further PCR was performed to check relative expression of *JAZ1* (AT1G19180), *VSP1*(AT5G24780) and *THI2.1*(AT1G72260) genes after the treatment of Me-JA using the following primers (Table 2.3) with *Actin1* as a control. All RT-PCR were repeated with two independent biological replicates.

Table 2.3 Primers for RT-PCR

S. No	Gene/ID	Sequence (5′-3′)	Size of amplicon (bp)
1	*JAZ1* (AT1G19180)	F- ATGTCGAGTTCTATGGAATG R- TCATATTTCAGCTGCTAAAC	762
2	*VSP1*(AT5G24780)	F- CCTACTACGCTAAATATGGA R- GATCCGTTTGGCTTGAGTAT	250
3	*THI2.1*(AT1G72260)	F- AAGGGAAGGTGTATGCAAGT R- CAGGTGGGACTACATAGCTC	250

2.11.2 QRT-PCR Analysis

Real time PCR was performed using SYBR® Green QRT-PCR kit to check expression level of *VSP1* after the spraying of 50 µM Me-JA on Col-0 and *atgnsor1-3* plants using *Tub2* housekeeping gene as a control.

RNA extraction and cDNA synthesis were done as stated earlier. The cDNA was diluted 5 times. All cDNA was diluted with nuclease free water. The Reaction was prepared as follows:

Reaction mix with and without cDNA template was transferred into each well on one 96-well plate. The reaction was processed in a Light Cycler® 480 Real-Time PCR system. Using the following parameters: 95°C for 05 min, 95°C for 10 sec, 60°C for 60 sec, 72° for 30 sec and final extension at 72°C for 05 min with 40 cycles. All reactions took place in triplicates. Gene specific and housekeeping primers like Tubulin were used in this study. The primer list is stated in (Table 2.4) QRT-PCR was repeated with two independent biological replicates.

Primers used for VSP1 gene expression are

Gene numbers AT5G2480.1

Table 2.4 Primers used in QRT-PCR

S.No	Gene/ID	Sequence (5′-3′)
1	VSP1	**F**-ATGGCATTGTCCCCTTGTAT **R**-ATTTAAGCAGTGGTGCCAAA
2	TUBULIN	**F**-CTCAAGAGGTTCTCAGCAGTACC **R**-TTTGTGCTCATCTTGCCACGGAAC

2.11.3 Expression of recombinant COI1

COI1 was assayed *in vitro* in *E. coli* to determine the possible role of protein S-nitrosylation on its function. *COI1* was amplified using a High Fidelity DNA polymerase such as the Phusion^{TM} High-Fidelity DNA polymerase (Finnzymes) and cloned into pMAL-c5X (Vector map is shown in appendix B).The primers used are stated in (Table 2.5).

A cDNA for Col-0 was PCR amplified. The primer set used for PCR is stated in table. The PCR products were separated on agarose gel. The PCR bands were excised and then purified using GeneJet^{TM} PCR product purification kit (Fermentas) according to manufacturer's instructions. Purified PCR products were digested using corresponding enzymes and then ligated into pMAL-c5Xwhich contains an N-terminal Maltose binding protein (MBP) tag. The ligated products were transformed into *E.coli* (strain XL1-Blue). Chemically competent *E. coli* were mixed with ligation products and transformed into *E. coli* by heat shock method.

After sequence confirmation, the recombinant plasmid was extracted from the transformed cells using GeneJet^{TM} Plasmid Miniprep kit (Fermentas) according to manufacturer's instructions and transformed into *E. coli* (Rosetta-gammi strain) for protein expression.

2.11.4 Expression of recombinant JAZ1

JAZ1 was assayed *in vitro* in *E. coli* to determine the possible role of protein S-nitrosylation on its function. A cDNA of Col-0 was PCR amplified using *JAZ1* primers. The primers set used for PCR are stated in (Table 2.5)-.The PCR product *JAZ1* was amplified using a High

Fidelity DNA polymerase such as the Phusion^{TM} High-Fidelity DNA polymerase (Finnzymes) and cloned into pMAL-c5-X The amplified gene product was cloned into the vector with an N-terminal MBP tag.

The PCR products were separated on agarose gel. The PCR bands were excised and then purified using GeneJet^{TM} PCR product purification kit (Fermentas) according to manufacturer's instructions. Purified PCR products were digested using corresponding enzymes and then ligated into pMAL-c5-X. The ligated products were transformed into *E.coli* (strain XL1-Blue) by heat shock method.

After sequence confirmation, the recombinant plasmid was extracted from the transformed cells using GeneJet^{TM} Plasmid Miniprep kit (Fermentas) according to manufacturer's instructions and transformed into *E. coli* (Rosetta-gammi strain) for protein expression.

Table 2.5 Primers used in recombinant protein expression

S.No	Primers	Sequence (5′-3′)
1	***COI1***	F-GCGGCCGCGATGGAGGATCCTGATATCAAGAG R-GCGTCGACGAGAGTCCTGAAGGAGCCAATATGA
2	***JAZ1***	F-AGCGGCCGCATGTCGAGTTCTATGGAATG R-AGAATTCTCATATTTCAGCTGCTAAAC
3	**pMal-c5X**	F- ACGCGCAGACTAATTCGAGC R- CGTTCACCGACAAACAACAG

2.11.5 Recombinant Protein Expression in *E. coli*

E. coli strain, (Rosetta-gami) was transformed by heat shock method. The resulting colonies were selected on 100 µg/ml ampicillin plates. The colonies were grown overnight in 5ml LB including antibiotics and sub-cultured in 200 ml-1000 ml LB media with antibiotics at 37°C for few hr until OD_{600} reached 0.7-0.8. Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM-1 mM. To enhance solubility, the induction was carried out at 30°C for 5 hr in case of COI1 protein and at 37°C

for JAZ1-MBP. The induced cultures were pelleted by centrifugation at 4500 g for 15 min. The harvested cells were stored at -80^{o} C for subsequent protein purifications.

2.11.6. Purification of MBP tagged Protein

2.11.6.1 Purification of MBP tagged Protein by using magnetic beads

MBP column binding buffer was prepared by using 200 mM NaCl, 20 mM Tris-HCL, 1 mM EDTA, 1 mM DTT (pH 7.4 @25°C). Magnetic beads (NEB, UK) were vortexed and thoroughly suspended. Aliquots of 100 µl of suspension beads were added to a sterile micro centrifuge tube. MBP binding buffer 500 µl was added to it and vortexed. To pull the beads magnet was applied for 30 sec. Washing of the beads was repeated twice. 200-500 µl of cell culture was added to the washed beads and incubated at 4°C for 1 hr with agitation after thoroughly mixing. Magnet was applied and supernatant was decanted and washed three times as before. At this point the purified MBP-fusion can be eluted from the beads or used directly for capture of target proteins. 50 µl of MBP column buffer containing 10 mM maltose (elution buffer) was added to the bead pellet, vortexed and incubated for 10 min at 4°C with agitation. Magnet was applied and eluted MBP-fusion protein (supernatant) was pipetted into a clean microcentrifuge tube. Additional 50 µl of elution buffer was added again to have multiple elutions.

2.11.6.2 Purification of MBP tagged protein using amylose resin

Amylose resin beads (NEB, UK) are equilibrated by 5 volumes of column buffer. Column buffer contains 200 Mm Tris-HCl (pH 7.4) 0.2 M NaCl, 1 mM EDTA, 1 mM DTT or 10 mM β-mercaptoethanol is optional. 1 ml of cell culture was added to the equilibrated amylose resin matrix in a column and incubated for 1 hr at 4°C with agitation. After agitation, the supernatant was decanted. Washing steps were carried out for 10 times by using column buffer to remove unwanted protein 50 µl of MBP column buffer containing 10 mM maltose (elution buffer) was added to the pellet, vortexed and incubated for 10 min at 4°C with agitation. MBP fusion protein was collected in a fresh 1.5 ml eppendorf tube from the column. Additional 50 µl of elution buffer was added again to have multiple elutions.

2.11.7 SDS PAGE AND WESTERN BLOT ANALYSES

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were carried out as described by Sambrook and Russel Molecular cloning 3rd cloning, CSHL Press (2001) with slight modifications. Crude protein extracts were supplemented with 4X SDS loading buffer (250 mM Tris-Cl pH 6.8, 40% glycerol, 8% SDS, 0.04% bromophenol blue) with or without 25 mM DTT. The samples were heated at 70°C for 15 min and separated by SDS-PAGE at 120 V for 2 hr. The gel was either stained with commassie brilliant blue solution (0.25% brilliant blue –R, 40% methanol, 7% acetic acid, 53% water) or transferred onto Nitrocellulose PVDF membrane in the trans blotting buffer (25mM Tris, 200 mM glycine and 20% Me-OH) at 100 V for 1 hr at 4°C. The transferred membranes were blocked in 25 ml blocking buffer (1X PBS containing 50 mM Tris-HCl pH 7.4 and 150 mM NaCl, 0.1% Tween-20 and 5% non-fat dried milk) or TBST for 1 hr on the tilting table at room temperature. The blots were incubated overnight at 4°C with the respective antibody (Table 2.6) at optimum dilutions in the blocking buffer. Next morning, the membranes were washed 3 times with TBS/T (1X TBS + 0.1% Tween-20). The immunodetection was carried out by using western blot detection kit (GE Healthcare, UK) which uses a non-radioactive chemiluminscent detection reagent(Amersham ECL™ GE Healthcare,UK) The blots were exposed to X-ray films (CL-XPosure Film, Thermo Fisher Scientific, UK) and autoradiographs were developed in an X-ray developer. The Commassie stained gels were washed in de-staining solution (10% methanol, 10% acetic acid, and 80% water) and were used as loading controls showing total assayed proteins.

Table 2.6 Antibodies used in western blot

Target	**Primary antibody**	**dilution**	**Secondary antibody**	**dilution**
Flag	Monoclonal anti-flag M2 mouse	1:2000	Monoclonal anti-mouse IgG HRP-conjugated	1:2500
Myc	Monoclonal mouse antibody (9E10) (Insight Biotechnology	1:1000	Monoclonal anti-mouse IgG HRP-conjugated	1:2500

	Limited, UK)			
Biotin	Monoclonal anti-biotin HRP-conjugated (Cell Signaling Technology, UK)	1:5000	-	-

2.11.8 SDS gel trypsin digestion and Mass spectrometry

Bands of desired protein size were tightly excised from SDS gel and placed in 1.5 ml eppendorf tube. The gel pieces were incubated in 200 mM NH_4HCO_3 (ABC) in 50% Acetonitrile (ACN) at room temperature for 30 min to remove SDSThe protein sample was incubated in 200 mM of ABC and 50% ACN at room temperature for 1 hr. Critically DTT was avoided in this step. Washing was carried out by 200 mM ABC and 50% ACN (instant wash 3X). Samples were stored using 50 mM Iodoacetamide (IAA) in dark for 20 min. washing steps were again repeated as above. Samples were covered with ACN till they turned white (Shevchenko et al., 2006). Gel pieces were allowed to dry and 0.4 µg of trypsin in 20 µL ABC solution was added and digestion was carried out overnight at 37^{o}C. Next day digested peptides were completely dried by speedvac concentrator.

HPLC-MS analysis was performed on an on-line system consisting of a micro-pump (1200 binary HPLC system, Agilent, UK) coupled to a hybrid LTQ-Orbitrap XL instrument (Thermo-Fisher, UK). Samples were reconstituted in 10 µl loading buffer before injection, and analyzed on a 1 hr gradient for data dependent analysis. MS data were searched using MASCOT Versions 2.2 and 2.3 (Matrix Science Ltd, UK). Mass spectrometry of recombinant COI1-MBP was performed by Dr. Andrew Cronshaw (COIL building University of Edinburgh, UK) and mass spectrometry of JAZ1-MBP was performed by Dr. Thierry Le Bihan (Waddington building, University of Edinburgh, UK)

2.11.9 Histochemical GUS staining

Histochemical GUS activity was carried out based on method described by (Jefferson et al, 1987). Ten-day-old seedlings from 35*S*::*JAZ1-GUS* transgenic lines were grown on MS

media. For NO treatments, seedlings were pre-treated with NO donors like GSNO and SNP for 1 hr (Gray et al., 2001b) prior to Me-JA treatment. pH was maintained by addition of MES buffer in MS media. After treatment seedlings were stained by using GUS staining solution (Table 2.7) from 2 hr to overnight at 37°C. After incubation the staining solution was discarded and seedlings were fixed in 90% acetone at -20°C for 10 min. Samples were cleared in 50%-100% ethanol for 30 min to overnight at room temperature. Samples were placed on a clean glass slide and photographs were captured using Nikon digital camera (Nikon, Japan).. Concentration used was 100 µM-(Me-JA, GSNO, SNP, GSH) 100 mM MES buffer. GUS histochemical analysis was repeated twice.

Table 2.7 Chemicals used in histochemical GUS staining assay

Chemical	Stock	Final volume
NaPO4 pH 7.0	1M	0.1M
EDTA	0.5 M	10 mM
TritonX-100	10%	0.1%
$K_3Fe(CN)_6$	50 mM	1.0 mM
X-Gluc	0.1M	2.0 mM
H_2O		

2.11.10 Flurometric GUS assay

10 µl protein sample extract and 130 µl of assay buffer were used. The reaction was carried out in a water bath in the dark at 37°C. After 20 minutes, 10 µl of the reaction was transferred to 190 µl Stop Buffer [200 mM sodium carbonate] in the plate.). The Fluorescence was measured at 460 nm when excited at 355 nm. Calculations were carried out to analyse the amount of liberated MU produced by each sample by determining a standard curve corresponding to 50, 25, 5, 2.5, 0.5, 0.25, and 0 µM 4-Methylumbelliferone (MU).

Values from the fluorescence assay obtained were converted to moles of MU/minute. Protein concentrations were determined by methods described by Bradford (1976). The GUS activity values were recorded as were recorded as pmoles MU/min/mg protein.

GUS extraction buffer- pH 7.0

150mM	Sodium Phosphate
10 mM	EDTA
10 mM	β-mercaptoethanol
0.1%	Triton X-100
0.1%	sarcosyl
140 µM	PMSF

Assay buffer-GUS extraction buffer+1.2 mM 4-methylumbelliferyl β-D-glucuronide (MUG)
Stop Buffer-200 mM sodium carbonate

2.11.11 Yeast two hybrid assay

Y2H assay was performed based on Matchmaker Gal4 system. The cDNAs of *JAZ1, COI1* and *MYC2* were cloned into appropriate destination vectors pDEST-32 and pDEST-22 from Clontech Company. Combinations of proteins-protein interactions are given in details in (Table 2.8) and primer sequence details are given in (Table 2.9). pDEST-32, pDEST-22 vectors (Clontech, www.clontech.com) maps are provided in appendix A. Yeast two-hybrid vectors pDEST32 and pDEST22 containing the different cDNAs were transformed into the yeast strain MaV203 (Clontech, www.clontech.com) by the lithium acetate method (Gietz et al., 1992). Handling of yeast cultures, plate growth assays were performed as described in the Clontech Yeast Protocols Handbook. Protein-protein interaction was determined by growth of mating on SD-Leu-Trp-His plates containing 3 mM 3-Amino-1-2-4-triazole (3- AT). For JAZ1-COI1 interaction plates were supplemented with 25 µM of Me-JA. To study the effects of NO donors on the protein-protein interactions plates were also supplemented with 100 µM of GSNO. Glutathione (GSH) 100 µM was used as control. Plates were allowed to incubate at 28°C for three days and results were observed and images were captured by using Nikon digital camera (Nikon, Japan). Y2H assay was performed twice with similar results.

Table 2.8 Combination of protein-protein interaction studied

Transformation of experimental interactions. LEU Plasmid (BAIT)	TRP Plasmid (PREY)	Experimental interaction
pDEST32-COI1	pDEST22-JAZ1	COI1-JAZ1
pDEST32-JAZ1	pDEST22-MYC2	JAZ1-MYC2

Table 2.9 Yeast two hybrid assay primers

S.No	Primers	Sequence (5′-3′)
1	*JAZ1*	**attB1**GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGAGTTCTATGGAATGTTCTGAGTT **attB2**GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATATTTCAGCTGCTAAACCGAGC
2	*MYC2*	**attB1**GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACTGATTACCGGCTACA **attB2**GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAACCGATTTTTGAAATCAAA
3	*COI1*	**attB1**GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGGATCCTGATATCAAGA **attB2**GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATATTGGCTCCTTCAGGA

2.11.11.1 Yeast cell transformation

A single yeast colony was used to make 5 ml YPD liquid culture at 30^{o}C overnight/shaking in a falcon tube. Next day 4 ml of this base culture was added to 40 ml YPD medium and incubated at 30^{o}C on shaker for 3 to 4 hr (until the O.D$_{600}$ = 0.2 – 0.3). The cells were then

pelleted at 1000 g for 5 min and re-suspended in 40 ml of autoclaved water twice. The cell suspension was centrifuged again and the harvested cells were re-suspended in 600 µl of 0.5xTE/1xLiAc solution to make 125 µl aliquots of yeast competent cells. Next, 10 – 15 µl of linearized DNA, 20 µl salmon sperm (denatured form; denature only once by heating for 5min at 95-100^{o}C), 600 µl of PEG/LiAc (1x) were added to a single aliquot of yeast competent cells on ice and vortexed for 10 sec. The suspension was incubated at 30^{o}C for 30 min at 1000 rpm shaker. 75 µl DMSO was added to the mixture and mixed well by inverting and given a heat shock at 42^{o}C for 40 min and then stored on ice. The transformed cells were then centrifuged at 14000 rpm for 5 sec and re-suspended in 200 µl autoclaved H_2O before plating on SD media plates. The plates were incubated at 30^{o}C overnight.

2.11.11.2 Yeast mating procedure

Fresh colonies of interest were picked and grown in 0.5 ml YPD medium in a 1.5 ml microcentrifuge tube for overnight at 30°C with shaking at 200 rpm. 100-µl aliquots were spread of the mating culture on the appropriate SD minimal media

Chemicals for yeast two hybrid assay

0.5xTE/1xLiAc solution:

H_2O (autoclaved)	1.7 ml
10x TE (pH 7.5)	100 µl
3 M Lithium acetate (LiAc) pH 7.5	200 µl

Buffer stocks

10x TE	100 mM Tris (pH 7.5)/10 mM EDTA
10x LiAc	1 M (pH7.5) Lithium acetate in autoclaved

Selection plates (-His) Recipe (1L)

Chemicals	To prepare 1 L of media
Synthetic complex drop-out medium (-His)	2 g/L
Difco Yeast nitrogen base	1.7 g/L
Glucose	20 g/L
Bacto agar	20 g/L
H_20	Upto 1L

PEG/LiAc solution (1x)

Final concentration	To prepare 10ml of 1x PEG/LiAc solution
PEG 40% TE b/f 1x LiAc 1x	8ml for 50% PEG 1ml of 10xTE, pH 7.5 1ml of 1-x LiAc, pH 7.5

2.11.12 Protein extraction from *Arabidopsis*

Plant leaf tissue of *Arabidopsis* (100 mg) was ground in liquid nitrogenLN_2 into fine powder. Ice-cold extraction buffer was added to the leaf powder and vortex vigorously for 1 min. Samples were centrifuged for 20 min at 13,000x g in 4°C and supernatant was collected. The protein concentration was determined by Bradford analysis (Bradford, 1976).

Extraction buffer

PBS	1 x
PMSF	1 mM
DTT	5 mM

2.11.13 Biotin- Switch Assay

Micro BiospinP6 (Zeba) column (Thermo Scientific, UK) was used to dialyze protein sample. The Zeba column was equilibrated by using HEN buffer (250 mM HEPES-NaOH pH7.1, 1m EDTA and 0.1 mM Neocuproine). Protein sample was recovered from the column and collected in fresh 1.5 ml eppendorf tube. Protein samples were treated with NO donors like CysNo, GSNO and GSH (control) and incubated in dark for 20 min at RT. Non-reactive NO donor was removed by zeba desalting column by centrifugation at 1000 g for 2 min. The NO treated protein samples were then ready for performing the biotin switch assay (Jaffrey and Snyder, 2001).

2.11.14 In Vivo Biotin switch assay

Typically biotin switch assay is carried out in three steps, blocking, labelling, and pull down. Figure 2.1 explains the mechanism of biotin switch assay technique. Protein sample 100 µl was mixed with 300 µl of blocking buffer (HEN buffer, 2.5% SDS and 20 mM S-methylmethanethiosulfonate (MMTS) in dark at 50^{o}C for 20 min. Protein precipitation was carried out using three volumes of ice cold acetone and was precipitated for 20 min at -20^{o}C and centrifuged at 10,000 x g for 10 min. Air-dried pellet was resuspended in 50 µl of HEN-S buffer (1% SDS in HEN buffer). Labelling of protein was carried out using 13 µl of Biotin-HPDP (N-[6-(Biotinamido) hexyl]-3'-(2'-pyridyldithio)-propionamide) in 5 mM in DMSO and 3 µl of sodium ascorbate (200 mM) for 1 hr at room temperature. Biotinylated protein was detected by anti-biotin antibody. In i*n vivo* assay proteins were pulled down by using streptavidin beads (Fluka) previously described by (Forrester et al., 2009a), which were pre-washed. Protein samples were rotated for 12-18 hr at 4^{o}C. Beads were collected by centrifugation at 200 g for 10 sec. The collected beads were washed using wash buffer for four times. Protein was eluted using elution buffer and mixed in non-reducing loading buffer which was heated at 95^{o}C for 5 min and analysed on SDS-PAGE. Anti-biotin antibody was used for western blot assay.

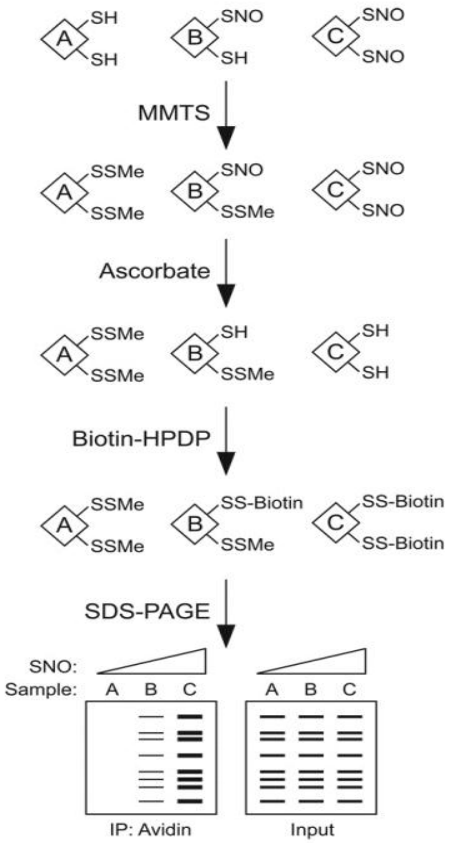


Figure 2.1 Mechanism of Biotin Switch assay (Forrester et al., 2009a).

HEN buffer	**Concentration**
HEPES	25 mM
EDTA	1 mM
Neocuproine	0.1 mM

Neutralization buffer	**Concentration**
HEPES	25 mM
NaCl	100 mM
EDTA	1 mM
Triton X-100	0.5 %

Anti-biotin antibody was used for western blot assay.

HEN/10 buffer diluted 10-fold in H2O, HENS/10 buffer with 1% SDS)

Wash buffer-Neutralization buffer + 600 mM NaCl,

Elution buffer-HEN/10 +1 % B-mercaptoethanol,

200 mM Sodium ascorbate in HEN buffer,

2.5 mg/ml biotin-HPDP in DMSO

2.11.15 Construction of transgenic *35S::FLAG-JAZ1*/*35S::MYC-COI1* plants

The full-length *JAZ1* gene was amplified from cDNA of wild type Col-0 and Gateway compatible vector were used according to the manufacturer's instructions. (Invitrogen, Carlsbad, CA). Entry clone was formed by flanking the *att*B site of PCR product with the *att*P site of donor vector using BP clonase enzyme mix and later transformed into *E. coli.* The *att*L recombinant site was recombined into destination vectors like pEG202 and pEG203 using LR clonase enzyme (Invitrogen) (Earley et al., 2006).

The tag is driven by cauliflower mosaic virus 35S promoter. pEG202/*35S::FLAG-JAZ1* vector was transformed into *Agrobacterium tumefaciens* strain GV3101 which was used to transform *jaz1* mutant plants by floral dipping (Bent, 2000). While the pEG203/*35S::MYC-COI1* were transformed into *coi1*-1 heterozygous plants. Basta herbicide was used for screening transgenic plants. A homozygous transgenic line with appropriate transgene expression was isolated by immunoblotting for FLAG and crossed into *atgsnor1-3* mutants.

Chapter 3

JA-mediated defence responses are attenuated due to high SNO accumulation

3.1 Background

Plants have evolved a sophisticated defence system to cope with different stress response like pathogen infection. Herbivores and pathogens have posed a great challenge for plants survival. Induced plant defence responses are mediated through JA signalling pathway. Two types of inducible defence responses are triggered by herbivores that are: direct defences and indirect defences. Direct defences are the one which inhibits the growth and development of the herbivores insects while indirect defence results in inducing plant volatiles that may cause damage to herbivorous by attracting its predator or parasites (Pare and Tumlinson, 1999; Walling, 2000). Plant key signalling molecules such as SA and ET plays a crucial role against biotic stresses (Fujita et al., 2006; Loake and Grant, 2007). SA is synthesised in plants upon pathogen infection to establish the systemic acquired resistance (SAR) and host cell death (Malamy et al., 1990; Yalpani et al., 1991). NO leads to the induction of defence responses and programmed cell death restricting the pathogen invasion reviewed by (Mur et al., 2013). NO may have a role in SA and ET hormone signalling pathway (Paris et al., 2013) where S-nitrosylation is thought to control the key steps involved in ET biosynthesis and SA signalling.

GSNOR is now well known to regulate the cellular level of SNOs and consequently GSNO content. In *A. thaliana*, mutation of *AtGSNOR1* modulates the cellular SNO level formation and turnover which regulates various modes of plant disease resistance (Feechan et al., 2005). Over accumulation of GSNO disturbs SA signalling by displaying the downregulation of *PATHOGENESIS-RELATED-1* (*PR-1*) transcript levels suggesting that SA signalling is affected (Feechan et al., 2005). NO accretion promotes S-nitrosylation of *A. thaliana* salicylic acid-binding protein (AtSABP3), which could result in the modulation of plant defence response (Wang et al., 2009).

A number of studies have provided information that JA signalling pathway is the central regulator of the defence responses against necrotrophic pathogens and herbivores attack (Glazebrook, 2005b; Howe and Jander, 2008; Kessler and Baldwin, 2002; Schmelz et al.,

2003; Thaler et al., 2012). Taking into account the previous studies we hypothesised that JA signalling pathway may be compromised due to high SNO accumulation and GSNOR may be a positive regulator of JA signalling pathway. The aim of study in this chapter is to elucidate the role of S-nitrosylation in JA signalling pathway under biotic stresses and to provide a new insight into the understanding of plant disease resistance through cellular redox regulation.

In this chapter, *atgsnor1-3* exhibits higher susceptibility towards *Pieris rapae* attack and *Botrytis cinerea* infection. *P. rapae* (small cabbage white butterfly) feeding is known to induce more than hundred genes that are defence related (Reymond et al., 2004). *P. rapae* is known as a pest in commercial agriculture which causes huge loss mostly in cabbage and mustard family crops (Mehrkhou et al., 2013). Detailed analysis showed a marked reduction in JA accumulation after mechanical wounding and transcriptional response of JA-regulated genes that are down-regulated in *atgsnor1-3* mutant.

Manipulating GSNOR1 activity may therefore, provide novel strategies for crop improvement. Further *atgsnor1-3* plants, can be used as useful tool to investigate the role of GSNOR in JA signalling.

3.*2 Atgsnor1-3* plants are susceptible against *Pieris rapae* attack

To investigate the effects of S-nitrosylation on JA signalling in response to herbivore attack, *P. rapae* larvae were allowed to feed on plant lines. Adults and caterpillars were collected from Oxenfoord Castle private garden, Pathead, Midlothian, UK (the Google map is stated in (Figure 3.1) and were reared at 27^{o}C on cabbage plants. Next generation larvae were used for feeding experiments. Caterpillars grind the leaf tissue with their mandibles and they use oral secretions to transport their food into their mouth parts. Recognition of the oral secretions by the plant and wounding as a result of feeding induces JA signalling and hence activates the defence mechanism (Howe and Jander, 2008; Schmelz et al., 2003).

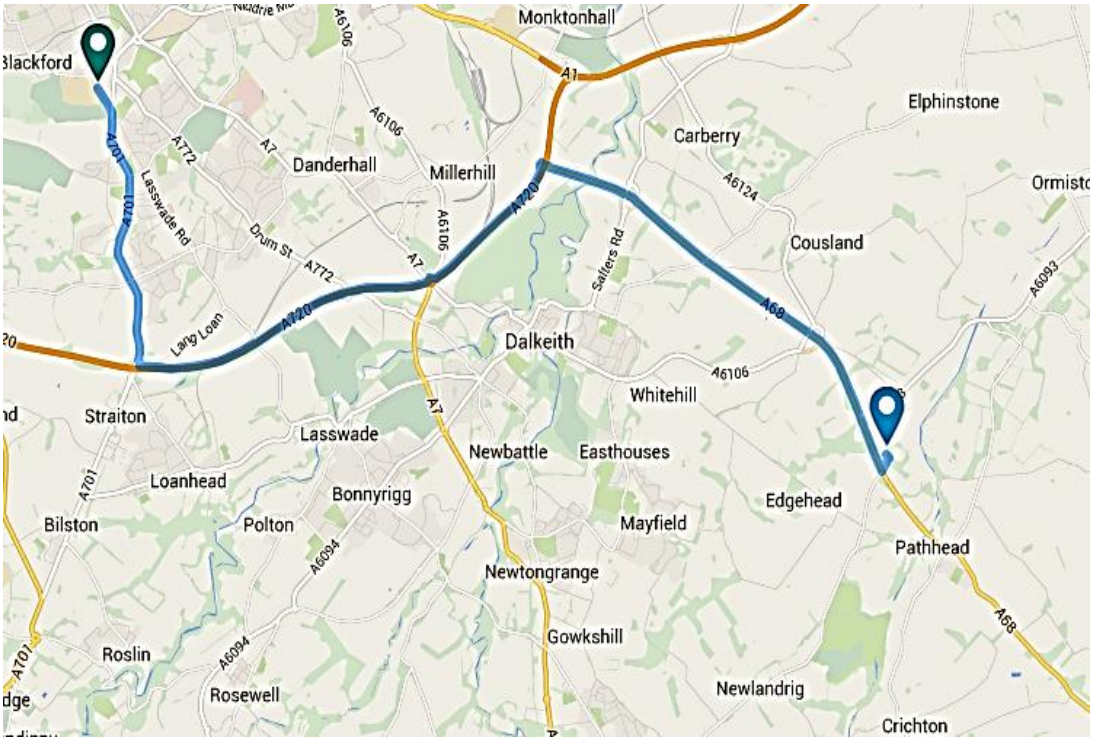


Figure 3.1 Google map of Oxenfoord Castle private garden from Kings Building, University of Edinburgh, UK.

JA application induces plants resistance, and it has been shown plethora of caterpillars, aphids and thrips growth are depleted (Lu et al., 2004; Thaler et al., 2001) To evaluate the role of JA defence response in *A. thaliana* against *P. rapae*, plants were treated by spraying 25 µM of Me-JA (Lu et al., 2004). Newly hatched larvae were placed individually on the leaves of control and Me-JA treated four-week-old Col-0, *atgsnor1-3* and *coi*1-*1* plants for a total of 10 days. Weight of the caterpillars was measured on 4^{th}, 7^{th} and 10^{th} day of the feeding period. The average weight of *P. rapae* caterpillars after feeding on Col-0 untreated plants was 0.017 g on the 4^{th} day while larvae infesting on Me-JA treated Col-0 has shown a weight of 0.008 g thus showing a significant weight difference (p=0.04). In comparison with *atgsnor1-3* average weight of caterpillars feeding on control was 0.028 g and average weight of the caterpillars feeding on Me-JA sprayed *atgsnor1-3* was 0.027 g. No significant difference was observed in both groups of caterpillars. Highest average weight was observed in caterpillars feeding on both *coi1*-1 Me-JA treated and untreated mutants which was 0.03 g and 0.04 g, showing no significance (p=0.10) (Figure 3. 2.c). Plants infested by caterpillars

and body mass of the caterpillars on the 4th day of feeding period are shown in (Figure 3. 2a, b).

Also 7th day feeding results have shown an elevated weight gain of caterpillars feeding on the *coi1*-1 mutant in Me-JA treated and control plants. *P. rapae* larvae devoured the majority of the leaves on *coi1* plants with no significant difference (p=0.057) (Figure 3. 3-a). *Atgsnor1-3* displayed a similar pattern to the *coi1*-1 mutant. In Col-0, a significant reduction in the weight of caterpillars was observed when fed on Me-JA treated plants compared to untreated plants (p=0.04) (Figure 3.3.c).

On the 10th day of feeding period, the feeding preference of caterpillars was evident. *Atgsnor1-3* and *coi1*-1 plants were completely eaten by the caterpillars and there was almost no green tissue left on the 10th day in both the mutants in Me-JA treated as well as untreated compared to wild type (Figure 3. 4.a). It is however worth mentioning that the weight was decreased of caterpillars feeding on *coi1*-1 plants, on 10th day compared with 7th day in untreated plants as very little green tissue from *coi1*-1 plants was left over after the 7th day of feeding. Body mass and weights of the caterpillars on the 10th day of feeding period are shown in (Figure 3.4.b, c) respectively. Thus, Col-0 plants supported less *P.rapae* caterpillars feeding vigorously. These plants showed weight of 0.04 g and 0.03 g in basal and Me-JA sprayed plants respectively.

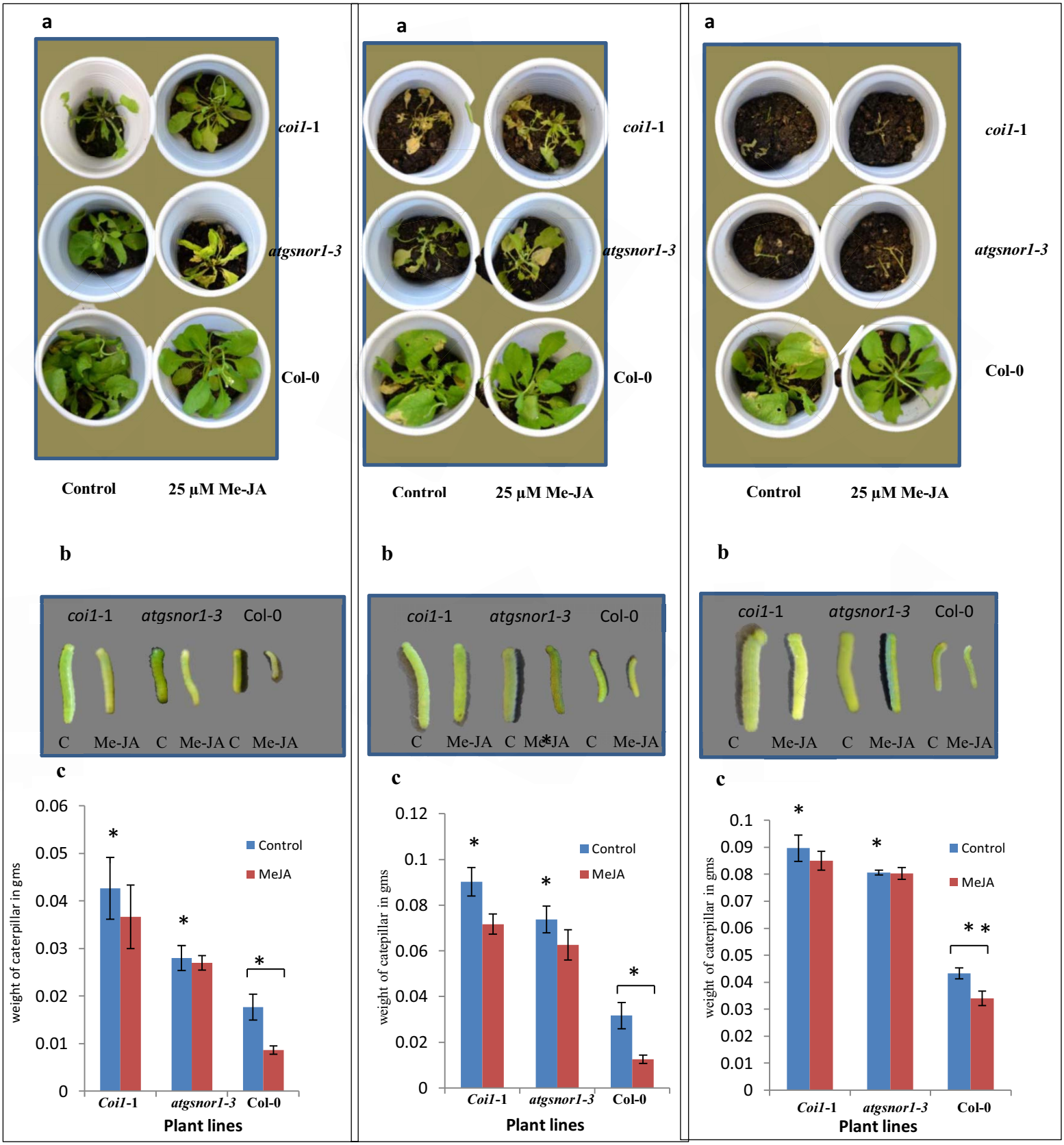

a
coi1-1
atgsnor1-3
Col-0
Control
25 µM Me-JA
b
coi1-1
atgsnor1-3
Col-0
C
Me-JA
c
0.06
0.05
0.04
0.03
0.02
0.01
0
weight of caterpillar in gms
Control
MeJA
*
Coi1-1
atgsnor1-3
Col-0
Plant lines
0.12
0.1
0.08
0.06
0.04
0.02
0.09
0.07
* *

Figure 3.2.a) *Arabidopsis* plants on 4th day of feeding period in control and after Me-JA treatment. **b)** Size comparison of representative caterpillars feeding on mutant and wild type plants before and after Me-JA treatment on 4th day. **c)** Average weight of caterpillars feeding on 4th day. Data points represent mean ± s.e of at least three biological replicates. (*n*=3, where *n* is the number of plants, analysed per biological replicate) student *t* test; *≤0.05, **≤0.01, showing significant difference in weights of caterpillars.

Figure 3.3.a) *Arabidopsis* plants on 7th day of feeding period in control and after Me-JA treatment. **b)** Size comparison of representative caterpillars feeding on mutant and wild type plants before and after Me-JA treatment on 7th day. **c)** Average weight of caterpillars feeding on 7th day. Data points represent mean ± s.e of at least three biological replicates. (*n*=3, where *n* is the number of plants, analysed per biological replicate) student *t* test; *≤0.05, **≤0.01, showing significant difference in weights of caterpillars.

Figure 3.4.a) *Arabidopsis* plants on 10th day of feeding period in control and after Me-JA treatment. **b)** Size comparison of representative caterpillars feeding on mutant and wild type plants before and after Me-JA treatment 10th day. **c)** Average weight of caterpillars feeding on 10th day. Data points represent mean ± s.e of at least three biological replicates. (*n*=3, where *n* is the number of plants, analysed per biological replicate) student *t* test; *≤0.05, **≤0.01, showing significant difference in weights of caterpillars.

3.3 *Atgsnor1-3* plants are susceptible to the necrotrophic fungus *Botrytis cinerea* infection

Changes in S-nitrosylation status impact resistance against a necrotrophic pathogen. Plants have evolved sophisticated mechanisms to sense and respond to pathogen attack. Necrotrophic pathogens benefit from host cell death (Glazebrook, 2005b) and one of the necrotrophic fungal pathogens *B. cinerea* causes extensive tissue damage in many plant species (Oirdi and Bouarab, 2007). *B. cinerea* is responsible for producing phytotoxin in plant cells and are capable of killing host cells at an early age. Phytotoxins are harmful substances produced by plant pathogens and are thought to play a significant role in plant disease development (Strobel, 1983). The sesquiterpene botrydial (sesquiterpene is a class of

terpenes which contains three units of isoprene (Bryant, 1969; Robert, 1972)) and the polyketide botcinic acid are the phytotoxins produced by *B. cinerea* (Dalmais et al., 2011).

B. cinerea is a necrotrophic pathogen characterised by its dark grey-brown spores (Figure 3. 5.a). A number of events get activated as soon as *B. cinerea* comes in contact with plants where these events develop into a process which leads to necrosis of the host (Elad, 1997). It leads to decaying leaves, which may cause to death of some plants.
The resistance against this fungus depends upon the ethylene response factor (ERF)-branch of JA signalling pathway branch (Lorenzo et al., 2003).We were interested in studying the *B. cinerea* challenge of *atgsnor1-3* plants.

To test possible JA mediated resistance of *atgsnor1-3* plants *B. cinerea* fungus was grown on ½ PDA (Potato Dextrose Agar) medium at 25^{o}C for 10 days and spores were collected in water. Four weeks old plants were selected for pathogen tests. Col-0 and *atgsnor1-3* plants were inoculated with 5µl of *B. cinerea* spores (5 x 10^{5} /ml) with the help of micropipette and were provided 100% humidity (Nurmberg et al., 2007).

a)

b)

Figure 3.5 (a) ***Botrytis cinerea*** **growing on PDA medium.**

Figure 3.5 Symptom development after infection of *B. cinerea* (b) Symptom development on leaves of plants of indicated genotype 3 days after infection with *B. cinerea*. Large lesion size can be easily observed in *atgsnor1-3* as compared to wild type.

c)

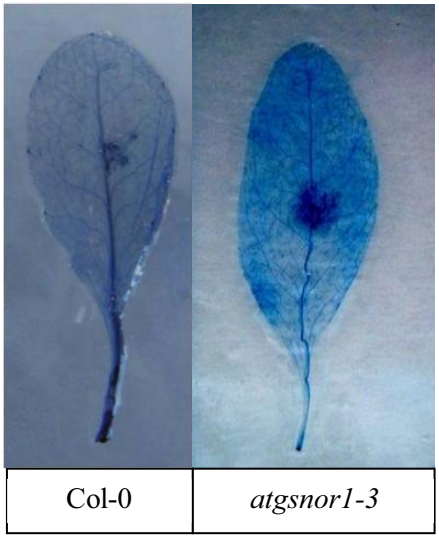


Figure 3.5 Trypan blue staining of dead cells (c) Trypan blue staining of dead plant cells show increased cell death on a*tgsnor1-3 leaves relative to wild type* after 3 days of infection with *B.cinerea*.

After three days of infection with *B. cinerea*, 5 times or 77% larger lesions were observed on the leaves of *atgsnor1-3* plants as compared to wild type (Figure 3.5.d). A comparison of lesion size on the leaves of mutant *atgsnor1-3* and wild type plants is shown in (Figure 3. 5.b). Furthermore, the leaves were also stained with trypan blue (which stains only dead or dying cells as the dead cells are permeable to uptake the trypan blue dye) shows a significantly high number of dead cells in a*tgsnor1-3* plants as compared to Col-0 (Figure 3. 5.c). For statistical analysis, cell death was measured using Adobe Photoshop CS Central European Version by using the pixel-histogram function (Hongzhe et al., 2011; Uribe et al., 2013). Collectively, this data implies that GSNOR plays a significant role in plant disease resistance by controlling the protein SNOs level. *Atgsnor1-3* plants have been found defective in their resistance against the necrotrophic pathogen *B. cinerea* implying a disrupted JA signalling.

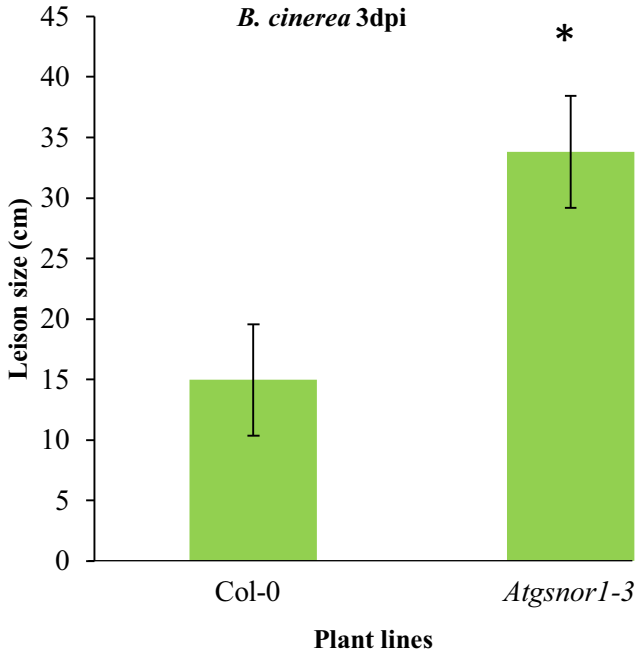
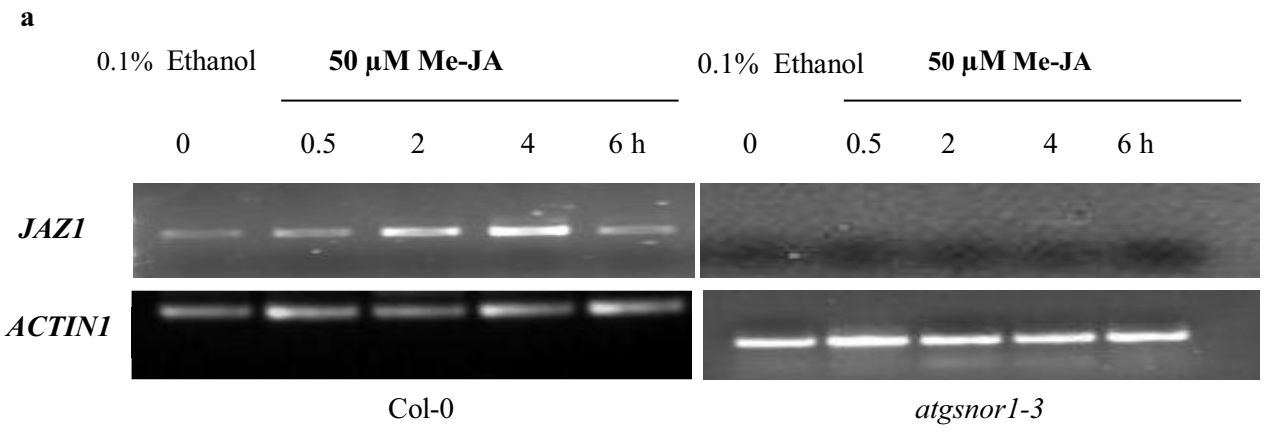
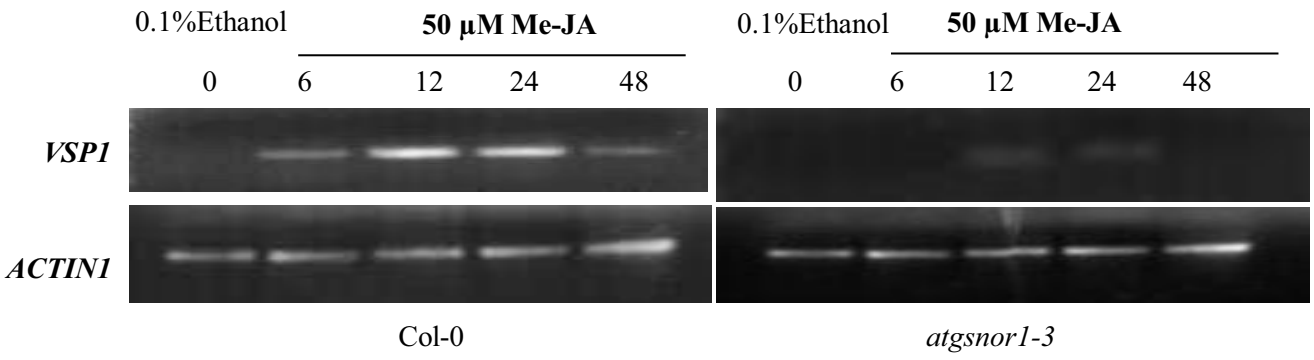


Figure 3.5 (d) **Lesion size after 3 days of infection with *B. cinerea*.** *Atgsnor1-3* plants shows larger lesion size being susceptible to the necrotrophic pathogen Data points represent mean ± s.e of at least three biological replicates. (*n*=5, where *n* is the number of plants analysed per biological replicate) student *t* test; *≤0.05, **≤0.01 showing significant difference in the lesion size.

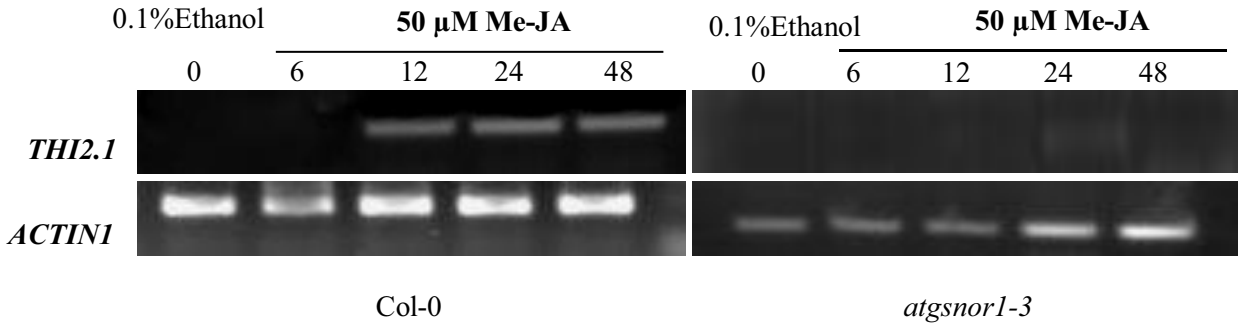
3.4 Early and late induced JA defence genes are downregulated in *atgsnor1-3* plants



b)


0.1%Ethanol
50 µM Me-JA
0.1%Ethanol
50 µM Me-JA
0
6
12
24
48
0
6
12
24
48
VSP1
ACTIN1
Col-0
atgsnor1-3

c


0.1%Ethanol
50 µM Me-JA
0.1%Ethanol
50 µM Me-JA
0
6
12
24
48
0
6
12
24
48
THI2.1
ACTIN1
Col-0
atgsnor1-3

d

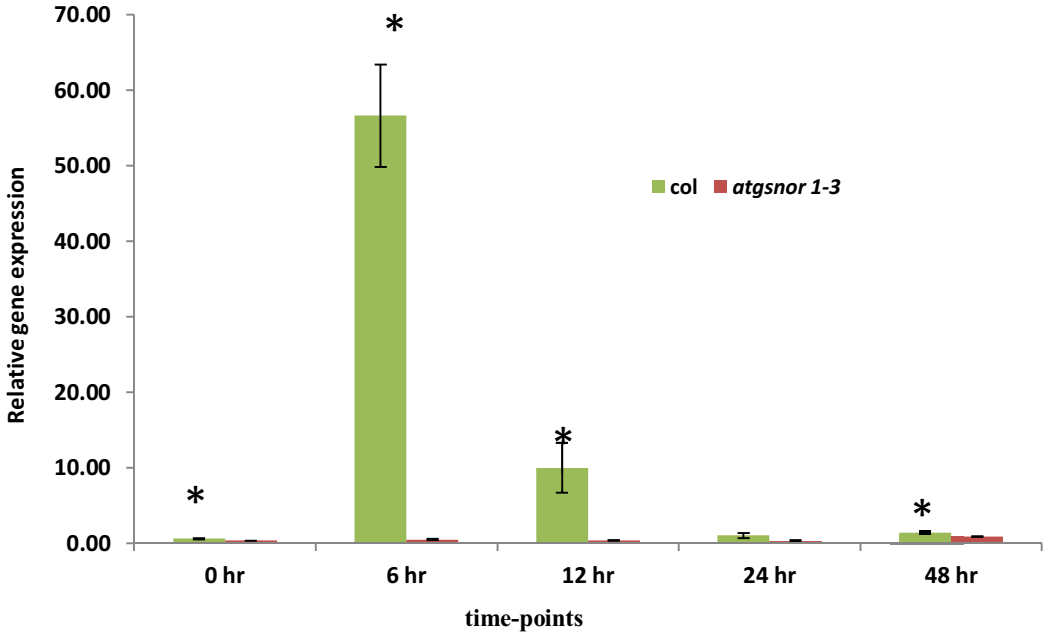

70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Relative gene expression
col
atgsnor 1-3
0 hr
6 hr
12 hr
24 hr
48 hr
time-points

Figure 3.6 Expression of JA-responsive genes in response to Me-JA. a) Expression levels of *JAZ1* in response to Me-JA in Col-0 and *atgsnor1-3* at early time points. b) Expression level of *VSP1* in response to Me-JA and **c)** Expression level of *THI2.1* in response to Me-JA in Col-0 and *atgsnor1-3* at late time points after treatment with 50 µM Me-JA. Control plants (0 h) were treated with 0.1% Ethanol. All RT-PCR experiments were repeated twice. **d) Q-RT PCR of VSP1 in response to Me-JA**. Graph of *VSP1* gene expression for Col-0 and *atgsnor1-3.* Data points represents mean ± s.e of at least two biological replicates (n=3, where n is the number of plants analysed per biological replicate) student t test; t *≤0.05, **≤0.01 showing significant difference in relative gene expression of *VSP1* in between Col-0 and *atgsnor1-3* plants. Time-points after 50 µM Me-JA treatment.

As JA levels in *atgsnor1-3* plants were reduced compared to wild type at 60 min of post-wounding, the increased susceptibility of *atgsnor1-3* plants to insect feeding and *B.cinerea* infection might reflect an additional impact of S-nitrosylation downstream of JA accumulation in the JA signalling pathway. To explore this possibility we compared the activation of JA-dependent gene expression in *atgsnor1-3* plants relative to wild-type. The function of COI1 is dependent on the degradation of JASMONATE ZIM-domain1 (JAZ1) (Thines et al., 2007). Another important factor is the protein complex SCF^{COI1}-JAZ1 which serves as a perception site of JA-Ile (Thines et al., 2007). The activation of *JAZ1* transcription occurs within the duration of 5 min in wounded leaf and is known as early responsive gene of JA signalling pathway (Chung et al., 2008; Koo et al., 2009). Along with JA pathway, JAZ1 is involved in phytochrome signalling and shade responses are integrated by JAZ1 (Robson et al., 2010). *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) is also used as a JA marker gene (Berger et al., 1995). *VSP1* is induced in response to herbivory attack and wounding (Benedetti et al., 1995; Berger et al., 1995; Berger et al., 2002) which is COI1 dependent as the *coi1*-1 fails to express *vegetative storage protein* (*VSP*) (Benedetti et al., 1995). It is also known to be regulated by external factors such as light, sugars and phosphates (Berger et al., 1995). The environmental factors such as photoperiod are involved in controlling VSP deposition (Noquet et al., 2003). *VSP1* also functions as a nutrient storage and is an acid phosphatase (Chen et al., 2007).

Thionin2.1 (*THI2.1*) are specifically activated upon necrotrophic pathogen attack or induced upon Me-JA treatment (Epple et al., 1995; Vignutelli et al., 1998). *THI2.1* consists of plant antimicrobial peptides with antibacterial and antifungal activities (Loeza-Ángeles et al., 2008).

Therefore, these three genes were selected for studying the transcript level in *atgsnor1-3* and Col-0 plants upon Me-JA treatment.

Col-0 plants were treated with 50 µM Me-JA and leaf samples were collected at 30 min, 2 hours, 4 hours and 6 hours time-points after treatment with Me-JA. Plants were treated with Me-JA dissolved in 0.1% ethanol and used to spray on control plants. The specific concentrations of Me-JA were used for these studies based on the concentration used in some previous studies to induce JA-related genes. Therefore, for studying JA-related gene transcript levels concentration of 50 µM of Me-JA (Huang et al., 1991; Staswick et al., 1991) was used and 25 µM of Me-JA for feeding assay was used.

A minor increase in *JAZ1* transcripts of *atgsnor1-3* was observed after 4 hours. However, the overall *JAZ1* expression in *atgsnor1-3* in response to Me-JA was negligible as compared to wild type (Col-0) which showed *JAZ1* induction after 30 min of treatment and reached its maximum level after 4 hours and then dropped back by 6 hours (Figure 3.6.a). This suggests an impaired JA signalling pathway in *atgsnor1-3* plants. To further confirm the downregulation of JA signalling pathway in *atgsnor1-3* we studied different gene expression. The JA signalling late marker genes *VSP1* and *THI2.1* were studied after treatment with 50 µM Me-JA.

Plants were treated with 50 µM Me-JA and samples were collected after 6 hours, 12 hours, 24 hours and 48 hours time points. Samples were collected at later time points as *VSP1* and *THI2.1* are induced later as compared to *JAZ1*. Results showed an overall negligible response of *atgsnor1-3* to the treatment with a very minor increase in *VSP1* transcripts after 12 hours and goes down again after 24 hours as compared to wild type plants which showed increase in *VSP1* transcripts after 6 hours with a maximum expression at 12 hours continued up to 24 hours (Figure 3.6.b). Similar expression pattern was observed for *THI2.1*. Expression levels were higher after 12, 24 and 48 hours of treatment in Col-0. However, very low levels of

THI2.1 transcripts were detected only after 24 hours in *atgsnor1-3* plants with a very low overall response to the treatment. Collectively the low response of *atgsnor1-3* plants to Me-JA treatment and low expression levels of JA marker genes such as *JAZ1*, *VSP1* and *THI2.1* suggest a disrupted JA signalling network in these plants (Figure3.6.a,b,c).

3.5 Expression profile of *VSP1* in response to Me-JA treatment- Real time PCR

Real-time polymerase chain reaction is also called quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or kinetic polymerase chain reaction (KPCR), which is used to amplify and simultaneously quantify a targeted DNA molecule (Higuchi et al., 1992). It enables both detection and quantification of gene expression from small amounts of RNA (Wong and Medrano, 2005). The number of cycles at which the fluorescence exceeds the threshold is called the cycle threshold; Ct value (Heid et al., 1996). SYBR Green is a dye used in qPCR, and as it binds the dsDNA, it fluoresces brightly (Rasmussen et al., 1998). According to the results of gene expression data by RT-PCR, *VSP1* and *THI2.1* marker genes has shown to be suppressed in *atgsnor1-3* plants. Further to detect and quantify the gene expression data real-time PCR was conducted.

A. thaliana consists of two different branches of the JA signalling pathway, MYC-branch and the ERF branch. The MYC2 branch is specifically regulated by bHLH TFs such as MYC2, MYC3 and MYC4 (Anderson et al., 2004; Fernandez-Calvo et al., 2011; Niu et al., 2011; Vos et al., 2013). They may play an important nutritional role during the plant development The cells expressing *VSP1* genes may display a higher level of endogenous JA (Huang et al., 1991). All leaf cells are capable of expressing *VSP1* gene upon Me-JA treatment (Huang et al., 1991). Therefore, we selected *VSP1* gene for q-RT-PCR analysis.

Two biological replicates are used for real-time PCR. *VSP1* gene primers used for gene expression analysis as stated in Chapter 2. PCR run stops after 40 cycles.

qRT-PCR results show a fifty-fold increase of *VSP1* gene expression in Col-0 (after treatment of 50µM Me-JA) at 6 hours. Later gene expression was decreased drastically at 12 hours in Col-0 plants and was much reduced at 24 hours and again slightly increased at 48 hours. However, no apparent increase in *VSP1* gene expression was shown in *atgsnor1-3*. The *VSP1* gene expression was low throughout all time-points as compared to Col-0 confirming that this gene is markedly down-regulated in *atgsnor1-3*. Sample collection time for q-RT-PCR was

different than the sample collection of RT-PCR. *VSP1* gene expression is accumulated at a high level at day hours, and sample collection timing may be the reason to observe differences in the kinetics of gene expression in between the two experiments (RT-PCR and q-RT-PCR) (Berger et al., 1995). Expression analysis by qRT-PCR -Data shown represents mean values obtained from at least three independent amplification reactions. Error bars depict standard error of the mean, (three references of sample replicates). Error bars are derived from standard deviations. Statistical analysis has shown that there is a significant difference in the overall gene expression of *VSP1* between Col-0 and *atgsnor1-3* plants after treating with 50 μM MeJA. Critical value p is 0.01963.

This data suggest that *VSP1* gene is activated significantly less in *atgsnor1-3* plant lines (Figure 3.6.d).

Collectively, this data shows that JA-regulated gene expression is diminished in *atgsnor1-3* plants relative to wild-type in response to Me-JA treatment.

3.6 JA levels are reduced after mechanical wounding in *atgsnor1-3* background

The JA signalling pathway is involved in resistance against the attack of insects and necrotrophic pathogens. To explore the molecular basis of the observed increased susceptibility of *atgsnor1-3* plants to insect feeding and *B. cinerea* infection, we were interested to study the endogenous concentration of JA levels in *atgsnor1-3*. The JA levels were measured by using Gas chromatography and mass spectrometry which was performed by Prof. Peter Morris at Herriot-Watt University, UK. Wounding in *A. thaliana* mimics insect feeding and induces JA accumulation (McCloud and Baldwin, 1998).To measure the JA levels in *atgsnor1-3* plants, a wounding experiment was conducted. *Coi1*-1 mutant plants (being insensitive to JA) and Col-0 were included as controls. JA production is triggered upon wounding and herbivory as cellular mechanisms convey a signal to plastids and thus activates constitutive enzymes to start the production of JA (Bonaventure and Baldwin, 2010).

After 15 min post wounding more than a 3 fold increase in JA level was observed in *atgsnor1-3* as compared to about 2.5 fold increase in wild type. After 30 min of mechanical wounding, JA concentrations increased further both in wild type and *atgsnor1-3* mutants however, the increase in JA levels of *atgsnor1-3* was only 1 fold as compared to a 2 fold

increase in wild type. A similar pattern in the increase of JA levels was observed after 60 min with a 2 fold increase for *atgsnor1-3* plants as compared to a 3 fold increase in Col-0 (Figure 3.7). Significantly, at this time point the levels of JA in *atgsnor1-3* plants relative to wild-type were reduced. Thus collectively these data suggest that *atgsnor1-3* plants do produce significantly lower amounts of JA in response to mechanical wounding relative to wild-type at 0, 15 or 30 mins post wounding. However, there is a relatively small but significant decrease in JA levels at 60 min post wounding compared to wild type plants. Thus *atgsnor1-3* plants are perturbed in the level of JA production.

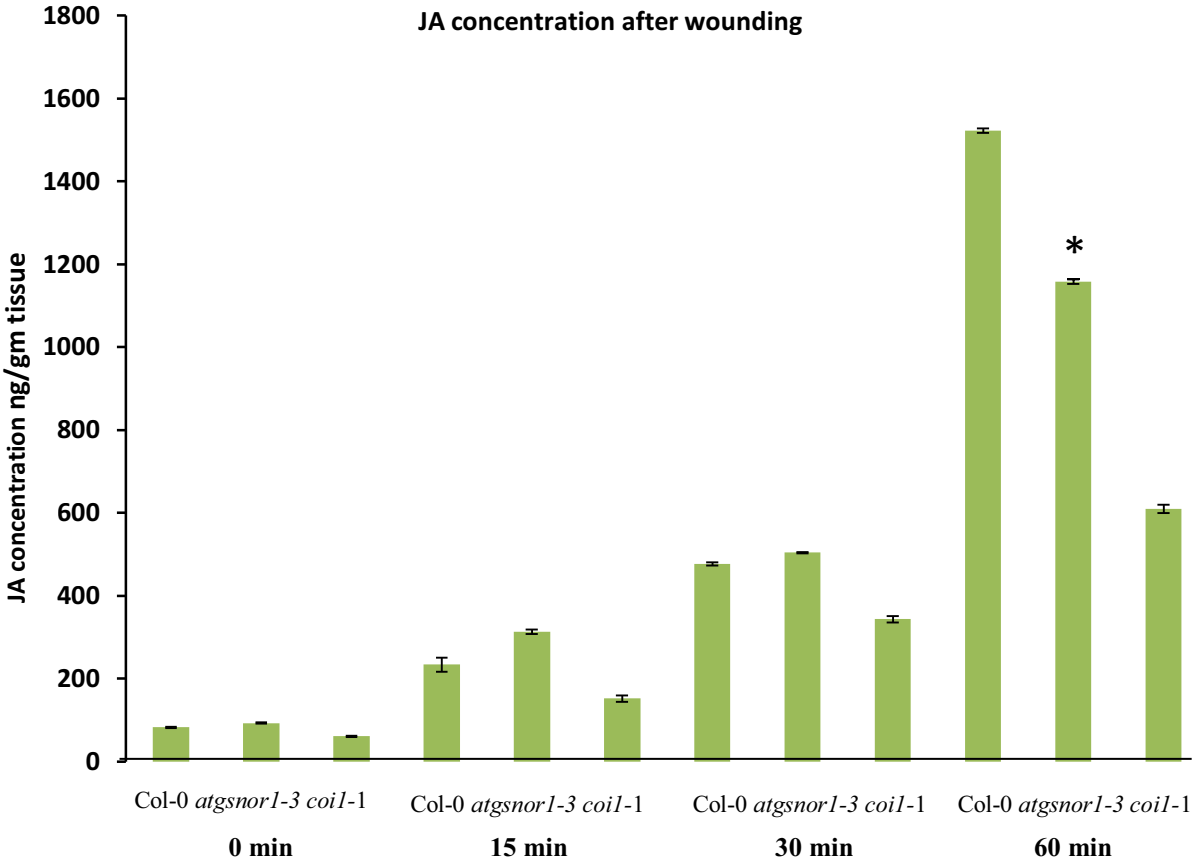


Figure 3.7 Measurement of JA levels in response to mechanical wounding. At 0, 15, 30 min time-point of wounding no significant difference was observed in Col-0 and *atgsnor1-3*. At 60 min of post-wounding a significant reduction in JA levels was observed in *atgsnor1-3* compared to Col-0. Data points represent mean ± s.e of at least two biological replicates. (n=2, where n is the number of plants analysed per biological replicate) student t test; *≤0.05, **≤0.01 showing significant difference in JA levels.

Thus collectively these data suggest that in *atgsnor1-3* plants there is a relatively small but significant decrease in JA levels at 60 min post wounding compared to wild type plants. *Atgsnor1-3* may be impaired in producing endogenous JA as compared to wild type and AOC is known to be S-nitrosylated at the cysteine which is proximal to the catalytic site (Delker et al., 2006). Therefore, S-nitrosylation may have an impact on the accumulation of JA levels in *atgsnor1-3* compared to wild type plants. Here our study suggests that *atgsnor1-3* plants have been found to be susceptible to infection by necrotrophic pathogens such as *B. cinerea* (Figure 3.5.a, b, c & d) and insect attack by *P. rapae* (Figure 3.2-Figure 3.4). It is likely that the relatively small decrease in JA levels observed at a later time point post wounding may not be sufficient to explain the increased susceptibility of *atgsnor1-3* plants to insect feeding and *B. cinerea* infection. Therefore, increasing the number of replication and studying the transcript of JA biosynthesis genes could provide with a better understanding of JA accumulation in *atgsnor1-3*.

3.7 Discussion

Jasmonates have been recognised as a vital defence-related hormone in the plant kingdom, similar to prostaglandins that function as the primary animal defence regulators (Mueller, 1998). *B. cinerea* is known to attack crops where more than 200 plant species are affected (Prins et al., 2000). *B. cinerea* infection may be dependent upon the timing and intensity of cell death or oxidative burst (Asselbergh et al., 2007; Yoshioka et al., 2009). Cell death leads to rapid necrosis in host plant cell which facilitates the pathogen infection of *B.cinerea* (Dickman et al., 2001; Govrin and Levine, 2000; Perchepied et al., 2010; Van Baarlen et al., 2007). To achieve the pathogenicity, *B. cinerea* may induce HR cell death in host plant cells in species like *A. thaliana* and tobacco (Dickman et al., 2001; Govrin and Levine, 2000; Kars et al., 2005). However, a controversy remains between cell death in HR and *B. cinerea* invasion (Asai and Yoshioka, 2009). In *ATGSNOR1,* a decrease in SNO level leads to increased resistance against microbial invasion (Feechan et al., 2005). The reduction in NO accumulation leads to susceptibility of pathogens (Delledonne et al., 1998; Zeidler et al., 2004). JA signalling mutants are susceptible against necrotrophic pathogen attack. The induction of induced systemic resistance (ISR) is mediated by JA/ET pathway which is also functional in Nah*G* an SA mutant (Choudhary et al., 2007; Pieterse et al., 1998). *B. cinerea* infection triggers the production of camalexin and transcription of genes belonging to JA/ET signalling pathway are activated (Govrin and Levine, 2002). VSPs are the only related genes to ISR while the transcriptional activation of genes consisting of antifungal properties are activated by JA/ET signalling pathway (Hammerschmidt, 1999; Pieterse et al., 1998).
Here, *atgsnor1-3* plants upon infection were not sufficient to suppress the lesion development and fungal growth. Therefore, *atgsnor1-3* failed to inhibit *B.cinerea* growth and its infection compared to wild type plants. Thus, *atgsnor1-3* is compromised in their resistance against the necrotrophic infection in comparison with wild-type plants.

JA insensitive mutant *coi1*-1 is reduced in responsiveness towards JA treatment and is thus extensively used to study the effects of JA signalling in various plant processes. Here in this study *coi1*-1 plants are included as a negative control to study the plant defence response. *coi1*-1 plants are also well known to be more sensitive to insect attack in different plant species like tobacco, tomato and *Arabidopsis*. *COI1* is the key regulator of genes involved in JA metabolism and systemic signalling after insect attack (Devoto et al., 2005). It is well established that *P. rapae* the specialist herbivore triggers a set of defence responsive genes

after its attack in *Arabidopsis*. A decline of 13% of crop loss is observed due to herbivory insect attack (Schoonhoven et al., 1998). Study of *A. thaliana* mutants has been revealed that the *VSP* genes are accumulated upon pathogen and insect attack (Ellis and Turner, 2001b). The study of *jin1-2* mutant shows an impairment of *MYC2* gene (Lorenzo et al., 2004). The gene expression of *VSP2* is less in this mutant upon *P. rapae infestation* while *PDF1.2* accumulates more. The transcript of *VSP2* is induced from MYC2 branch of JA response. Therefore, *MYC2* is involved in inducing plant defence against herbivory insect (Pieterse et al., 2012a; Pre et al., 2008). No-choice experiment has shown that *coi1*-1 plants are highly susceptible to *P. rapae* attack. Me-JA treated Col-0 plants were capable of inducing defence responses while *atgsnor1-3* plants showed higher susceptibility against insect attack. MYC2 has four cysteine residues and is a potential candidate of getting S-nitrosylated. It may be the S-nitrosylated proteins could modulate the signalling pathway in plants (Paris et al., 2013). *Nicotiana attenuata* RNAi based gene silenced plants accumulate more GSNO. Silencing *NaGSNOR* has shown an increased susceptibility towards insect herbivore *Manduca sexta* attack (Wunsche et al., 2011). Thus, collectively these data suggest GSNOR plays an essential role in plant defence against specialist insect herbivore *P. rapae*.

We used JA marker gene expression as an indicator for active JA signalling pathway. Induced Me-JA gene expression was studied in Col-0 and *atgsnor1-3*. Early and late responsive marker genes like *JAZ1* and late responsive genes like *VSP1* and *THI2.1* were expressed strongly and rapidly in response to Me-JA treatment in wild type plants whereas gene expression was relatively weak and delayed in *atgsnor1-3*. Study in *A. thaliana* has been shown that NO changes the transcription level upon the treatment of gaseous NO and sodium nitroprusside (SNP) (Huang et al., 2002; Polverari et al., 2003). The process of S-nitrosylation may lead to change the protein from their subcellular localisation and may lead to export or import in the nucleus (Malik et al., 2010; Qu et al., 2007). S-nitrosylation of nuclear proteins leads modulation of transcription in plants (Mengel et al., 2013). Study of microarray analysis in *GSNOR* null mutant has shown downregulation of expression of genes where one-third of them are related to pathogen resistance (Xu et al., 2013). Loss of GSNOR has demonstrated to influence calcium signalling, redox and pathogen response (Xu et al., 2013). Therefore, here our data illustrates the role of GSNOR1 as a positive regulator of JA mediated defence response in plants. Here, *atgsnor1-3* plants after the infection were not sufficient to suppress the lesion development and fungal growth. Therefore, *atgsnor1-3* failed

to inhibit the *B.cinerea* growth and its infection compared to wild type plants. Therefore *atgsnor1-3* plants are compromised in their resistance against the necrotrophic infection in comparison with the wildtype plants.

The conversion of methionine to *S*-adenosylmethionine (*S*-AdoMet) is catalysed by *S*-AdoMet synthases (SAMSs), and *S*-AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) that is mediated by ACC synthases (ACSs). The last step is the oxidation of ACC, which is catalysed by ACC oxidase (ACOs) to form ET (Wang et al., 2002). SAMSs and also methionine adenosyltransferases (MATs) are the enzymes involved in the biosynthesis of ET. Significantly some of these enzymes have identified as targets of S-nitrosylation. Thus, NO may regulate ET biosynthesis can controlling the activity of its biosynthetic enzymes (Lindermayr et al., 2006; Lindermayr et al., 2005).

Similarly in our studies here, *atgsnor1-3* has displayed a significant decrease in JA levels after 60 min of post wounding compared to Col-0. *Atgsnor1-3* plants accumulate more SNO, which may nitrosylate the biosynthetic genes involved in JA pathway and in return may decrease their activity. It is known as one of the enzymes involved in oxylipin pathway for JA biosynthesis, (AOC) identified to be a nitrosylating target (Romero-Puertas et al., 2008). Subsequently, this could establish a link in between NO and JA biosynthesis (Romero-Puertas et al., 2008). It could be one of the reasons for a disturbance in the synthesis of JA levels in *atgsnor1-3* plants compared to wild type plants. However, repetition of the experiment with considerable number of replicates might produce more significant results. Thus our results state that GSNOR has an important consequence in plant resistance against insects and necrotrophic pathogens.

Chapter 4

GSNO modulates JA signalling protein interactions in yeast

4.1 Background

Previous studies have been shown JAZ1 is a substrate of SCF^{COI1} ubiquitin ligase and physically interacts with COI1 in yeast two hybrid assays. The interaction was dependent remarkably on JA-Ile but not any other jasmonate precursors such as Me-JA, OPDA (Thines et al., 2007). The requirement of JA-Ile or coronatine is not unique for COI1-JAZ1 but can be extended to other JAZ family proteins (Melotto et al., 2008). Researchers were also interested to study this interaction in other plant species such as *Solanum lycopersicum* where SICOI1 and SIJAZ1 interaction was stimulated only by JA-Ile (Thines et al., 2007). Modification of cysteine is known to influence the function of protein in association with another protein (Jia et al., 2012). Here we hypothesised that NO might influence protein-protein interactions involved in JA signalling pathway. Therefore to study the impact of S-nitrosylation on JA signalling; we used NO donors externally in a Y2H assay. Different combinations of proteins-protein interactions were tested for this study such as COI1-JAZ1 and JAZ1-MYC2.

Protein-protein interactions can be determined by biochemical techniques such as crosslinking, co-immunoprecipitation and co-fractionation by chromatography (Fields and Song, 1989). Among these the Y2H assay is one of the most frequently used methods to study protein-protein interactions and was developed by Fields and Song (Fields and Song, 1989). GAL4 is a transcriptional activator of *Saccharomyces cerevisiae* (Brent and Ptashne, 1985). The GAL4 protein consists of two separate domains; the C -terminal domain and the N-terminal domain. The DNA binding domain (BD) is at its amino terminus (Keegan et al., 1986) and an activation domain (AD) at its carboxy terminus (Brent and Ptashne, 1985). A gene transcript process occurs only in the presence of both the domains. Thus, the proteins of interests are fused to either the AD or the DNABD. Here in our study we used Clontech vectors pDEST-32 and pDEST-22. The protein attached to the DNA-BD is the “bait” (pDEST-32), while the protein fused to AD is a “prey” (pDEST-22). The GAL4 protein activates transcription only after physical interaction between the “bait” and “prey” proteins which brings the GAL4 AD into association with its DNA BD (Fields and Song, 1989). This

can be detected by yeast growth on specific media or marker enzyme activity, depending upon the auxotropic growth markers.

HIS3 is the most sensitive reporter gene but in most yeast strains selection can be leaky and least selective. Thus a histidine analogue, 3-amino-1, 2, 4-triazol (3-AT) is added in the media. 3-AT is a competitive inhibitor of imidazole glycerol phosphate dehydratase, a biosynthetic enzyme of histidine (Hilton et al., 1965; Klopotowski and Wiater 1965) and therefore limiting biosynthesis of HIS and helps in reducing the background growth (Lentze and Auerbach, 2008; Phizicky and Fields, 1995; Topcu and Borden, 2000).

COI1 contain LRRs and a degenerate F-box motif (Xie, 1998). JAZ proteins, the targets of the Skp1/Cullin/F-box (SCF) complex and *JAZ* transcripts are induced upon wounding or JA treatment (Chini et al., 2007; Thines et al., 2007). The SCF complex functions as an E3 ubiquitin ligase, these enzymes target specific protein substrates for ubiquitination and subsequent degradation by the proteasome (Morgan, 2006). Upon perception of JA-Ile COI1 F-box recognises JAZ proteins. This recognition leads to ubiquitination and degradation of JAZ proteins (Chini et al., 2009c; Chung and Howe, 2009; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007; Yan et al., 2009). MYC2 TF is released upon degradation of JAZ proteins. (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). MYC proteins belong to the basic helix–loop–helix (bHLH) domain-containing family of TFs (Lorenzo et al., 2004). A small clade of *Arabidopsis* bHLH TFs (bHLH003, bHLH013 and bHLH017) are phylogenetically closely related to MYC2, MYC3 and MYC4 and all these TFs can interact with JAZ proteins *in vivo* shown by the Tandem Affinity Purification (TAP) (Fonseca et al., 2014) . However, MYC2 is thought to be the most prominent and a master regulator in the control of JA-Ile-mediated defence and developmental responses (Kazan and Manners, 2013).

4.2.1 PCR optimization and cDNA amplification

The full length Arabidopsis *JAZ1*, *COI1* and *MYC2* were amplified by PCR from a cDNA library of Col-0 wild type plants: *JAZ1* (762 bp), *COI1* (1779 bp) and *MYC2* (1872 bp). The amplification products were achieved in the range of 58°C for *JAZ1*, *MYC2*, while 60°C was used for *COI1*. The PCR products were analysed by agarose gel electrophoresis (Figure 4.1) which showed DNA product bands of the corresponding sizes. The PCR products were further used for cloning reactions.

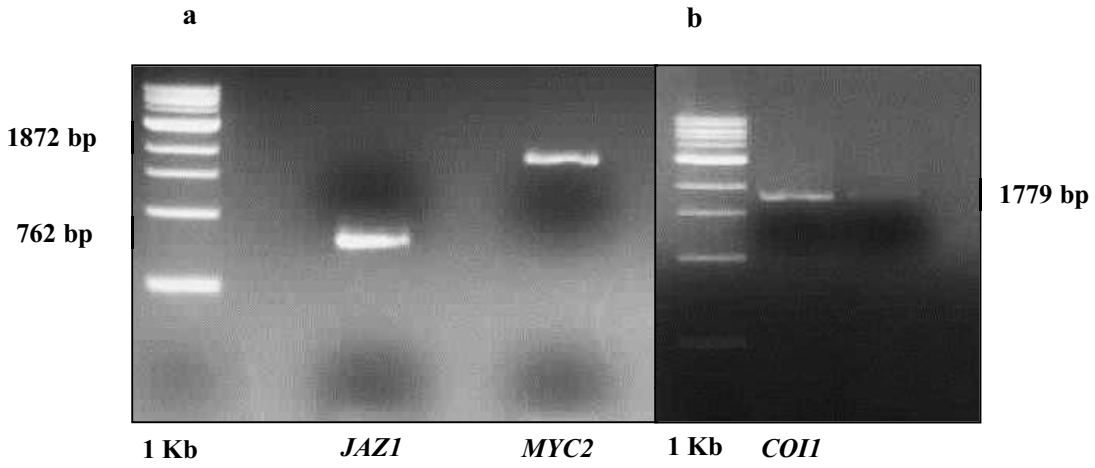


Figure 4.1.a) PCR product of *JAZ1* (762 bp) and *MYC2* (1872 bp). **b)** PCR product of *COI1* (1779 bp). 1.2 % agarose gel used. To analyse the PCR product size 1 kb NEB DNA ladder was used..

4.2.2 Generation of entry clones and expression clones for the study of yeast two hybrid assay

PCR products were cloned into the pDONR221 entry vector with a Gateway BP II kit using BP clonase mix (Nakagawa et al., 2008) (Invitrogen, http://www.invitrogen.com) and the clones were sequenced at The GenePool, University of Edinburgh, UK using gene specific primers and the primer sequence is stated in Chapter 2.

Different colonies were picked and results of the entry clone were analysed by 1.2% agarose gel electrophoresis (Figure 4.2. a, b, c) that showed the product bands with their correct sizes.

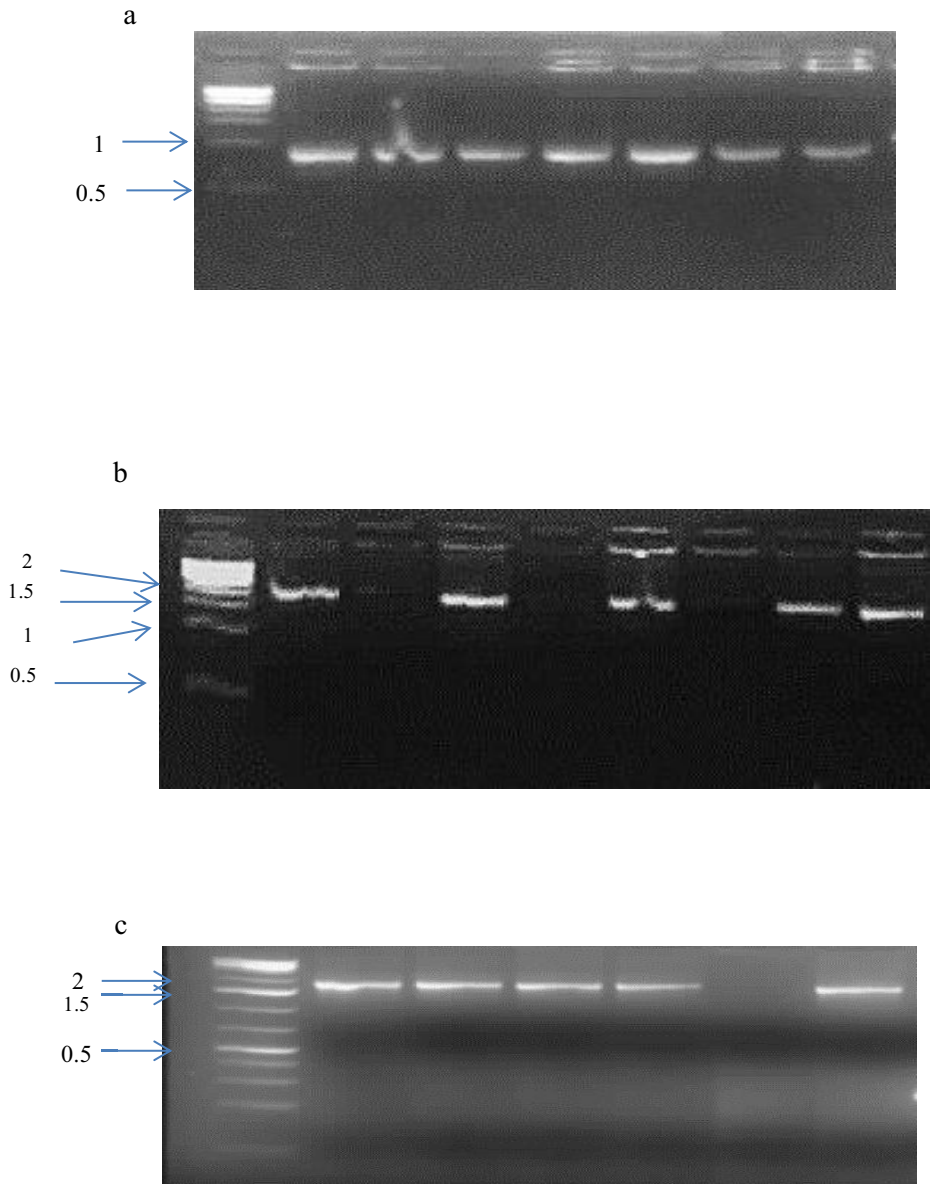


Figure 4.2 Colony PCR of entry clone of a) *JAZ1* (765 bp), b) *MYC2* (1872 bp) and c) *COI1* (1779 bp) on 1.2% agarose gel. 1 kb DNA ladder used (NEB) for *JAZ1* and *COI1*, while 1 kb Plus DNA ladder was used for *MYC2* (GeneRuler^{TM}).

Further the pDONR221 constructs described above were used in Gateway LR (Invitrogen) reactions, in combination with the destination vector pDEST-22-gateway (Gal4 AD) and pDEST-32 gateway (Gal4 DNA BD) (Curtis and Grossniklaus, 2003) in which the gateway

cassette were generated. In one combination *COI1* was cloned in pDEST-32 as "bait" *and JAZ1* cDNA was fused in pDEST-22 as a "prey". To access the protein interactions in between JAZ1 and MYC2, *JAZ1* was cloned in pDEST-32 (Gal4 BD), and *MYC2* into pDEST-22 (Gal4 AD). The protein fused in appropriate vectors were confirmed by colony PCR (Figure 4.3. a, b, c, d).

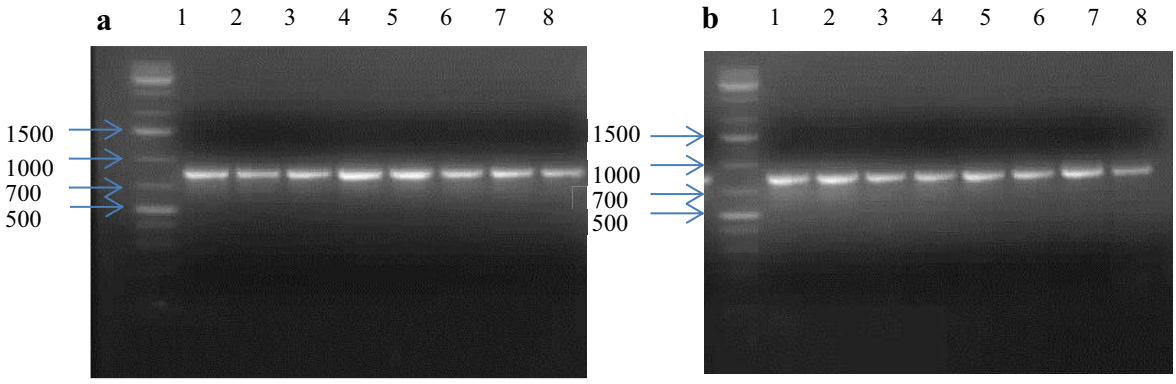


Figure 4.3 Colony PCR for expression clones. a) pDEST22-*JAZ1* and b) pDEST32-*JAZ1* by using gene specific-primers. The product size is 762 bp. 1 kb plus DNA ladder used (GeneRuler^{TM}).

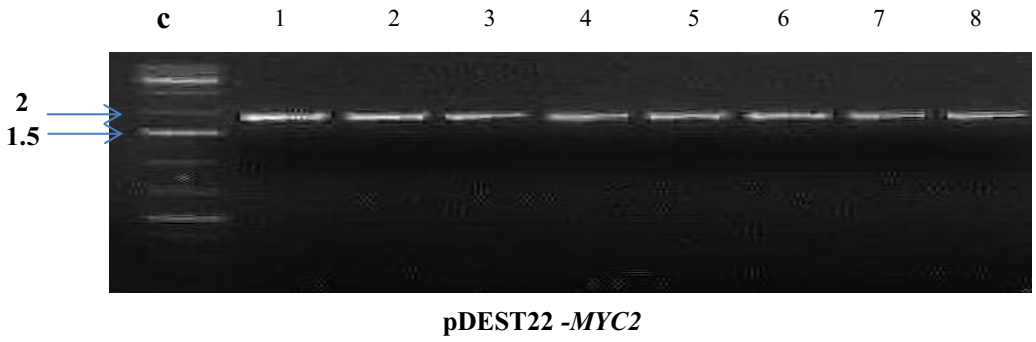


Figure 4.3.c Colony PCR for expression clones of pDEST22-*MYC2* by using gene specific primers. The product size is 1872 bp. 1 kb DNA ladder used (GeneRuler^{TM}).

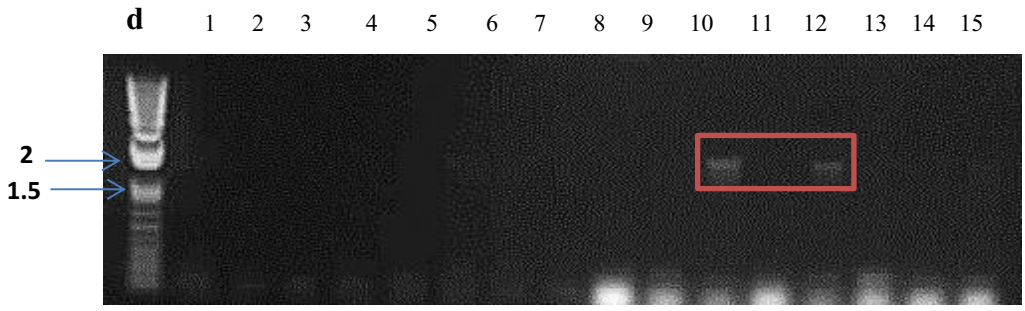


Figure4.3.d Colony PCR for expression clone of *COI1* in pDEST-32.The red marked bands highlights the right size bands of *COI1* while the other colonies do not show any positive insert. *COI1* gene specific primers were used. 1 kb (GeneRuler^{TM}) DNA ladder used.

To study protein interactions, the corresponding constructs were transformed into *S. cerevisiae* Mav203 strain by lithium acetate yeast transformation method (Gietz and Woods, 2002).

Cloned-insert DNA was confirmed by screening yeast transformants and a colony PCR was performed that shows the cloned insert were successfully transformed into Mav203 strain. The yeast colony PCR for all the expression clones of JAZ1, MYC2 and COI1 are shown in the figure below (Figure 4.4.a, b).

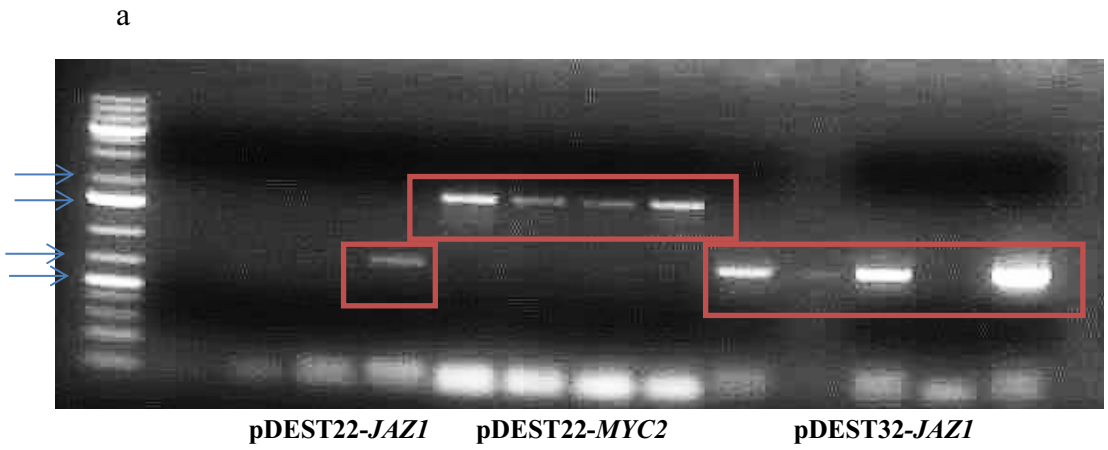


Figure 4.4 Yeast colony PCR for pDEST22-*JAZ1* pDEST22-*MYC2* and pDEST32- *JAZ1*
(a) Yeast colony PCR on 1.2% agarose gels for pDEST22-*JAZ1***(762 bp)**pDEST22-*MYC2* **(1872 bp)** and pDEST32- *JAZ1* **(762 bp)**. 1 kb DNA ladder used (GeneRuler^{TM}).

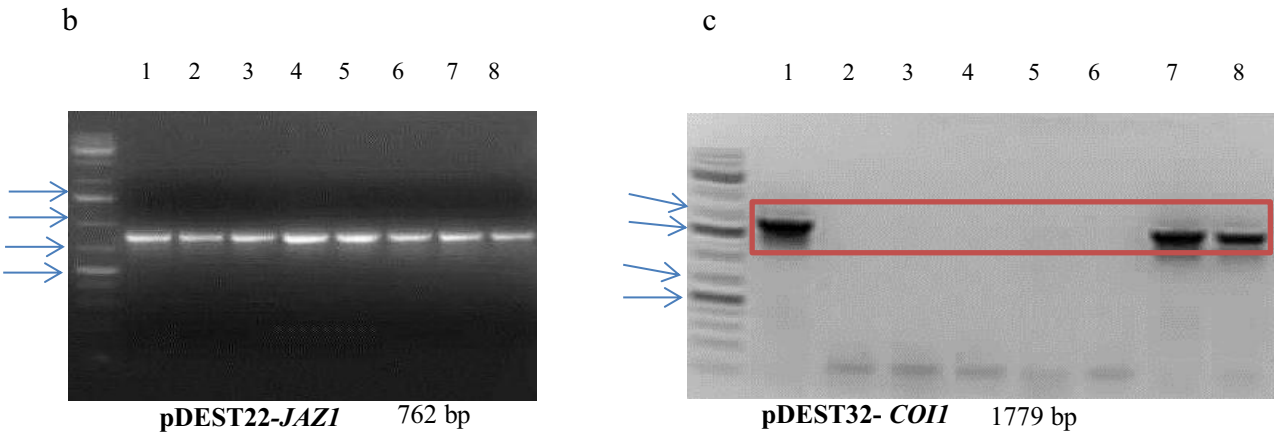


Figure 4.4 Yeast colony for pDEST22-*JAZ1*, pDEST32-*COI1* (b) Yeast colony for pDEST22-*JAZ1* with a size of 762 bp and (c) red marked band shows pDEST32-*COI1* transformed in yeast cells.1 kb DNA ladder used (GeneRuler^{TM}).

4.3 NO blocks interaction of COI1 and JAZ1

S. cerevisiae MaV203 contains auxotrophic mutations that are complemented by following the interaction of "bait" and "prey" proteins (Noel et al., 2014).

The transformed yeast cells were plated on appropriate (SD) media. The plates were incubated at 30°C for 3 days. Three different plates were used as a control, glutathione (GSH) and S-nitrosoglutathione (GSNO), and all three were lacking the amino acids leucine (Leu) and tryptophan (Trp). The combinations of "bait" and "prey" constructs transformed into *S. cerevisiae* MaV203 are described in table 4.1.

Table 4.1 Shows a combination of proteins used as "bait" and "prey" for testing protein-protein interactions.

Transformation of experimental interactions. LEU Plasmid (BAIT)	TRP Plasmid (PREY)	Experimental interaction
pDEST32-COI1	pDEST22-JAZ1	COI1-JAZ1
pDEST32-JAZ1	pDEST22-MYC2	JAZ1-MYC2

Both the fused proteins are important in Y2H assay as alone the expression construct is not sufficient to initiate the reporter gene transcription, therefore yeast cells fail to grow. The plate SC (-leu-trp) was the control plate, merely indicating that the cells contained both "bait" and "prey" plasmids.

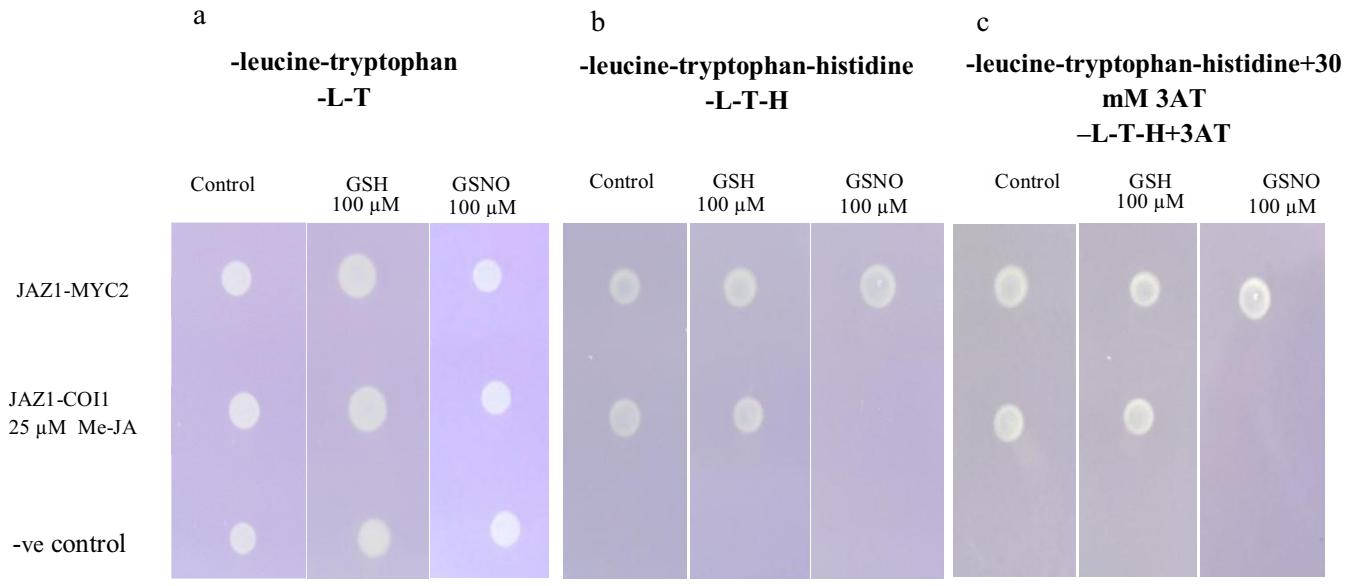


Figure 4.5.a Y2H assays of JAZ1-MYC2 and JAZ1-COI1. Cells were grown on selective media for 3 days at 30^{o}C. AD and BD served as negative control. pDEST32-JAZ1 (bait) and pDEST22-MYC2 (prey) grow on –Leu-Trp control, 100 µM GSH and 100 µM GSNO plates.

pDEST32-COI1 (bait) and pDEST22-JAZ1 (prey) grow on control, 100 μM GSH and also 100 μM GSNO plates in presence of 25 μM Me-JA.

b) Protein-protein interaction on –Leu-Trp-His plates**.** pDEST32-JAZ1 (bait) and pDEST22-MYC2 (prey) grow on –Leu-Trp-His control, 100 μM GSH and 100 μM GSNO plates. pDEST32-COI1-pDEST22-JAZ1 grow on control, 100 μM GSH but no growth was observed on 100 μM GSNO plates in presence of 25 μM Me-JA. The *HIS3* reporter gene was not activated in negative control.

c) JAZ1-MYC2 interaction was observed on –L-T-H+30 mM 3AT control, GSH and GSNO plates. COI1-JAZ1 interaction was observed only on control and GSH plates.100μM GSNO inhibits COI1-JAZ1 interaction. No yeast growth was observed in negative control. 25 μM concentration of Me-JA was used.

.

JAZ1 and MYC2 as the "bait" and "prey" after mating were capable of growing on yeast SD media lacking leu and trp. Similarly a combination of pDEST32-COI1 and pDEST22-JAZ1 were capable of growing on SD (–L-T) in presence of 25 μM Me-JA. A fusion of pDEST32-COI1 (BD) and empty vector pDEST22 served as negative control. Growth of the yeast cells of fusion of JAZ1-MYC2 protein combination and also in COI1-JAZ1 "bait" –"prey" combinations have grown on SD (-L-T) plates containing 100 μM of GSH, an antioxidant. Addition of 100 μM of GSNO to the SD (-L-T) yeast media did not suppress yeast cell growth in strains containing pDEST32-COI1 or pDEST22-JAZ1 (Figure 4.5.a).

Interactions between the combinations of proteins were observed by monitoring the growth of yeast cells on growth medium lacking HIS. Yeast SD media plates like (–L-T-H) are used to test the activation of *HIS3* reporter gene. The protein-protein interaction drives the expression of *HIS3* reporter gene. 3-AT is a competitive inhibitor of HIS and also suppresses self-activation ((Van Criekinge and Beyaert, 1999)thus it was used in –L-T-H plates to increase stringency in for protein-protein interactions.

Optimal concentration of 3-AT allows visualising the expression of protein-protein interactions by minimising the background and eliminating weak interactions (Van Criekinge and Beyaert, 1999)(Criekinge & Beyaert, 1992).

. JAZ1 fused as "bait" and MYC2 fused as "prey" were capable of driving the expression of *HIS3* reporter genes. These proteins show a strong protein-protein interaction. In the presence of 100 μM GSH the two proteins were capable of interacting. Addition of an NO donor such as 100 μM

GSNO did not show any significant alteration in JAZ1-MYC2 interaction therefore activating the *HIS3* reporter gene. Interestingly the yeast SD (–L-T-H) and (–L-T-H +30 mM 3AT) plates supplemented with 100 μM of GNSO were capable of preventing the yeast cell growth and were inefficient to activate the reporter genes expression. Therefore this study concluded that the COI1 and JAZ1 interaction in the presence of 25 μM of Me-JA was blocked by the application of NO donor but not in the plates consisting of 100 μM GSH.

4.4 *JAZ1* degradation is inhibited by NO in histochemical GUS analysis but not in fluorometric GUS activity

JA enhances the removal of JAZ proteins (Zhu et al., 2011b) and this allows the engagement of JA signalling. JA enhances the interaction of JAZ1-COI1 (Thines et al., 2007). This promotes the ubiquitination of JAZ1, leading to the degradation of these proteins, once they have interacted with the COI1 in a SCF^{COI1} complex (Thines et al., 2007). To investigate the role of degradation of JAZ proteins, transgenic plants expressing a JAZ1-GUS fusion protein under the control of the CaMV*35S* promoter were used which were generated in John Browse's Lab (Thines et al., 2007). The *E. coli uidA* gene encodes β-glucuronidase (GUS) (Jefferson, 1987). GUS is one of the most popular reporter genes used in plant transformation for assessing promoter activity. In our study to explore impact of NO on JAZ stability we utilised these transgenic lines.

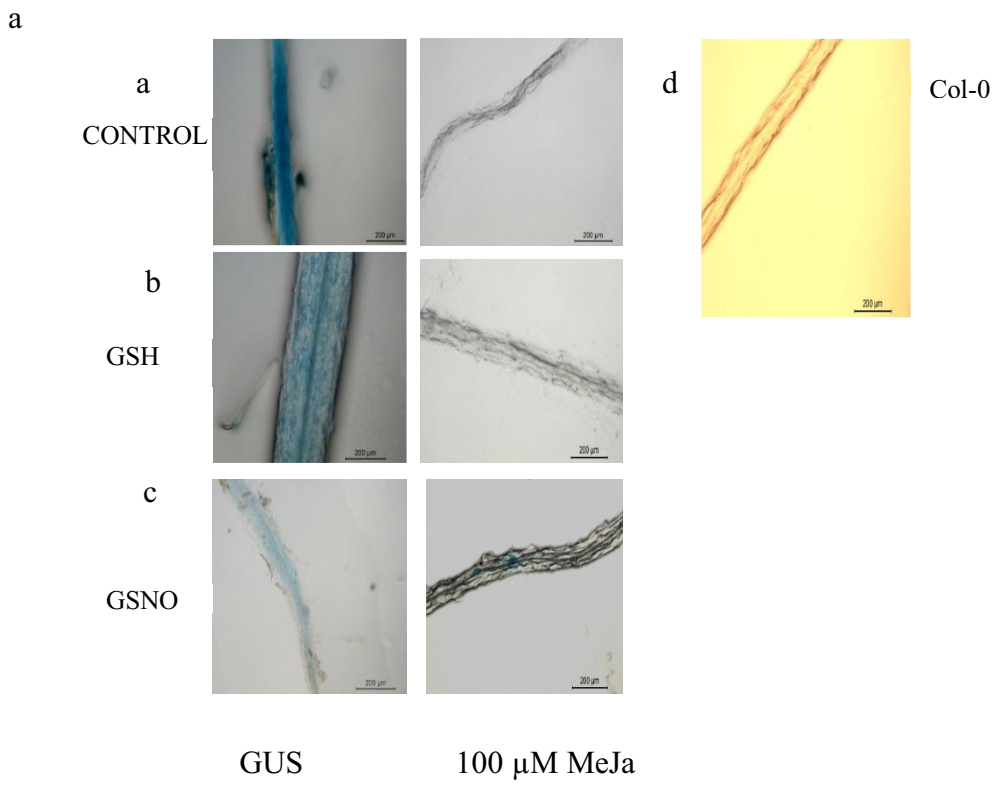


Figure 4.6.a Histochemical staining of 35S-*JAZ1-GUS*. Histochemical staining of GUS activity in the 10-day-old 35S-*JAZ1-GUS* transgenic plants upon Me-JA treatment. a) 100 µM Me-JA degraded GUS activity in *35S-JAZ1-GUS* seedlings. b) 100 µM GSH treated seedlings exhibits inhibition of GUS activity upon Me-JA treatment. c) 100 µM GSNO treated seedlings are resistant to MeJA treatment. d) Col-0 plant serves as a negative control. This experiment was repeated atleast with two biological replicates (*n*=3, where n is the number of plants analysed per biological replicates).

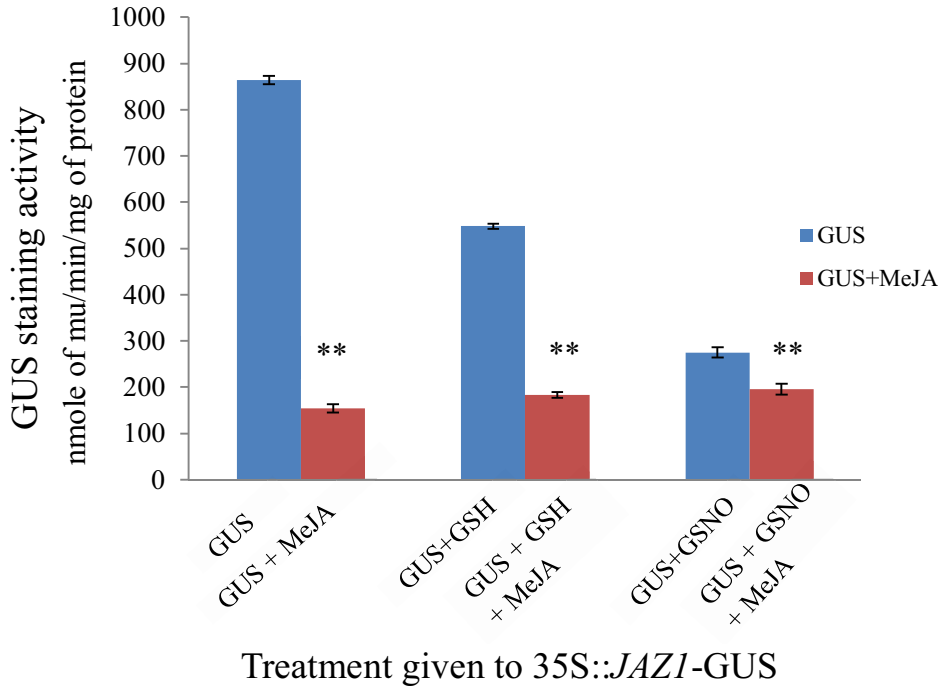


Figure 4.6.b Fluorometric GUS activity of 35S::*JAZ1*-GUS with treatment of GSH and GSNO with and without MeJA. Data points represent mean ± s.e of at least three biological replicates. (*n*=3, where *n* is the number of plants, analysed per biological replicate) student *t* test; *≤0.05, **≤0.01, showing significant difference in GUS activity degradation.

The CaMV*35S::JAZ1-GUS* lines, after staining exhibits strong β-glucuronidase activity, producing a blue colouration. Previous studies have shown treatment of Me-JA degrades JAZ1-GUS reducing GUS activity, most effectively in the root tissue of seedlings (Thines et al., 2007).

Our data suggests that the seedlings when treated with 100 μM Me-JA for 1 hour before histochemical GUS staining have resulted in complete loss of GUS activity in *35S-JAZ1-GUS* seedlings.. The 35S-*JAZ1*-GUS seedlings when pre-treated with 100 μM GSH for one hour and then stained with GUS staining solutions exhibited a blue colouration. A Similar pattern of degradation of GUS activity was observed after addition of Me-JA. GSH is used as a negative control in this experiment.
Seedlings treated with GSNO preceding Me-JA did not exhibit any substantial decrease in GUS reporter activity (Figure 4.6.a). Taken together these findings show that NO may stabilize JAZ1 in the presence of Me-JA.

In contrast to the above results the flurometric assay data here demonstrates that GUS protein activity was significantly reduced after Me-JA treatment in 100 μM GSNO treated seedling samples similar to the seedlings treated with GSH and control plants (Figure 4.6.b). Therefore from the flurometric assay it has shown that JAZ1 is degraded after MeJA treatment even in the presence of NO donor like GSNO.

4.5 Discussion

Cellular signal transduction depends upon the protein-protein interaction complex. The role of S-nitrosylation in regulating the protein-protein interaction was firstly identified by Matsumoto and co-workers (Hess et al., 2005). A modified Y2H assay was utilised for exploring the role of NO in protein-protein interaction. They identified the binding in procaspase-3 and the acid sphingomyelinase (ASM) that significantly increased following exogenous application of NO in mammalian cells (Matsumoto et al., 2003).

Juglone the 5-hydroxy-1,4-naphthoquinone is the inhibitor of peptidyl-prolylisomerase *PIN1* (Chao et al., 2001). The juglone and the *N-ethylmaleimide* (NEM) could inhibit the SFC-TIR1-Aux-IAA interaction by forming the cysteine adducts (Kepinski and Leyser, 2004). In case of auxin signalling pathway, the redox based PTM of E3 ubiquitin ligase in plants due to NO which could be one of the important aspect in the S-nitrosylation action involved into the interaction in between TIR1 and auxin ligand (Terrile et al., 2012a). Similarly this could be applicable in case of the interactions in between COI1 and JAZ1. The effects of S-nitrosylation on the binding of COI1 and JAZ1 could prove to be versatile in the JA signalling pathways.

The TF *MYC2* plays a significant role in plant growth and development and acts as a regulatory hub within hormonal signalling (Kazan and Manners, 2013).

Previous studies have shown MYC2 interacts with the C-terminal JAS domain of JAZ proteins through the JAZ interaction domains JID, in the amino terminal domain (Chini et al., 2007; Fernandez-Calvo et al., 2011). JAZ proteins repress JA-mediated responses under low JA levels. JAZ also binds the TF MYC2. Upon stress responses such as insect feeding or necrotrophic pathogen infection, bioactive forms of jasmonates like JA-Ile facilitate the interaction between JAZ repressors and COI1, an F-box protein. This interaction targets JAZ repressors for degradation via the 26S proteosome degradation pathway and subsequently releases the TF MYC2 from JAZ repression, allowing the induction of JA responsive gene transcription.

In our studies AD-MYC (AD: activation domain) and BD-JAZ1 (BD: GAL4 DNA binding domain) after co-expressing in yeast were able to interact and enable the yeast host strain to

grow on medium lacking histidine even in the presence of 30 mM 3AT. The data shows that JAZ1 interacts with MYC2 in absence of jasmonate precursors.

NO donors did not inhibit JAZ1-MYC2 interactions, as the yeast cells grow well on – (L-T-H) and (–L-T-H+3-AT). Thus this reconfirms that JAZ1-MYC2 interaction is independent of JA precursors and GSNO does not inhibits the negative regulator JAZ and MYC2 from interacting in an Y2H assay.

The JAZ proteins were found to interact with COI1 in a JA-Ile dependent manner (Katsir et al., 2008; Thines et al., 2007). The F-box protein has the ligand binding pockets that are represented by the LRR and the loop structure of proteins. The crystal structure of COI1 reveals that JA-Ile is in interaction with the 11 residues of COI1 in a vertical orientation (Sheard et al., 2010). Three residues are responsible in forming a cavity that holds the active isomers like 3R, 7S but does not accommodate the inactive form of JA-Ile (Sheard et al., 2010). JA carboxyl group and the keto group of Ile are exposed while the JA-Ile ligand is buried due to the COI1 residues. The exposed JA and the Ile are available for the interactions with the JAS domain (Sheard et al., 2010). The region of the JAZ1 that binds with the COI1 and JA-Ile displays a bipartite structure (Bulatov and Ciulli, 2015; Sheard et al., 2010).

Coronatine which is a mimic of JA-Ile was known to be a sufficient precursor for inducing interactions between JAZ1-COI1 (Monte et al., 2014). In our studies we used 25µM Me-JA for observing JAZ1-COI1 interaction. Thus full length fused COI1 in pDEST32 as a “bait” and JAZ1 fused in pDEST22 as “prey" ,transformed into yeast Mav203 strain were capable of growing on SD-media lacking Leu, trp, his and also on SD –L-T-H +30 mM 3AT. Strikingly SD (-L-T-H) and SD (-L-T-H+3AT) containing 100 µM GSNO inhibited the JAZ1-COI1 interaction. This suggests a possibility of NO dependent regulation of JAZ1-COI1 protein-protein interactions. A significant impact of NO on JAZ1-COI1 protein-protein interactions might provide further insights into the molecular mechanisms of JA signalling.

S-nitrosylation is a redox based post-translational modification process which is important in signal transduction (Hess et al., 2005). S-nitrosylation can modify protein activity, protein-protein interactions and also protein localization. JAZ/COI1/MYC2 are rich in cysteine residues. Rare, highly reactive cysteines are potential target for S-nitrosylation. Upon necrotrophic pathogen or insect attack *atgsnor1-3* plants were compromised in their disease resistance. JA signalling genes like *JAZ1*, *VSP1*, *THI2.1* transcript accumulation was reduced

in *atgsnor1-3* plants. Further JA accumulation was reduced in *atgsnor1-3* plants suggesting JA signalling is downregulated in high SNO levels. Therefore, S-nitrosylation might be one of leading cause of inhibit of protein interactions integral to JA signalling

Previous studies have shown JA-Ile is sufficient to degrade JAZ1-GUS. Here we found that NO is capable of inhibiting the JA-mediated degradation of JAZ proteins in histochemical GUS staining. Due to the stability of the JAZ1 protein, there may be suppression of JA responsive genes. Thus, NO accumulation may downregulate JA signalling by blocking the interaction between COI1 and JAZ proteins, thereby preventing the degradation of JAZ1 and by extension possibly other JAZ proteins.

In contrast to these results, the fluorometric GUS activity has shown that the degradation of JAZ1 is not blocked by the application of 100 μM GSNO. It is worth mentioning that the protein extraction was from whole seedlings of *35S::*JAZ1-GUS whereas the GUS histochemical analysis was observed in the root tissues. This could be one of the reasons for observing a different pattern of JAZ1 degradation upon GSNO treatment.

Chapter 5

Modification of JA signalling proteins due to S-nitrosylation

5.1 Background

JASMONATE ZIM DOMAIN (JAZ1) and CORONATINE INSENSITIVE1 (COI1) are key components of the JA signalling (Robson et al., 2010) pathway and regulates plant development and defence responses. They are relatively rich in cysteine (Cys) residues, target sites for S-nitrosylation. NO reacts covalently with the cysteine residues and a single cysteine residue in protein may suffice the proteins to get S-nitrosylated. The hypothesis of this chapter is JA signalling proteins might get S-nitrosylated. The elevated SNOs are associated with comprising plant disease resistance (Feechan et al., 2005). Genotypic and phenotypic results supports the hypothesis stated in Chapter 3 that *atgsnor1-3* has a high accumulation in SNO, may display a decrease in disease resistance and an attenuated JA signalling. Therefore, the data so far provides significant evidence that *atgsnor1-3* plants exhibit abnormal JA signalling. It may be possible the function of JA signalling pathway proteins might be regulated by SNO formation. To explore this possibility we therefore sought to first express recombinant COI1 and JAZ1 and examine if these proteins are S-nitrosylated *in vitro*. JAZ proteins are classified as negative regulators of the JA signalling pathway. JAZ proteins contain two functionally conserved domains, ZIM with TIF [F/Y] XG motif and C-terminal sequence $SLX_2FX_2KRX_2RX_2PY$, which is known as JAS motif (Chini et al., 2007; Thines et al., 2007). The *JAZ* family contains 12 members from *JAZ1* to *JAZ12* in *A. thaliana* (Howe, 2010). COI1 has an F-BOX and 18 LRRs in a large LRR domain (Xie et al., 1998). A high-quality structural model of COI1 was developed by Yan and co-worker using Accelrys Discovery Studio 1.7 software (Yan et al., 2009). The structure of COI1 consists of a central cavity in the solenoid fold assembled to LRR and it consists of three loops, loop2, loop12 and loop14. Loop 2 occupies the top surface of the central cavity that associates to LRR-2 and 3_{10}-helix with other loop12 and loop14 (Yan et al., 2009).

The structure of COI1 has four distinctive pockets like P1, P2, P3 and P4, which consist of different residues and surface properties. P1 and P2 form the bottom surface and consist of positively charge arginine residues such as Arg-440, Arg-85 and Arg-407, while P2 pocket is

hydrophobic as it is surrounded by Met-88, Phe-89 and Try-467. The P3 and P4 pockets are hydrophilic and hydrophobic and P3 consists of Glu-355 and Arg-446 while P4 consist of Leu-445 and Leu-469. A bottleneck area is formed in the surface pocket due to the presence of tyrosine residues such as Try-386 and Try-444 into the channels, which connects all the four pockets.

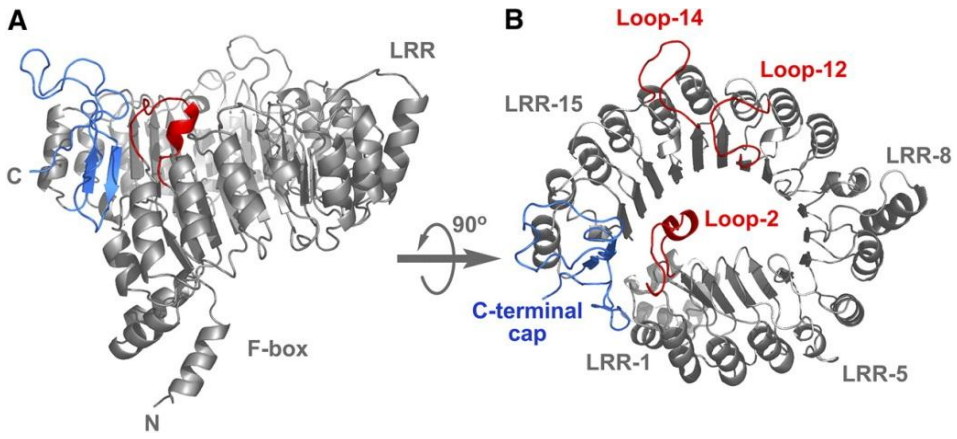


Figure 5.1 Structural model of COI1. **a)** Displaying the ribbon diagram showing the C and N terminal, F-box and LRR in grey. **b)** Displays the various LRR domains. The figure is obtained from (Yan et al., 2009).

The α helix or loops in β-sheets of COI1 are important region of LRR as the amino acid substitution in G369E (*coi1*-4), G155E (*coi1*-7), D452A (*coi1*-9) and L490A (*coi1*-10) disrupts COI1 stability compared to substitution of amino acids in G98D (*coi1-5*). *Coi1*-5 is at the end of loop2 and E-543K is at the end of LRR domain. E-543K (*coi1*-8) and *coi1*-5 accumulated low level of COI1 protein while *coi1*-10 and *coi1*-7 do not exhibit any COI1 protein in comparison to wild type plants. They show a complete loss of JA response, similar to *coi1*-1 plants. Therefore, LRR domain has a very significant role in stability of COI1 (Yan et al., 2009).

The other domain of COI1 protein is the F-box. It is important as it is capable of interacting with several proteins such as JAZs (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007) ASK1, ASK2, cullin and Rbx1 (Wang et al., 2005; Xu et al., 2002). Almost 700 F-box proteins are present in *A. thaliana* (Xu et al., 2009). Not only hormonal signalling but also F-box leads a significant role in circadian clock, cell cycle and defense response (del Pozo et al., 2002; Gray et al., 2001a; Lechner et al., 2006; Santner and Estelle, 2010; Stirnberg et al., 2002).

Post-translational modification of proteins can change the physical and chemical properties of a protein. S-nitrosylation regulates dynamic processes such as pathogen response and metabolism (Grennan, 2007). Over the past decade, S-nitrosylation has been involved in signalling systems, structural proteins and metabolic processes in animals (Hess et al., 2005). Recently, S-nitrosylation has found to control various cellular functions in plants (Wang et al., 2006). Initially, S-nitrosylated proteins were analysed on the basis of acid-base consensus motif (Stamler et al., 1997). Reactive cysteine thiols when present in between the flanking acid–base motif, the nucleophilic thiolate (RS^-) is formed due to the electrostatic interactions. Another feature of S-nitrosylated cysteines is their occurrence in hydrophobic pockets (Greco et al., 2006a), where the radicals get stabilised (Nedospasov et al., 2000). According to the studies proposed by Stamler for the acid-base motifs, analysis of SNO motifs in Arabidopsis protein database have been obtained numerous hits and these proteins are involved in cell signalling, cell cycle and metabolic processes. However, none of them have been S-nitrosylated *in vivo* (Wilson et al., 2008b). Therefore, laboratory based investigations may be more reliable in this end (Hao et al., 2006). A yeast two-hybrid assay showed JAZ1 -COI1 physical interactions were impaired by external applications of NO donors (Chapter 4). These findings may suggest interaction of JAZ1 and COI1 proteins involved in JA pathway might be regulated by S-nitrosylation. Therefore to determine if the interaction of these proteins might be controlled by S-nitrosylated, recombinant proteins of JAZ1 and COI1 were expressed.

5.2 Construct of *JAZ1*-MBP and *COI1*-MBP recombinant protein in *E. coli*

The full coding cDNA fragments of *JAZ1* and *COI1* were amplified by PCR from the cDNA library of WT plants (Col-0) with sizes of 762 bp (*JAZ1*), 1779 bp (*COI1*) using phusion®

High-fidelity DNA polymerase (Figure 5.2.a, b). Any cloned gene of interest is inserted downstream of malE that encodes Maltose-binding protein (MBP). MBP has a molecular weight of 42 kDa. The PCR product fragments of *JAZ1* were then digested by *Not*1 and *EcoR*1 while the PCR products of *COI1* were digested with *Not*1 and *Sal*1 restriction enzymes and cloned into expression vector pMAL-c5X for expression in *E. coli*

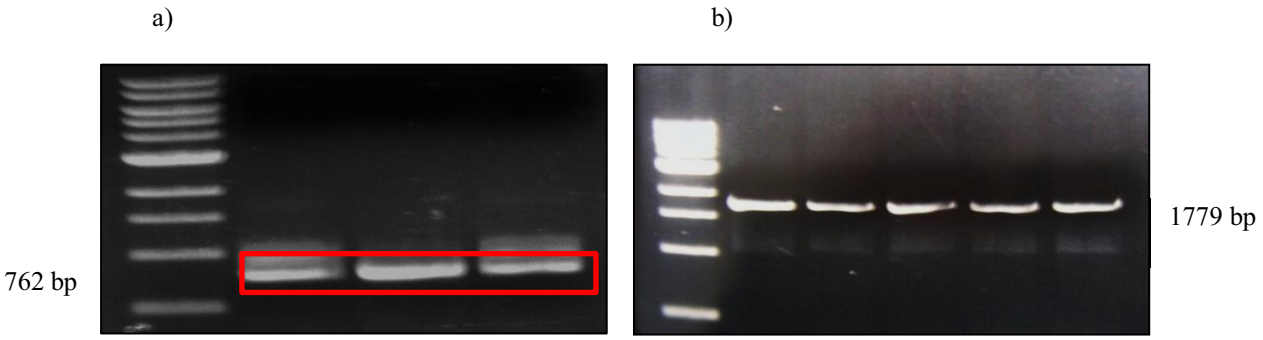


Figure 5.2 a) Bold red shows the amplified *JAZ1* of 762 bp using High fidelity phusion polymerase. **b)** Amplification of *COI1* with High fidelity phusion polymerase showing a size of 1779 bp. PCR products were separated on 1.2% agarose gel. 1kb ladder used (NEB).

Colony PCR confirmed positive clones for the two construct (*JAZ1*-pMAL-c5X) and (*COI1*-pMAL-c5X) stated in (Figure 5.3.a, b). Gene specific primers were utilised for sequencing, and the primer sequence detail is provided in Chapter 2.

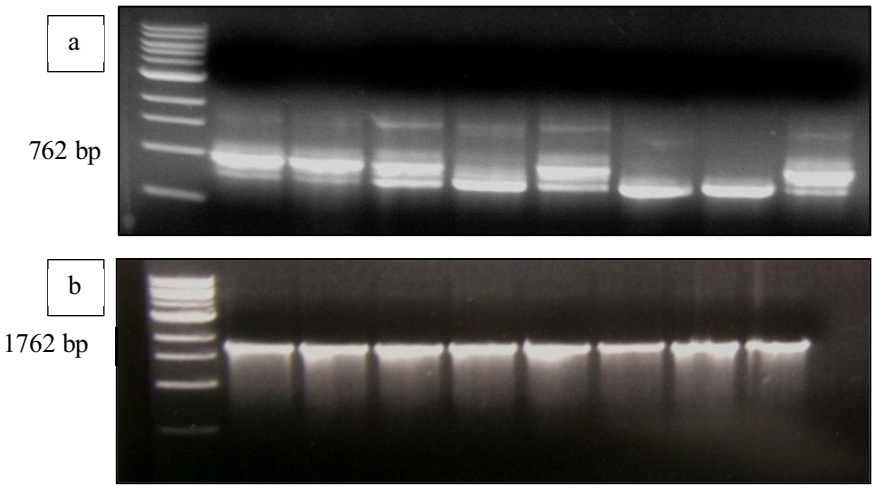


Figure 5.3 Colony PCR screen of transformation for selection of positive clones (a) Colony PCR for *JAZ1***.** (b) Colony PCR for *COI1*. Eight different clones were selected and 1.2% agarose gel was used. 1 kb ladder used (NEB).

5.3 Expression and purification of JAZ1-MBP and COI1-MBP recombinant protein in *E. coli*

A) Recombinant JAZ1-MBP

JAZ1 (AT1G19180) is a nuclear-localised protein involved in JA signalling. JAZ protein functions as repressors of JA signalling, by binding to the bHLH transcriptional activator MYC2 (At1g32640) and inactivating its function (Chini et al., 2007). *JAZ1* transcript levels rise in response to a jasmonate stimulus. MBP tag has a size of 42 kDa (Figure 5.4). A vector map of pMAL-c5X is provided in Appendix B, and here the details of restriction sites according to the size are provided. A single base change in the lacIq increases the expression of lacI gene about 10-fold. The lacI is a repressor that inhibits the transcription in *E. coli* by binding it to the lac operator, which can be resumed by the addition of IPTG or lactose. The promoter known as tac is a strong promoter in *E. coli*. The Xa factor is for the recognition and a cleavage site. AmpR confers the resistance to antibiotic ampicillin. Origin of replication is the ori where the DNA replication begins. This enables plasmid to reproduce as to survive in the cells.

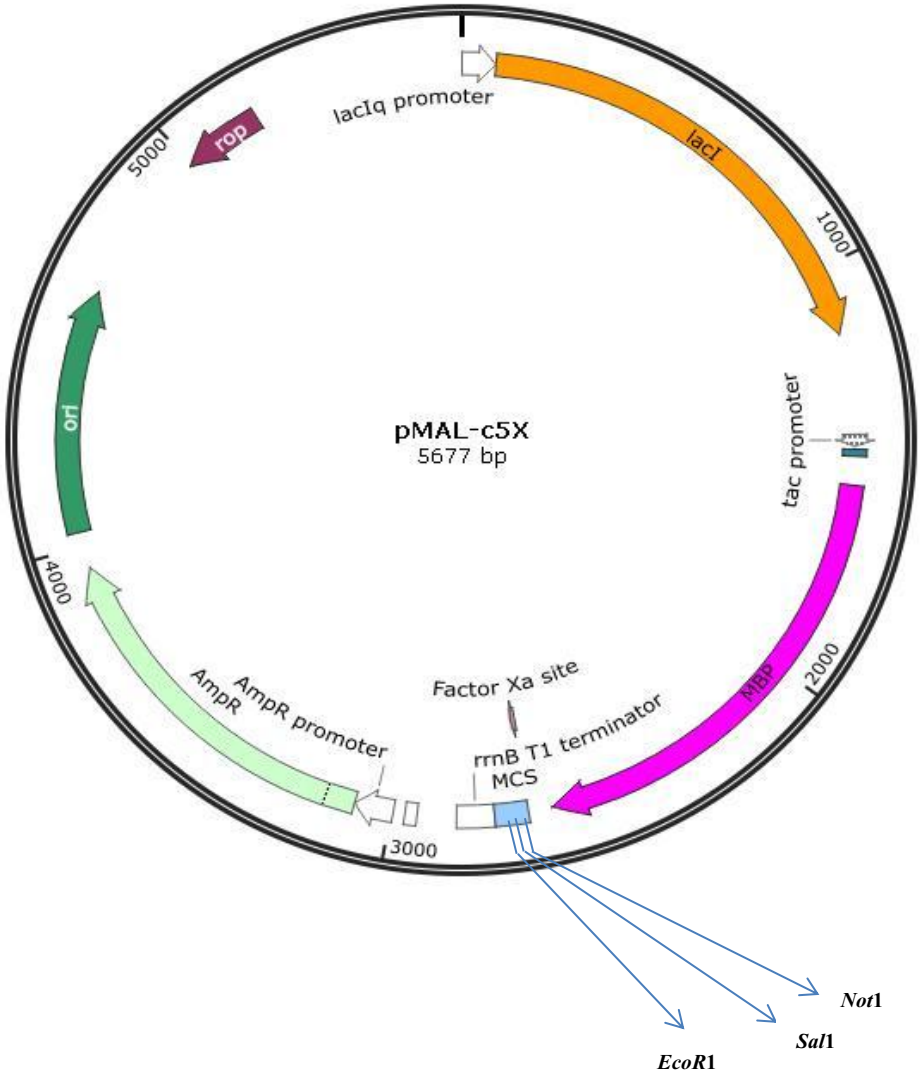


Figure 5.4 pMAL-c5X-MBP protein expression vector map displaying features and restriction sites.

JAZ1 has a molecular weight of (27.60 kDa) and has 253 amino acids. It was expressed in *E. coli* by using Rosetta-gami host cells. Rosetta-gami cells is a combination of Origami and Rosetta and the strain is manipulated to enhance both the eukaryotic proteins expression and the formation of target protein disulphide bonds in the bacterial cytoplasm (Bessette et al., 1999) . The 0.5 mM and 1 mM concentration of IPTG was used to induce JAZ1-MBP fusion protein which encodes a size of 69.60 kDa (Figure 5. 5. a). Both concentrations of IPTG resulted in similar protein expression level.. Purification of JAZ1-MBP (Figure 5.5. b) was

carried out using amylose resins according to the manufacturer's purification protocol. The details of the purification methods are provided in Chapter 2.

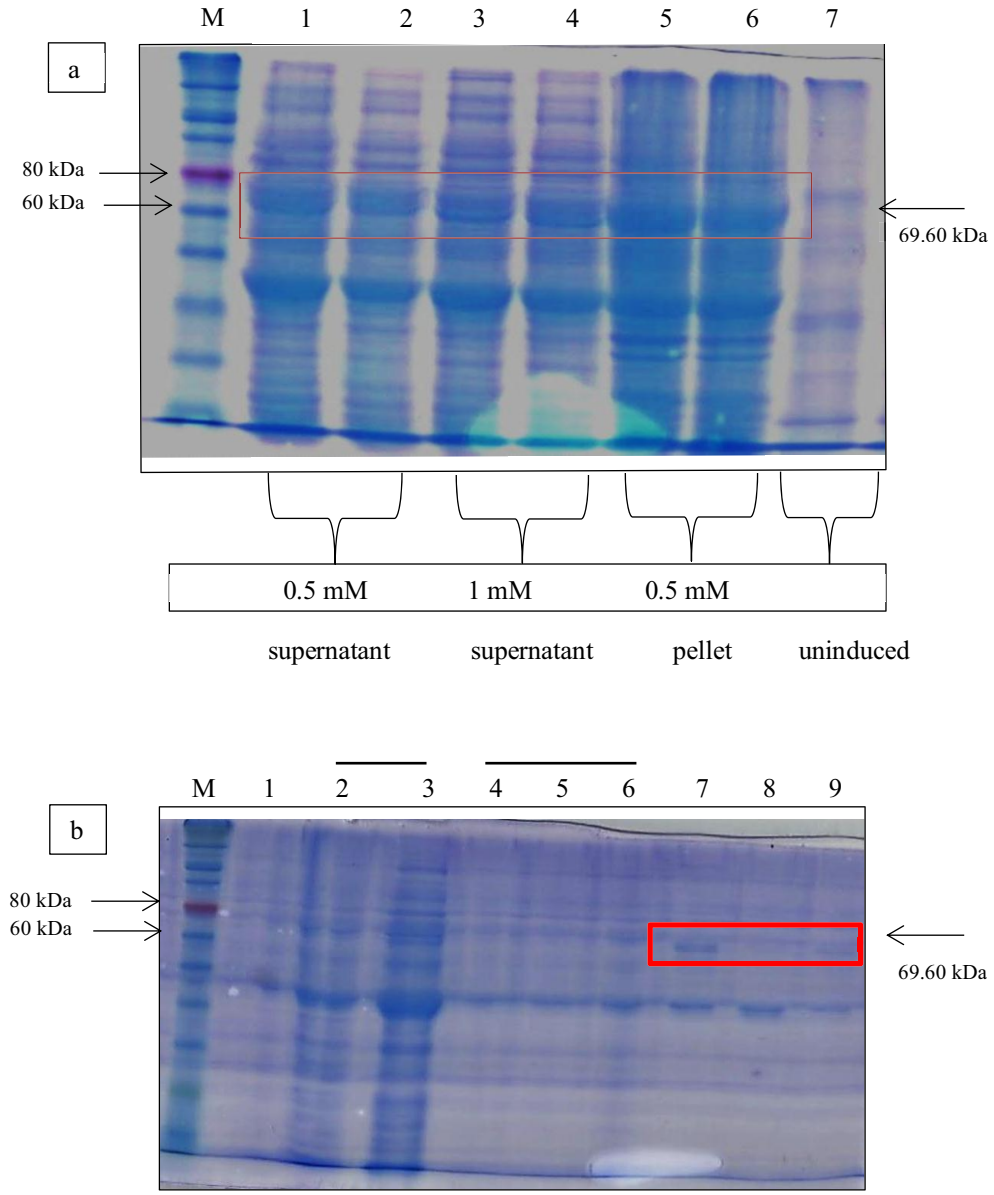


Figure 5.5 SDS-PAGE analysis for protein expression and purification of JAZ1-MBP (a) JAZ1-MBP was expressed in pMAL-c5X in *E. coli* in R. gami cells. M, protein molecular weight marker, lane 1, 2 and lane 3, 4 display supernatant protein induced by 0.5 mM and 1 mM IPTG, lanes 5, 6 shows JAZ1-MBP pellet and lane 7 is an uninduced supernatant. (b) M, protein molecular weight marker, lane 1 show uninduced cells, lane 2-3 show induced cells, lane 4-6 are washes using column buffer and bold red marked lane 7-9 shows purified elution Ist, II nd and IIIrd. The purification of JAZ1-MBP was carried out using amylose resins.

B) Recombinant COI1-MBP

.

COI1 an F-box protein was expressed with MBP tag in *E. coli* to determine if it is S-nitrosylated and is responsible for the impairment of JA signalling in *atgsnor1-3* plants. COI1 has 19 cysteine residues and at least 3 of them are solvent exposed and have a good chance of being post-translationally modified such as by S-nitrosylation and according to careful predictive calculations using Group-based prediction system (GPS-SNO 1.0), the 13th cysteine residue of COI1 is a potential candidate target for S-nitrosylation (http://sno.biocuckoo.org/). GPS-SNO is computational prediction software implemented in JAVA and available freely on the website http://sno.biocuckoo.org/. This software is utilised to predict the S-nitrosylated sites of proteins (Xue et al., 2010). GPS.SNO 1.0 is the software were four parameters such as sensitivity (*Sn*), specificity (*Sp*), accuracy (*Ac*) and Mathew correlation coefficient (*MCC*) are the evaluating measurement used in the prediction performance of GPS.SNO 1.0. Three thresholds such as high, medium and low threshold are available in this software. The FASTA format of the protein sequence is occupied and pasted into the prediction column, and submit button is clicked to run the program, therefore yielding a predicted S-nitrosylated sites column (Xue et al., 2010).

The position of this residue in the COI1-crystal structure further strengthens the possibility of it being S-nitrosylated, as it is highly solvent exposed (PyMOL software analysis) and is present in a LRR domain where COI1 attaches to SKP1 (Figure 5.6). Any change in the structure of the protein as a result of S-nitrosylation of Cys 13, might have significant effects on the overall function. Therefore to identify the status of S-nitrosylation COI1-MBP and JAZ1-MBP proteins were expressed in *E. coli*.

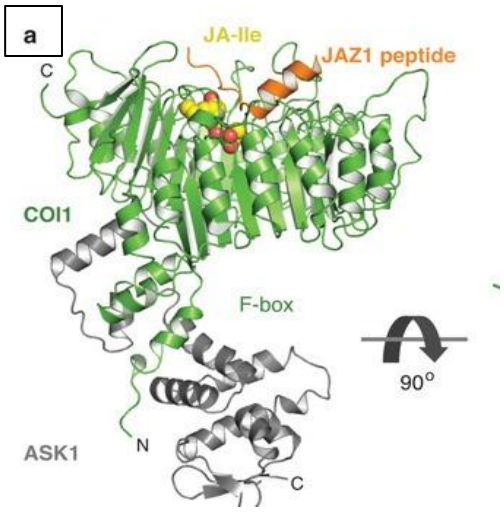


Figure 5.6. Crystal structure displaying attachment of COI1 to ASK1. This figure is obtained from (Sheard et al., 2010).

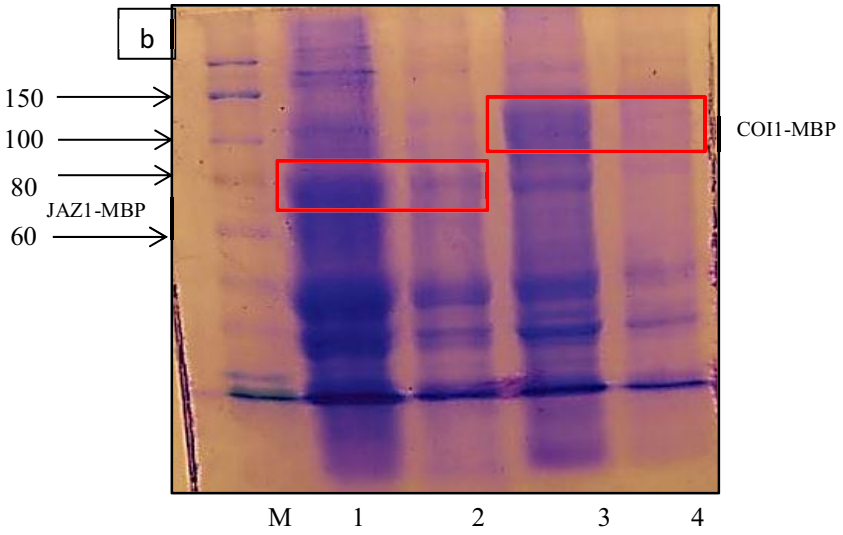


Figure 5.7,M, protein molecular weight marker. Lane 1 and lane 2 shows recombinant JAZ1-MBP protein expression and purified protein yielding a size of 69.60 kDa. The 3rd lane shows COI1-MBP protein of 110 kDa expressed in *E. coli* (Rosetta- gammi cells) induced by 0.5 mM IPTG. Lane 4th displays purified COI1-MBP by amylose resin purification method.

COI1-MBP a fusion protein was expressed in *E. coli* and was induced at the concentration of 0.5 mM of IPTG at 37° C for four hours. The size of COI1 protein is 67.66 kDa and the size

of MBP tag is 42 kDa. COI1-MBP protein size is 110 kDa. The third lane (Figure5.7) shows the supernatant protein and the fourth lanes display the purified COI1-MBP protein using amylose resin. This protein was further cleaved and was treated with trypsin to analyse the peptide sequence of COI1-MBP by mass spectrometry assay.

5.4 Mass spectrometry analysis of recombinant COI1-MBP

Trypsin is a serine protease that specifically cleaves the carboxyl terminus of amino acid residues lysine (K) and arginine (R), resulting in cleaved peptides with K or R at the C-terminal (Thiede et al., 2000). Protein band with expected size was excised from a SDS-PAGE gel and was dehydrated in methanol. Later the samples were treated as according to the protocol stated in Chapter 2. In-gel digestion of protein sample was carried out by trypsin overnight at 37°C. The dried samples were used for analysis after mixing them into loading buffer. Peptide sequence was obtained after the samples were analysed by mass spectrometry and the peptide sequence data was obtained through the MASCOT Versions 2.2 and 2.3. MASCOT is an online search server which provides peptide mass obtained from the digested protein by trypsin and helps in identifying the protein (Matrix Science Ltd, UK)The mass spectrometry experiment and further analysis were performed by Dr. Andrew Cronshaw, Centre Optical Instrumentation Laboratory (COIL), Michael Swann Building**,** The University of Edinburgh, UK.

Protein sequence coverage: 40%

Matched peptides shown in ***bold red***.

```
  1 MEDPDIKRCK LSCVATVDDV IEQVMTYITD PKDRDSASLV CRRWFKIDSE
 51 TREHVTMALC YTATPDRLSR RFPNLRSLKL KGKPRAAMFN LIPENWGGYV
101 TPWVTEISNN LRQLKSVHFR RMIVSDLDLD RLAKARADDL ETLKLDKCSG
151 FTTDGLLSIV THCRKIKTLL MEESSFSEKD GKWLHELAQH NTSLEVLNFY
201 MTEFAKISPK DLETIARNCR SLVSVKVGDF EILELVGFFK AAANLEEFCG
251 GSLNEDIGMP EKYMNLVFPR KLCRLGLSYM GPNEMPILFP FAAQIRKLDL
301 LYALLETEDH CTLIQKCPNL EVLETRNVIG DRGLEVLAQY CKQLKRLRIE
351 RGADEQGMED EEGLVSQRGL IALAQGCQEL EYMAVYVSDI TNESLESIGT
401 YLKNLCDFRL VLLDREERIT DLPLDNGVRS LLIGCKKLRR FAFYLRQGGL
451 TDLGLSYIGQ YSPNVRWMLL GYVGESDEGL MEFSRGCPNL QKLEMRGCCF
501 SERAIAAAVT KLPSLRYLWV QGYRASMTGQ DLMQMARPYW NIELIPSRRV
551 PEVNQQGEIR EMEHPAHILA YYSLAGQRTD CPTTVRVLKE PI
```

Figure 5.8 Mass spectrometry results of COI1-MBP. Bold red shows the peptides of COI1 generated after cleavage by trypsin. Mass spectrometry was performed by Dr. Andrew Chronshaw, The University of Edinburgh, UK

In the Figure 5.8 bold red marked peptides are the identified and matched peptide obtained through the mass spectrometry data displaying 40% coverage with the COI1-MBP protein sequence.

5.5 Biotin switch assay of JAZ1-MBP

JAZ1 is an important protein in JA signalling pathway, and it contains five Cys residues, thus this protein is potential for getting S-nitrosylated. Therefore, the Biotin Switch Technique was used (Forrester et al., 2009b) that detects S-nitrosylated proteins. Biotin switch assay helps to utilise the tagging of protein-SNO such as biotin to be identified as an individual protein-SNO by using anti-biotin even in a complex mixture. It has proved to be an advantageous method for studying specific or a class of proteins (Forrester et al., 2009b) .

JAZ1-MBP proteins were treated with 1 mM of GNSO, the NO donor and BSA was performed. MMTS is a blocking agent which blocks the free thiols and minimises the biotinylation background by increasing the sensitivity of the experiment (Forrester et al., 2009b). The addition of sodium dodecyl sulphate (SDS), a detergent helps in in denaturing

the protein and losing its native shape (Gudiksen et al., 2006). SDS also ensures and promotes the MMTS accessibility to each thiol (Kenyon and Bruice, 1977). Therefore, in this assay protein sample containing SDS was treated as a positive control.

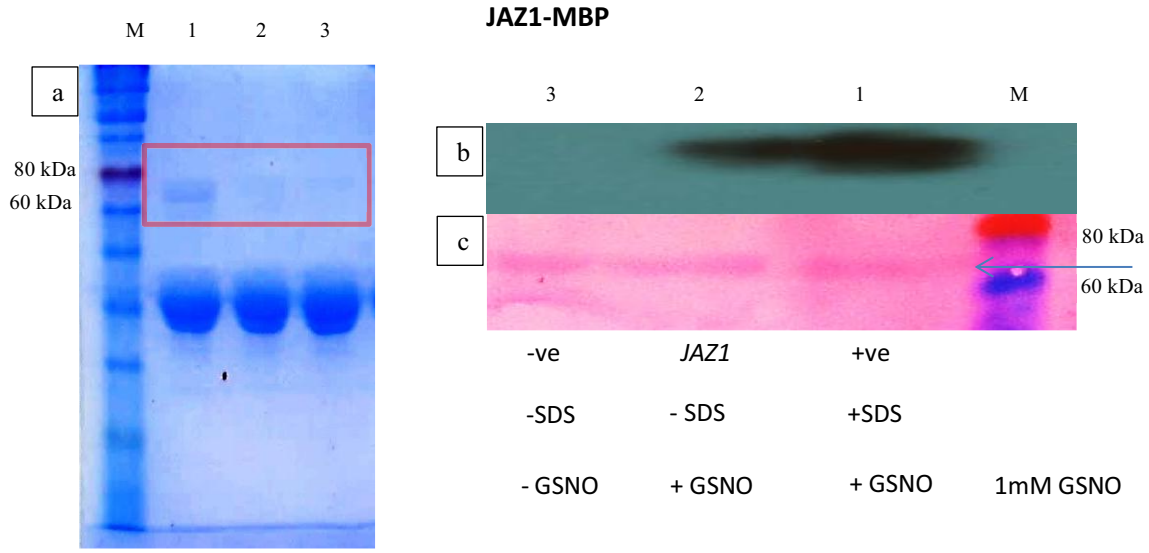


Figure 5.9 S-nitrosylation of JAZ1-MBP *In vitro* (a) M, protein molecular weight marker, lane 1 -3 are biotin switch samples of JAZ1-MBP of 69.60 kDa.. (b) Western blot of JAZ1-MBP using anti-biotin antibody at a concentration of (1:1000). M, protein molecular weight marker. Lane 1 is a positive control showing a robust signal with SDS. Lane 2 is without SDS giving a signal with the utilised antibody. Lane 3 exhibits no signal as it is a negative control. (c) Ponceau staining for the loading control of JAZ1-MBP.

. Reduction of S-nitrosylated cysteines is achieved by using ascorbate. The newly formed free thiols are covalently coupled with the biotin-HPDP. Details of the protocol are mentioned in Chapter 2. A robust signal was observed when recombinant JAZ1-MBP was treated with an NO donor like GSNO. Gradually an enhanced signal was observed when protein samples were treated with GSNO in addition to SDS, showing an exposure of all cysteine residues and giving a positive signal using the anti-biotin antibody at a concentration of 1:1000. No signal was visualised in the negative protein sample which lacked GSNO. Collectively, these findings imply that JAZ1-MBP is S-nitrosylated *in vitro*.

The S-nitrosylation of JAZ1-MBP is concentration dependent (Figure 5.10). JAZ1-MBP protein samples were treated with GSNO with an increasing concentration. Protein treated with GSNO at a concentration of 1 mM exhibits a robust signal, while the signal goes on reducing as the concentration of NO donors were reduced. JAZ1-MBP when treated with 0.1 mM GSNO failed to display a signal against the anti-biotin antibody. Another NO donors Cys-NO also formed SNO-JAZ1. Dithiothreitol (DTT) is a reducing agent and has an ability to destabilise SNO and is capable of removing the biotin tag. Therefore, the biotinylation reaction can be reversible due to the addition of DTT (Forrester et al., 2009b). Here in our results, the addition of DTT has reversed the formation of SNO-JAZ1 and no signals were found in this protein sample. It is worth mentioning, that the ponceau staining has not displayed JAZ1-MBP protein but only MBP tag alone. Upon the exposure of chemiluminscent reagents right size signal of JAZ1-MBP was observed. Therefore, collectively this data confirms that JAZ1-MBP is S-nitrosylated *in vitro* and the S-nitrosylation of JAZ1-MBP is concentration dependent.

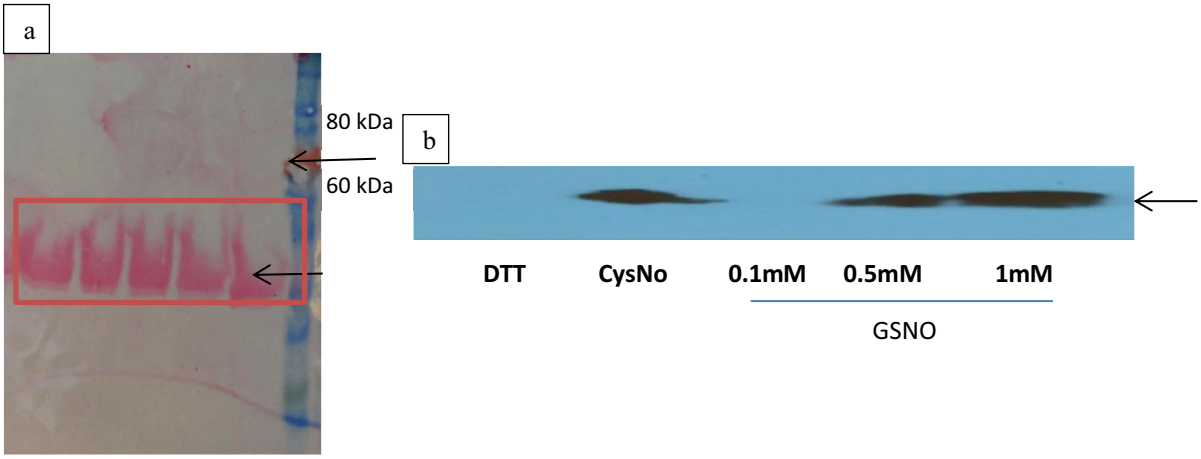


Figure 5.10 Concentration dependent S-nitrosylation of JAZ1-MBP a) Ponceau staining for biotin switch samples of JAZ1-MBP**. b)** Biotin switch assay of JAZ1-MBP using different concentration of GSNO, Cys-NO (1 mM). S-nitrosylation of JAZ1-MBP is reversible after addition of DTT.

5.6 Identification of S-nitrosylated sites by mass spectrometry of *JAZ1*-MBP

In this study it was confirmed that JAZ1-MBP was S-nitrosylated *in vitro* with the help of biotin switch assay. We were interested to identify the site of S-nitrosylation therefore mass spectrometry of GSNO treated JAZ1-MBP was performed. The detailed method of mass

spectrometry is provided in Chapter 2. Recombinant JAZ1-MBP protein was purified and the biotin switch assay was performed. We used Iodoacetamide (IAA) instead of biotin for labelling as biotin is sometimes heavy for the peptides with NO donor to identify. The Biotinylated samples were treated with trypsin for cleavage overnight at 37°C and further utilised for MS.

```
         10          20          30          40          50
MSSSMECSEF VGSRRFTGKK PSFSQTCSRL SQYLKENGSF GDLSLGMACK
         60          70          80          90         100
PDVNGTLGNS RQPTTTMSLF PCEASNMDSM VQDVKPTNLF PRQPSFSSSS
        110         120         130         140         150
SSLPKEDVLK MTQTTRSVKP ESQTAPLTIF YAGQVIVFND FSAEKAKEVI
        160         170         180         190         200
NLASKGTANS LAKNQTDIRS NIATIANQVP HPRKTTTQEP IQSSPTPLTE
        210         220         230         240         250
LPIARRASLH RFLEKRKDRV TSKAPYQLCD PAKASSNPQT TGNMSWLGLA

AEI
```

PLTIFYAGQVIVFNDFSAEKAKEVINL- ZIM domain

ELPIARRASLHRFLEKRKDRVTSKAPYQLCDPAKASSNPQTTGNMSWLGLAAEI- Jas domain.

Figure 5.11 Amino acid sequence of JAZ1 protein showing cysteine's highlighted in green. The ZIM domain is highlighted pink. The jas domain is highlighted in yellow. The amino acid sequence is derived from www.uniport.org.

Carbamidomethyl modifications were found in peptide sequence "APYQLCDPAK" and "FTGKKPSFSQTCSR", but only "APYQLCDPAK" sequence has shown methylation modification. This experiment was performed by Dr. Thierry Le BihanSynthSys-Systems and Synthethic Biology,_CH Waddington Building, The University of Edinburgh, UK. GPS.SNO 1.0 prediction website suggests the 7th and 27th cysteine residues are potential sites of S-nitrosylation. Here the mass spectrometry results suggest a peptide sequence containing the 229th cysteine is getting modified with the help of IAA. Cys 229 is the S-nitrosylated site in JAZ1-MBP as it exhibits methylation and also carbamidomethyl modifications. Cys 27 failed to show methylation modification in positive controls suggesting that only Cys 229 is

the S-nitrosylated site in JAZ1-MBP. Cys 229 is present in the JAS domain of JAZ proteins. The JAS domain is conserved throughout JAZ proteins and is important for interaction with COI1 and also for many other functions integral to JA signalling Table 5.1 shows the mass spectrometry data for JAZ1-MBP. S-nitrosylation status for COI1-MBP remains unknown in this study as very less concentration of protein was yielded.

Table 5.1 Peptide sequence of COI1-MBP showing modification with IAA in mass spectrometry

Peptide sequence		IAA modification
APYQLCDPAK	A	Carbamidomethyl (C)
KPSFSQTCSR	L	Carbamidomethyl (C)
MSSSMECSEFVGSR	R	Carbamidomethyl (C)
MSSSMECSEFVGSR	R	Carbamidomethyl (C); Oxidation (M)
MSSSMECSEFVGSR APYQLCDPAK	R A	Carbamidomethyl (C); Oxidation (M) Methylthio (C)

Table. 5.1 Cysteine site identification of JAZ1-MBP through mass spectrometry. The experiment was performed by Dr. Thierry Le Bihan, The University of Edinburgh, UK.

5.7 Discussion

To increase protein stability tags such as MBP are found helpful. Here in our studies, we used MBP tag. But it is one of the heaviest tags at 42 kDa (Bessette et al., 1999). COI1 has 19 cysteine residues and consists of two domains: a small N-terminal F-box domain and a large LRR domain. COI1 is an F-box protein that functions as the substrate-recruiting module of the Skp1–Cul1–F-box (SCF) ubiquitin E3 ligase complex. "The LRR domain includes 18 LRRs, which adopt a tandem packed structure of staggered α-helices and β-sheets. These LRRs assemble into a solenoid fold with a horseshoe-like shape. COI1 is exclusively encircled by amino acid side chains. Many of the pocket-forming residues on COI1 are large in size and carry a polar head group" (Sheard et al., 2010; Yan et al., 2009)**.** COI1 has LRR domains important for protein-protein interactions. Each LRR domain containing proteins that expressed in *E. coli* produces insoluble inclusion bodies (Afzal and Lightfoot, 2007).. Various parameters such as reducing cytoplasm environment, reduced half-life and improper interaction with the chaperones may be involved in the formation of inclusion bodies (Idicula-Thomas S. and Balaji, 2005). One of the examples of an LRR protein is RHG1-LRR from soybean (*Glycine max* L. Merr) where buffered urea was used for solubilising this protein (Afzal and Lightfoot, 2007). The refolding was carried out in the presence of arginine and reduced/oxidised glutathione after removing urea (Afzal and Lightfoot, 2007). Post-translational modification is essential for the proper folding and functional activity. The inability to perform post-translational modification is one of the disadvantages of host cell *E. coli*. The other mode of host cell to express protein is the yeast cells. The yeast cells grow rapidly with high cell densities. *S. cerevisiae* is the commonly used yeast strain for protein expression (Frommer and Ninnemann, 1995).Yeast cells are capable of performing post-translational modification mechanism similar as in *planta*. The limitations of using the yeast as host cell are they could have cell stress due to foreign bodies, low yield, and hyperglycosylation (Schmidt and Hoffman, 2002). Manipulating the baculoviruses is another method for recombinant protein expression in insect cells. This system includes the homology recombination through which the nonessential genes are replaced by the target proteins, and the baculovirus can infect the insect cells, therefore, expressing the heterologous genes (Yesilirmak and Sayers, 2009). AUX1 was expressed in baculovirus-

insect cell system with high expression level to study the interactions with IAA (Carrier et al., 2008). The recombinant protein produced in this system is post-translationally modified, soluble and biologically active and functional (Beames & Summers, 1998). Highly efficient laboratory skills and complex facilities, difficulties in constructing an expression vector, and a short period after infection are the technical disadvantages in using baculovirus system (Yesilirmak and Sayers, 2009). Sometimes proteins may not fold into their native conformation even in the presence of MBP tag, in most cases of passenger proteins (http://mcl1.ncifcrf.gov/waugh). In this study, COI1-MBP protein yield was low, and no significant amount of protein was obtained to perform the BSA. In contrast to our studies, previous research has shown a significant expression level of COI1 protein *in vitro* and in *planta*. Devoto and co-workers expressed COI1 in *E. coli* BL21 as a fusion protein using a histidine tag (Devoto et al., 2002).

To study the pharmacology of jasmonate perception, COI1 and ASK1 were co-expressed as a glutathione transferase (GST) in high five (Hi5) suspension insect cells (Sheard et al., 2010). The recombinant protein expression of a full-length coding sequence of COI1 was obtained by expressing in BL21 cells (DE3)pLysE fused with GST tag using the pDEST^{TM}15 (Invitrogen) (Potuschak et al., 2003).

After many repetitions of the experiment with different inducing conditions, e.g. changing the IPTG concentration, induction time and also changing the protein expression strain no significant COI1-MBP could be produced. Therefore, biotin switch assay was not performed for COI1-MBP. Therefore, a change in the choice of host cells and tag could help in expressing COI1 protein for future studies.

COI1 has a sequence similarity with the TIR1 protein, a receptor for auxin. Interestingly, TIR1 has been shown to be S-nitrosylated recently (Terrile et al., 2012a). COI1 depends upon a reducing environment to be functional. Thus, COI1 is a potential candidate for S-nitrosylation (Terrile et al., 2012a).

On the other hand, JAZ proteins have a conserved JAS domain which is important and necessary for the interaction with LRR domain of COI1 (Chini et al., 2007; Melotto et al., 2008; Sheard et al., 2010; Thines et al., 2007). A degron which consists of 21 amino acids in the JAS motif is sufficient for making JAZ1-COI1 complexes (Sheard et al., 2010). Several studies have shown that JAZ proteins like JAZ1, JAZ3 and JAZ10 when lacking a functional JAS domain, are resistant against degradation upon JA-Ile accumulation and the corresponding lines are JA-insensitive. Therefore, it has shown that JAS domain of JAZ proteins is important for the JA signalling pathway (Chini et al., 2007; Chung and Howe,

2009; Chung et al., 2008; Thines et al., 2007; Yan et al., 2007). JAZ1 consists of 253 amino acids and has five cysteine residues. JAZ1 is S-nitrosylated *in vitro* and the JAS domain has a cysteine residue which is the site of S-nitrosylation that was shown through mass spectrometry assay. This suggests how the interactions between COI1-JAZ1 could be blocked following NO accumulation. Thus, SNO formation of Cys 229 within the JAZ1 JAS domain might disrupt JAZ1-COI1 interactions, preventing ubiquitin and subsequent degradation of JAZ1, enabling MYC2 to promote the expression of JA-dependent genes. In *atgsnor1-3* plants high accumulation of SNO results in increased susceptibility to insect feeding and necrotrophic pathogens. Thus, collectively our findings suggest disruption of JA signalling in *atgsnor1-3* plants, may be due to S-nitrosylation of JAZ1.

Chapter 6

JAZ1 is S-nitrosylated *in vivo*

6.1 Background

JAZ1 is involved in many different plant physiological processes such as plant defence and development (Robson et al., 2010). GSNOR controls multiple modes of plant development and disease resistance (Feechan et al., 2005). In plant defence signalling NO acts as an important chemical messenger against microbial pathogen (Delledonne et al., 1998; Durner et al., 1998). RNS and NO have a tendency to oxidise and nitrosylate proteins and NO modulate directly the plant proteins, therefore, regulating the biological events in plants (Stamler et al., 2001). Recent studies have shown a few plant proteins are S-nitrosylated such as metacaspase, S-adenosylmethionine synthetase (SAMS), peroxiredoxin, NPR1 and TIR1 (Belenghi et al., 2007; Lindermayr et al., 2006; Romero-Puertas et al., 2007a; Tada et al., 2008; Terrile et al., 2012b) under stress conditions. Increased SNOs disable the plant defence responses to microbial invasions. Previous studies have shown *atgsnor1-3* has reduced SA accumulation and increased plants susceptibility towards pathogenic microorganisms (Feechan et al., 2005). GSNO negatively regulates the expression of the genes and gene encoding the proteins that are involved in SA biosynthesis (Malik et al., 2011).As changes in cellular redox status are common features during immune function (Tada et al., 2008)we were therefore interested to explore the possible role of NO and associated S-nitrosylation in the JA signalling during the plant immune response. Here in this study, it was shown that in *atgsnor1-3* plants immunity is compromised against necrotrophic pathogen infection and insect attack. The hypothesis of our study was the JA signalling may be disrupted due to high SNO accumulation and proteins involved in JA pathway may be the potential targets of S-nitrosylation.

Therefore, we investigated the status of S-nitrosylation of JAZ1 *in vivo*. To study the JAZ1 S-nitrosylation status, we developed transgenic plants.

6.2 Generating a construct of pEG202-*JAZ1-FLAG* and pEG203 -*COI1- MYC*

Full-length of *JAZ1* (762 bp) was amplified from wild-type Col-0 plants by using Phusion^{TM} High-Fidelity DNA polymerase (Figure 6.1.a). The PCR product was then cloned into pDONR-221 (Invitrogen) vector to make an entry clone by using BP clonase enzyme mix.

Colony PCR results are shown in (Figure 6.1.b). The cloned gene insertion into entry vector was confirmed through sequencing using gene specific primers and M13-forward and M13-reverse primers. Using LR clonase (Invitrogen), *JAZ1* sequence flanked by the entry vector's *att*L recombination site was recombined using the pEG202-*FLAG* vector. Colony PCR results confirmed an insert into destination vector as expected size of *JAZ1* (762 bp) was observed on a 1.2% agarose gel (Figure 6.1.c). Positive colonies were selected for plasmid extraction and clones were confirmed by sequencing using gene- specific primers and destination vector primers. Similarly, construct was generated for *COI1* gene using pENTRY-223 as a donor vector and pEG203-*MYC* as a destination vector. Results of *COI1* gene amplification and entry clone are mentioned in Figure 6.2.a, b. accordingly. Gene-specific and pEG203-*MYC* primers were used for sequencing to confirm the insertion of *COI1* into pEG203-*MYC*. The results of colony PCR for expression clone of construct pEG203- *COI1-MYC* are stated in Figure 6.2.c. All the primer sequence details are described in Chapter 2. Constructs were later transformed into *Agrobacterium tumefaciens* strain GV301 by freeze-thaw method (Chen et al., 1994).

Note- (construct of *pEG202-JAZ1-FLAG* was generated by Dr. Byung-Wook Yun).

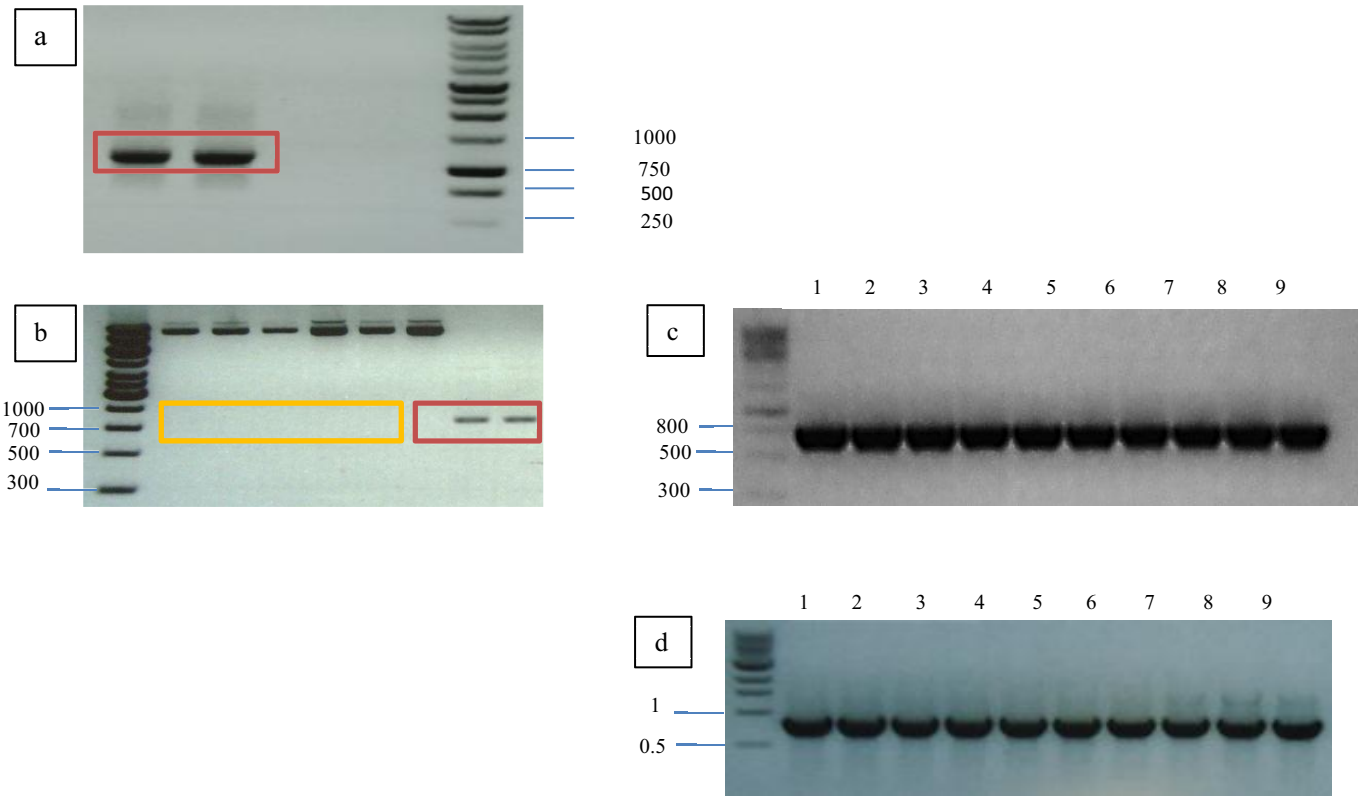


Figure 6.1 (a) **Amplification of *JAZ1*** (762 bp) by phusion polymerase. 1 kb DNA (Gene ruler ^{TM}) ladder was used (b) **Colony PCR of entry clone pDONR221-*JAZ1*.** Out of eight only two were pisitive transfomants**.** 1 kb DNA ladder (Fisher bioreagent) was used (c) **Colony PCR of pEG202-*JAZ1-FLAG* in Top-10 cells.** Colony PCR after LR reaction that achieved right size PCR product *JAZ1* (762 bp). 1 kb plus^{TM} DNA ladder was used (d**) Colony PCR of pEG202-*JAZ1-FLAG* in *A. tumefaciens.*** Colony PCR of pEG202-*JAZ1-FLAG* after transformation in *A. tumefacien*. 1 kb DNA (NEB) ladder was used. Gene specific primers were used for all PCR.

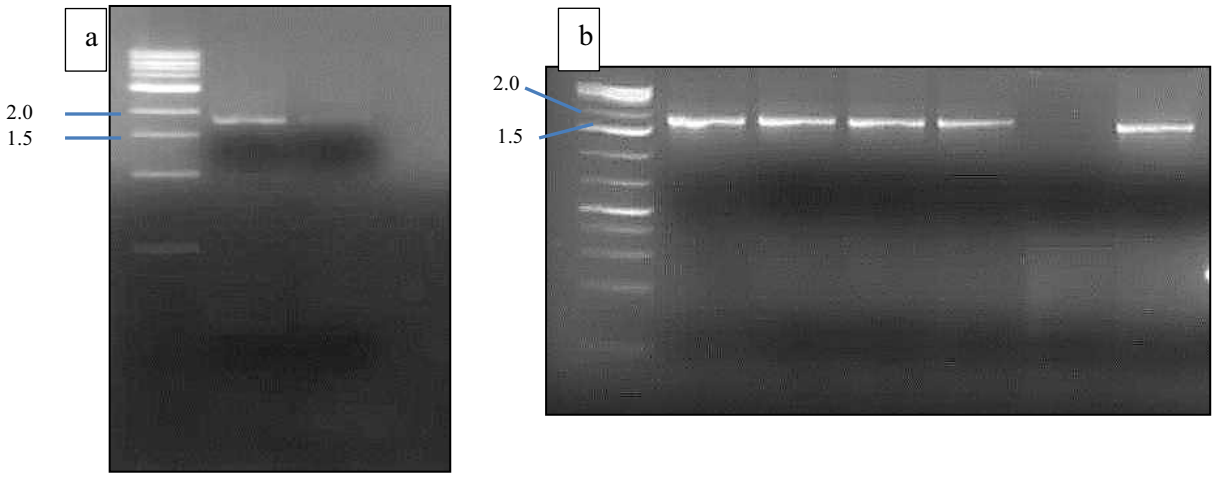
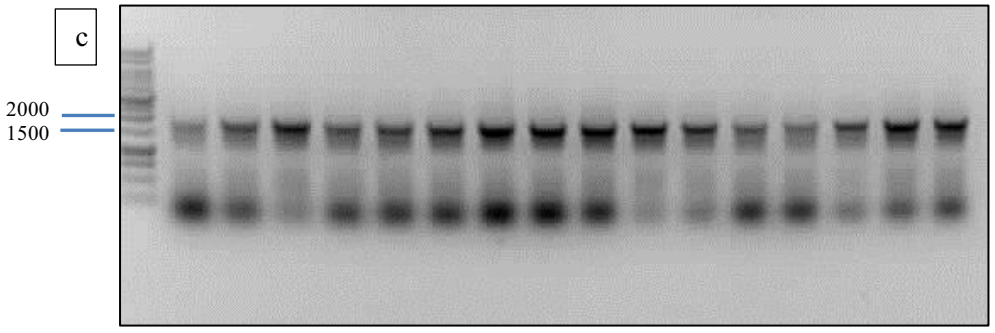


Figure 6.2 (a) **Amplification of *COI1* by phusion polymerase**. *COI1* shows a size of 1779 bp. 1 kb DNA ladder (NEB) was used (b) **pEntry223-*COI1* (1779** bp**) colony PCR after BP reaction.** 1 kb plus DNA ladder (GeneRuler ^{TM}) was used.



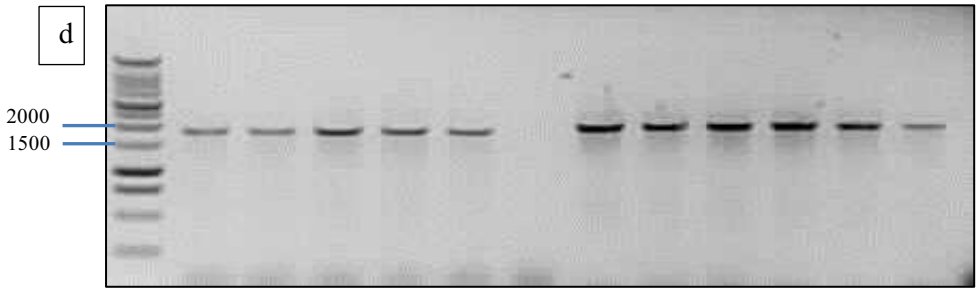


Figure 6.2 Colony PCR of transformation of pEG203-*COI1-MYC* in Top-10 cells for selection of positive clones (c) Colony PCR of LR reaction for *COI1* (1779 bp). (d) **Colony PCR of pEG203-*COI1-MYC* in *A. tumefaciens*** . *Agrobacterium* transformed colony PCR result of pEG203-*COI1-MYC*. PCR product size is (1779 bp). All colonies show successful insert except 6th colony. 1 kb DNA (Invitrogen) ladder was used.

```
   1 ATATTGGAGG TAGGAAGAAG AACTCTGCAA CCAAACCAAC CAACCCCAAA
  51 GCCAAACAAA GTTTTATAGA GACCTTCCAT TTCTCCCTCT CGTGACAAAC
 101 GCAATTTGCA GAGAAGCAAC AGCAACAACA AGAAGAAGAA GAAAAAGATT
 151 TGAGATTACT TTGTATCGAT TTAGCTATTC GAGAAACTCT TGCCGTTTGA
 201 AAGTTTTAAT TGTTAAAGAT GTCGAGTTCT ATGGAATGTT CTGAGTTCGT
 251 CGGTAGCCGG AGATTTACTG GGAAGAAGCC TAGCTTCTCA CAGACGTGTA
 301 GTCGATTGAG TCAGTATCTA AAAGAGAACG GTAGCTTTGG AGATCTGAGC
 351 TTAGGAATGG CATGCAAGCC TGATGTCAAT GGTAAGAAAC CTTCTCTTTC
 401 TCCTAGATCC ACTTCTTTTT TCGTTTTCTC TGTTTTTTAT TTCTTGAATC
 451 TTGATCTTGA AAACTTTTCA AGAAAATTTT GAATCGATTT CAAAGAAATT
 501 AGGGAGAGTT AGTTTGCTAA ATTTTGACAT AGAAAATGAT TGGAGAGAGT
 551 TCTAACTTTT GGATCATATA TATTTGCAGG AACTTTAGGC AACTCACGTC
 601 AGCCGACAAC AACCATGAGT TTATTCCCTT GTGAAGCTTC TAACATGGAT
 651 TCCATGGTTC AAGATGTTAA ACCGACGAAT CTGTTTCCTA GGCAACCAAG
 701 CTTTTCTTCC TCATCTTCCT CTCTTCCAAA GGAAGATGTT TTGAAAATGA
 751 CACAGACTAC CAGATCTGTG AAACCAGAGT CTCAAACTGC ACCATTGACT
 801 ATATTCTACG CCGGGCAAGT GATTGTATTC AATGACTTTT CTGCTGAGAA
 851 AGCCAAAGAA GTGATCAACT TGGCGAGCAA AGGCACCGCT AATAGCTTAG
 901 CCAAGAATCA AACCGATATC AGAAGCAACA TCGCTACTAT CGCAAACCAA
 951 GTTCCTCATC CAAGAAAAAC CACAACACAA GAGCCAATCC AATCCTCCCC
1001 AACACCATTG ACAGAACTTC CTATTGCTAG AAGAGCTTCA CTTCACCGGT
1051 TCTTGGAGAA GAGAAAGGAC AGAGTTACGT CAAAGGCACC ATACCAATTA
1101 TGCGATCCAG CCAAAGCGTC TTCAAACCCT CAAACCACAG GCAACATGTC
1151 GTGGCTCGGT TTAGCAGCTG AAATATGAAT GCTAACCACC CTCAAGCCGT
1201 ACCAAGAAAT TCTTTTGACG ACGTTGCTTC AAGACAAGAT ATAAAAGCTC
1251 CTATCTTCAT GCTTTTTGAT TTAAGATACA AACTACTCAA TGATTAGGAA
1301 ACTTCATATA TTTGTATGTA TTGATTAGTG ATCAATTATT GTTAGTATTC
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1351 GTTATAGTCT GTTTTTCTAC TAGTTATTGT CGCCTGTCTA AATCCCCTTG
1401 CTATGGGTTA TCTCAAAATT AGTTTCGTAT GTAACTAATT TTGTAAGAAC
1451 AATAATTTTT GTTGACGAAC CATACTATCA AATACTCTAA ATTATATCTT
1501 GATAAATCTA CCTATCAGGT AAGTAGG
```

```
5'UTR = 1-218                    Intron = 382-579

EXON = 1-381                     T-DNA insertion = 835-1501

Exon = 580-1527                  3'UTR = 1179-1527

Start codon = ATG
```

Figure 6.3 Genomic DNA sequence of *JAZ*1 and detail analysis of resulting constructs T-DNA insertion.

JAZ1 has a T-DNA insertion in an exon at 835 bp of *jaz1* on chromosome 1 and a homozygous plant line for this insertion in *JAZ1* was selected. T-DNA insertion coordinates are found at coordinates (COOR) W/6622928-6623594 and Figure 6.3 provides the details of T-DNA insertion in the exon region of *jaz1* mutant. A single band was obtained in PCR reaction when LBb1.3/RP primers were used (Figure 6.4). This confirms *jaz1* is a homozygous plant line as generally a single band is obtained after performing PCR if the plant line is homozygous, using LBb1.3/RP primer. Details of the construct are provided in appendix D.

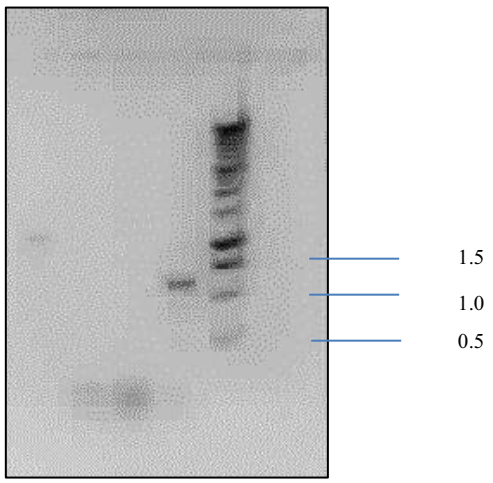

Figure 6.4 Genotyping PCR reaction for confirmation of *jaz1* as a homozygous line giving a product size of (1172 bp). 1 kb DNA ladder (NEB) was used.

JAZ1 mutant (At1G19180) seeds (SALK_011957 C) were ordered from Nottingham Arabidopsis Stock Centre (NASC). These plants do not show any phenotypes as *JAZ* genes are functionally redundant. No phenotype was observed in this study, and neither any information related to *jaz1* phenotype is available in TAIR (https://www.arabidopsis.org).

JAZ1 is an important protein in the JA signalling pathway that interacts with most of the target proteins like MYB21, MYB24, TPL, EIN3, GL3 and TT8 (Pauwels et al., 2010; Qi et al., 2011; Song et al., 2011a; Zhu et al., 2011b). The main purpose of generating the transgenic plants was to study the S-nitrosylation of *JAZ1* in *atgsnor1-3* plants a high SNO background mutant. FLAG epitope tag is driven by cauliflower mosaic virus *35S* promoter, and it is a constitutive promoter which causes a high level of gene expression (Tepfer et al., 2004). The *35S* promoter is used on a large scale to generate recombinant protein in plants (Saidi et al., 2009).

The jaz1 mutant SALK_011957C line (Figure 6.5) was selected for floral dipping. The *Agrobacterium* construct of pEG202-*JAZ1-FLAG* was transformed into the *jaz1* mutant plant line by floral dip method (Clough and Bent, 1998) to generate *35S::JAZ1-FLAG*.

Figure 6.5 (a) Wild type Col-0 plant (b) *jaz1* mutant SALK_011957 from NASC.

The pEG202 and pEG203 vectors have a basta herbicide resistance gene.. The transgenic *35S::JAZ1-FLAG* were subsequently selected by repeated spraying with basta. Resistant seedlings were grown and one-week-old plants were selected for extracting genomic DNA. PCR was performed for the purpose of genotyping by using the BASTA resistant (*BAR*) gene primers. Col-0 and *jaz1* are used as a negative control as they do not have the selection marker *BAR* gene. The genotyping PCR gave a product size of 1368 bp (Figure 6.6.) therefore giving confirmation of successful insertion of pEG202-*JAZ1-FLAG* construct into the *jaz1* mutant.

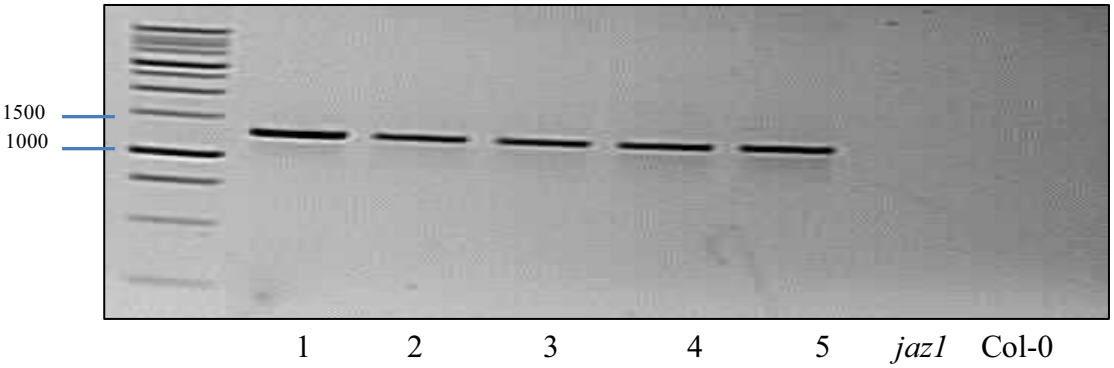

Figure 6.6 Genotyping PCR for F1 generation of *35S::JAZ1-FLAG line* . *jaz1* and Col-0 were used as negative controls. A PCR product of 1368 bp was produced by using *BAR* primers. 1 kb DNA ladder (Promega) used for DNA size comparison.

Further, to detect the expression of JAZ1 protein a Western blot assay was performed. Western blot is a sensitive method in which the antibody-antigen reaction helps in identifying the specific protein of interest from a mixture of complex proteins (Towbin et al., 1979). This assay helps to confirm the protein produced from the inserted transgene of interest. In this assay antibodies are used to detect the protein fused with tag and they bind only to the protein of interest (Mahmood and Yang, 2012). Using these methodologies; we could select those plant lines with a suitable amount of protein to cross with the other mutants. Plant proteins were extracted by grinding plant material into ice cold extraction buffer. Extracts from a *35S::GSNOR1-FLAG* line was used as a positive control (transgenic plants *35S::GSNOR1-FLAG* made by Michael J. Skelly in Loake lab). To indicate the procedure is optimised and working properly and to verify the results, a positive control from the known and tested transgenic plant line was used. JAZ1 protein consists of 27.60 kDa and flag tag consists of 1012 Da. A signal of a size of 28 kDa was detected by using anti-FLAG M2 clone antibody at (1:2,000) dilution (Figure 6.7).

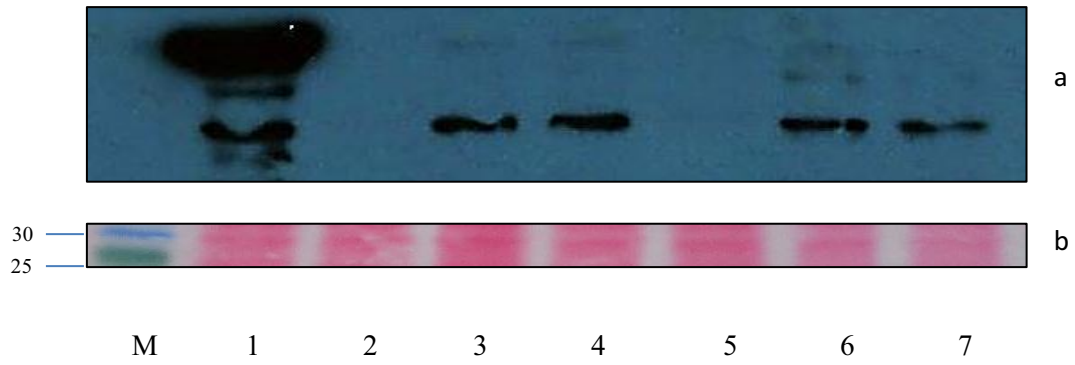

Figure 6.7 (a) **Western blot analysis for *35S::JAZ1-FLAG*.** M, protein molecular weight maker. Lane 1 is a positive control *35S::GSNOR1-FLAG*. Lane 2 and lane 5 are *jaz1* and Col-0 included as a negative control. Lanes 3, 4, 6 and 7contain protein extracts from *35S::JAZ1-FLAG* lines under test displaying signals against anti-FLAG antibody. (b) Ponceau staining shows total protein levels confirming equal protein loading.

The COI1 receptor is a key component of the JA signalling pathway and is required for JA responses in plant. *coi1* mutant was isolated by selecting plants that were resistance against the bacterial toxin, coronatine. The coronatine is structurally and functionally similar to JA-Ile (Feys et al., 1994).. COI1 encodes an F-box protein and *coi1*-1 mutants are male sterile and also displays defects in the synthesis of secondary metabolites, pathogen resistance and responses towards wounding (Bohlmann et al., 1998; Feys et al., 1994; McConn et al., 1997) *COI1* cDNA sequence has 1779 nucleotides that encode 592 amino acids. *coi1*-1 is a result of a conversion of a single nucleotide 467 (W codon) into a translation stop codon (Xie et al., 1998). The *coi1*-1 homozygous phenotype is insensitive to root growth inhibition after JA treatments (Feys et al., 1994). For the normal function of COI1 a reducing environment is required which is also important for JA-Ile induced interaction in COI1 and JAZ1 (Yan et al., 2009). In this study we have observed a markedly down-regulation of the JA signalling pathway in *atgsnor1-3* plants therefore we were interested in investigating the formation of SNO-COI1 in *planta*.

A construct was made for *COI1* gene using pENTRY223 as a donor vector and pEG203-MYC as a destination vector. Results for generating a construct using gateway cloning are

mentioned in Figure 6.2 accordingly. The *35S::COI1-MYC* construct was fused into *coi1*-1 mutant background and further the transgenics were crossed with *atgsnor1-3*.

As *coi1*-1 homozygous plants are male sterile, the construct was subsequently introduced into the *coi1*-1 heterozygous plants. *coi1*-1 (GL-1) is a single mutant generated by backcrossing with the original *coi1*-1 (Xie et al., 1998) to Col-0. The mutant *coi1*-1(GL-1) lacks in trichomes (Yoshida et al., 2009). *coi1*-1 heterozygous produces anthocyanin pigmentation. Therefore taking advantage of this feature heterozygous plants were screened on MS plants supplemented with 25 µM Me-JA.

The resulting pEG203-*COI1-MYC* vector was transformed into *A. tumefaciens* strains GV3101, which was subsequently used to transform *coi1*-1(AT2G39940) heterozygous plants by floral dipping to generate *35S::COI1-MYC* transgenics.

A) B)

Figure 6.8 (a) *coi1*-1 homozygous plant lacking developed siliques **(b)** A heterozygous *coi1*-1 mutant plant.

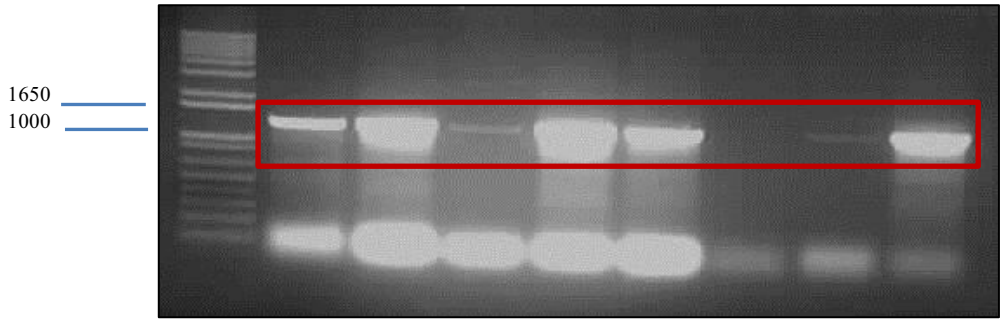

Figure 6.9 Genotyping PCR of *35S::COI1- MYC* using *BAR* gene primers. PCR product size is 1368 bp.1 kb plus (Thermo Fisher Scientific) ladder was used.

After performing several western blots we were unable to detect the attachment of a MYC tag to the COI1 protein.

Further experiments beyond the scope of this thesis will be required using alternative tags MYC to generate a tagged COI1 line which will be important for further studies.

6.3 JAZ1 protein is S-nitrosylated *in vivo*.

To determine whether JAZ1 is S-nitrosylated *in vivo* during the wound response, to that end transgenic *35S::JAZ1-FLAG* were crossed with *atgsnor1-3* that resulted in line *atgsnor1*-3 *35S::JAZ1-FLAG.* The *atgsnor1-3 35S::JAZ1-FLAG* seeds were sterilised and screened on MS medium plates containing 10 µM sulfadiazine which marks the presence of the T-DNA insertion in *atgsnor1-3*. Sulfadiazine is one of the effective selective agent in plant screening (Guerineau et al., 1990). The F1 seedlings which were resistant towards sulfadiazine screening were selected and grown under normal light conditions (16 hours light, 8 hours night). F2 generation mutants were selected in 1:3 ratio. Further genotyping PCR was performed for selection of homozygous plant lines using sets of gene specific primers. Figure. 6.10 shows the genotyping results of *atgsnor1-3-35S::JAZ1-FLAG* plants. The expected size PCR product was obtained using 35S -forward, *JAZ1* gene specific and

atgsnor1-3 (LP, RP) primers confirming the transgenic plant line is homozygous and successfully crossed.

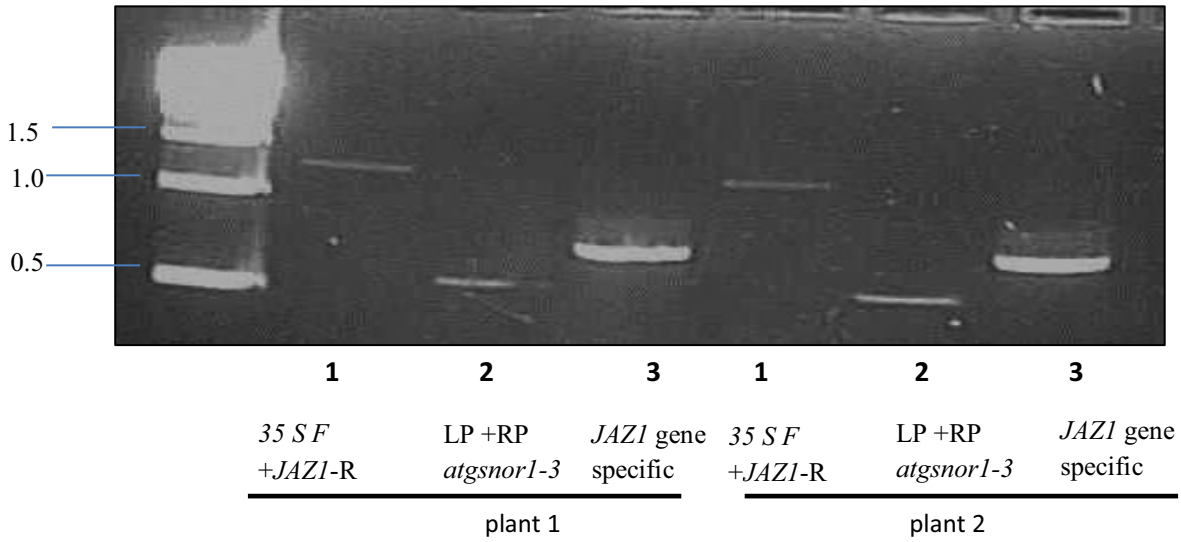

Figure 6.10 Genotyping PCR of candidate *atgsnor1-3-35S::JAZ1-FLAG* plants. 1=1219 bp 2=500 bp 3=762 bp. Genomic DNA was extracted from two individual plants. 1 kb DNA (NEB) ladder was used.

Further, to test whether JAZ1 is S-nitrosylated *in vivo*, total protein was extracted from wild-type Col-0, *35S::JAZ1-FLAG* and *atgsnor1-3-35S-JAZ1-FLAG* plants with or without wounding. Plant protein samples were subjected to the biotin switch assay and the biotinylated proteins were purified with the help of streptavidin beads. Streptavidin is a tetrameric biotin-binding protein and isolated from *Streptomyces avidinii* (Suter et al., 1988). It consists of a mass of 60,000 Daltons and displays a very high affinity towards biotin (Jones and Kurzban, 1995). A Western blot was carried out using an anti-FLAG antibody. The identification of JAZ1 S-nitrosylation *in vivo* was displayed by signals obtained after performing the Western blot assay of the *atgsnor1-3-35S::JAZ1-FLAG* protein extracts by using an anti-FLAG *antibody*. No signal was detected in protein extracts of wild-type and *35S::JAZ1-FLAG* plants as it served as a negative control in this assay. However the signal indicats the *in vivo* S-nitrosylation in *atgsnor1-3-35S::JAZ1-FLAG* lines. Further, this signal was only detected in the presence of ascorbate indicates that this signal was specific for S-nitrosylation. Collectively, these data indicate that JAZ1 is S-nitrosylated *in vivo* (Figure

6.11). Here in this experiment, the marker was overlapped, and the bands were too faint. Therefore, a repetition of this experiment will be necessary.

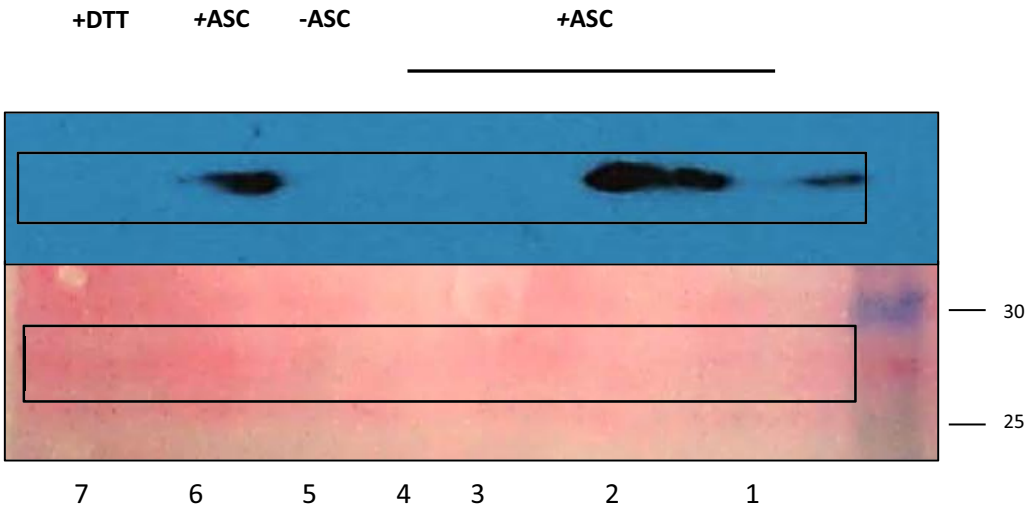

Figure 6.11 *In vivo* **S-nitrosylation of JAZ1 using *atgsnor1-3-35S::JAZ1-FLAG***. S-nitrosylated proteins were pulled down using streptavidin beads. SNO-JAZ1 was analysed by using anti-FLAG antibody. Sample no.1, 2, 5, 6 and 7 are *atgsnor1-3-35S*::*JAZ1-FLAG*. Sample 3 *is* Col-0 and sample 4 is *35S::JAZ1-FLAG* as negative control. Plant samples were mechanically wounded, except sample 6th. Sample 5th is without ascorbate while sample 7th contains DTT. Concentration of ascorbate used is 100 mM and DTT 1 mM. (b) Ponceau staining shows total protein levels confirming equal protein loading.

6.4 Discussion

This studies here have provided a novel mechanism in between NO and the JA signalling pathway in *Arabidopsis*. *JAZ1* is a repressor of the JA signalling that is degraded upon JA-Ile perception (Chini et al., 2007; Thines et al., 2007). Once JAZs are degraded they release MYC2 which can then drive JA-dependent gene expression (Chini et al., 2007; Lorenzo et al., 2004; Pauwels et al., 2008; Thines et al., 2007).As we have shown NO accumulation can potentially down regulates the JA signalling, proteins involved in JA signalling are therefore potential targets for S-nitrosylation. *A. tumefaciens* mediated transformation was used to generate a *35S::JAZ1-FLAG* transgene in a *jaz1* mutant background using pEarly Gate vector under the control of cauliflower mosaic virus *CaMV 35S* promoter. JAZ1 was not detected in Col-0 background plants by using anti-FLAG antibody thus we selected *jaz1* mutant plants.

Arabidopsis atgsnor1-3 plants display elevated NO levels. The phenotype exhibits an impaired flower development and also displays compromised plant immunity (Feechan et al., 2005; Kwon et al., 2012). *35S::JAZ1-FLAG* transgenics were backcrossed with a *atgsnor1-3* line for studying the status of S-nitrosylation of JAZ1 protein in high SNO background. Plant protein extracts from *atgsnor1-3-35S::JAZ1-FLAG* were subjected to biotin switch assay and later biotinylated proteins were pulled down by using streptavidin beads, signals were detected using an anti-FLAG antibody in Western blot assay.

The identification of JAZ1 S-nitrosylation *in vivo* was displayed by the signals obtained after performing the Western blot assay. The JAZ1 S-nitrosylation was identified in *planta* during the wounding response. The protein of interest is involved in stress response, signalling and plant development.

Similar like other phytohormones JA may also be regulated by cellular redox status (Feng et al., 2011). NO stabilises repressor DELLA of GA signalling pathway (Lozano-Juste and Leon, 2010) therefore it may also stabilise repressor JAZ1. Here we provide evidence suggesting the S-nitrosylation attenuates the JA signalling pathway in *atgsnor1-3* plants due to the JAZ1-SNO formation *in vivo*.

Jai3 is one of the most dominant JA resistant phenotype which is less studied (Chini et al., 2007). In *jai3* mutant, the splicing of the JAS domain disrupts the JAZ interaction with COI1, thus generating JA-insensitive phenotypes (Chung et al., 2010; Yan et al., 2007). Similarly

here in this study, it may suggest S-nitrosylation of cysteine residues in JAS domain of JAZ1 may be one of the reasons for inhibiting the interaction in between JAZ1-COI1. The S-nitrosylation of JAZ1 might modify its binding with MYC2 of the JA signalling pathway. JAZ proteins are functionally redundant as the sensitivity towards Me-JA is countered by the remaining eleven JAZ proteins. Multiple sequence alignment analysis of JAZ proteins has shown the JAS domain is highly conserved throughout the 12 JAZ members (Chini et al., 2009b).

Till date, GSNOR has a certain function in JA signalling (Wunsche et al., 2011). Decreased accumulation of herbivory induced JA was observed in *GSNOR*-silenced plants upon *Manduca sexta* attack. In *Nicotiana attenuata NaGSNOR*-silenced plants, decreased activity of secondary metabolites was observed such as trypsin proteinase inhibitor (TPI), caffeoylputrescine and diterpene glycoside (Wunsche et al., 2011). This can contribute to the fact that GSNOR positively regulates the JA signalling pathway (Wunsche et al., 2011).

Consequently, the S-nitrosylation of JAZ1 could be a control point of the JA signalling pathway. It may also affect the binding with the COI1. Similarly, the other proteins involved in JA signalling pathway may also be subjected to a redox-based modification by NO.

In our research the phenotype of *35S::JAZ1-FLAG* was not observed and neither the *atgsnor1-3-35S::JAZ1-FLAG*. It may be of interest to study the phenotypic characteristics of these transgenics in future studies.

COI1 requires a reducing environment to be functional and here in our studies we used anti-MYC antibody in the immunoblots assay for *35S::COI1-MYC* protein which failed to express COI1. TIR1 has an F-box and LRR region and the Cys 140 present in LRR on loop 4 is post-translationally modified (Terrile et al., 2012b). Similarly, COI1 has 19 cysteine residues and each one of them is important for post-translational modification by NO and 16 cysteine residues are situated in the LRR domain (http://www.uniprot.org). Here in our study, the COI1 S-nitrosylation status remained unknown.

Chapter-7

7.1 General Discussion

Plants are equipped with numerous defence mechanisms to counteract invading pathogens. Some defence mechanisms are pre-existing while others are induced upon pathogen attack. To minimise the fitness cost, plants have adapted effective mechanisms and co-ordinated strategies for the activation of specific defences (Pieterse and Dicke, 2007). JA is a key plant hormone one that is prominent in various physiological processes. Upon the formation of JA-Ile, this molecule binds to COI1 and promotes the binding of JAZ to SCF-COI1. JAZ proteins are then subjected to ubiquitination and destroyed by the 26S proteasome. MYC2 is thereby released from suppression and is free to activate transcriptional events in JA signalling (Chini et al., 2009a; Gfeller et al., 2010; Staswick, 2008). JAZ proteins are bound to MYC2 in a resting state and the domain TIFY of JAZ proteins also interacts with NINJA. The NINJA protein has an EAR motif. This EAR motif is known to recruit the TOPLESS protein which functions as a transcriptional repressor (Pauwels et al., 2010).

A ubiquitous signalling process exerted by NO through a post-translational modification process is termed S-nitrosylation (Gonzalez et al., 2009). Increasing evidence has shown that NO functions as a regulator of plant physiological processes (Grun et al., 2006). S-nitrosylation can impact protein activity by altering or activating enzyme activities. It can also obscure protein-protein interactions and influence protein localisation (Nakamura et al., 2013). In response to an array of microbial pathogens, S-nitrosylation controls the expression of disease resistance (Feechan et al., 2005). Here we address questions of how GSNOR1 regulates JA signalling during plant immune responses.

JAZ proteins are localised in the nucleus, and have a highly conserved JAS domain at the C-terminus (Chung et al., 2009; Thines et al., 2007; Yan et al., 2007). A study has revealed that mutations in *JAZ1*, *JAZ3* and *JAZ10* disrupt the function of the JAS domain are insensitive towards degradation upon JA-Ile or coronatine treatment and disrupt JA signalling. They also exhibit JA-insensitive phenotypes (Chini et al., 2007; Chung et al., 2010; Chung et al., 2008; Thines et al., 2007; Yan et al., 2007). A single receptor of the JA pathway, COI1, has an F-

box and COI1 forms a SCF-COI1 complex. JAZ1 is degraded by the 26S proteasome in a COI1 dependent manner. *COI1*, *JAZ*, *MYC2* therefore play an important role in regulating plant defence responses. Not only are they important in defence but these proteins also help in regulating various plants developmental processes (Cheng et al., 2011; Dombrecht et al., 2007).

Our emerging data highlights that high SNO levels can downregulate JA signalling. Through this research we have highlighted the significant role of GSNOR in plant disease resistance.

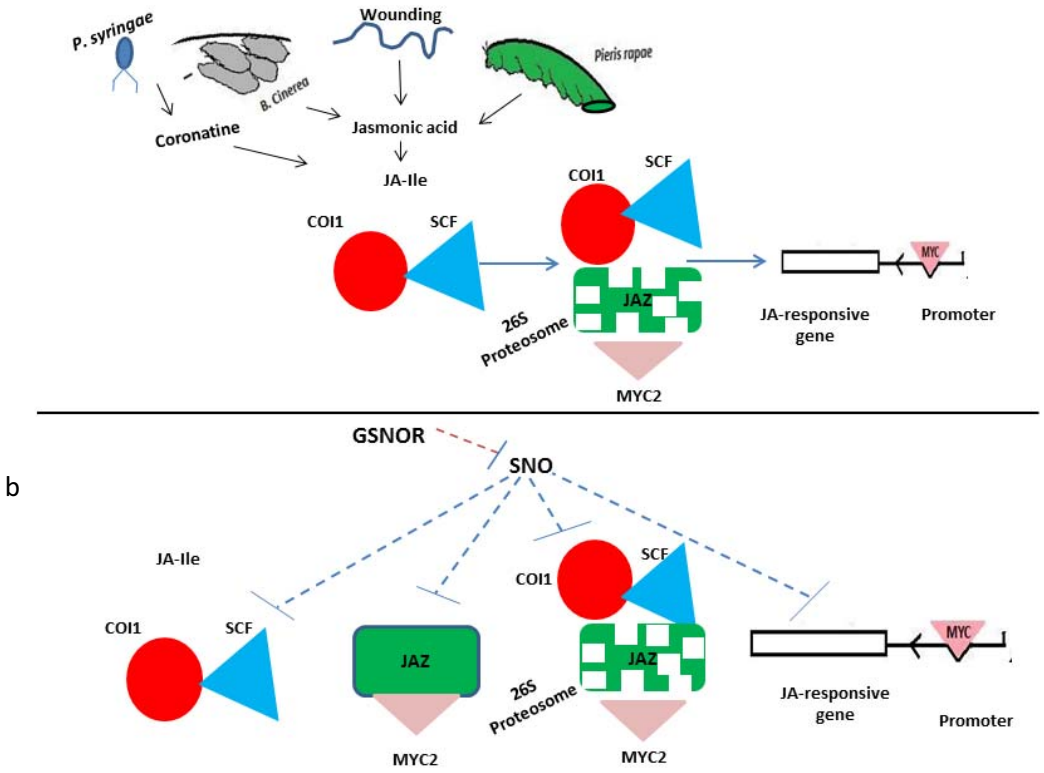

Figure 7.1 a) Activated JA signalling pathway. **b**) Possible mechanisms for the disruption of JA signalling due to increased SNO formation in *atgsnor1-3*.

7.2 *GSNOR* is involved in plant disease resistance

Increasing evidence has shown that NO substantially functions as a key regulator in plant physiological processes including plant defence (Grun et al., 2006). Prior studies have noted that decrease in SNO level leads to increased resistance against microbial invasion in *ATGSNOR1* (Feechan et al., 2005).The JA signalling pathway is the most important signal transduction pathway that is involved in the defence mechanism against insect attack (Turner et al., 2002).

Upon *B. cinerea* infection JA is rapidly induced in the wildtype plant cells (Kang et al., 2006; Li et al., 2005). Here we reported that conspicuous disease symptoms developed on *atgsnor1-3* plants as lesions induced by *B. cinerea* were of increased size relative to those found on wild-type plants. *atgsnor1-3* plants have been shown to compromise disease resistance against *B. cinerea* infection and also *Pieris rapae a* generalist herbivore-infested more *atgsnor1-3* plants compared to wild-type plants. These results may suggest a negative effect of NO on plant resistance against necrotrophic pathogens and insect feeding. SNO might be an important component in JA signalling triggered response to *B. cinerea* and *P. rapae* infection. GSNOR may protect the plant cells and may be a key regulator in plant-pathogen infection during JA signalling response. These findings indicate that *AtGSNOR1* function is required for establishing defence resistance against various microbial pathogens.

Upon damage caused by insect herbivory and fungal infection, linoleic acid and linolenic acid is released by lipase from the chloroplast membrane (Bargmann and Munnik, 2006; Schaller et al., 2005) and further JA is produced. Knock out *atgsnor1-3* plants are high in SNO accumulation and the proteins involved in biosynthesis of JA are the potential targets of S-nitrosylation. It is possible that *atgsnor1-3* plants over-accumulating a source of NO may nitrosylate certain JA biosynthetic enzymes and thus decrease their activity. OPDA contain six cysteine residues while AOC and AOS contain three cysteine residues (https://www.arabidopsis.org). It is a known fact that AOC involved in biosynthesis of JA through oxylipin pathway was found to be S-nitrosylated (Romero-Puertas et al., 2008). Due to S-nitrosylation of any of these proteins, production of JA must be decreased in *atgsnor1-3* plants compared to wild-type plants. So we speculate that *AtGSNOR1* is required for

mechanical wounding-induced accumulation of phytohormone such as JA. However, S-nitrosylation status of AOC and OPDA should be further examined.

It can thus be suggested from our findings that high SNO content in *atgsnor1-3* interferes in defending the fungal elicitors by disrupting the biosynthesis of JA content in *atgsnor1-3* plants upon mechanical wounding. Therefore we conclude that the susceptibility of *atgsnor1-3* plants towards the *B. cinerea* infection and *P. rapae* attack may be due to insufficient production of JA as the phytohormones.

Me-JA treatment induces *JAZ1* gene expression in wild-type plants. The organisation of induced defence response is interconnected by a network of signal transduction pathways in which phytohormones such as JA, SA and ET are involved (Kazan and Manners, 2008; Pieterse and Dicke, 2007). We observed difference in the Me-JA induced gene transcript level in *atgsnor1-3* plants compared to wild-type. *AtGSNOR1* knockout may compromise the activity of JA signalling cascade by disturbing the receptor COI1 or the SCF^{COI1} complex in turn altering the *JAZ1* gene expression. The new and rapid synthesis of JAZ proteins may be disrupted by high SNO level thus disrupting the downstream JA signalling. It may also be possible that high SNO levels in *atgsnor1-3* are not producing sufficient quantity of bioactive JA in turn interfering in transmitting a signal that triggers an SCF^{COI1} or 26S proteosome–mediated destruction of JAZ repressor and therefore down regulating the JA early or late responsive gene expression.

7.3 S-nitrosylation has an impact on protein-protein interactions involved in JA signalling pathway

JAZ/COI1/MYC2 are key components in JA signalling pathway. The reduction of JA accumulation , higher susceptibility of *atgsnor1-3* plants towards invading pathogens and downregulation of JA marker gene expression leads to a clue towards a disruption in JA signalling protein complexes. In yeast cells, NO donors affected the interaction in between JAZ1-COI1 in presence of Me-JA analysed by yeast two-hybrid system. Hence, NO attenuates interaction between JAZ1-COI1 but has no effect on JAZ1 and MYC2 interaction. COI1 dependent degradation of JAZ upon the perception of JA-Ile is important for the activation of JA defence response (Thines et al., 2007). Single amino acid substitution in JAZ

proteins may lead to disruption in interaction with COI1 (Melotto et al., 2008). We speculate that S-nitrosylation could modify the ability of JAZ1 to bind COI1. JA signalling pathway proteins may be subject to redox based modification by NO. NO may form a cysteine adduct, especially in the JAS domain hence blocking the JAZ1-COI1 interaction.

JAS and ZIM domain are involved in protein-protein interactions. JAZ1 is a repressor of JA signalling pathway and S-nitrosylation of JAZ1 may favour its interaction with MYC2 thereby, binding it and attenuating its release and in turn inhibiting its JA transcriptional activation responses.

ZIM domain of JAZ has a significant role in forming homomeric and heteromeric interactions in JAZ family. JAZ-JAZ interactions have relevance in JA signalling pathway. The redox-based post-translational modification process may interfere to some extent with homomeric or heteromeric interactions, therefore, downregulating JA signalling. However, further experiments will be required in support to this hypothesis.

The synthesis of new negative regulator JAZ proteins ensures inhibition of activation of JA responsive genes (Thines et al., 2007) therefore, degradation of JAZ proteins is necessary which releases MYC2 TFs and in turn, activates JA defence responses against pathogen and insects.

The fluorometric assay of CaMV *35S::JAZ1-GUS* transgenic plants upon the application of NO donors like GSNO do not show significant inhibition in the degradation of JAZ1 however the histochemical analysis displays that JAZ1 stability is promoted by GSNO. It is worth mentioning the stability of JAZ may be due to homomeric and heteromeric interactions in JAZΔJas (Chini et al., 2009b) and JAZ may be destabilised by JAS domain upon JA-Ile perception (Chung et al., 2009). However, the concentration of plant protein and the vegetative tissues of transgenic plants would have an influence on the emission of fluorescence and GUS activity. Therefore, repetition of this experiment may be helpful in understanding the effect of NO on JAZ1 stability..

7.4 Proteomic approach leads to an understanding of modulation of proteins involved in JA signalling by S-nitrosylation

Proteomic studies have proved advantageous to discover numerous proteins getting S-nitrosylated. Protein S-nitrosylation in plants is recognised as an emerging topic of interest. Most of the proteins are linked to plant defence responses (Astier and Lindermayr, 2012).

Peroxiredoxin II E (PrxII E) is known to S-nitrosylate, which results in the attenuation of peroxynitrite ONOO- reductase activity (Romero-Puertas et al., 2007b). S-nitrosylation of PrxII E in *A. thaliana* occurs upon the *P. syringae* infection (Abat et al., 2008). Another example of plant hormone signalling pathway is the SA pathway. Upon the challenge SABP3 undergoes S-nitrosylation at Cys 280 residues, impacting the decrease of CA activity and SA binding properties (Wang et al., 2009). Similarly, upon pathogen attack, ATRBOHD at Cys 890 residue undergoes S-nitrosylation therefore, modulating ROS production (Yun et al., 2011a).

Another interesting example is S-nitrosylation of CDC48 in tobacco plants. CDC48 is the protein involved in cell division, differentiation growth, development, degradation of protein and plant disease resistance (Bae et al., 2009; Park et al., 2008). External application of NO on the recombinant protein of NTCDC48 has been shown to be S-nitrosylated at Cys 526 by changing and decreasing the protein activity *in vitro* (Besson-Bard et al., 2008c; Lamotte et al., 2004).

In JA signalling pathway JAZ1 has five cysteine residues. Cysteine residues are potential candidates for getting S-nitrosylated. These cysteine residues are highly conserved in almost all JAZ family members except JAZ10 (https://www.arabidopsis.org). To identify potential protein S-nitrosylation during the plant immune response JAZ1-MBP recombinant protein was used for biotin switch analysis. This analysis revealed that JAZ1 is S-nitrosylated *in vitro*. JAZ1-SNO formation is also concentration dependent. Cys 229 was identified as target sites for S-nitrosylation. Cys 229 is in the JAS domain and this domain is conserved throughout other JAZ members. JAS motif is also necessary for binding of JAZ and MYC2 and the binding is hormone independent (Chini et al., 2007; Katsir et al., 2008; Melotto et al., 2008). We demonstrated the formation of JAZ1-SNO *in vivo* as JAZ1 is S-nitrosylated in *atgsnor1-3-35S::JAZ1-FLAG* background. The current data highlights that S-nitrosylation of JAZ1 in vitro and in vivo consequently attenuates JA signalling this may lead to uncovering the molecular mechanism for the perception of a redox signal by the JA signalling

components, thereby coordinating plant defence. We speculate that a negative feedback loop may establish in between the JA and NO signalling due to the high SNO burst that may act to deactivate JA signalling through S-nitrosylation.

In support of the existing literature that S-nitrosylation has an impact on phytohormones signalling pathway such as auxin and gibberellin, we illustrate that S-nitrosylation also has an impact on JA signalling pathway. S-nitrosylation degrades PIN1 in auxin signalling pathway while the DELLA are stabilised in gibberellin signalling pathway. In a similar manner, our study states that S-nitrosylation may regulate JAZ1 protein.

Therefore, this study could bring into insight that *atgsnor1-3* may compromised in its resistance against the necrotrophic fungus, herbivory attack and also has a disrupted JA response due to S-nitrosylation of JAZ1 at Cys 229 residue. Further investigation needs to carry out to decipher the status of S-nitrosylation of COI1 and MYC2.

7.5 Future work

The results so far provided in this work, provides a strong framework for future work in protein-protein interaction *in vivo* to determine the impact of high SNO on JAZ1 and COI1 through immunoprecipitation, therefore, leading to uncovering the effects of redox regulation in *planta.*

Generating *35S::COI1-MYC* and backcrossing them with *atgsnor1-3* to produce *atgsnor1-3 35S::COI1-MYC* will be of interest. S-nitrosylation of JAZ1 and the identification of target site of Cys 229 through mass spectrometry have provided a strong foundation to analyse site directed mutagenesis and verify the abolishing S-nitrosylation of JAZ1 confirming Cys 229 as a target site *in vivo*. Based on the fact that COI1 has 19 cysteine could also be S-nitrosylated along with MYC2 and this possibility should be investigated.

7.6 Conclusion

The thesis aimed to understand the role of S-nitrosylation in JA signalling pathway during the plant-pathogen interactions by manipulating the mutant *atgsnor1-3*. S-nitrosylation is an emerging field where a number of target proteins are identified to be S-nitrosylated that leads a role in plant immunity. This study has provided a strong framework for further analysis of immunoprecipitation assay in between COI1 and JAZ1 therefore, leading to uncovering the effects of redox regulation in *planta*.

S-nitrosylation of JAZ1 *in vivo* might support the disruption of JA signalling. It may suggest the ability of GSNO to negatively regulate the expression of genes encoding proteins involved in JA biosynthesis and signalling. GSNOR, therefore, might be a positive regulator of the JA signalling pathway. The emerging data suggest that JAs underpin a broad sweep of activities integral to plant growth, development and immunity. Also, a molecular framework for how JA-Ile is perceived and subsequently regulates JA-dependent gene expression has enabled a paradigm shift in our understanding of JA signalling. Functional redundancy of *JAZ1* is one of the limitations for studying the characterisation of phenotypes. In this context, COI1 is thought to be the JA receptor suggesting a remarkable similarity between auxin-signalling and JA-signalling. Therefore, it shows SNO formation may target multiple nodes of this defence signalling network. Manipulating cellular levels of S-nitrosylation may provide novel opportunities for the control of both insect pests and fungal pathogens.

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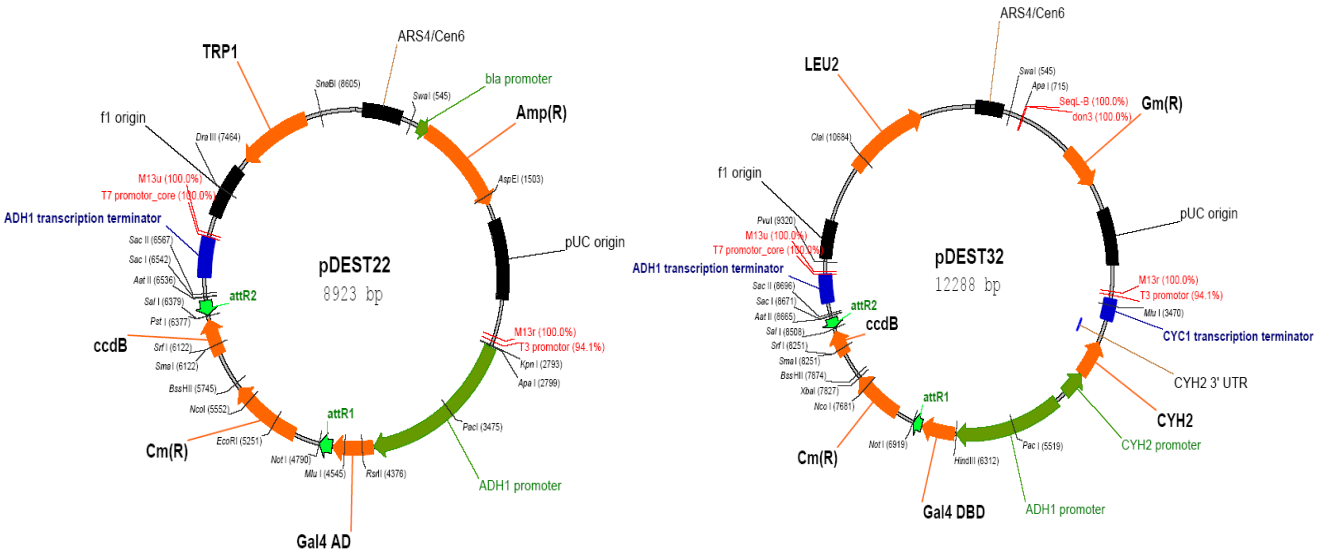
Appendix A

1) Yeast two hybrid assay vectors



Gateway cloning map of destination vectors pDEST-22 and pDEST-32 used in Y2H assay. pDEST-32 has a GAL4 DNA Binding domain and pDEST-22 has a GAL4 DNA Activation domain that is ampicillin resistance and contains Tryptophan. pDEST-32 is gentamycin resistance and contains leucine amino acid.

2) Gateway cloning reaction

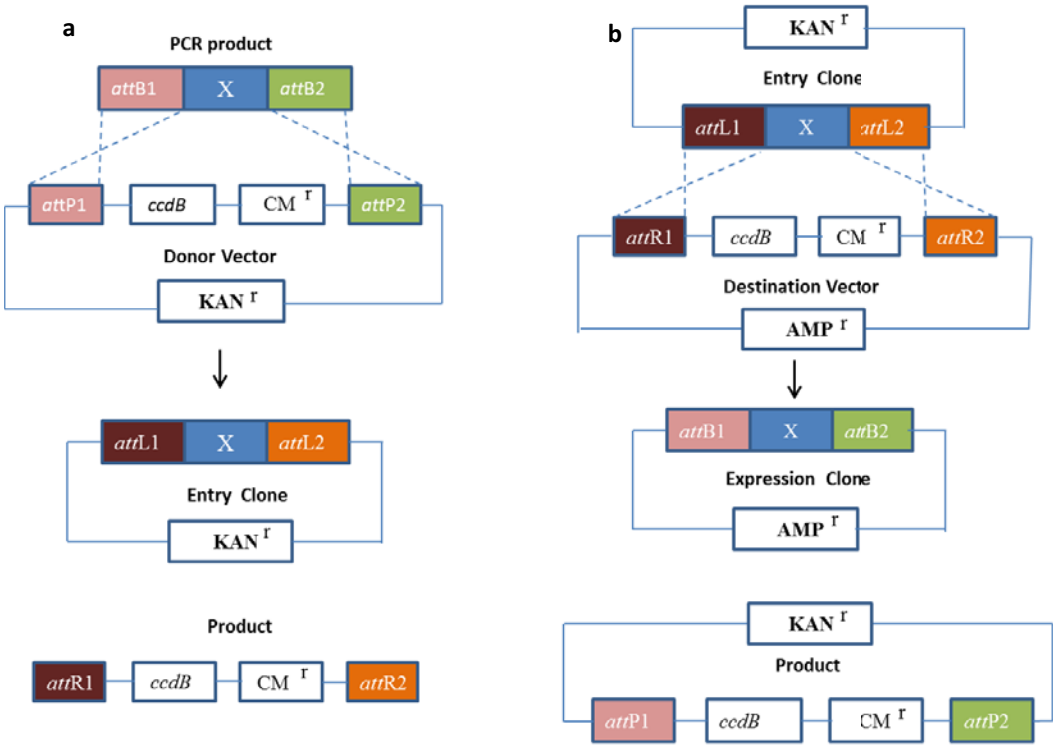
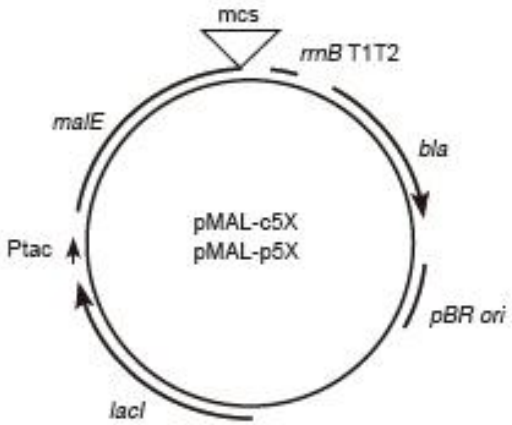


Figure 2.Gateway cloning mechanism. a) In a BP reaction PCR product X is flanked by *att*B site into the donor vector forming an entry clone. b) LR reaction is the combination of entry clone containing the X DNA sequence with the destination vector (Magnani et al, 2006).

Appendix B

Vector used for recombinant protein expression



pMAL-c5X Polylinker:

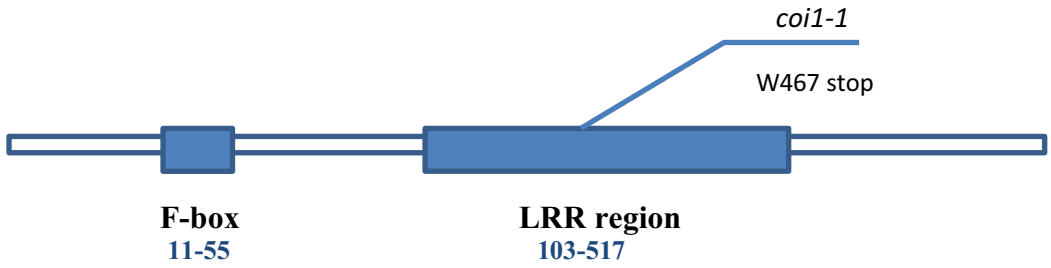
```
                    SacI                                                                  XmnI
5' malE...TCG AGC TCG (AAC)n AAT AAC AAT (AAC)n CTC GGG ATC GAG GGA AGG ATT TCA
  NdeI    NcoI     NotI     EcoRV    SalI     BamHI    EcoRI    SbfI
CAT ATG TCC ATG GGC GGC CGC GAT ATC GTC GAC GGA TCC GAA TTC CCT GCA GGT

AAT TAA ATA A...
```

pMAL-c5-X vector with MBP tag at N-terminal used for recombinant protein expression for COI1 and JAZ. MBP tag proteins are expressed cytoplasmically. MBP tag is designed for tighter binding to amylose resin. A vector polylinker is present with restriction sites. A gene or open reading frame fused into restriction site of the polylinker in the same translational reading frame as malE. It also has XmnI site coding for four amino acids like Ile-Glu-Gly-Arg that allows protein of interest to be cleaved from MBP tag using Xa factor (NEB).

Appendix C

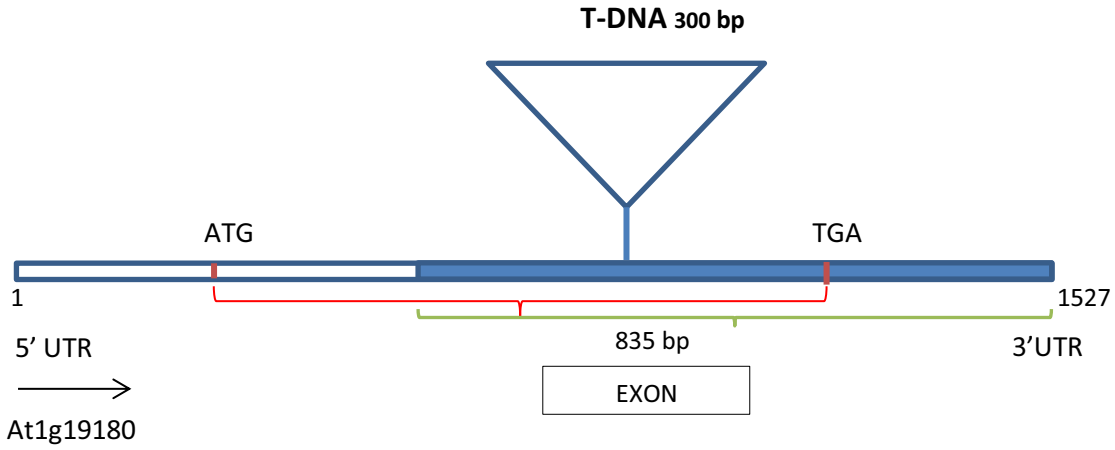
Mutation of *coi1-1*



***Coi1-1* mutation occurs at codon 467 that is converted to a translational stop codon. At position 1401, a single nucleotide is substituted from G to A leading to conversion into a stop codon.**

Appendix D

Scheme of T-DNA insertion in *jaz1* (At1g19180).



SALK T-DNA Insertion Sites Map. Length of DNA sequences (1-1527 bp). T-DNA Insertions in exon at 835 bp on chromosome 1 and at coordinates 6622928-6623594.