THE UNIVERSITY OF WESTERN AUSTRALIA

New Roles for Old Enzymes:

Characterizing *Arabidopsis* nitrilases

This thesis is presented for the Degree of Doctor of Philosophy of the University of Western Australia

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Declaration

The work presented here in this thesis is my own work except where stated. This work was carried out in the School of Chemistry and Biochemistry, Faculty of Life and Physical Sciences, at the University of Western Australia. The material presented in this thesis has not been presented for any other degree.

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Acknowledgments

In my honours thesis, I used the analogy of a rollercoaster to describe my experience during honours. Continuing that theme, I would say doing a PhD was like spending a day at a theme park. I got to try many things, see what I would like (and not) and ultimately have lots of fun (and lots of excruciating demoralizing times). It was an amazing experience but it is almost closing time for this theme park and I have to leave and try another park elsewhere.

I would like to express my utmost gratitude to my supervisors, Thomas and Harvey, both who have been very supportive of me and to my research. Their guidance has been amazing and without them, I would not have been able to finish all of this and come out with some sanity intact. All the past and present members of the Martin lab – thanks for instilling numerous bad habits which I will most likely never shake off. Many thanks to Adriana Pruzinska and Julia Grassl for their help during my PhD. Lastly, the staff at CMCA for their amazing help and advice especially when errors kept coming up on the microscopes.

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To sum up my experience in doing a PhD: "It was the best of times, it was the worst of times."

Scientific abstract

Arabidopsis nitrilases are well characterized nitrile degrading enzymes which are reportedly involved in glucosinolate catabolism. Here, interactors were determined using affinity purification coupled with mass spectrometry (AP-MS) and Bimolecular Fluorescence Complementation (BiFC) for the *Arabidopsis* nitrilases. The GRFs (14-3-3 protein), AtMKK2 and ERF1 were all found to be interactors for the *Arabidopsis* nitrilases. Aside from determining these interactors, it was also established that the nitrilases are able to form both homo-and heterodimers.

GFP fusion studies for the four nitrilase isoforms showed that they have both nuclear and cytosolic localization despite lacking typical nuclear localization signals. Nitrilase dimers also showed the same localization pattern (chapter 3). Interaction with AtMKK2 was shown to be cytosolic whereas with ERF1 it was both nuclear and cytosolic. The localization of ERF1-GFP was traced to be mostly nuclear and the shift upon interaction with nitrilases suggests a regulatory role of the nitrilases. Further investigation into the 14-3-3 interaction utilized BiFC and showed that the NIT1 and NIT 4 isoforms interact with both the epsilon and non-epsilon 14-3-3 isoforms. The other isoforms were shown to interact with kappa 14-3-3. Upon interaction with the 14-3-3 proteins, the localization shifts from to being exclusively cytosolic. This suggests that the regulatory role for the 14-3-3s is to control the localization of the nitrilases and sequester them to the cytosol. Initial investigation into the binding site for 14-3-3s in the NIT1 protein suggests that the binding site is within the first 69 amino acids of the protein.

The interactors, AtMKK2 and ERF1, are involved in JA signalling as well as abiotic stress responses. Thus, a link between *Arabidopsis* nitrilases and Jasmonic (JA) signalling was investigated (chapter 4). Using qRT-PCR, it was shown that the expression of NIT1 homologues were upregulated in response to MeJA whereas the expression of NIT4 was downregulated. The expressions of the two JA signalling pathway marker genes, *VSP1* and *PDF1.2*, were investigated in the 35:NIT1 and 35SNIT4 mutant lines using qRT-PCR. *VSP1* was upregulated in both lines. However for *PDF1.2*, it was downregulated in 35S:NIT4 but upregulated in 35S:NIT1. JA-dependent phenotypes were also looked into using root growth inhibition assays for the 35:NIT1 and 35:NIT4 lines comparing with Col-0 (chapter 5). It was found that the 35S:NIT4 line was more sensitive to MeJA than the other lines. A role in abiotic stress

was also looked into for the nitrilase. 35:NIT1, 35S:NIT4 and Col-0 seeds were germinated on salt and ABA and increased sensitivity was observed when NIT1 was over-expressed, suggesting involvement in abiotic stress responses. Finally, resistance to nitrile herbicides, bromoxynil, atrazine and DCMU was investigated for 35S:NIT1. Germination assays showed that NIT1 confers specific resistance to bromoxynil; no resistance was observed for atrazine and DCMU.

List of Abbreviations

Arabidopsis	*Arabidopsis thaliana*
BiFC	Bimolecular Fluorescent complementation
bp	Base pairs
BSA	Bovine Serum albumin
cDNA	Complementary DNA
DAPI	4', 6-diamidino-2-phenylindole
ddH_20	Distilled water
DNase	Deoxyribonuclease
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid mix
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra-acetic Acid
EtBR	Ethidium bromide
GFP	Green fluorescent protein
HA	haemagglutin
LB	Luria-Bertani (broth/agar)
MeJA	Methyl jasmonate
MS	Murashige and Skoog
$MgCl_2$	Magnesium chloride
OD_{600}	Optical density at λ=600nm
ORF	Open reading frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RFP	Red fluorescent protein
RPM	Revolutions per minute
qRT-PCR	Quantitative reverse transcription PCR
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDW	Sterile distilled water
TAE	Tris-acetic acid-EDTA
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 1% Tween 20
TEMED	Tetramethylethylenediame
TRIS	tris (hydroxymethyl) aminomethane
TFB	Transformation buffer
YC	c-terminal fragment of the YFP
YFP	Yellow fluorescent protein
YN	n-terminal fragment of the YFP
UTR	Untranslated region

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Chapter 1

General Introduction

1.1 Secondary metabolites and plant defence

Being sessile, plants not only need to deal with environmental stresses such as drought, high salinity or fluctuating temperatures but also pathogen and herbivory attacks. Thus plants have developed an arsenal of defences to deal with such stresses. Whilst plants lack the mammalian adaptive immune system, they have an innate immune system and a systemic signalling system which respond to infections. Recognition of microbial or pathogen associated molecular patterns (MAMPs or PAMPs) form the basis of the plant innate immune system. MAMPS and PAMPs include fragments of bacterial cell wall, flagellin and the elongation factor EF-Tu. When challenged, PAMPs/MAMPs are recognised by transmembrane pattern recognition receptors (PRRs) triggering PAMP-triggered immunity (PTI). A second side of the innate immune system involves disease resistance proteins (R proteins). R proteins detect effector molecules which are secreted by pathogens into plant cells to aid in the infection process. Both paths halt further colonization by the pathogen. However, pathogens can secrete effectors which supresses PTI, inducing disease in the plant. This results in effector triggered susceptibility (ETS) which activates effector-triggered immunity (ETI), the second phase in the immune response in plants (*1*). This whole process allows the plant to develop resistance to the pathogen and restrict its growth.

Herbivores can induce a similar immune response to MAMPs/PAMPs which can systemically deter insects from further attacking the plant and ward off future attacks. Oral secretions, saliva and oviposition fluids can act as elicitors not dissimilar to MAMPs/PAMPs and are referred to as herbivore-associated molecular patterns (HAMPs) (*2*). In response to HAMPs, a defence response is elicited and one of those responses is the release of defence compounds. The catabolism of secondary metabolites in plants leads to the release of these defence compounds which form the basis of defence against herbivores.

Plant secondary metabolites are a large group of structurally diverse compounds, derived from primary metabolism or from biosynthetic intermediates. Secondary metabolites are well studied and many are part of plant defence (*3*). Secondary metabolites involved in plant defence can be classified into: phytoanticipins and phytoalexins. Phytoanticipins are defined as compounds which are preformed or formed

from precursors that are released following elicitation. As for phytoalexins, these are compounds which are synthesised and accumulate in plants following pathogen attack (*4*). Plants have a great diversity of defence compounds, providing a single plant with defences against different herbivores with differing feeding methods (*4*). Interestingly, plant secondary metabolites are often restricted to particular families or genus. An example of this are glucosinolates, a group found only in the Capparales order which includes the Brassicaceae family (*5*). This shows that the secondary metabolites and their catabolism has evolved in different plants to deal with the different types of herbivores.

1.1.1 Glucosinolates

Glucosinolates are sulfur and nitrogen containing secondary metabolites, deriving from glucose and amino acids. Due to sulfonation during synthesis, glucosinolates contain at least two sulfur atoms and are thus considered as potential storage proteins for sulfur in plants (*6*). Glucosinolates have a large structural diversity; they can be aliphatic or aromatic depending on the precursor amino acids. In *Arabidopsis*, there are at least 30 types of glucosinolates with 4-methythio, 4-methylsulfinylbutyl and indoyl-3ylmethylglucosinolates being the predominant glucosinolates (*7*). They can be catabolised into more than 100 breakdown products after hydrolysis by myrosinases (*8*). Whilst found in all types of plant tissue, the concentration and type of glucosinolate in each tissue is dynamic. The variation depends on the type of tissue, age, developmental stage and even occurs between accessions in a single species (*9*). Furthermore, environmental factors such as light, biotic and abiotic stresses affect glucosinolate levels.

Glucosinolates are chemically stable and biologically inactive compounds which are stored in sulfur-rich S-cells. However, when plant tissue is disrupted through wounding or other environmental stimuli, glucosinolates are rapidly hydrolysed into unstable aglycones by myrosinases. Myrosinases are β-thioglucoside glucohydrolases which are kept physically separate from glucosinolates to prevent spontaneous hydrolysis. They are compartmentalised in glucosinolate-free cells called myrosin cells which often sit next to the S-cells (*10*). The aglycones degrade into either isothiocyanates (ITCs), thiocyanates or nitriles. The final product the aglycone degrades into depends on the structure of the side chain, pH, ferrous ion concentration and the presence of

epithiospecifier proteins (ESP) (*5*). Due to this, the breakdown products of glucosinolates are varied and provides a diverse chemical arsenal for the plant.

Whilst glucosinolate degradation products generally act as deterrents for insects, they can also act as an attractant. Specialist feeders such as *Pieris rapae* use ITCs as host cues and have developed methods to detoxify ITCs (*11*). In addition to deterring herbivores, the degradation products of glucosinolates can also restrict microbial pathogen growth *in planta*. Using mutants of key genes involved synthesising and catabolising indole glucosinolates in *Arabidopsis*, it was shown that glucosinolate metabolism also has a role in providing fungal and bacterial resistance. *Arabidopsis* mutants devoid of the atypical myrosinase PEN2 had higher levels of indolic glucosinolates as compared to the wild type and were more susceptible to fungal pathogens (*12*). Flg22, a bacterial elicitor induces indolic glucosinolate biosynthesis and catabolism through the MYB51 transcription factor and PEN2 myrosinase (*13*). This shows that glucosinolates may be involved in defending against bacterial infections.

The breakdown products of glucosinolates are not only toxic to the herbivores but also to the plants themselves. Thus, once the threat of a herbivore is gone, there is a need to detoxify such compounds. The nitriles and ITCs derived from glucosinolates are degraded into carboxylic acids and ammonia *via* C-N hydrolases such as nitrilases. The degradation products can be remobilised into synthesising other amino acids and essential metabolites. As stated earlier, glucosinolates are considered as potential sulfur and nitrogen sinks. In the absence of wounding, glucosinolates are reported to be endogenously catabolised to release sulfur and nitrogen during specific developmental stages such as seedling growth (*6*). The potential sulfur storage role is supported by the decline of glucosinolate levels during sulfur deprivation (*14*). Furthermore, transcriptomic data have shown that myrosinases are upregulated during sulfur deprivation and that genes involved in the synthesis of glucosinolates such as the MYB transcription factors are downregulated (*14-16*). Thus, it appears that glucosinolates not only have a role in plant defence but also in the remobilisation and storage of important macronutrients such as sulfur and nitrogen.

1.2 The nitrilase family

The nitrilase superfamily consist of thiol enzymes of eukaryotic and prokaryotic organisms. There are 13 branches of the nitrilase superfamily; with branch one defined as the true nitrilases. True nitrilases are bi-functional; they simultaneously catalyse the hydrolysis and hydration of both nitrile and cyanic substrates (*17*). All branches of the nitrilase family have a conserved catalytic triad consisting of the amino acids glutamic acid, cysteine and lysine. The cysteine residue is important for enzyme activity as demonstrated using site-directed mutagenesis; mutation of the cysteine residue to an alanine resulted in the loss of nitrilase activity (*18*). The different branches of nitrilases can be distinguished by the motif in proximity to the active-site cysteine. For branch one nitrilases, there is a Cys-Trp-Glu motif at the active site cysteine (*17*).

1.2.1 Structure of nitrilases

Whilst the structure of plant nitrilases hitherto have not been solved, structures of bacterial and archael nitrilases have been solved *via* x-ray crystallography (*19, 20*). Solved crystal structures suggest that despite the nitrilase superfamily being diverse, they all share a similar αββα sandwich fold secondary structure. This sandwich fold forms the basis for nitrilase dimerization, whereby the proteins form an αββα-αββα super-sandwich scaffold which can further oligomerize. The oligomerization of nitrilases is well studied in bacterial nitrilases (*21*). Whilst the formation of a dimer *via* the super-sandwich scaffold is common between the diverse nitrilases, the oligomerisation state varies between the diverse nitrilases. Nitrilase oligomers can be quite large, consisting of 6 to 26 subunits (*22*). Furthermore, such oligomers arranged themselves as filamentous structures. Using electron microscopy, bacterial and fungal nitrilase oligomers were shown to form left-handed helical spiral filamental structures (*21, 23*) Oligomer formation in nitrilases seems to be important for enzymatic function and dependent on substrate binding. An example is the nitrilase from *Nocardia*. When purified in absence of its substrate, the nitrilase does not form oligomeric structures. However, the enzyme oligomerizes into its active form when co-purified with its substrate (*24*). Given the homology between plant and bacterial nitrilases (60 to 70%), plant nitrilases most likely have similar oligomeric features (*22*). Indeed, Doskocilova *et al* showed that NIT1 form similar oligomeric structures of about 440 KDa (*25*). Using

electron microscopy, they further showed that NIT1 forms highly ordered linear filamentous structures similar to the structures observed with bacterial nitrilases.

1.2.2 Plant nitrilases

Plant nitrilases were first described in 1964; they were said to be enzymes involved in the biosynthesis of auxin, a major phytohormone (*26*). Nitrilases are ubiquitously distributed in the plant kingdom; they are found from mosses to higher plants (*27*). Thus it can be said that the nitrilase gene in plants is ancient. Plants can have more than one nitrilase isoform; *Zea mays* (maize) has two isoforms (ZmNIT1 and 2), *Sorghum bicolor* (sorghum) has three (SbNIT4A, B1 and B2) and *Arabidopsis thaliana* has four (NIT1 to 4). NIT1 to 3 (At3g44310, At3g44300, At3g44320) have up to 82% identity in their amino acid sequence whereas NIT4 (At5g22300) has only 65% identity with NIT3. Maize nitrilases shares a higher homology on the amino acid level with NIT4 (69%) than with the other *Arabidopsis* nitrilases (*28*). The sorghum nitrilases are also homologues of NIT4, sharing about 71 to 75% amino acid sequence identity to NIT4 (*29*). Similarly, nitrilases found in other plant species are also said to be homologues of NIT4 (figure 1.1). Interestingly, NIT1 and its homologues (NIT2 and 3) are limited to the Brassicaceae family. This suggests that NIT1 and its homologues are a result of a duplication event of the NIT4 gene early on in the phylogeny of the Brassicaceae. The chromosomal positioning of the NIT1 homologues reflects this; whilst NIT4 is located on chromosome five, NIT1 to 3 are all located on chromosome three.

The high sequence similarities even between the NIT4 and NIT1 homologues indicate that all plant nitrilases share a common origin. As NIT4 homologues are common to all plans whereas NIT1 homologues are limited to one family, it appears that the primordial nitrilase is a homologue of NIT4. The wide distribution of the NIT4-like homologues in the plant kingdom allows the speculation that these nitrilases share a common and ancient biological role which is vital for plant survival. The lack of visible phenotype of NIT4 knockout mutants under normal growth conditions argues against such an essential role for the NIT4 homologues. However, it can be imagined that the important role of the NIT4-like homologues is more obvious under abiotic or biotic stress conditions reflecting the environment plants face in nature. Putting aside the limitation of being found only in the Brassicaceae family, one could argue that the high sequence

similarities of the NIT1 homologues to each other and to the other plant nitrilases may indicate a level of functional redundancy.

It appears that the comparison of distribution and sequences of the plant nitrilases is limited in determining the biological roles of nitrilases. An indication for shared or specific roles may be found in the analysis of expression patterns or regulation of the plant nitrilase genes and/or in the enzymatic activities of these proteins.

1.2.3 Expression patterns of the *Arabidopsis* nitrilases

The four *Arabidopsis* nitrilases are differentially expressed during plant development. *NIT1* and *NIT2* are constitutively expressed during all stages of development whereas *NIT3* and *NIT* 4 are expressed during specific developmental stages. According to microarray data, *NIT3* is strongly expressed during early and late developmental stages with its highest expression during senescence whereas NIT4 is expressed mostly during the flowering stage and silique formation (figure 1.2A) (*30*). Interestingly, *NIT1* and *NIT2* also have high expression during senescence. The localization of the expression of the four *Arabidopsis* nitrilases also differ; *NIT1* and *NIT2* are expressed in all tissue whereas high expression levels for *NIT3* and *NIT4* are limited to a few tissues (figure 1.2B) such as the hypocotyl, radicle, root tissue (*NIT3*) and senescent and cauline leaves (*NIT4*).

Whilst sequence similarities suggest a level of redundancy between the *Arabidopsis* nitrilases, the spatial and developmental differences in their expression indicate that there may be different biological roles for the four isoforms. These could be linked to the requirement for plants to respond dynamically to the various environmental stimuli. Currently, there are a few studies linking *Arabidopsis* nitrilases' expression profile and environmental stimuli. Such studies looked into stresses like drought, nutrient starvation and senescence and saw changes in the expression profile of the nitrilases (*31-33*). The diversity of conditions impacting on *Arabidopsis* nitrilase gene expression suggests the involvement of different stimuli acting *via* various signalling cascades which can be under the regulation of hormone signalling networks. Indications that this is the case for the regulation of *Arabidopsis* nitrilase gene expression have emerged in the recent years. For example, cDNA macroarrays, performed to generate an expression profile for *Arabidopsis* seedlings, showed that NIT1 and 2 are upregulated in response to the

phytohormone, MeJA (*34*). Similarly, abscisic acid (ABA), a phytohormone involved in abiotic stress responses, was shown to upregulate NIT1 and 2 on a protein level (*31*). A hormonal impact on gene expression is not limited to the NIT1 homologues as it was shown that NIT4 is upregulated in response to ethylene (ET) treatment (*35*).

1.2.4 Enzymatic diversity within the plant nitrilases

Initially, the biological role for NIT1 to 3 was defined as catalysing the final step in auxin biosynthesis, the conversion of indole-3-acetonitrile (IAN) into indole-3-acetic acid (IAA, auxin). However, enzymatic studies showed that IAN is a poor substrate for NIT1 (*18, 36*). Indeed, NIT1 mutants did not have any auxin starved phenotype leading to the conclusion that there was either functional redundancy between the nitrilases or that they do not have a major role in auxin synthesis.

Like the NIT1 homologues, glucosinolates, are restricted to the Brassicaceae family. Catabolism of glucosinolates results in the production of nitriles which are potential substrates for nitrilases. Thus it was hypothesised that NIT1 to 3 may be involved in detoxifying the metabolites derived from glucosinolate catabolism. This hypothesis was supported when it was shown that NIT1 to 3 accept a variety of aliphatic and aromatic nitriles with a preference for nitriles derived from glucosinolates and their analogues (*18*). Further research showed that nitrilases from other members of the Brassicaceae family also have preference for nitriles derived from glucosinolates. *Brassica rapa* nitrilases, NIT-T1 and T2, accept a variety of nitriles as substrates. However, the greatest activity observed is towards 3-phenylproprionitrile, a nitrile derived from gluconasturiin (*37*). *Capsella rubella*, a close relative of *A. thaliana*, contains two long-chain glucosinolates and one short chain glucosinolate. In this species, there are two NIT1 homologues which have evolved to be specific for these types of glucosinolates (*38*).

The wide distribution nitrilases similar to NIT4 and the restriction of glucosinolates to Brassicaceae suggests that there is a more ancient role for plant nitrilases, preceding the glucosinolate-myrosinase catabolism pathway. As with NIT1, several studies have shown that there is little conversion of IAN to IAA by NIT4 and its homologues (*18, 36*). This coincides with the fact that IAN-hydrolysis *via* nitrilases is not common in plants despite NIT4 homologues found in most plant species. A different detoxification

role for NIT4 and its homologues was suggested based on an enzymatic study on the substrate preference of NIT4. This study showed that its preferred substrate is β-cyanoalanine, a by-product of cyanide detoxification during ET synthesis (*39*). During the last step of ET synthesis, cyanide is produced when its precursor 1-aminocyclopropane-1-carboxylic acid is formed into ET with the concomitant release of cyanoformic acid, which degrades into CO_2 and HCN. The cyanide produced is then converted into β-cyanoalanine *via* the β-cyanoalanine synthase (β-CAS). NIT4 converts β-cyanoalanine into asparagine, aspartic acid and ammonia thus recycling the nitrogen.

As cyanide production is quite widespread in plants, it suggests that primary and original function of NIT4 and its homologues is cyanide detoxification. This assumption is further supported by germination assays using β-cyanoalanine on knockout and over-expressing mutants of NIT4 which showed that this nitrilase is involved in the breakdown of β-cyanoalanine (*27*). It was shown that whilst wild type *Arabidopsis* is unaffected by low concentrations of β-cyanoalanine (0.05, 0.1, 0.5 and 1mM), NIT4 knockout mutants have only a 50% survival rate at the same concentrations, indicating β-cyanoalanine sensitivity of these mutants (*40*). Both studies indicate a connection between NIT4 and β-cyanoalanine detoxification. However, the lack of a visible phenotype of NIT4 knockout mutants under normal growth conditions implies that NIT4 is not essential for the plant under normal growth conditions. Despite not being essential to the plant, the fact that NIT4 homologues are present from mosses to higher plants and has not been lost during the evolution process suggests a requirement for NIT4 and its homologues.

The enzymatic functions of *Arabidopsis* nitrilases point to a clear division of substrate preferences and activities between NIT1 and NIT4-like enzymes where NIT1 homologues are involved in glucosinolate catabolism and potentially also a minor role in IAN hydrolysis; NIT4 homologues detoxify β-cyanoalanine and contributes to nitrogen recycling. The same substrate specificities are not necessarily found in other plant nitrilases. ZmNIT1 and 2 are homologues of NIT4 yet they do not use the same substrates as NIT4. Both maize isoforms do not have much activity with β-cyanoalanine as substrate (*28*). Sorghum nitrilases also show a similar lack of activity with β-cyanoalanine (*29*). In addition to NIT4 substrates, ZmNIT1 is generally inactive for

substrates of NIT1 and its homologues. In contrast to ZmNIT1, ZmNIT2 is able to catalyse IAN hydrolysis seven to 20 times more efficiently than the NIT1 homologues.

Furthermore, ZmNIT2 also shows high catalytic activities for various nitrile substrates (*28*). Thus, ZmNIT2 display NIT1 homologue activity despite being considered as an NIT4 homologue. This is also observed for sorghum nitrilases which also take on IAN and nitriles as substrates despite being homologues to NIT4.

Like bacterial nitrilases, maize nitrilases are also able to form oligomers. Using yeast two hybrid assays, Kriechbaumer *et al* showed that the two maize nitrilase isoforms able to interact with themselves and with each other (*41*). Interestingly, the combination of nitrilase as dimers had an impact on substrate preference. As discussed above, the individual maize nitrilases cannot take β-cyanoalanine as a substrate. However, as heteromers, maize nitrilases are able to use it as a substrate (*41*). This suggests that the enzymatic function of hydrolysing various nitrile substrates is conserved between all plant nitrilases but that different strategies have evolved to take substrates. Whilst maize nitrilases evolved to form heteromers to use both IAN and β-cyanoalanine as substrates, *Arabidopsis* nitrilases have evolved to split their functional roles so that NIT1 homologues take on IAN and NIT4 uses β-cyanoalanine as a substrate.

Given the high sequence homology between maize and *Arabidopsis* nitrilases, it can be postulated that the *Arabidopsis* nitrilases are also able to form homo- and heteromers. Whether this is the case and how it impacts on substrate preference remains as open questions and has yet to be determined. In addressing this, one would need to consider the spatial distribution of the enzyme. It is well described that *Arabidopsis* nitrilases are only co-expressed under certain environmental and developmental conditions or specific set of organs or tissues. Thus, even if the *Arabidopsis* nitrilases interact with each other, the relevance of this may be quite limited to specific conditions.

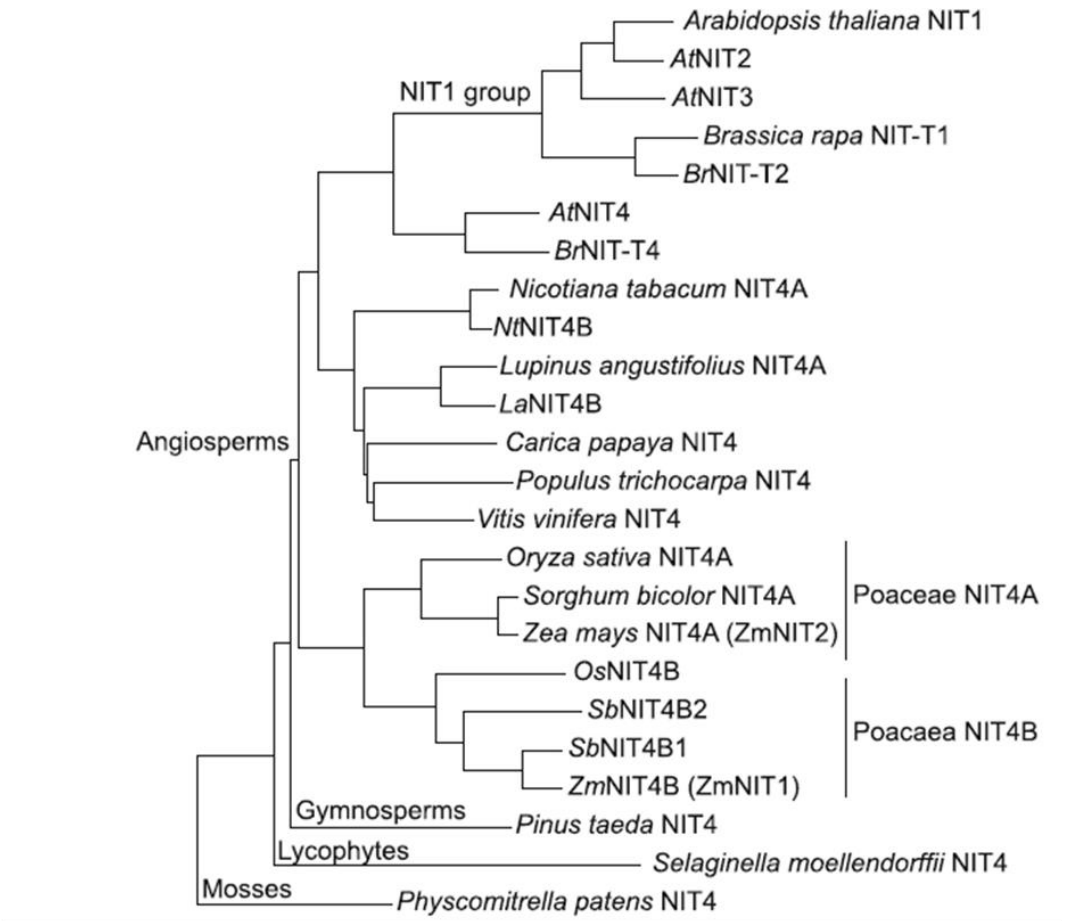


Figure 1.1 Phylogenetic representation of the plant nitrilases
Nitrilases are found in many plant species, ranging from mosses to higher plants such as maize and rice. The phylogenetic relationships indicate two groups of nitrilases. Of the *Arabidopsis* nitrilases, AtNIT 4 is the closest relative to the primordial nitrilases. Furthermore, the AtNIT1 homologues(NIT1/2/3group) are separated from the other plant nitrilase, found only in the Brassicaceae family, indicating occurance of the AtNIT1 homologues is a relatiely new evolutionary event and specific for this family

Image from Piotrowski *et al* (2008) *(27)*

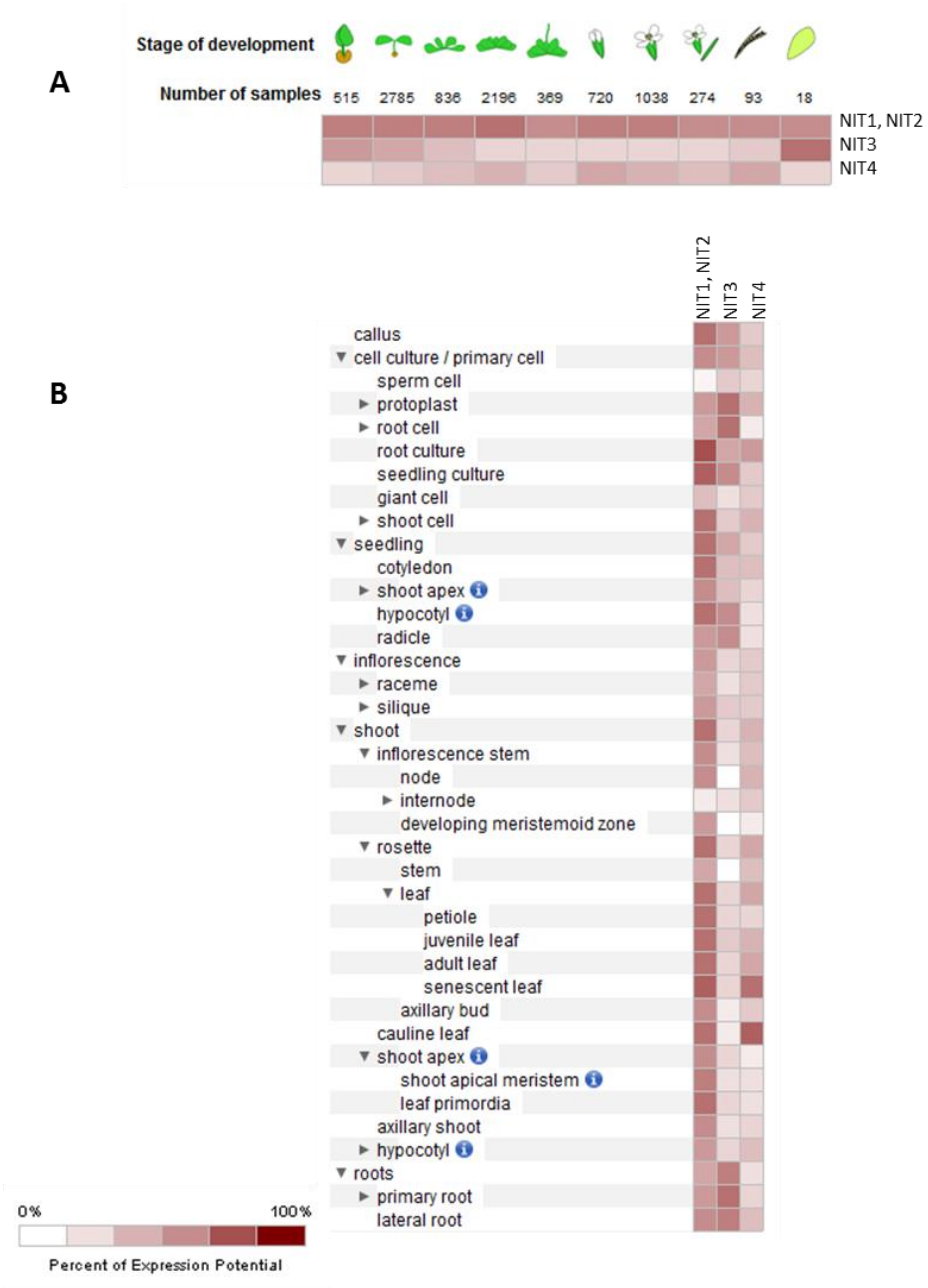


Figure 1.2 Developmental and anatomical expression patterns for nitrilases in *Arabidopsis* tissue

Expression data retrieved from Genevestigator shows developmental (A) and anatomical differences (B) in the expression of the four *Arabidopsis* nitrilase isoforms. *NIT1* and *NIT2* are not differentiated on the ATH1 affymetrix chip used for the microarray data. **(A)** *NIT1* and *NIT2* are constitutively expressed during all stages of developmental. Conversely, *NIT3* and *NIT4* are expressed mostly during specific developmental stages. **(B)** *NIT1* and *NIT2* are expressed in almost all tissues whereas *NIT3* and *NIT4* expression is limited to fewer tissues. The percent of expression potential represents the top percentile of all expression values for the gene of interest.

An explanational key of the developmental stages can be found in appendix figure 4.

1.2.5 Biological roles of *Arabidopsis* nitrilases

In *Arabidopsis*, there are four isoforms with distinct and discrete enzymatic functions yet there are generally only one or two nitrilase isoforms found in other plant species. The necessity of having four nitrilase isoforms in *Arabidopsis* has not been fully elucidated, begging the question: why the requirement for four isoforms in *Arabidopsis*? The four isoforms catalyse the same redundant enzymatic function albeit localized to different tissues. Furthermore, the enzymatic function does not need four isoforms to be carried out. Thus, it is plausible that novel biological roles are associated with *Arabidopsis* nitrilases that go beyond their enzymatic function. These new roles may have evolved more recently corresponding to the relatively recent duplication event of the NIT1 homologues

In *Drosophila melanogaster* and *Caenorhabditis elegans*, *NIT1* is fused to a fragile histidine triad (FHIT) gene which encodes for a member of the histidine triad protein family. This fusion connects nitrilase to Fhit specific functions such as regulating cell-cycle progression, apoptosis and tumourgenesis (*17*). Thus mammalian *NIT1* was hypothesised to have similar biological functions as the FHIT gene. This was supported by the over-expression of mammalian *NIT1* which led to the activation of the caspase-3-dependent apoptosis, whereas loss of *NIT1* promoted cell growth and resistance to DNA damage stress (*42*). The effects of *NIT1* on cell growth and cell-cycle progression is independent of enzymatic activity as shown with an enzymatically inactive NIT1 mutant with a C203A mutation. Such a mutant showed similar suppression of cell viability as the wild type *NIT1*(*42*). Further supporting evidence came from microarray analysis on *NIT1* null cells which showed that the expression of genes involved in cell cycling, check point and apoptosis were altered compared to the wild type (*42*). Similar roles were investigated in NIT1 which has the highest homology to bacterial and mammalian nitrilases. Doskocilova *et al* showed that NIT1 is involved in cell proliferation and differentiation, cell-cycle exit and normal cytokinesis (*25*). Furthermore, a yeast two hybrid study showed that mammalian NIT1 interacts with RAD23, involved DNA repair and proteosomal protein degradation (*25*). Similar to mammalian NIT1, it was found that NIT1 interacts with RAD23A which is also involved in DNA repair and proteosomal dedgradation (*43, 44*).

NIT1 has also been implicated in regulating programmed cell death (PCD). NIT1:GFP fusion proteins form wounding induced aggregates which are an early marker of PCD (*45*)., *Arabidopsis* nitrilases are also implicated in leaf senescence. This type of senescence is a highly ordered and complex process, involving internal and external cues such as phytohormone signalling, abiotic and biotic stresses. Transcriptomics performed on leaves under senescing conditions showed that the four nitrilases are upregulated during leaf senescence (*33, 46*). A proteomic study looking into stay green mutants, plants which maintain green-ness despite leaf senescence occurring normally, showed that protein levels of NIT1 and 3 are upregulated in stay green mutants as compared to wild type *Arabidopsis (47, 48).*

It is a well-established hypothesis that NIT1 to 3 are involved in the glucosinolate-myrosinase catabolism pathway through their ability to degrade nitriles. Furthermore, NIT4 is said to be involved in ET synthesis; ET being one of the major phytohormones involved in defence signalling. Thus, one of nitrilases' biological roles could be mediating plant defence mechanisms. Macroarray and microarray studies have shown that NIT1 and 2 are upregulated in response to MeJA, a phytohormone which plays a major role in defence signalling. Furthermore, yeast two hybrid analysis showed that TbNIT4, a nitrilase from *N.tabacum*, interacts with Ethylene Responsive Element Binding Protein 2 (EREBP2) through yeast two hybrid (*49*). EREBP2 is part of the AP2/ERF transcription factor family involved in ET signalling. Another family of transcription factors, the NAM/ATAF/CUC (NAC) family are reported to interact with *Arabidopsis* nitrilases. The NACs are also well established as proteins involved in mediating plant defence as well as responses to biotic and abiotic stresses (*50*). In particular, ATAF2 is induced in response to wounding and several phytohormones known to mediate plant defence responses (*51*). Apart from biotic stresses, ATAF2 also responds to abiotic stresses such as salinity. Thus, it has been suggested that ATAF2 may integrate wounding with pathogen responses. A connection between nitrilases and ATAF2 was demonstrated when it was shown that NIT3 is upregulated in ATAF2 over-expressor lines and that ATAF2 binds to the promoter region of NIT2 (*51, 52*). This promoter binding is apparently required for the expression of NIT2.

Cyanide production can be a result of water stress, a stress condition which can induce ET synthesis (*53*). As discussed previously, NIT4 was implicated in cyanide

detoxification. This may link NIT4 to water stress responses and ET. In a water withholding experiment using β-CAS and NIT4 knockout mutants, Machingura *et al* showed that NIT 4 mutants has the highest decrease in relative water content along with the highest increase in tissue cyanide (*54*). In addition, the relative chlorophyll content and the photosynthetic capacity decreases in NIT4 knockout mutants as the cyanide levels increase decreases during water deficiency. Thus NIT4 may have a role in providing drought resistance through cyanide detoxification. Despite being involved in the second step of cyanide detoxification, there is no difference in the enzymatic activity of NIT4 between the wild type plants and the knockout mutant (*54*). Furthermore, knocking out NIT4 also has a concomitant influence on β-CAS activity, with a 71% decrease in β-CAS activity in the knockout line as compared to the wild type plant (*54*). In addition to providing drought resistance, NIT4 may also have a regulatory role in β-CAS activity. NIT4 could be regulating β-CAS activity through negative feedback regulation, affecting the first step of the cyanide detoxification pathway. These experiments along with the sensitivity of NIT4 knockout lines to KCN and β-cyanoalanine show that NIT4 may have more than just a simple enzymatic role in cyanide detoxification.

Reverse genetics approaches, microarrays and transcriptomic data have started to provide more clues about the biological roles of nitrilases. The current research has now pointed a novel direction, suggesting biological roles in plant defence and stress responses. Thus, *Arabidopsis* nitrilases just as mammalian nitrilases may have biological roles that are independent of their enzymatic activity. These may be roles that *Arabidopsis* nitrilases carry out in addition to their enzymatic function or instead of an enzymatic function.

1.3 Project hypothesis and aims

1.3.1 *Arabidopsis* nitrilases: do enzymatic functions define their biological role?

The literature presented so far highlights an interesting problem: do enzymatic functions of *Arabidopsis* nitrilases define their biological role, *i.e.* the biological context of the protein's function? To determine how well enzymatic function correlates with a biological role, one has to consider the molecular and physiological contexts to provide a more holistic understanding of the protein. The molecular context is determined by the

protein's localization (*i.e.* where it is expressed), the timing of its expression and interacting partners. Over-expressing or knocking out the gene of interest and testing for phenotypic differences under different conditions such as pathogen resistance defines a physiological context. For the *Arabidopsis* nitrilases, enzymatic function might not necessarily translate into a biological role. I hypothesise that the overall biological roles of *Arabidopsis* nitrilases' are broad, encompassing plant defence and stress response and that this breath is defined by nitrilase expression and interaction partners, not just their enzymatic function.

1. Novel roles have not been thoroughly investigated in the four nitrilase isoforms. Association with transcription factors such as ATAFs which are involved in defence and stress responses indicate that *Arabidopsis* nitrilases may be more than just nitrile degrading enzymes. Furthermore, transcriptomic analyses showed that *Arabidopsis* nitrilases respond to MeJA and ABA, two major plant hormones involved in defence and stress responses. Thus, it is hypothesised that *Arabidopsis* nitrilases have a more direct role in stress response and plant defence than just nitrile degradation.

2. Mammalian nitrilases are not involved in nitrile degradation despite enzymatically still able to catalyse the degradation of nitriles. Similar to mammalian nitrilases, NIT1 may be involved in cellular development. This suggests that plant nitrilases may also have biological functions not related to their enzymatic ability.

3. Bacterial nitrilases are well characterised to be acting as oligomers, either forming homo- or heteromers. It is often required for functionality and to take on certain substrates. Whilst it is known that NIT1 exists in oligomeric states and that it forms filamentous structures like bacterial nitrilases (*25*), heterodimerization has not been observed. Furthermore, the dimerization ability of the other *Arabidopsis* nitrilases has not been investigated.

1.3.2 Aims

Based on the above statements, the overall aim of this project is to provide a deeper understanding in the biological roles of the *Arabidopsis* nitrilases particularly in plant defence and stress responses on genetic, proteomic and physiological levels. This work will:

1. Identify novel interactors which bind with the *Arabidopsis* nitrilases, using affinity purification coupled with mass spectrometry (AP-MS) and bioinformatic approaches. Putative interactors will be confirmed using Bimolecular Fluorescence Complementation (BiFC). These tests will also address if novel interactions are conserved across the *Arabidopsis* nitrilases.

2. Investigate the roles *Arabidopsis* nitrilases may have in MeJA regulated plant defence responses. This will be achieved by using quantitative real time PCR (qRT-PCR) to investigate whether there is gene expression change for the *Arabidopsis* nitrilases in response to MeJA.

3. Determine whether there are phenotypic responses to the various phytohormones involved in plant defence and stress responses using NIT1 and 4 over-expressing *Arabidopsis* lines.

4. Determine the capacity of the four *Arabidopsis* nitrilases to form homo- and heteromers *in planta* using BiFC.

This project will provide the groundwork for determining novel roles of *Arabidopsis* nitrilases by establishing an *Arabidopsis* nitrilase interactome. Aside from establishing new roles, it will also extend the concept that proteins have roles which go beyond their enzymatic functions and that their biological functions are not necessarily dictated by the enzymatic functions of the isolated proteins. New insight will also be developed into how nitrilases may influence plant defence and stress responses.

Chapter 2

Materials and methods

2.1 Plant growth

2.1.1 Seed sterilisation

Arabidopsis thaliana seeds were surface sterilised prior to sowing onto Murashige and Skoog (MS) agar plates to prevent the growth of bacteria or fungi. To sterilise the seeds, 1 ml of the sterilisation solution was added to an aliquot of seeds in a 1.7 mL microfuge tube and incubated for five minutes with occasional inversion. The sterilisation solution was then removed and seeds were washed twice with 95% ethanol. Ethanol was removed and the tubes were left to dry the seeds in a laminar flowhood. After the seeds were completely dried, the tubes were kept under dark conditions and at room temperature until required.

2.1.2 Seed sowing and plant growth

Two plant species were used; *Arabidopsis thaliana* (L.) Heynh. (ecotype Col-0) and *Nicotiana benthamiana*. *Arabidopsis* plants were either grown in compost, on ½ MS agar in Petri plates or in magenta vessels on a wire mesh that was placed over ½ MS liquid medium.

The compost used to germinate and grow both *A. thaliana* and *Nicotiana benthamiana* consisted of seven parts of Shamrock soil (Seeds and modular propagating medium, Scotts, UK) and three parts of vermiculite (Perlite and Vermiculite Factory, WA, Australia). Compost was prepared and lightly compressed into pots of various sizes. Pots were then treated placed into with an aqueous solution (3 % v/v) of 30ml (in one litre of water) of Intercept 70MG insecticide (Scotts) and left in the solution for one hour to allow for uptake of the insecticide by the compost. *Arabidopsis* or *Nicotiana* seeds (50-100) were then scattered onto the compost and pots were covered with plastic wrap to create a humid environment to encourage germination. If *Arabidopsis* plants were later used for transformation *via* floral dipping (2.3.2), fly screen was placed over the pots prior to covering them with plastic wrap to prevent plants and compost from falling into the transformation suspension (2.3.2). These were transferred into a 4°C °C in the dark for three days to stratify the seeds. Pots were then transferred into a growth cabinet with growth conditions as described above for *Arabidopsis*. *N. benthamiana* seeds were not stratified. Instead, pots were directly placed into a growth cabinet under long day growth conditions (16 hour day/8 hour night cycle, ~80-100µE/cm2, cool

fluorescent white light, 22 °C). The plastic wrap was removed once seedlings were well established.

Half strength liquid MS medium was prepared by dissolving 2.15 g/L MS basal mixture (Phytotech labs) in SDW and adjusting the pH to 5.8 using KOH. For germination and growth in magenta vessels, the ½ MS liquid media was autoclaved along with the magenta vessels and a wire mesh that was placed inside the vessel so that it was just above the liquid surface. After autoclaving, the mesh was covered with agar (0.8 %) to obtain a sealed surface for the seeds. Sterilised *Arabidopsis* seeds (2.1.1) were sown onto the agar surface covering the mesh. The Magenta vessels were placed in the dark at 4 °C for three days to stratify the seeds to ensure synchronised germination. After stratification, the Magenta vessels were placed into growth cabinets with the same growth conditions mentioned previously.

For germination of seeds on ½ MS agar, 0.8 % agar (w/v, Amresco) was added to the liquid MS liquid medium prior to autoclave sterilization. Sterilised *Arabidopsis* seeds (2.1.1) were sown onto ½ MS agar in Petri plates. The plates were sealed with Micropore tape (3M) and transferred to stratification conditions as described above. After stratification, plates were transferred to a growth cabinet with the growth conditions as described for magenta vessel growth.

2.2 Bacterial manipulations

2.2.1 Competent cell preparation for *Escherichia coli*

E. coli (DH5α) cells were streaked onto LB agar plates from glycerol stocks to obtain single colonies. Plates were incubated overnight at 37 °C. A single colony was selected to inoculate 5 mL of LB broth and was grown overnight at 37 °C. Following overnight growth, a 1 mL aliquot was used to inoculate 100 mL of LB broth which was grown to an OD_{600} of 0.4-0.6, also at 37 °C. Once the desired density was reached, cells were pelleted *via* centrifugation (Beckman-Coulter, Australia) at 5000 g for five minutes at 4 °C. The supernatant was discarded and the pellet was gently resuspended in 40 mL of ice-cold TFBI (30 mM potassium acetate, 100 mM rubidium chloride, 10 mM $CaCl_2$, 15% (w/v) glycerol) solution on ice. Resuspended cells were centrifuged at 5000 g for five minutes. The supernatant was again discarded and the pellet was resuspended in 4

mL of ice-cold TFBII solution (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% (w/v) glycerol). The cells were then aliquoted into 100 or 200 µL batches and snap frozen in liquid nitrogen and stored at -80°C till required.

2.2.2 Transformation of competent *E. coli* cells

For the transformation, an aliquot (50 µL) of competent cells were partially thawed on ice. Plasmid DNA (5 ng) was added to the competent cells, mixed gently and left on ice to incubate for five minutes. Next, the cells were heatshocked at 42 °C for 45 seconds and returned to ice immediately. LB broth (500 µL) was added to the cell mixture and then incubated with shaking (800 rpm) at 37 °C for 45 minutes. After the incubation, the cell mixture was centrifuged briefly and 400 µL of the supernatant was removed. The cell pellet was then resuspended in the remaining 100 µL. The resuspended cells were plated on the LB agar plates supplemented with 50 µg/mL of kanamycin. The bacteria were then grown overnight at 37°C until colonies of 1 mm in size had grown.

2.2.3 Competent cells preparation for *Agrobacterium tumefaciens*

A.tumefaciens (GV3101) cells which contains the pSoup vector were streaked onto LB agar plates supplemented with rifampicin (25 µg/mL), gentamycin (5 µg/mL) and tetracycline (2 µg/mL). The plates were grown at 30 °C for three days till colonies were 1mm in size. A single colony was selected to inoculate 3 mL of LB broth supplemented with antibiotics (rifampicin: 25 µg/mL and gentamycin: 5 µg/mL) and grown overnight at 30 °C. From this overnight culture, 100 µL was used to inoculate a 50 mL LB broth supplemented with the same antibiotics. This cultures was grown overnight at 30 °C till the OD600 reached 0.75. Cells were first chilled on ice for 10 minutes, then harvested *via* centrifugation (3000 g, 10 minutes, 4 °C). The supernatant was discarded and the pellet was resuspended in 1 mL of ice-cold 20 mM $CaCl_2$. Another round of centrifugation was performed to wash the cells and remove excess antibiotics. The pellet was resuspended in 1 mL of ice-cold 20 mM $CaCl_2$ and aliquotted out into either 50 µL or 100 µL. The aliquots were then snap frozen in liquid nitrogen and stored at -80 °C till required.

2.2.4 Transformation of competent *A.tumefaciens* cells

To transform, 20 ng of DNA plasmid was added to 20 µL of competent *A.tumefaciens* cells. The mixture was gently mixed and placed on ice for five minutes. Next, it was

incubated in liquid nitrogen for five minutes. Lastly, the cells were incubated at 37 °C for five minutes. For recovery, 400 µL of LB broth was added to the cells and cells were incubated at 29 °C for two hours with shaking (850rpm). Finally, 150 µL of cells were plated onto LB agar plates supplemented with 50 µg/mL kanamycin and incubated for three days at 29 °C.

2.3 Transformation of plant material

2.3.1 Transient transformation of *N. benthamiana*

A mixed *Agrobacterium* suspension was prepared for the transient transformation of four to six week old *N. benthamiana* leaves. Overnight cultures were made by inoculating LB broth supplemented with antibiotics (rifampicin 25 µg/mL, kanamycin 50 µg/mL and gentamycin 5 µg/mL) with single colonies of transformed *A.tumefaciens* strains. For the analysis of the subcellular localisation of GFP fusion proteins, cultures of an Agrobacterium strain containing a plasmid encoding for the GFP fusion protein and a strain containing the p19 vector were prepared. The p19 plasmid encodes for the P19 protein of the Tomato bushy stunt virus which supresses RNA silencing thereby enhancing the transient expression of proteins of interest (*55*). For the analysis of protein interactions using Bimolecular Fluorescence Complementation (BiFC) three cultures were prepared; one encoding a protein of interest fused to an N-terminal YFP fragment (YN), one encoding a protein of interest fused to a C-terminal YFP fragment (YC) and a culture containing the p19 plasmid.

After overnight growth, 1 mL of the cells was pelleted via centrifugation (max speed, 1 min). The pellet was resuspended in infiltration buffer (10 mM MES.KOH, 10 mM MgCl2) to an OD600 of 0.75. Acetosyringone (200 mM) was added to the diluted cell suspensions and left to incubate for an hour at room temperature. Working suspensions were then made by mixing the appropriate agrobacteria suspensions. For GFP analysis, agrobacteria encoding the GFP fusion protein were mixed with agrobacteria containing the p19 plasmid in a 1:1 ratio. For BiFC, the agrobacteria encoding for the YN and YC fusion proteins and Agrobacteria containing the p19 plasmid were mixed in a 1:1:1 ratio. The mixed *A.tumefaciens* suspensions were co-infiltrated into the abaxial air space of the leaves of *N. benthamiana* using a 1 mL syringe. Infiltrated plants were returned to the growth cabinet and grown under long day conditions for three to four days as

described (2.1.2). Leaf samples were then prepared and observed for fluorescence in the lower epidermal cell layer *via* fluorescent or confocal laser scanning microscopy (CLSM) (2.6).

2.3.2 Transformation of *A. thaliana*

For the stable transformation, *A. thaliana* (Col-0) seedlings were densely grown in pots as described (2.1.2). After 5 weeks, the first bolts of the inflorescence were removed to encourage greater proliferation of flowers. Once the subsequent inflorescence had proliferated, A. tumefaciens strains containing the genes of interest cloned into pG179NS-GFP vectors were grown in 5 mL of LB broth containing kanamycin (50 µg/mL), rifampicin (25 µg/mL) and gentamycin (5 µg/mL) overnight at 30 °C. An aliquot of the overnight culture was used to inoculate 100 mL of LB broth containing the antibiotics and under the growth conditions as stated above until the optical density was 1.5 to 2. The 100 mL culture was then harvested by centrifugation (4000 g, 4 °C, 10 minutes). The harvested cells were resuspended in a 5 % sucrose solution to give a final density of OD600 of 0.8. Once the cells were resuspended, Silwet L-77 solution was added to the resuspension to a final concentration of 0.01 % (v/v). Flowers of *Arabidopsis* plants were immersed in the bacterial suspension by dipping the plants up to the rosette leaves into the bacteria and gently agitating for 10 seconds. Plants were then covered with plastic bags and transferred back to the growth cabinets. The plastic bags were removed after two days and plants were grown until the siliques had begun to mature. The siliques were then placed inside paper bags to collect the seeds.

Once siliques had fully matured, seeds (T1) were harvested and transferred to 1.7 mL microtubes. The constructs used to transform *Arabidopsis* contained a resistance to hygromycin gene. Thus, T1 seeds were firstly surface sterilised as described in section 2.1.1 and sown onto ½ MS agar plates supplemented with 20 µg/mL of hygromycin and germinated as described (2.1.2). Seedlings which had green leaves and extensive primary root growth by the second week were determined to be transformed with the construct and were transplanted onto soil to grow into maturity. Further confirmation of transformation was obtained either by detecting fluorescence of GFP using microscopy or by PCR to detect the transgene. Seeds (T2) were collected from the T1 generation and again screened for the transformation event as described above. Confirmed transgenic plants were grown to produce T3 seeds. Plants of the T3 generation were

screened for homozygous, stable transformants using the same procedures described above.

2.4 DNA manipulations

2.4.1 Polymerase chain reaction

PCR was used to generate DNA fragments (high fidelity PCR) as the first step of cloning as well as to detect specific DNA sequences in bacteria and plants (low fidelity PCR). The type of DNA polymerase differed depending on the type of PCR performed.

For high fidelity cloning PCR, the 2x Accuzyme mix (Accuzyme DNA Polymerase, $MgCl_2$ and dNTPs, Bioline) was used to yield blunt ended amplicons. A standard reaction contained 25 µL of the mix, 10 pg of forward and reverse primers, 1-5 ng of template DNA and SDW to a final volume of 50 µL. The reactions were vortexed to mix, centrifuged briefly and loaded into a thermocycler (MJ research Inc, PTC-100). Low fidelity PCRs were set up in a similar manner, except that the BioTaq Polymerase (Bioline) was used. The standard low fidelity PCR consisted of 1.25 U of the BioTaq polymerase, 1xNH4 buffer, 2.5 mM MgCl2 and 200 µM dNTPs in a final volume of 50 µL. The thermocycler programmes used for both types of PCRs are summarised in table 2.1.

2.4.2 Purification of PCR products and DNA

Digested DNA required for downstream cloning applications was purified in a similar manner. Firstly, the digested DNA was separated by size through agarose gel electrophoresis as described (2.4.5). Following electrophoresis, the DNA was stained with 0.02 % (w/v) methylene blue for an hour, washed in water to remove excess dye until the DNA bands were visible. Slices of agarose gel containing DNA as visualised by the stain were excised using a scalpel blade and transferred to a 1.7 mL microfuge tube. To extract the DNA from the gel slice, the PCR/Gel clean-up kit (Promega) was used following standard instructions except that the gel slice was first dissolved in the membrane binding solution before proceeding as described above for the purification of PCR fragments.

2.4.3 Genomic DNA extraction from plant tissue

Leaf tissue from 4 week old *A. thaliana* rosettes were harvested and homogenised in 500 µL of DNA extraction buffer (0.1M TRIS, 0.05M EDTA, 0.5M NaCl and 1% PVP) using a drill bit. SDS (66 µL, 10%, w/v) was then added to the homogenised tissue and mixed *via* inversion. Potassium acetate (166 µL, 5M) was then added and mixed *via* inversion. The solution was the centrifuged at 16000 g for 15 minutes. The supernatant was then transferred into a new microfuge tube containing 460 µL of isopropanol, the centrifugation step was then repeated. This time, the supernatant was discarded and the resulting pellet was washed with 70% ethanol. Another centrifugation step was performed (16000 g at five minutes). The ethanol was then removed and the pellet was left to dry in the microfuge tube for about 30 minutes. The pellet was then resuspended in 100 µL of SDW and stored at -20°C till required.

2.4.4 Miniprep for DNA purification from bacteria

Minipreps were performed using the Promega "Wizard Plus SV Miniprep DNA Purification System", following the standard protocol. Briefly, *E. coli* transformed with the plasmid of interest was plated onto LB agar plates supplemented with 50µg/ml of Kanamycin and incubated at 37 °C overnight to obtain single colonies. A single colony was then selected to inoculate 5ml of LB broth (50µg/mL Kanamycin). Inoculated cultures were then grown overnight at 37 °C with shaking. A 1.5 mL aliquot of the liquid culture was harvested and pelleted down *via* centrifugation (16000 g, 30 seconds). The supernatant was removed and the pellet was resuspended in 250 µL of the Cell Resuspension Solution. Next, 250 µL of the Cell Lysis Solution was added to the resuspended cells and gently mixed *via* inversion. The Neutralization Solution (350 µL) was then added and also gently mixed which resulted in the precipitation of cellular debris. This was then pelleted down through centrifugation for 10 minutes. The resulting supernatant was then transferred to a spin column and collection tube, and centrifuged for one minute to capture the DNA onto the silica membrane. The DNA was then washed twice with the Column Wash Solution. Excess ethanol on the column was dried off by centrifugating for five minutes at low speed. To elute the DNA off the column, 50 µL of nuclease-free water was added to the column and centrifuged at maximum speed for one minute. The purified DNA was then stored at -20 °C till required.

2.4.5 Agarose gel electrophoresis

Agarose gels (1 % agarose, w/v) containing 0.5 µg/mL of ethidium bromide were used to separate DNA fragments based on size. DNA samples were mixed with 10x Orange G loading dye (3 mL glycerol, 20 mg Orange G) and loaded into the wells of the agarose gel. Electrophoresis was performed in 1xTAE buffer (tris, acetic acid, EDTA pH8.0) in a Sub-Cell or Mini-Sub Cell GT gel tank (Bio-Rad) at either 70 V or 120 V depending on the size of the gel. Images of the gels were taken after electrophoresis under UV light with a ChemiDoc XRS Gel Documentation System using the Quantity ONE software (Bio-Rad).

2.4.6 Restriction digestion

Restriction enzymes and required buffers were purchased from New England Biolabs (NEB). Restriction digests were performed in 1.7 mL microfuge tubes containing 80 to 100 ng of plasmid DNA, 1x restriction enzyme buffer and 10 units of the required enzyme(s) in a final reaction volume of 20 µL. The reaction mixtures were then briefly vortexed and spun down to collect the liquid and incubated for two to three hours at 37 °C. Results of the digests were visualised using agarose gel electrophoresis (section 2.4.5).

2.4.7 Ligation

The ligation reaction consisted of 1x T4 DNA ligase buffer, 10 units of T4 DNA ligase (NEB), the vector and insert DNA in a 1:3 ratio in a final volume of 10 µL. The reaction mix was vortexed and centrifuged shortly before being incubated at either room temperature (one hour) or at 4 °C (overnight) to allow the ligation reaction to occur. Following ligation, the mix was transformed into competent *E. coli* cells as described in section 2.2.2

2.4.8 DNA sequencing

To confirm that the correct DNA sequences were obtained in PCR reactions and cloned into plasmid vectors, recombinant plasmids were prepared (2.4.4) and used for automated DNA sequencing using the BigDye Terminator Cycle sequencing protocol (Applied Sciences). Each sequencing reaction contained 0.5 µL of template DNA, 1 µM of sequencing primer, 2 µL of 2.5x BigDye reaction buffer, 2 µL of the BigDye reaction mix and SDW to give a final volume of 10 µL. The reactions were then transferred to a

thermocyler (programme as per table 2.1). The sequenced samples were purified prior to submission for the sequencing run by first adding EDTA (25 mM, pH 8.0). The DNA was then precipitated by adding three volumes of 100 % ethanol, thorough mixing via vortex and incubation at room temperature for 15 minutes. The precipitated DNA was pelleted by centrifugation at 2500 g for 30 minutes. The supernatant was removed and the pellet was washed with 150 µL of 70 % ethanol. The centrifugation and wash step was repeated. After the second wash step, the supernatant was removed and the pellet was left to air-dry at room temperature for 30 minutes. The DNA pellet was then sent off to the Australian Genome Research Facility (AGRF) for the analytical separation of DNA fragments produced during the sequencing reaction.

2.4.9 RNA extraction

Three to four week old *A. thaliana* seedlings were harvested from ½ MS agar plates and were ground into a fine powder under liquid nitrogen using a mortar and pestle. The powder was transferred to a 1.7 mL microfuge tube. Total RNA was extracted from the plant powder (100 mg) using the Qiagen RNeasy Plant Mini Kit following the manufacturer's standard protocol. An on-column DNase treatment (Qiagen) was performed as recommended by the manufacturer. Firstly, a working solution was made by adding 10 µL of the DNase solution (1500 Kunitz units) to 70 µL of RDD buffer and thorough mixing through inversion. This DNase working solution was transferred to a spin column loaded with RNA and incubated at room temperature for 30 minutes. Following this treatment, the RNA was eluted off the column as per the manufacturer's instructions. A second DNase treatment was performed after elution. For this, the Ambion Turbo DNase protocol was used: 0.1 volume of Ambion Turbo DNase buffer and 1 µL of Ambion Turbo DNase were added to the eluted RNA and incubated at room temperature for 30 minutes. The DNase was inactivated by adding 5 µL of inactivation slurry and incubation for five minutes at room temperature. The slurry was precipitated by centrifuging at 10000 g for one minute. The supernatant containing RNA was transferred into a new 1.7 mL microfuge tube and stored at -20 °C until required.

The quality of the RNA was determined by comparing the ratios of the absorbances measured at both 260 nm and 280 nm. The concentration of the RNA was determined from the absorbance value at 260 nm. RNA with a 260/280 ratio of 1.8 – 2.0 was

considered sufficiently pure for downstream applications. The absorbance and concentration were determined using an Eppendorf Spectrophotometer.

2.4.10 cDNA template synthesis

Complementary DNA (cDNA) used as template for qRT-PCR analysis was synthesised using the Bio-Rad iScript kit following the manufacturer's instructions. In summary, the template synthesis reaction consisted of 1 µg of total RNA, 4 µL iScript Buffer and 1 µL enzyme in a final volume of 20 µL. The reaction was then transferred to a thermocycler to perform the reverse transcription reaction (five minutes at 25 °C, 30 minutes at 42 °C, five minutes at 85 °C and finally kept at 4 °C). Following reverse transcription, the cDNA was stored at -20 °C. No reverse transcriptase (NoRT) controls were set up as stated for cDNA synthesis except that 1 µL of RNase-free water was added instead of the reverse transcriptase.

2.4.11 qRT-PCR

Quantitative reverse transcribed PCR (qRT-PCR) was used to determine the relative gene expression of genes of interest in wild type and transgene over-expressing *Arabidopsis* lines subjected to various treatments described in the result sections. Briefly, each qRT-PCR reaction consisted of 1 µL of cDNA (2.4.10), 1 µL of each forward and reverse primers (2.5 µM each), 10 µL of sso Advanced Universal SYBR Green qPCR Supermix (Bio-Rad) and made up to a final volume of 20 µL with UV-sterilized milliQ H2O. Fluorescence due to binding of SYBR Green to double stranded DNA was monitored using the Bio-Rad CFX-96 Real Time PCR detection system (Bio-Rad CFX-1000 thermocycler). The thermocycler programme used is summarised in table 2.2. To assess the quality of the reaction, both melt curve analysis and agarose gel electrophoresis (2.4.5) were used.

2.5 Protein assays and procedures

2.5.1 Total protein extraction for *N. benthamiana* leaves

Extraction buffer (50 mM Tris pH7.4, 150 mM NaCl, 2 mM MgCl2, 1 mM DTT, 20 % glycerol (v/v), 1 % Igepal (Sigma Aldrich), 1 % protease inhibitor (Thermo Fischer Scientific) was added to the leaf tissue (1 mL per 1 g leaf tissue). The tissue was then homogenised using a mortar and pestle on ice. The homogenate was transferred to a

chilled 1.7 mL microfuge tube and cellular debris was removed through two steps of centrifugation for 20 minutes (16000 g, 4 °C). The resulting total protein extract was then transferred to a new 1.7 mL microfuge tube.

The concentration of total protein was determined using the Bradford Assay using a BSA standard curve. The standard curve was generated by applying the Bradford Assay to a dilution series of BSA (0.1 to 1 mg/mL). The assay was conducted by first diluting an aliquot of total protein extract or of a BSA dilution with water (1:100, v/v). Diluted Bradford Reagent (900 µL of 1/5 dilution) (Bio-Rad) was added to the diluted protein sample. The samples were briefly vortexed and incubated at room temperature for one minute. The absorbance was then measured at 595 nm using an Eppendorf Spectrophotometer.

2.5.2 Affinity purification

Three to four week old *Arabidopsis* seedlings were harvested from ½ MS agar plates. Harvested seedlings were homogenised using a mortar and pestle in extraction buffer (2.5.1, 1 g of tissue per mL of buffer) at 4 °C. The extraction buffer consisted of 50 mM Tris pH 7.4, 100 mM NaCl, 10 % glycerol (v/v), 0.01 x protease inhibitor cocktail (Halt Protease, Thermo Scientific). Two centrifugation steps were performed (16000 g for 20 minutes at 4 °C) to clear the cellular debris following homogenisation. The concentration of the resulting total protein extract was then determined using the Bradford assay (2.5.1). To perform the Co-IP, 25 µL of GFP antibodies coupled to sepharose beads (Abcam) was added to 10 mg total protein extract and incubated at 4 °C for 3 hours with rotation (20 rpm). The beads were then pelleted down by low speed centrifugation (2500 g) for 10 minutes. The supernatant was transferred into a new 1.7 mL microfuge tube and the pellet was washed in 500 µL of wash buffer (100 mM NaCl, 50 mM Tris pH7.4, 0.05 % Triton-X100). This wash step was repeated at least five times, each time the supernatant was kept and the pellet was gently resuspended in the wash buffer. To assess how well the beads were washed and to determine the success of the Co-IP, SDS-PAGE was done.

2.5.3 SDS-PAGE

Protein samples were separated based on molecular weight using SDS-PAGE. A 2x SDS loading buffer (20% w/v SDS, 20% v/v glycerol, 120mM Tris, 0.02% w/v bromophenol blue, 5% v/v β-mercaptoethanol) was added to diluted protein samples and mixed *via* vortex. Samples were then boiled at 95 °C for 10 minutes to denture the samples. Samples and a pre-stained protein ladder (Precision Blue, Bio-Rad) were loaded onto any KDa precast gels (Bio-Rad) and separated at 200 V until the dye front reached the bottom of the gel. Following electrophoresis, the gels were either used for transblotting or stained with Coomassie blue to visualise protein bands. Gels were stained in Coomassie blue for an hour and then washed to remove excess Coomassie stain until the background stain was thoroughly removed.

2.5.4 Western Blot

Western blots were done to determine whether YN and YC fusion proteins were expressed in *Nicotiana benthamiana* tissues during BiFC experiments. Proteins separated by SDS gel electrophoresis (2.5.3) were transferred onto PVDF membranes (Amersham Hybond-P). The membrane was cut to the size of the protein gel; pre-wetted with methanol, rinsed in ddH2O and incubated in transfer buffer (2.42 g glycine, Tris, 20% w/v SDS, 500 mL methanol) until required. The transfer itself was performed using the Bio-Rad Transblot SD semi dry transfer cell, following the manufacturer's protocol. Proteins were transferred onto the membrane using 25 mA per gel for 90 minutes. Transfer efficiency was assessed by the transfer of a pre-stained protein ladder onto the membrane.

After transfer, membranes were incubated in a blocking buffer (5 % skim milk powder in 1x TBS-T) (TBS: 50 mM Tris, 150 mM NaCl, pH7.6) for an hour at room temperature. Membranes were then briefly rinsed twice in 1x TBS-T. Primary antibodies were diluted in 1 in 2000 (c-Myc or HA antibodies, Abcam) or 1 in 5000 (anti-GFP) with 1x TBS-T and incubated with the membrane for one hour under gentle rocking at room temperature. Following this, the membranes were washed with the wash buffer once at room temperature for 15 minutes and then three more times for five minutes with fresh changes of 1x TBS-T. For the secondary antibodies, a 1:12500 (anti-mouse HRP, Abcam) or 1:10000 (anti-rabbit HRP, Abcam dilution was used. The membranes were incubated in the presence of the secondary antibody as described for

the primary antibody. The membrane was then washed as described above, excess wash buffer was drained off and the membrane was placed protein side up onto plastic film. The antibody detection solution consisting of Immobilon chemiluminescent substrate (Millipore), mixed in a 1:1 ratio, was warmed to room temperature and added to the membrane (0.125 mL per cm2 of membrane). The membrane was then incubated for one and half minutes at room temperature. The detection solution was drained off and the membrane (protein side up) was placed between two sheets of transparencies and air bubbles were removed. The chemiluminescent substrate indicating the position of proteins of interest was detected using the LAS-100 LumiImager (Fujifilm). The exposure time for detection was empirically determined based on the first exposure attempt.

2.5.5 Trypsin digest and protein extraction for mass spectrometry analysis

Following separation on a SDS gel, the separated proteins were excised and extracted from the gel and treated with trypsin. Whole lanes were excised from the gel using a razor blade to ensure all proteins were analysed. The gel slice was then further divided up into smaller pieces to aid the extraction process. To extract, the gel slices were washed with a buffer consisting of acetonitrile (ACN) (100 % v/v) and AmBic (20 mM) in a 50:50 ratio until completely destained. ACN was then added to dehydrate the gel pieces. After dehydration, the ACN was removed and the gel pieces were further dried using a Speed Vac until all the ACN was evaporated.

For the trypsin digest, a 10 ng/μL trypsin solution was made in 10 mM AmBic. For the samples, 300 μL of the trypsin solution was added and incubated on ice for 30 minutes. Then a further 300 μL of 10 mM AmBic was added to the sample and incubated overnight at 37 °C. The digested gel pieces were then extracted with 200 μL of a buffer consisting of 80 % (v/v) ACN and 1 % TCA and shaken at maximum speed for five minutes. The resulting supernatant was then dried via vacuum centrifugation. Samples were then kept at -20 °C until analysis.

Before loading samples onto the Q-TOF, the dried peptides were resuspended in 20 μL of loading buffer (5 % (v/v) ACN and 0.1 % (v/v) trifluoroacetic acid (TFA)). The resuspended peptides were loaded onto a filter column (0.2 μm, Millipore) and centrifuged at 5000 x g for two minutes. A total of 5 μL of filtered samples were loaded

onto a C18 high-capacity nano LC chip (Agilent Technologies) using a 1200 series capillary pump (Agilent Technologies). Following loading, samples were eluted from the C18 column directly into a 6550 series quadrupole time-of-flight mass spectrometer (Agilent Technologies) with a 1200 series nano pump using the following buffer B (0.1 % formic acid in Acetonitrile) gradient: 5-35 % in 35 min, 35-95 % in 2 min and 95-5 % in 1 min. Parameter setting in the mass spectrometer was as described per Nelson *et al*., 2014 (*56*). Mascot generic file peak lists were searched against an in-house *Arabidopsis* database comprising ATH1.pep (release 10) from The *Arabidopsis* Information Resource (TAIR) and the *Arabidopsis* mitochondrial and plastid protein sets (33621 sequences; 13487170 residues), using the Mascot search engine version 2.3 and utilizing error tolerances of 100 ppm for MS and 0.5 Da for MS/MS; "Max Missed Cleavages" set to 1; variable modifications of oxidation (Met) and carbamidomethyl (Cys) (*57*).

2.5.6 *In silico* analysis of protein-protein interactions

In silico prediction of protein-protein interactions was performed using several prediction databases. *Arabidopsis thaliana* protein interaction network (AtPIN - http://atpin.bioinfoguy.net/cgi-bin/atpin.pl), *Arabidopsis thaliana* Protein Interactome Database (AtPID - http://www.megabionet.org/atpid/webfile/), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING - http://string-db.org/) and Predicted *Arabidopsis* Interactome Resources (PAIR - http://www.cls.zju.edu.cn/pair/) were used to perform the in silico prediction. The default settings wherever applicable were selected to perform the analysis.

2.6 Microscopy

Leaf material of transiently transformed *N. benthamiana* leaves (2.3.1) was prepared for microscopy by excising small sections using a scalpel blade. These small sections were then mounted onto glass slides and fixed with water. Confocal laser scanning microscopy (CLSM) was performed using a TCS SP2 AOBS confocal microscope (Leica, Germany) through a 40x objective lens. Confocal images were collected using

the Leica confocal software. The following excitation lasers and emission channels were used:

Fluorescent tag	**Excitation wavelength (nm)**	**Emission wavelength (nm)**
GFP	488	510-540
YFP	488	530-550
Chlorophyll (autofluorescence)	514	680-700
DAPI	358	461

Table 2.1 : Various thermocycler programmes used for amplification

	ORF amplification (high fidelity PCR)	**cPCR amplification (low fidelity PCR)**	**BigDye (sequencing PCR)**
Initial denaturation	96 °C for 30 seconds	96 °C for 5 minutes	96 °C for 1 minute
Amplification	35 cycles	30 cycles	25 cycles
Denaturation	96 °C for 30 seconds	96 °C for 30 seconds	96 °C for 10 seconds
Annealing	52 °C for 30 seconds	52 °C for 30 seconds	50 °C for 5 seconds
Extension	68 °C for 1Kbp per minute	72 °C for 30 seconds	60 °C for 4 minutes
Final extension	68 °C for 5 minutes	72 °C for 2 minutes	-
Hold	15 °C indefinitely	15 °C indefinitely	4 °C indefinitely

Table 2.2: Thermocycler programme used for qRT-PCR

Step	Temperature	Time	Repeats
Initial denaturation	95 °C	3 minutes	
Denaturation	95 °C	15 seconds	13 cycles
Annealing	85°C -2 °C /cycle	30 seconds	
Denaturation	95 °C	15 seconds	40 cycles
Annealing	58°C + plate read	30 seconds	
Extension	72°C + plate read	30 seconds	
Melt curve	70 °C + 0.5 °C + plate read	15 seconds	50 cycles
Hold	4 °C	Indefinite	

Table 2.3: Primers used for PCRs and qRT-PCR

Target DNA	Forward primer (5'-3')	Reverse primer (5'-3')	PCR used
NIT1	ATGTCTAGTACTAAAGATATGTCAAC	TCTCGGATCCTTTGTTTGAGTCATCCTCAGC	ORF and cPCR amplification
NIT2	ATGTCAACTTCAGAAAACACTCCG	CAACGGATCCCTTGTTTGAGTCATCTTCC	
NIT3	ATGTCTAGTACTGAAGAAATGTCATC	ATCTGGATCCTTTGTTTGATTCATCCTC	
NIT4	ATGTCCATGCAACAAGAAACGTC	TCATGGATCCGACGGATTCATCTTCC	
NIT1DelA	N/A	GATAGGATCCGATAAACCCCTCCG	
NIT1Del B	AGATGTTTATCGGTGGCTATC	AAAGGAATCCGCAATAGAGGGTATACC	
NIT1Del C	CCATGTATTGCACAGTTCTTTTC	CACAGGATCCCTCAATGCCTTTG	
NIT1Del D	CATGGGCATTGAGCTTTATTG	GTGAGGATCCGACACTTCCACCTTG	
NIT1Del E	GTATGAGTGTCATTATTTCACCTTTG	N/A	
CYFPsp (for sequencing)	N/A	AGCTCAGGTAGTGGTTGTC	sequencing PCR
AtMKK2	ATGAAGAAAGGTGGATTCAGC	CGATCTCGAGCACGGAGAACGTACCAG	ORF and cPCR amplification
ERF1	ATGGATCCATTTTTAATTCAGTCCC	CTTCTCGAGCCAAGTCCCACTATTTTC	
ACT2 RT	GTATGATGAGGCAGGTCCAG	CACAAGTGCATCATAGAAACG	qRT-PCR
ATPase RT	GAGAGAGGCGAAATCAAGAC	CCATAATAAGCACAGTTTTCCC	
AOS RT	GATCCATCGGAGCCTAAACAC	CGGTAAACCAATAGAGAGTAATGGAT	
OPR3 RT	ATGTGCCTGGAATCTATTCAG	GGAACCATCGGGCAACA	
NIT1 RT	GGTGGAAGTGTCATTATTTCAC	ATCCTCAGCTTTCTCCACC	
NIT2 RT	CGTTTACGACACTCCGATTGG	GGGAAATCTTTACGAAGGCAG	
NIT3 RT	AAAGTCATGCCCACATCTTTG	TACCGCAATGTGAATCATCG	
NIT4 RT	TGGAGATGGATCAACCATCC	GACGACAAAACTGGTTAGCTGAC	
VSP1	AAATATGGATATGGGACCGAG	TCTGTCAGAGATTATGATGGG	
PDF1.2	CACACGATTTAGCACCAAAG	CACACGATTTAGCACCAAAG	

Chapter 3

Novel functions of *Arabidopsis* nitrilases based on subcellular localization and interactors

3.1 Introduction

3.1.1 Determining new roles for *Arabidopsis* nitrilases

Discerning roles of a new protein or novel roles of an established protein can be achieved through combining several approaches such as enzymatic, genetic, and proteomic characterization. For the *Arabidopsis* nitrilases, much of the focus has been on the first two types of characterization.

Enzymatic profiling is often the first step in determining the function of a novel protein. From this, how the protein fits in the whole biological context of the organism can often be determined. Enzymatic characterization by Thimann *et al* defined *Arabidopsis* nitrilases as IAN degrading enzymes (*26*). Within the nitrilase superfamily, there are members with biological roles beyond their enzymatic function. Mammalian nitrilases are an example of this. They are known to be involved in cell cycle regulation and cell growth, independent of their enzymatic function (*42*). A similar role has been postulated by Doskocilova *et al*, having shown that NIT1 has similar cell cycle regulatory roles (*25*). Thus, determining the biological role of plant nitrilases through their enzymatic function alone is not always enough to determine their overall biological role in the cell.

Genetic characterization is a very powerful approach to discern biological roles based on mutant analysis. Here, a phenotype is determined and associated with a gene (forward genetics) or the gene of interest is knocked out or over-expressed and phenotypic differences are observed (reverse genetics). From there, roles can be ascertained from a biological perspective. So far, not much has been determined about *Arabidopsis* nitrilases from forward genetics; there are no known phenotypes associated with nitrilase expression disturbances. However, point mutations in NIT1 have been shown to have a root inhibition phenotype in the presence of IAN, a precursor of IAA (*58*). Neither knocking out or over-expressing the nitrilases leads to any observable phenotype. However, when challenged with drought conditions knockouts for NIT4 show a more sensitive response than the wild type which is manifested in a more pronounced decrease of the photosynthetic capacity (*54*). These examples demonstrate that the analysis of nitrilase mutants, when challenged with appropriate conditions, can reveal otherwise hidden functions of these proteins.

Interactomics can be defined as a form of functional proteomics; it is the study of the physical molecular interactions on either a cellular or organelle level. Proteins rarely exist in isolation; their biological role is dependent on the interaction with other proteins or nucleic acids. Whilst specific functions can be attributed to a protein or a protein family, interaction with a multitude of other proteins defines aspects of its role in some form or another. Thus, a protein's role may depend on the formation of stable or transient protein complexes. As such, any single protein will have its own interactome, with its own specific binding partners dependent on the cell expression state at the time. Biological processes such as signal transduction, homeostasis control, plant development and growth, stress response and plant defence require physical interaction between proteins. On a molecular level, protein-protein interactions (PPIs) form the basis for post-translational modifications such as phosphorylation, enzyme activation/inactivation and transcriptional co-factor recruitment.

3.1.2 An interactome for *Arabidopsis* nitrilases

As suggested earlier, the biological functions of nitrilases may not be tied to their enzymatic abilities. It has been shown that transcription factors such as EREBP and ATAFs which are involved in plant stress responses and hormone signalling interact with plant nitrilases (*49, 52*). This suggests a role beyond nitrile detoxification. Furthermore, RAD23A have been shown to interact with NIT1 which is involved in cell cycle regulation *via* proteosomal protein degradation (*43, 44, 59*). Using affinity purification coupled with mass spectrometry, NIT1 and 2 were co-immunoprecipitated with an isoform of the 14-3-3 protein family (*60*). Protein interaction studies coupled with enzymatic analysis revealed that interaction partners can influence the activity of plant nitrilases. Like their bacterial counterparts, maize and sorghum nitrilases form multimers and depending on the combination of the isoforms which make up the multimers, the substrate specificity alters (*29, 41*). These examples demonstrate that by identifying interaction partners for a protein allows the protein to be positioned into new networks or pathways hitherto not considered.

For an interaction to take place, the two proteins need to converge on a spatial and temporal level. Thus, a protein's subcellular localization can provide the initial clues to its roles. The localization of a protein of interest can help in the interpretation of PPI predictions and experiments when deciding which of the putative interactions observed

may be biologically relevant. Localization studies make use of fluorescent proteins such as GFP. Proteins of interest are fused to GFPs and then the localization is traced *in vivo* using confocal laser scanning microscopy. This has been shown with *Arabidopsis* enolase; GFP fusion studies traced the localization to be both nuclear and cytosolic, supporting the dual role of this protein in glycolysis and gene regulation (*61*). Investigation of the protein sequences of the *Arabidopsis* nitrilases showed that they lack typical nuclear localization signals (NLSs) or any other type of organelle localization signal. However, subcellular localization database for *Arabidopsis* protein (SUBA3) which curates localization data from predictions, MS/MS and GFP data predicts that the four *Arabidopsis* nitrilases could be localized to the cytosol, plastid, plasma membrane, nucleus, mitochondrion or the peroxisome (*62*). Tracing the localization of the *Arabidopsis* nitrilases *via* GFP fusion will show which of these predictions apply and provide more information towards establishing an interactome for the *Arabidopsis* nitrilases.

Initial steps towards generating an interactome involve *in silico* analysis using several PPI databases and prediction tools to determine known and predicted interactomes of the *Arabidopsis* nitrilases. *In silico* modelling of PPIs can be achieved using several methods. The 3D structures and physio-chemical properties of putative interacting proteins can be analysed to predict domain-domain interactions (*63*). Protein docking algorithms are also used to predict protein-protein binding interfaces. Such prediction methods can be quite limiting because of their reliance of accurate 3D structures which we lack for many proteins. In addition, transient protein interactions cannot be predicted through these methods. Accurate x-ray structures of the four *Arabidopsis* nitrilases have not been determined with only structures of bacterial nitrilases known. Thus, such methods are not useful for the *Arabidopsis* nitrilases. Protein complexes are often structurally and functionally conserved during the evolution process. Thus, another way to predict interactions is to use both experimental and predicted data; co-expression and co-localization data, and functional categories are used to determine the likelihood for interaction. Predicting interologs is one of the most commonly used methods to generate interactomes. Interologs are generated based on the conservation of the protein interaction between organisms. The interaction is inferred from the orthologs for each protein partner with the presence/absence of the interaction in other species indicating functional relationship (*64*). This approach assumes conservation of the interaction

between organisms which does not work well if the model species are compared with newly annotated genomes or those with unknown functions. In *Arabidopsis*, almost 45% of the predicted interolog proteins have unknown functions (*65*). This questions the reliability of the interaction networks generated for *Arabidopsis* proteins using this approach.

Dynamic interactions cannot be detected by *in silico* methods which predict static PPI networks. In addition, as mentioned above, prediction by interologs is unreliable for *Arabidopsis* meaning *in silico* prediction needs to be complemented with experimental methods. Affinity purification coupled with mass spectrometric identification (AP-MS) is a technically easy and common experimental method. Here, the bait protein is fused to an affinity tag such as a His, Flag or TAP tag and expressed *in vivo*. Using antibodies to purify the tagged bait, the bait protein is then pulled down along with other proteins bound to it. The bait and the bound protein are then identified by using mass spectrometry. Being an *in vivo* system gives AP-MS an advantage over yeast two hybrid (Y2H) experiment of being able to determine multi-component protein complexes in the native organism of the protein. Furthermore, AP-MS is conducted under a native state, giving a closer representation of what occurs on the cellular level. It also allows the experiment to be performed under different conditions, allowing the dynamics of the interactions to be probed and to potentially determine interactors not identified under normal conditions.

The increasing amount of data available from proteomic studies, coupled with data analysis and mining tools enables PPIs to be predicted using database interrogation if they are backed-up by experimental approaches. The development of experimental interaction methods such AP-MS and Y2H, and the development of *in vivo* protein interaction techniques such as Föster Resonance Energy Transfer (FRET) and Bimolecular Fluorescence Complementation (BiFC) have greatly advanced the field of interactomics (*63*). Despite the power of PPI studies and the data mining tools available, not much has been published to date in regards to interactomes of plant nitrilases which would provide a more holistic view in how nitrilases are involved in the plant's biology. Thus, this thesis will attempt to discover novel interactors of nitrilase proteins from *Arabidopsis* and generate an interactome.

3.1.3 Interaction with 14-3-3 proteins

Using a monoclonal antibody raised against an exposed domain of several *Arabidopsis* 14-3-3s, Paul *et al* precipitated a pool of 14-3-3 isoforms and along with them, co-immunoprecipitating a diverse and large number of 14-3-3 interactors (*60*). Within this large and diverse pool of interactors, NIT1 and 2 were identified to be putative interactors of 14-3-3s.

The 14-3-3 proteins themselves are also known as general regulatory factors. The 14-3-3 protein family have been shown to be ubiquitous in all eukaryotes, regulating a wide range of cellular processes such as cell cycle, apoptosis, metabolism and gene transcription regulation. Interactomics studies in humans and *Arabidopsis* identified a diverse and large number of proteins which interact with 14-3-3s (*60*, *66*). These regulatory factors bind to client proteins through recognition of a phosphorylated motif (*67*). This suggested that not only are 14-3-3s important in signalling transduction but that phosphorylation may play a role in coordinating protein-protein complexes. Subsequently, it has been shown that phosphorylation is not always required for 14-3-3:protein interaction; 14-3-3s are also able to recognise unphosphorylated motifs (*68*).

Arabidopsis has 13 expressed 14-3-3 isoforms which share highly conserved amino acid sequences between each other and with 14-3-3s of other species (*69*, *70*). The high conservation of their amino acid sequences suggested redundancy in 14-3-3 function. This was supported in yeast knock-out experiments where the loss of one of the two 14-3-3 genes, bmh1 and bmh2, did not result in a visible phenotype. However, a double knock-out resulted in a lethal phenotype which was recovered when complemented with the 14-3-3 plant homologues (*71*). Such experiments highlight the importance of 14-3-3s for an organism as well as the functional redundancy within and across species. Variation of the 14-3-3 proteins between species does exist and is mainly at the N- and C-terminal regions. The 13 expressed 14-3-3 isoforms are divided into two distinct clades (epsilon and non-epsilon) based on phylogenetic analysis and gene structure (*72*). It has been suggested that this isoform diversity is related to the ubiquitous nature of 14-3-3s; the diversity ensures that fundamentally 14-3-3s are present in all cell types if required. Another possibility could be that different isoforms are required to interact

with different client proteins and are a reflection of the co-evolution of 14-3-3 binding sequences of target protein and the specific 14-3-3 isoform it interacts with.

The identification of 14-3-3 proteins as putative interactors of both NIT1 and 2 through AP-MS suggests regulation by the 14-3-3s involve several ways: enzymatic activity, localization, protein-protein interaction and or protein stability. Investigating this interaction may provide new insights into the biological roles of the *Arabidopsis* nitrilase family. Confirmation of this interaction is required as one of the disadvantages of AP-MS is false positives due to non-specific interactions (*63*). Interactions can be confirmed using BiFC, the split fluorescent protein assay. For BiFC, two putative interactors are fused to either the N-terminal or C-terminal halves of a fluorescent protein. The interaction is then determined by whether fluorescence is observed due to the functional reformation of the split fluorescent protein. BiFC can also be used to see where the interaction occurs as the fluorescence can be traced *via* confocal laser scanning microscopy. Within the *Arabidopsis* nitrilase proteins, there are many serine and threonine residues which can serve as phosphorylation targets and thus binding sites for the 14-3-3s. However, the *Arabidopsis* nitrilases lack known 14-3-3 consensus binding motifs, of both phosphorylated or non-phosphorylated binding. This suggests that the nitrilases could have a novel 14-3-3 binding motif. The knowledge of such a binding site opens up the possibility to generate mutant nitrilases unable to interact with 14-3-3 proteins. This would allow further investigation of the biological role of 14-3-3s binding to nitrilases which in turn will shed light onto the biological roles of this family of enzymes.

3.1.4 Aims and hypotheses

The novel interaction between NIT1 and 2, and 14-3-3s as identified by Paul *et al* (*60*) may provide an interesting insight into the biological role of these enzymes as hitherto no novel interactors for the four nitrilases have been identified. Given that the pull down data identified both NIT1 and 2 as being targets of 14-3-3s and the four *Arabidopsis* nitrilases are fairly conserved, it can be hypothesised that not only NIT1 and 2 interact with 14-3-3s *in planta* but also NIT3 (*60*). As NIT4 is the least conserved within the *Arabidopsis* nitrilase family, there is a possibility that it does not interact with 14-3-3s.

Studies on maize and sorghum nitrilases have shown that plant nitrilases have the ability to form homo-and heterodimers. It is hypothesised that the four *Arabidopsis* nitrilases are able to dimerize like maize and sorghum nitrilases. Apart from identifying the catalytic domains, further characterization of their regulation and other possible roles have not been fully investigated for the *Arabidopsis* nitrilases. As mentioned above, aside from 14-3-3s, there are little data on putative interactors for the four *Arabidopsis* nitrilases. By identifying novel interactors for *Arabidopsis* nitrilases, novel biological roles or confirmation of established roles can be determined.

Thus, to study the interactions of *Arabidopsis* nitrilases and their functional context, the following aims were made :

1. Determine the subcellular localization of the four *Arabidopsis* nitrilases by generating *Arabidopsis* nitrilases GFP fusion constructs and observe their localization *via* transient transformation in *Nicotiana benthamiana* using confocal laser scanning microscopy.

2. Generate an interactome using *in silico* analysis and AP-MS for the *Arabidopsis* nitrilases. Several interactome databases will be used to determine putative interactors for *Arabidopsis* nitrilases. In parallel, AP-MS will be performed for *Arabidopsis* plants over-expressing NIT1 and 4.

3. Define the subcellular location of the 14-3-3:nitrilase interaction and to evaluate the specificity of nitrilase and 14-3-3 isoforms in such interactions. To achieve this, BiFC will be used to provide an *in planta* context and to show where the interaction occurs on a subcellular level.

4. Identify the 14-3-3 binding site in *Arabidopsis* nitrilases. To fully characterize the interaction and thus determine the functional consequence of the 14-3-3 interaction, the binding site needs to be identified. Deletion constructs of NIT1 will be generated and tested for loss of interaction with 14-3-3 *via* BiFC. This will determine which regions of the nitrilase are required for the interaction.

3.2 Results

3.2.1 Subcellular localization of the *Arabidopsis* nitrilases

The four NIT isoforms were cloned into the pG179NS-GFP vector to generate NIT-GFP fusion proteins (2.2, 2.3 and 2.4) . These vectors were used to transform *Agrobacteria* which were then infiltrated into the epidermal leaf cells of *N. benthamiana* (2.3.1). Fluorescence was observed three days post transformation *via* fluorescence microscopy (2.6). The fusion protein was observed using GFP emission; DAPI emission was used to view the nucleus after DAPI staining. Autofluorescence due to the chloroplasts was observed using RFP emission channel. Cytosolic and nuclear fluorescence was observed for the NIT1-GFP fusion in the GFP channel (figure 3.1).The DAPI staining highlighted the nucleus as seen in the DAPI channel in figure 3.1. The merged image of the three channels shows the overlap of the DAPI stained nucleus and the nuclear fluorescence observed in the GFP channel, confirming the nuclear localization of NIT1. This was similarly observed for the NIT2, 3 and 4 GFP fusion proteins. The cytosolic and nuclear fluorescence observed for all four fusion proteins was not due to autofluorescence as they did not match with the fluorescence observed under the RFP emission channel. Furthermore, the nitrilases were absent from the chloroplasts as determined from the overlay. The nuclear localization of the *Arabidopsis* nitrilases was a novel and surprising outcome of this study and was not previously reported to localize to the nucleus using an *in vivo* approach.

3.2.2 *In silico* analysis of potential *Arabidopsis* nitrilase interactors

An *in silico* analysis of putative interactors for *Arabidopsis* nitrilases was performed using several PPI databases to predict plant protein interactions (2.5.6). *Arabidopsis thaliana* Protein Interactome Database (AtPID) generates interactomes by a Naïve Bayesian approach, integrating interologs, gene expression data, phylogenetic profiles (*63*). AtPID predicts that the four isoforms interact with each other based on phylogenetic profiling (data not shown). No other interactors were identified aside from the nitrilase isoforms themselves.

Arabidopsis thaliana Protein Interaction Network (AtPIN), another predicted interactome database, combines information on PPIs, ontology and subcellular localization to predict interactions (*73*). Similar to AtPID, AtPIN predicts that the

Arabidopsis nitrilases interact with each other. Predicted interactors for the *Arabidopsis* nitrilases include a ribosomal protein (At4g39200), a serine/threonine kinase (At1g50370), an ion transporter (At4g19690) and a ubiquitin protease (At4g39910). Further predicted interactors for the four isoforms are tabulated in table 3.1.

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (*74*) integrates genomic context, experimental data, co-expression data and previous knowledge (database mining) to predict functional linkages and PPIs. Proteins involved in the auxin/glucosinolate synthesis pathway such as the cytochrome P450s, the YUCCA proteins and myrosinases were predicted to be functional partners for NIT1, 2 and 3. For NIT4, cysteine synthase C1 which is involved in β-cyanoalanine synthesis, is a predicted functional partner. Amidase 1, a C-S lyase (Superoot 1) and several cytochrome P450s, enzymes which are involved in auxin and its derivatives' synthesis, were also predicted to be functional partners for NIT4. Other predicted functional partners are tabulated in appendix table 1.

Predicted *Arabidopsis* Interactome Resourses (PAIR) similarly uses the same data as AtPID to predict interactomes for *Arabidopsis* (*63*, *75*). Similar to AtPID, PAIR predicted the interaction of NIT 2, 3 and 4 with themselves. No further interaction were predicted for the four isoforms (data not shown).

3.2.3 Dimerization of *Arabidopsis* nitrilases

Nitrilases form multimers to be functional; maize and sorghum nitrilases are well known to form dimers in order to take a variety of substrates (*28, 29*). Furthermore, interaction databases like AtPIN and AtPID predict the four nitrilase isoforms interact with each other. The ability to form homo- and heterodimers has not been investigated for *Arabidopsis* nitrilases. Thus it was decided to investigate whether the *Arabidopsis* nitrilases behave similarly to maize and sorghum nitrilases and form dimers of different isoform combinations. To achieve this, the four NIT isoforms were cloned into both pG179NS-YC and YN vectors. The ability to homo-and heterodimerize was then determined by testing all possible dimer combinations for interaction using BiFC. The different combinations of NIT-YC and YN were transformed into *A.tumefaciens* and subsequently transiently transformed into *N. benthamiana* epidermal leaf cells. Strong YFP fluorescence was observed for all the tested combinations of NIT dimers (figure

3.2). This indicated that *Arabidopsis* nitrilases were able to form both homo- and heterodimers. The fluorescence in every case was localised to both the cytosol and nucleus. Localization to the nucleus was confirmed with the DAPI staining which highlighted the nuclear DNA. Nuclear localization was confirmed with the DAPI staining highlighting nuclear DNA. Furthermore, comparing the localisation of YFP fluorescence with fluorescence observed under the RFP channel confirmed that the YFP fluorescence was not due to cellular autofluoresence. The localization results here agree with that obtained from the nitrilase-GFP localization studies, indicating that the dimerization does not affect localization of the *Arabidopsis* nitrilases. In addition, the BiFC analysis confirmed the AtPID, AtPIN and PAIR predictions that the *Arabidopsis* nitrilases form dimers with each other.

3.2.4 AP-MS for NIT-GFP over-expressing plant lines

Out of the four nitrilase isoforms, NIT1 and 4 are the most different in sequence and are representative of the two branches of the plant nitrilase family. Thus it was decided that a point of focus was to determine the interactors for NIT1 and NIT4. *Arabidopsis* lines over-expressing 35S:NIT1-GFP and 35S:NIT4-GFP were generated for this purpose. Transgenic seeds of these two lines, as well as Col-0 and of a 35S:GFP line were grown in soil (2.1.2). Leaves of three week old plants from each line were harvested and used to extract total protein (2.5.2). The NIT1-GFP and NIT4-GFP fusion proteins were immunoprecipitated from the protein extracts using anti-GFP antibodies. Immunoprecipitation was also performed for the 35S:GFP and wild type (Col-0) *Arabidopsis* line. Both the 35S:GFP and Col-0 lines serve as negative controls to ensure that interaction candidates observed for the nitrilases are not due to interactions with GFP or to non-specific immunoprecipitated proteins. Immunoprecipated proteins were resolved on SDS-PAGE gel. The separated proteins were excised and extracted from the gel. The gel extracts were then prepared for mass spectrometry and then analysed by mass spectrometry (MS) to identify the immunoprecipitated proteins (2.5.5). From the MS analysis, a list of identified proteins was obtained. Putative interactors of NIT1 and 4 were selected from this list based on them being unique to the over-expressing mutant lines, *i.e.* were they absent from the negative control samples and their identification scores being significant. From that, a pool of interactors of NIT1 and 4 were determined and tabulated in table 3.2.

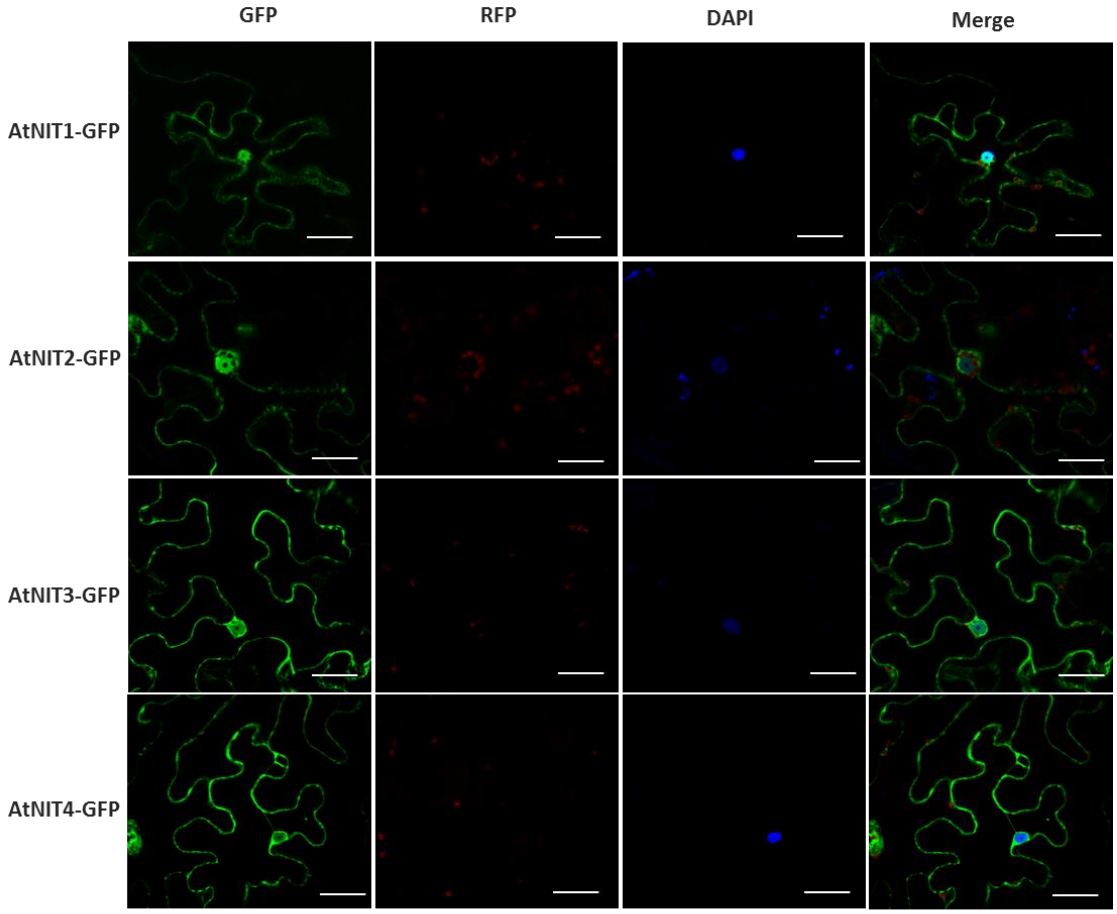


Figure 3.1 AtNIT 1 to 4 have both nuclear and cytosolic subcellular localization pattern
AtNIT-GFP fusions were generated to investigate the localization of the four *Arabidopsis* nitrilase isoforms. The AtNIT-GFP fusion constructs were transiently transformed into *N.benthamiana* epidermal leaf cells. Expression was analysed three days post infection using confocal laser scanning microscopy. Green fluorescence and autofluorescence was determined using GFP and RFP channels respectively. Nuclei were shown using DAPI staining and detected with the DAPI channel. The overlay of all three channels confirm the nuclear localization of the GFP fusion protein and that GFP fluorescence is not due to autofluorescence of organelles such as chloroplasts.

Scale bar represents 35µm.

Table 3.1: Predicted interactors identified using AtPIN for the four *Arabidopsis* nitrilase

AtPIN uses ontology, subcellular localization and experimental data to predict PPIs *(73)*. Using the default settings, AtPIN predicted that the four AtNITs will form heteromers but not homodimers. Whilst there are no predicted proteins which interact with all four AtNITs, ribosomal proteins, serine/threonine kinases and ion transporters are commonly predicted as interactors for the nitrilases.

	AT gene number	Predicted interactors
AtNIT1		
	AT3G44300	AtNIT2
	AT3G44320	AtNIT3
	AT5G22300	AtNIT4
	AT4G39200	40S RIBOSOMAL PROTEIN S25
AtNIT2		
	AT3G44300	AtNIT1
	AT3G44320	AtNIT3
	AT5G22300	AtNIT4
	AT3G12800	SHORT-CHAIN DEHYDROGENASE-REDUCTASE B
	AT1G26160	METAL-DEPENDENT PHOSPHOHYDROLASE HD DOMAIN-CONTAINING PROTEIN
	AT1G02500	SAM1 (S-ADENOSYLMETHIONINE SYNTHETASE 1) METHIONINE ADENOSYLTRANSFERASE
	AT2G44510	P21CIP1-BINDING PROTEIN-RELATED
	AT3G51895	SULTR31 (SULFATE TRANSPORTER 31)
	AT4G39910	ATUBP3 (ARABIDOPSIS THALIANA UBIQUITIN-SPECIFIC PROTEASE 3) UBIQUITIN-SPECIFIC PROTEASE
	AT2G18230	ATPPA2 (ARABIDOPSIS THALIANA PYROPHOSPHORYLASE 2) INORGANIC DIPHOSPHATASE/ PYROPHOSPHATASE
	AT1G50370	SERINE/THREONINE PROTEIN PHOSPHATASE PUTATIVE
	AT2G26695	BINDING / ZINC ION BINDING
	AT2G34180	CIPK13 (CBL-INTERACTING PROTEIN KINASE 13
	AT4G34880	AMIDASE FAMILY PROTEIN
AtNIT3		
	AT3G44300	AtNIT1
	AT3G44310	AtNIT2
	AT5G22300	AtNIT4
	AT3G14420	S)-2-HYDROXY-ACID OXIDASE PEROXISOMAL PUTATIVE / GLYCOLATE OXIDASE
	AT3G03920	GAR1 RNA-BINDING REGION FAMILY PROTEIN
	AT5G63980	SAL1 3(2)5-BISPHOSPHATE NUCLEOTIDASE/ INOSITOL OR PHOSPHATIDYLINOSITOL PHOSPHATASE
	AT3G48040	ROP10 (RHO-RELATED PROTEIN FROM PLANTS 10) GTP BINDING
	AT4G19690	IRT1 (IRON-REGULATED TRANSPORTER 1
	AT4G28860	CKL4 (CASEIN KINASE I-LIKE 4) ATP BINDING
	AT3G13460	ECT2 PROTEIN BINDING
	AT5G02730	ALLERGEN V5/TPX-1-RELATED FAMILY PROTEIN
	AT1G06090	FATTY ACID DESATURASE FAMILY PROTEIN
	AT2G47970	NPL4 FAMILY PROTEIN
	AT4G16370	ATOPT3 (OLIGOPEPTIDE TRANSPORTER)
	AT5G15240	AMINO ACID TRANSPORTER FAMILY PROTEIN
AtNIT4		
	AT3G44310	AtNIT1
	AT3G44300	AtNIT2
	AT3G44320	AtNIT3

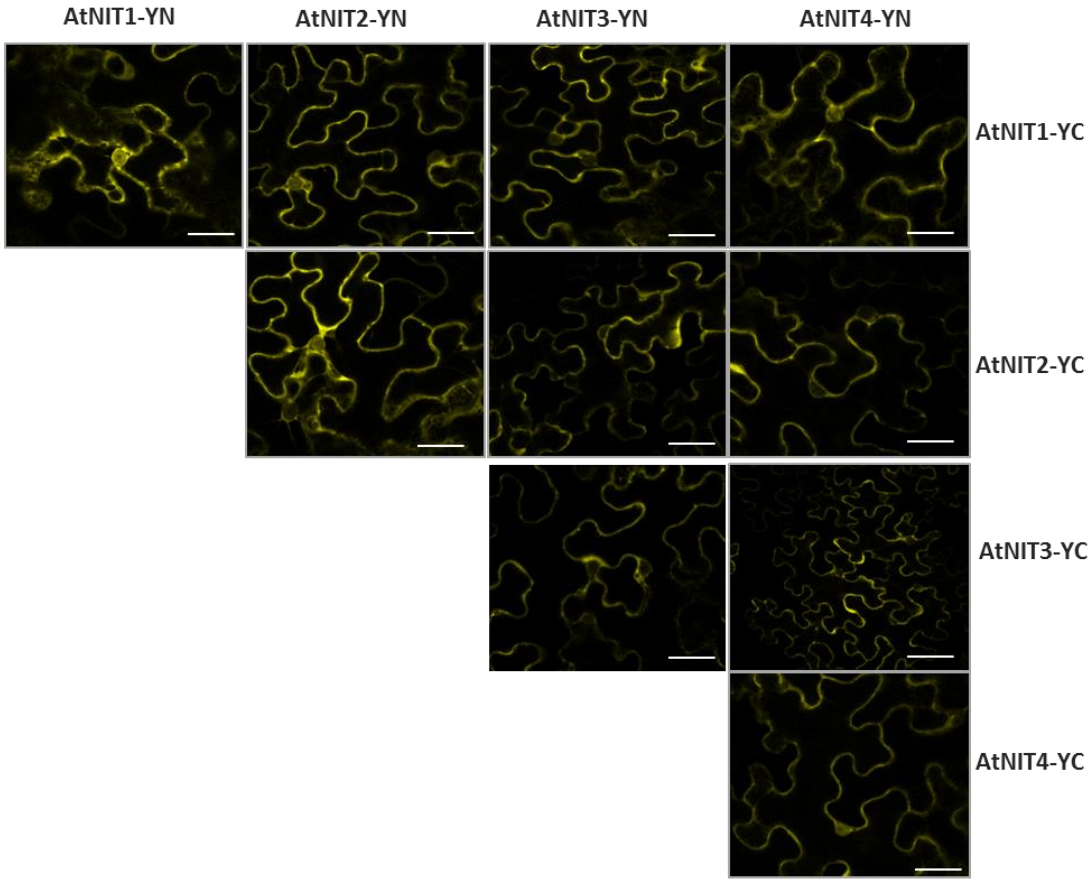


Figure 3.2 Homo-and heterodimerization of the *Arabidopsis* nitrilases
AtNIT1 to 4 were cloned into pG179NS YC and YN vectors. To test for dimerization, all possible dimer combinations were considered and tested for interaction using BiFC. Combinations of the YC and YN AtNIT fusions were transiently transformed into *N.benthamiana* epidermal leaf cells. Expression and reformation of the split YFP was determined three days post infection using confocal laser scanning microscopy. Yellow fluorescence was determined using the YFP channel.

Scale bar represents 35µm.

Table 3.2: Putative interactors identified through mass spectrometry for AtNIT 1 and 4
Putative interactors were determined using AP-MS for the two AtNIT-GFP overexpressing plant lines. Interactors were selected based on the protein identification score (Mascot protein score) and uniqueness (i.e. whether they appeared in the negative controls) and whether they were common contaminants.

		Interactors			
		Protein name	Protein functions	Mascot scores	Number of peptides (number of significant matches)
AtNIT1					
	AT3G44310.1 AT3G44300.1 AT3G44320.1	Nit1 Nitrilase 1 NIT2, AtNIT2 NIT3, AtNIT3	C-N hydrolyase activity C-N hydrolyase activity C-N hydrolyase activity	4092 110 1373	235 (80)
	AT4G09000.1 AT3G02520.1 AT1G78300.1 AT2G42590.1 AT5G10450.1	GRF1, GF14 CHI GRF7, GF14 NU GRF2, 14-3-3OMEGA GRF9, GF14 MU GRF6, AFT1, 14-3-3lambda	general regulatory factor 1 general regulatory factor 7 general regulatory factor 2 general regulatory factor 9 G-box regulating factor 6	151 130 120 116 91	20 (7) 13 (4) 14 (5) 9 (2) 9 (3)
	At5g17990.1	TRP1 Tryptophan biosynthesis 1	IAA synthesis pathway	28	2 (1)
	At5g54810	TSB1 (TRP2) Tryptophan synthase beta subunit 2	IAA synthesis pathway	50	2 (1)
	AT4G29810.1	AtMKK2 MAPK Kinase 2	Interacts MPK – activates Involved in cold stress and ABA signalling	25	1 (1)
	AT5G35750.1	AHK2 histidine kinase 2	Cytokinin receptor ; negative role in cold signalling via ABA response inhibition	25	2 (1)
AtNIT4					
	AT5G22300.1 AT3G44310.1 AT3G44320.1	NIT4, AtNIT4 NIT1, ATNIT1, NITI NIT3, AtNIT3	C-N hydrolyase activity C-N hydrolyase activity C-N hydrolyase activity	429 161 144	27(18) 18 (5) 18(2)
	AT5G16050.1 AT1G35160.1 AT3G02520.1 AT5G38480.1 AT4G09000.1 AT1G78300.1 AT5G10450.1 AT5G65430.1 AT1G22300.1 AT1G26480.1	GRF5, UPSILON GRF4, 14-3-3PHI, PHI GRF7, NU GRF3, RCI1 GRF1, CHI GRF2, 14-3-3OMEGA, GRF6, AFT1, 14-3-3lambda GRF8, 14-3-3KAPPA, GRF10,14-3-3 EPSILON, GRF12, IOTA	general regulatory factor 5 general regulatory factor 4 general regulatory factor 7 general regulatory factor 3 general regulatory factor 1 general regulatory factor 2 general regulatory factor 6 general regulatory factor 8 general regulatory factor 10 general regulatory factor 12	61 61 61 61 61 61 61 61 61 61	36 (1) 18 (1) 18 (1) 18 (1) 19 (1) 17 (1) 20(1) 20 (1) 10 (1) 9 (1)
	AT5G64240.1	AtMC3 Metacaspase 3	Cell death related	36	19 (1)
	AT2G46520.1	CAS Cellular apoptosis suspectibility protein	Putative importin re-exporter	28	13 (1)
	AT4G29810.1	AtMKK2 MAPK Kinase 2	Interacts with MPKs – activates Involved in cold stress and ABA signalling	26	6 (1)
	AT4G23210.3	CRK13 Cysteine rich receptor like protein (RLP) kinase 13	Induces defence in response to SA Pathogen defence Overexpression leads to pathogen resistance	20	29 (1)
	AT1G17950.1	AtMyb52	ABA signalling, drought tolerance	21	15 (1)
	AT3G61440.1	ATCYSC1 Cysteine synthase C1	B-cyanoalanine synthesis	60	11 (2)

Figure 3.3 A comparative analysis of the co-expression of the *14-3-3* and *NIT* isoforms in *Arabidopsis* tissue

A comparative gene expression analysis was performed to determine for the 11 *14-3-3* isoforms and the *NIT*s using data retrieved from Genevestigator *(29)*. The Affymetrix *Arabidopsis* ATH1 Genome array platform was used to perform this analysis. The darkness of the red colour indicates the linear percentage of expression potential with white indicating zero expression. All four NITs and 14-3-3 isoforms were found to be expressing in the same tissue, albeit of varying levels of expression.

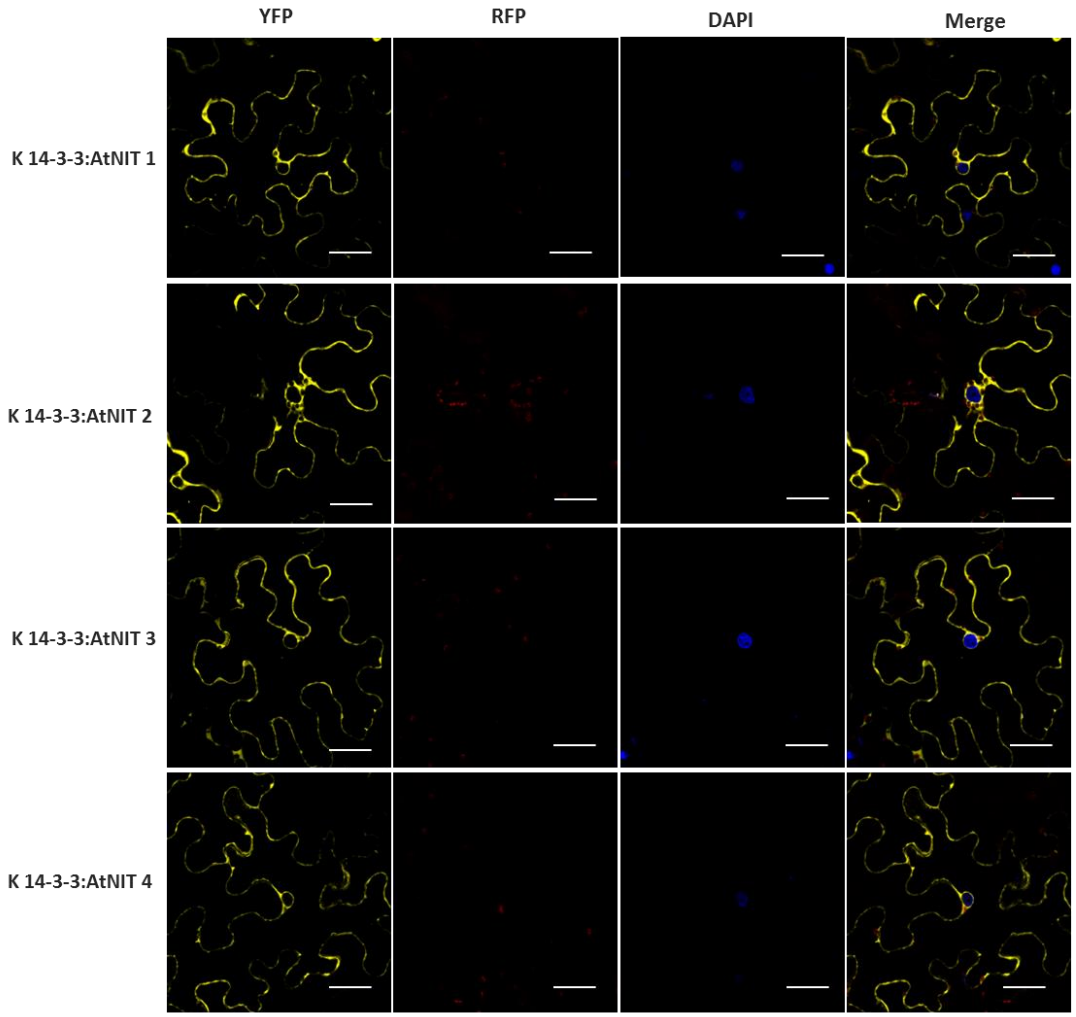


Figure 3.4 Interaction of the AtNITs with 14-3-3 proteins using BiFC analysis
The interaction of the four AtNITS as YC fusions with Kappa 14-3-3 as YN fusions were tested in transiently transformed *N.benthamiana* leaf epidermal cells. Expression and reformation of the split YFP was analysed three days post infection using confocal laser scanning microscopy. Yellow fluorescence was detected using the YFP channel whereas the RFP channel was used to detect autofluorescence. Nuclei were shown with DAPI staining and detected using the DAPI channel. Images were merged to show all three channels in one image.

Scale bar represents 35µm.

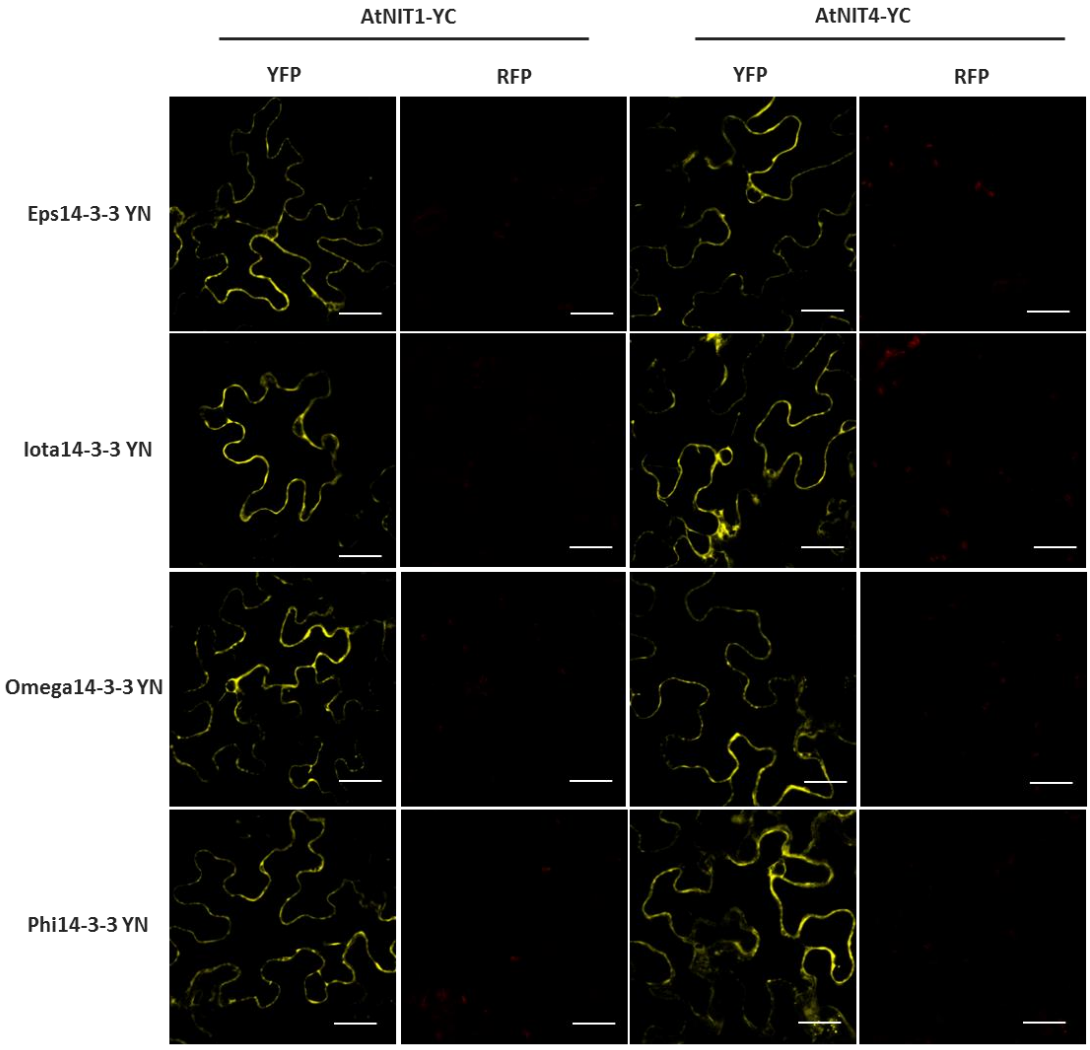


Figure 3.5 AtNIT interacts with both epsilon and non-epsilon 14-3-3 isoforms.
AtNIT1 and 4 YC fusions were tested for interaction with several 14-3-3 isoforms representing the two clades of the 14-3-3 isoforms. AtNIT1 or 4 YC fusions were transiently transformed with a 14-3-3 isoform YN fusion in *N. benthamiana* epidermal leaf cells. Expression and reformation of the split YFP was analysed using confocal laser scanning microscopy. Yellow fluorescence was detected using the YFP channel whereas the RFP channel was used to detect autofluorescence.

Scale bar represents 35µm.

For NIT1, several interactors of interest were identified: histidine kinase 2 (AHK2 - At5g35750.1, tryptophan synthase beta subunit 2 (TSB1- At5g54810), tryptophan biosynthesis 1 (TRP1 - At5g17990.1). Similarly, a number of interactors of interest were identified for NIT4, namely metacaspase 3 (AtMC3 - At5g64240.1), cysteine rich receptor like protein (RLP) kinase 13 (CRK13 - At4g23210.3) and myb domain protein 52 (AtMYB52 - At1g17950.1) and cysteine synthase C1 (At3g61440.1). The *Arabidopsis* nitrilases themselves (Mascot score: 110 to 4092), the 14-3-3s (Mascot score: 61 to 151) and the MAPK kinase 2 (AtMKK2 - At4g29810.1) (Mascot score: 25 to 26) were identified as common interactors for both NIT1 and 4. Collectively, these putative interactors had low mascot scores, between 20 to 50 as seen in table 3.2. However, these proteins did not appear in the negative controls nor are they considered to be abundant proteins such Tubulin, Rubisco and ribosomal proteins which are typically excluded from analysis (*76*) .

The AP-MS approach confirmed the predicted and experimentally tested (BiFC analysis) nitrilase dimer interactions (3.2.2 and 3.2.3). This gave validity to the AP-MS method and showed that all three approaches chosen are able to demonstrate the same interactions. Furthermore, several 14-3-3 isoforms were precipitated with both *Arabidopsis* nitrilases. The 14-3-3 proteins were identified with high Mascot scores, ranging from 61 to 151 (table 3.2). Interaction between 14-3-3s and NIT1 and 2 has been previously shown experimentally *via* affinity chromatography using a 14-3-3 exposed domain shared by multiple isoforms (*60*). This AP-MS experiment presented here suggested that NIT1 may interact with a smaller number of 14-3-3 isoforms as compared to NIT4 (table 3.2). Furthermore, mu 14-3-3 (GRF9) was the only isoform to be detected as an interactor for NIT1 but not for NIT4. These results raised the possibility that the interactions between the *Arabidopsis* nitrilases and 14-3-3 proteins may be isoform specific in both protein families. Together, the results presented here also give credibility to the accuracy of the AP-MS approach used here.

3.2.5 *Arabidopsis* nitrilases interact with the 14-3-3 protein family

As the work presented here and by Paul *et al* (*60*) was based on the same principle, *i.e.* the co-immunoprecipitation of one protein with another, there is the possibility that the interaction between *Arabidopsis* nitrilases and 14-3-3s are due to non-specific binding or artefacts. Thus, interaction between the 14-3-3 proteins and the *Arabidopsis* nitrilases

needed to be confirmed *via* an independent interaction assay such as BiFC. Furthermore, whether the interaction between 14-3-3 and *Arabidopsis* nitrilases is limited to a subset of the two protein families was investigated by testing all four nitrilase isoforms for interaction with a subset of 14-3-3 isoforms. Given that NIT4 shares the highest homology with the nitrilases from other plant nitrilases, conserved interaction may indicate that the 14-3-3 interaction is common to all plant nitrilases.

The AP-MS experiment presented here indicated that NIT1 and 4 may interact with a number of 14-3-3 isoforms (3.2.4, table 3.2). This further pointed to the possibility that interactions between the NIT isoforms and 14-3-3 proteins may be isoform specific. Data mining of publicly available microarray data was used to determine expression of the two sets of proteins (*30*). The expression data were used to perform a comparative analysis for 12 out of the 13 expressed 14-3-3 isoforms and the four *Arabidopsis* nitrilases to determine the expression of the two families (figure 3.3). With the exception of the iota isoform, an overlap of expression was observed across the 14-3-3 isoforms for NIT1, 2, 3 and 4. The most significant overlap of four *Arabidopsis* nitrilases was found with kappa and epsilon 14-3-3 isoforms especially in epidermal cells, guard cells, cotyledons, hypocotyls and seeds.

The kappa 14-3-3 isoform was selected for interaction with NIT1 to 4 as it was co-expressed with all four *Arabidopsis* nitrilases. The four NIT ORFs were previously cloned into the pG179NS-YC vectors (3.2.3). Several 14-3-3 isoforms including the kappa isoform were available in the complementary pG179NS-YN vector as lab stocks (*77*). The NIT and kappa 14-3-3 vectors were transiently transformed into epidermal leaf cells of *N. benthamiana*. Interaction between the individual nitrilase isoforms and 14-3-3 kappa was investigated three days after transformation using confocal laser scanning microscopy. To exclude that observed fluorescence is due to non-specific reformation of the split YFP protein, an N-terminally truncated 14-3-3 kappa YN was used as a negative control (data not shown). N-terminally truncated 14-3-3s are unable to dimerize which is a requirement for the ability of 14-3-3 proteins to interact with a client protein (*78*). Strong fluorescence was observed for the four *Arabidopsis* nitrilases co-expressed with kappa 14-3-3 (figure 3.4). Conversely no fluorescence was observed when co-expressed with the truncated kappa 14-3-3 YN construct, demonstrating that the fluorescence observed for the full length 14-3-3 protein and the *Arabidopsis*

nitrilases was due to specific protein-protein interactions. A lack of expression was confirmed to be not the reason for the lack of interaction observed using western blotting (2.5.4, appendix figure 1).

The results obtained here using an *in planta* system confirmed the outcome of the AP-MS experiment. It further showed that there is no discrimination in interaction between the nitrilase isoforms and kappa 14-3-3. This suggested that the *Arabidopsis* nitrilases and kappa 14-3-3 interaction is conserved and could be extended to other plant nitrilases. The cellular distribution for the interaction was exclusively cytosolic for the four *Arabidopsis* nitrilases. This was in contrast to the nitrilase-GFP localization studies which showed *Arabidopsis* nitrilases localized to both the nucleus and cytosol nuclear (figure 3.1). This may indicate that the interaction of *Arabidopsis* nitrilases with 14-3-3s retains the enzyme in the cytosol.

The results further showed that kappa 14-3-3 does not discriminate between the four *Arabidopsis* nitrilases. A second BiFC study was performed to determine whether the *Arabidopsis* nitrilases discriminate between 14-3-3 isoforms. To this end, four 14-3-3 isoforms were selected to be tested: two of those that have highly correlated expression with *Arabidopsis* nitrilases during many developmental stages (epsilon and phi 14-3-3) and two that are co-expressed with *Arabidopsis* nitrilases during limited developmental stages *e.g.* during flowering (iota and omega 14-3-3). This selection also took into account that two of the isoforms belong to the epsilon clade (epsilon and iota) and the other two belong to the non-epsilon clade (omega and phi) of the 14-3-3 family. This allowed for an analysis of evolutionary similarities and differences. Only NIT1 and 4 were used for this interaction analysis given the redundancy of the interaction and that NIT1 and 4 represent the two branches of plant nitrilase family. As described previously, NIT-YC and 14-3-3-YN constructs were co-transformed into *N. benthamiana* leaf cells *via Agrobacteria* infection. Interaction was determined using confocal laser scanning microscopy three days after transformation. Strong, cytosolic YFP fluorescence was observed in leaf cells for each of the 14-3-3 and NIT combinations (figure 3.5). The observations here are consistent with that observed with the kappa 14-3-3 isoform and the four nitrilases. Thus it appears that neither *Arabidopsis* nitrilases nor the 14-3-3 proteins show discrimination for specific isoforms. Given that five 14-3-3 isoforms were tested, it can be postulated that all twelve 14-3-3 isoforms will interact with the

Arabidopsis nitrilases, *i.e*. that there is a high degree of redundancy at least under the conditions tested here.

3.2.6 Analysis of NIT1 14-3-3 binding site

The interaction of nitrilases with 14-3-3 proteins could have several biological consequences including regulation of nitrilase activity, subcellular localisation or interaction with other proteins. The differences in subcellular localisation of nitrilase-GFP fusions and of nitrilase multimers on one hand (cytosolic and nuclear) and of NIT:14-3-3 interactions (cytosolic) on the other hand allowed the postulate that 14-3-3s may capture or direct nitrilases to the cytosol. To address this further it is desirable to obtain nitrilase mutants that are unable to interact with 14-3-3 proteins. This could be achieved by mutating the nitrilase region required for interaction with 14-3-3 proteins. A first step towards this is the identification of such nitrilase regions and of the contained 14-3-3 binding motifs. As all four nitrilase isoforms interact with 14-3-3 proteins, it was assumed that the binding motif is conserved in nitrilases. Thus, as a starting point, NIT1 was used to determine regions of the nitrilase proteins that are able to interact with 14-3-3 proteins.

Most 14-3-3 binding motifs reported so far contain a phosphorylated serine or threonine residue. As NIT1 lacks a typical 14-3-3 binding motif, the focus was on phosphorylated amino acid residues. The first step was to determine whether there are phosphorylated amino acids for NIT. PhosPhAT, a phosphorylation predictor and database, indicated that within NIT1, serine 329 is phosphorylated based on MS data (*79*). NIT1 contains a catalytic triad at amino acids 64, 152 and 185 which serves an important role in its enzymatic function. This region may be of importance in regards to the 14-3-3 binding site if 14-3-3s regulate the catalytic activity of NIT1. The phosphorylated serine 329 and the catalytic triad were used as starting points to determine the potential 14-3-3 binding site. The NIT1 protein was divided into five similarly sized fragments (figure 3.6) and generated by amplifying the coding regions from NIT1 cDNA *via* PCR. The five fragments were cloned into the pG179NS-YC vectors and then co-expressed in *N. benthamiana* leaf cells along with kappa 14-3-3 YN. Fluorescence was monitored after three days using epifluorescence microscopy. The analysis showed that only one fragment (amino acids 1 to 69) interacted with kappa 14-3-3 (figure 3.6, appendix figure 2). Again, the interaction was exclusively cytosolic.

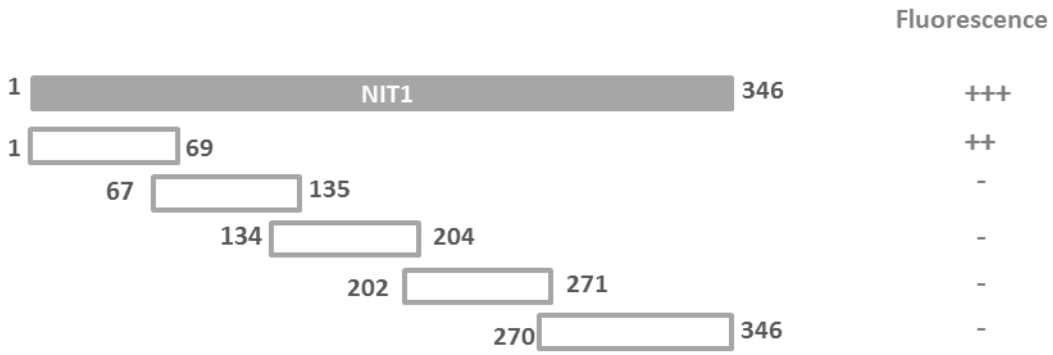


Figure 3.6 A schematic map of fragments used to determine which part of the Arabidopsis nitrilase 1 interacts with 14-3-3 proteins.
Interaction of the YN and YC fusion proteins for AtNIT1 and Kappa 14-3-3 as described in appendix figure 2 using BiFC. The fluorescence intensity was scored as strong (++), weak (+) or non detected (-). A detailed anaylsis where five fragment peptides of AtNIT1 were tested against Kappa 14-3-3 was performed. The analysis indicated that the binding site residues between amino acid 1 to 69.

The remaining four fragments did not interact with kappa 14-3-3. This analysis indicated that the binding site for 14-3-3 is most likely within the N-terminal end of NIT1, between amino acid residues 1 to 69.

3.3 Discussion

3.3.1 Summary

Here, investigations into the novel biological roles for the *Arabidopsis* nitrilase family were initiated through localization studies and by generating an interactome for the *Arabidopsis* nitrilases. The subcellular localization of the four nitrilase isoforms was determined by generating GFP fusions of the *Arabidopsis* nitrilases. The NIT-GFP fusions showed that all four isoforms are localized to both the cytosol and nucleus. An *in silico* analysis performed here using several PPI databases predicted that the *Arabidopsis* nitrilases can interact with each other like their maize and sorghum counterparts (*29, 41*). It was confirmed *in planta* that the *Arabidopsis* nitrilases are able to form such homo and heterodimers of all possible combinations when co-expressed. Similar to the nitrilase-GFP fusions, the subcellular localization of the dimers was both cytosolic and nuclear. Potential interactors were determined using both *in silico* and experimental methods. Several PPI databases for *Arabidopsis* were used to predict interactors for the *Arabidopsis* nitrilases. However, there was little consensus between the predictions of the different databases. AtPIN predicted the most unique interactors (36 in total) whereas AtPID and PAIR predicted only the *Arabidopsis* nitrilases as interactors. AP-MS had already shown that 14-3-3 proteins may interact with NIT 1 and 2 (*60*). The interaction with 14-3-3 proteins was confirmed here *in planta* using BiFC and it was further shown that NIT3 and 4 also interact with 14-3-3s. This indicated that the 14-3-3:nitrilase interaction may be conserved in plants. There was no isoform specificity between 14-3-3 and NIT isoforms. All four isoforms were able to interact with 14-3-3s of both the epsilon and non-epsilon clades. Unexpectedly, the interaction between 14-3-3s and *Arabidopsis* nitrilases was restricted to the cytosol. This was in contrast to the nuclear and cytosolic localization of the NIT-GFP fusion proteins and the NIT dimers. This is puzzling as the 14-3-3s themselves are also known to have nuclear and cytosolic localization (*77*). The localization of NIT:14-3-3 to the cytosol may indicate sequestration as a biological role for the interaction. The interaction of NIT1 and kappa 14-3-3 was mapped to the first 69 amino acids of NIT1 using small peptide fragments covering the nitrilase protein in combination with *in planta* BiFC analysis.

3.3.2 Subcellular localization of the *Arabidopsis* nitrilases

An important point of consideration when determining biologically relevant PPIs is whether interacting partners are spatially together *in vivo* allowing the interaction to occur. Whilst there have been GFP fusion studies for NIT1 which showed NIT1 localized to the nucleus as well as the cytosol, the localization patterns of the other three isoforms was not known prior to this thesis (*25*, *45*). Using GFP fusions, it was shown that the localisation of the other three *Arabidopsis* nitrilases be identical to NIT1, *i.e*. cytosolic and nuclear (3.2.1). The nuclear localisation is somewhat puzzling as *Arabidopsis* nitrilases lack typical mono or bipartite nuclear localisation signals and do not have lysine rich regions which are typically found in such signals. However, an analysis of plant nitrilase sequences as done part of this thesis shows that the *Arabidopsis* nitrilases have a LGKHRK motif which is quite conserved within plant nitrilases (figure 3.7) (*80*). This motif resembles an atypical NLS (LGKR(K/R)(W/F/Y)) which was identified by Kosugi *et al* as a motif binding to the minor groove of the nuclear import receptor importin α (*81*). Thus, it may be possible that nitrilase import into the nucleus uses an atypical NLS. It has to be kept in mind that small proteins (< 60 KDa) such as GFP and small GFP fusion proteins may diffuse into the nucleus without the aid of an import receptor protein (*82*). As NIT-GFP fusion proteins are around 60 KDa in size, such diffusion may have occurred, especially when considering that expression under the control of the strong CaMV 35S promoter may have led to high nitrilase protein levels usually not found in cells. However, the restriction of similarly small NIT:14-3-3 interactions to the cytosol argues against a migration of nitrilases into the nucleus due to size (3.2.5) despite the small sizes of these proteins. Furthermore, the *Arabidopsis* nitrilases homodimers (3.2.3, figure 3.2) have a combined total size greater than 60KDa yet also show the same cytosolic and nuclear localization pattern as the nitrilase-GFP fusion proteins. Thus, passive diffusion may not be the reason behind the nuclear localization. This question could be resolved in future work by determining the localisation of nitrilase mutant proteins in which the putative atypical NLS is changed. Such mutant proteins would also be useful for investigations into the biological significance of the nuclear localisation of the *Arabidopsis* nitrilases.

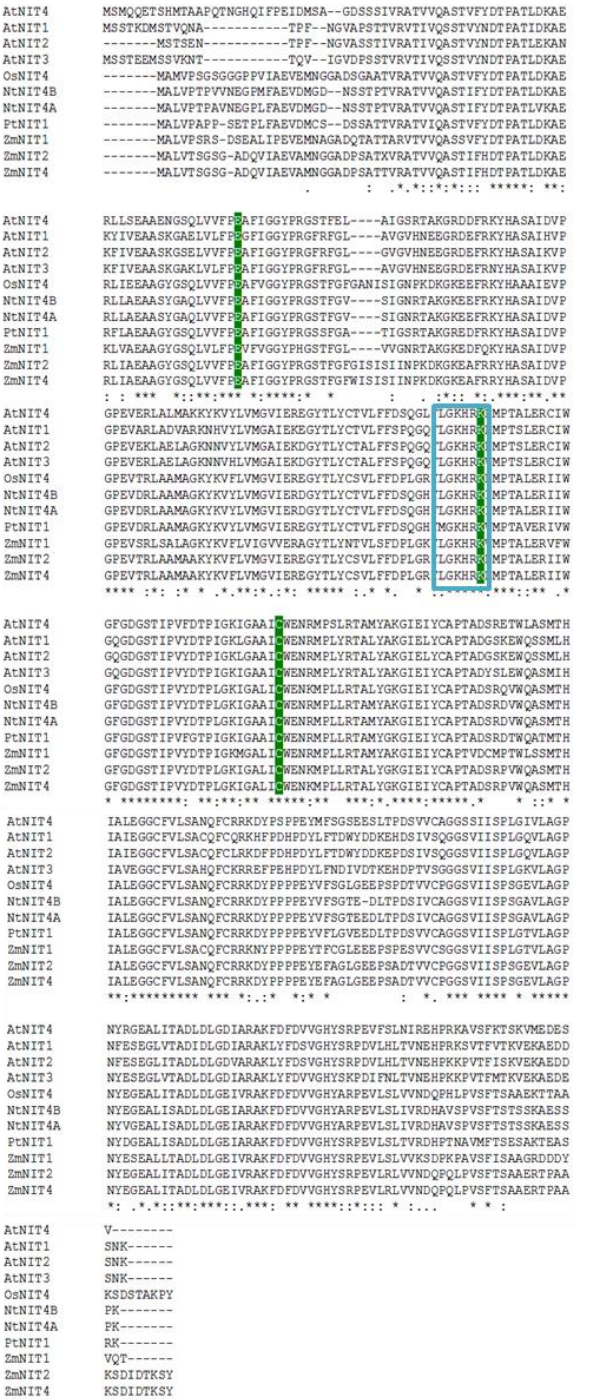


Figure 3.7 A multiple sequence alignment of AtNIT 1 to 4 and their homologues from other plant species showing the conservation of a putative nuclear localization signal

The alignment shown here using ClustalO sequence alignment programme represents the amino acid sequences of the AtNIT1 to 4 proteins and nitrilase homologues of Maize (Zea Mays), Rice (Oryza sativa), Tobacco (Nicotiana tabacum) and Populus (Populus trichocarpa) (http://www.ebi.ac.uk/Tools/msa/clustalo/). Amino acid sequences for the four AtNITs were retrieived from TAIR (http://www.arabidopsis.org/) whereas sequences for the other plant nitrilases were retrieved from UniProt (http://www.uniprot.org/).The catalytic triad within the nitrilase enzyme is hightlighted in green and the highly conserved putative NLS is highlighted in the blue box.

3.3.3 *Arabidopsis* nitrilases form both homo- and heterodimers

Studies involving bacterial nitrilases have shown that functionality requires oligomerization and that this is important in substrate preference and binding. Formation of nitrilase oligomers and the dependence of substrate preference and enzymatic activity on composition of such oligomers were also observed for maize and sorghum nitrilases indicating similarities between plant and bacterial nitrilases (*29*, *41*). Furthermore, it was demonstrated that NIT1 can form large oligomeric structures similar to those observed for bacterial nitrilases (*25*). Using an *in planta* method it was shown here (3.2.3) that *Arabidopsis* nitrilases form homo and heterodimers. Furthermore, it was observed that all nitrilase dimers studied occur in the cytosol and nucleus resembling the localisation of NIT-GFP fusion proteins (3.2.1). Whilst the studies performed here cannot allow for interpretation of the results beyond dimerization, it can be postulated that all *Arabidopsis* and possibly plant nitrilases may form larger oligomeric structures with various subunit compositions.

Maize and sorghum nitrilases are considered to be dual function enzymes; they are involved in both auxin synthesis and cyanide detoxification. The dimerization state affects their substrate selection and thus alters their enzymatic role. With this in mind, it can be further postulated that the ability to form dimers of various nitrilase subunit composition indicates that the enzymatic role of *Arabidopsis* is regulated by their subunit composition. If this is true, then one would assume that *Arabidopsis* nitrilases are also dual function enzymes like their maize and sorghum counterparts, capable of hydrolysing nitriles derived from a wider range of metabolic pathways such as the glucosinolate-myrosinase pathway, auxin synthesis, and cyanide detoxification. Specificity and determination of biological function may then be determined by the spatial and temporal regulation of their expression. This possibility is supported by the analysis of microarray data performed here that showed that NIT1 and 2 are expressed in most tissues whilst expression of NIT3 and NIT4 was restricted mostly to root and floral tissues respectively (3.2.1, figure 1.1). The duality of *Arabidopsis* nitrilases could be explored by generating *Arabidopsis* mutants that are over-expressing different combinations of the *Arabidopsis* nitrilases and performing metabolomic profiling to determine whether metabolites involved in auxin, cyanide and glucosinolate synthesis is altered. Another aspect which should be further investigated is how the homo- and

heterodimeric states of the *Arabidopsis* nitrilases impacts on the interaction of nitrilase oligomers with other proteins and how this determines functionality.

3.3.4 Establishing an interactome for *Arabidopsis* nitrilases

In this study, an interactome for the *Arabidopsis* nitrilases was to be established using a combination of bioinformatic data mining and experimental methods (3.2.2, table 3.1). Several protein interaction prediction databases were used to determine potential interaction partners for the four *Arabidopsis* nitrilases. From the *in silico* investigation, no consensual interaction partners were found across the different programmes which is most likely due to the different algorithms and datasets used by these programmes. However, the bioinformatic analysis does provide some insight into potential functional networks of the *Arabidopsis* nitrilases. For example STRING predicted functional interactions of the NIT 1 homologues (NIT1 to 3) with enzymes involved in the auxin synthesis or glucosinolate catabolism (3.2.2). These interactors would be in agreement with nitrilases' assumed involvement in auxin synthesis and glucosinolate detoxification. The STRING based analysis further predicted that NIT4 may also interact with proteins involved in auxin/glucosinolate synthesis such as the YUCCA protein, myrosinases as well as cysteine synthase C1 which is involved in cyanide detoxification. This suggest that NIT4 could be involved in pathways similar to the NIT1 homologues and further supports the hypothesis that the *Arabidopsis* nitrilases are dual function enzymes. AtPID and AtPIN predicted the formation of NIT heteromers which was confirmed experimentally using BiFC (3.2.3, table 3.1). AtPIN also predicted that *Arabidopsis* nitrilases may interact with several nuclear/nucleolar localized proteins such as the P21CIP1-binding protein-related protein which is believed to be involved in cell-cycle regulation through inhibiting cyclin-dependent kinases. An interaction with cell cycle regulating proteins would be in agreement with reports that NIT1, similar to mammalian nitrilases, participates in the regulation of cell proliferation and cell division (*25*). Gar1 RNA-binding region family protein, another nuclear/nucleolar localized protein were predicted to interact with NIT3. Interaction with nuclear/nucleolar proteins would lend credibility to the localisation of nitrilases in the nucleus which was shown here (3.2.1, 3.2.3) and for NIT1 in previous publications (*25, 45*). Further predicted interactors of *Arabidopsis* nitrilases are ATUBP3 and NPL4 family protein (NIT2 and 3 respectively). These are involved in ubiquitin-dependent protease activity. These predicted interactions may be true as NIT1 is known to be ubiquitinated and to interact with

RAD23 which is involved in DNA repair and proteosomal degradation (*43, 44*) A sulfate transporter, SULTR3.1, is predicted to interact with NIT2. This would be in agreement with transcriptomic and localisation data that were interpreted by suggesting a role of nitrilases in sulfur starvation responses (*15, 83*). ROP10, a small GTPase which negatively regulates ABA signalling, was predicted by AtPIN to interact with NIT3 (3.2.2, table 3.1) (*84*). This may indicate a role for nitrilases in abiotic stress responses as ABA is one of the main phytohormones in these response pathways. Whilst the effect of ABA on NIT3 is not known, protein levels of NIT1 and 2 are known to be upregulated in response to ABA further supporting the hypothesis that nitrilases are involved in abiotic stress responses (*31*).

An experimental approach based on AP-MS was chosen as a second way to identify potential interactors of nitrilases, (3.2.4). This approach found that NIT1 and 4 interact with the other *Arabidopsis* nitrilases. The *in silico* predictions of NIT dimerization and the experimental confirmation of such interactions by BiFC and AP-MS (3.2.2. 3.2.3, 3.2.4) validate each other and demonstrate that these interactions are true. Furthermore, AP-MS also identified cysteine synthase C1 as an interactor for NIT4 (3.2.4, table 3.2) which was predicted by STRING to be a functional partner for NIT4 (3.2.2). Aside from confirming NIT4's involvement in β-cyanoalanine synthesis, it also validates the methodology in which a combination of bioinformatic datamining and experimental approaches are used to determine the biological roles of a protein.

Some of the experimentally identified NIT1 interactors, such as TSB1 and TRP1 which are involved in auxin and glucosinolate synthesis, could be expected as they fit into the presumed roles of nitrilases. Other putative interactors indicate that *Arabidopsis* nitrilases may have additional functions. For example, a few of the putative interactors are said to be involved in ABA or SA signalling based on TAIR annotations (AtMKK2, CRK13, AHK2 and AtMYB52). This suggests that *Arabidopsis* nitrilases may also be involved in ABA and SA signalling. ROP10, a predicted interactor for NIT3, is involved in the negative regulation of ABA signalling. Thus it can be inferred that the involvement in ABA may be conserved between the four *Arabidopsis* nitrilases. At the current time, many of the interactions implied here are still uncertain as Mascot scores for the peptide used to identify interacting proteins are quite low. The Mascot score is based on a probability based scoring system that enables judgment on whether an

obtained result is significant or a random event. The Mascot scores for the proteins discussed above are quite low. However, as the Mascot score is a probability score, it is somewhat open to interpretation. To achieve a higher degree of certainty about the interaction of nitrilases with the proteins discussed, these putative interactors would need to be tested for interaction using independent interactions assays such as Y2H or BiFC which has been used in this thesis to explore the dimerization of the *Arabidopsis* nitrilases.

3.3.5 Conserved NIT and 14-3-3 interaction

In addition to the above discussed putative interactors, the AP-MS approach identified a number of 14-3-3 proteins as interactors for the *Arabidopsis* nitrilase proteins. The Mascot scores for these interactors were high, lending credibility to the interactions (table 3.2). Further experimental evidence for the interaction of *Arabidopsis* nitrilases with 14-3-3 proteins was obtained using BiFC studies which showed that all four *Arabidopsis* nitrilases can interact with kappa 14-3-3. This confirmed the previous reported identifications of NIT1 and 2 with 14-3-3 proteins (*60*) and further extends this to all four *Arabidopsis* nitrilases.

NIT4 is considered to be the primordial nitrilase, sharing the highest sequence identity with nitrilase of other plant species (*27*). The interaction of 14-3-3 proteins with the four *Arabidopsis* nitrilases and especially with NIT4 suggests that interaction of nitrilases with 14-3-3 proteins may be conserved in other plant nitrilases. The 14-3-3 family is large and evolutionarily conserved which suggests functional and isoform specificity (*85*). However, it was found that 14-3-3 proteins behave like redundant proteins. For example, several *Arabidopsis* 14-3-3 isoforms were able to complement lethal yeast 14-3-3 double mutant (*71*). Thus, 14-3-3 specificity may be derived from their spatial and temporal expression patterns and their subcellular localisations. This is supported by GFP fusion protein studies which showed that the varying 14-3-3 isoforms can have differential localization patterns (*86*). Furthermore, transcriptomic data analysed here showed that the expression patterns of twelve 14-3-3 proteins differ from each other, for example iota 14-3-3 is mostly expressed in floral tissue whilst kappa 14-3-3 is found in most *Arabidopsis* tissues (figure 3.3). The formation of 14-3-3 homo and heterodimers may further support the notion that 14-3-3 proteins *in vivo* may have specificity with individual dimers tailored to different target proteins. A co-expression analysis

performed here showed that not all 14-3-3 isoforms are co-expressed with each other and with *Arabidopsis* nitrilases (3.2.5). On the other hand, all tested 14-3-3 proteins (epsilon, iota, omega and phi) from the epsilon and the non-epsilon 14-3-3 clades were able to interact with the four nitrilases (3.2.5). These results further support the hypothesis that 14-3-3 proteins are redundant with regards to their ability to interact with target proteins and that specificity is mediated by expressional regulation and localisation rather than by the 14-3-3 amino acids sequence and structure. In the debate about redundancy and specificity of 14-3-3 proteins one also has to take into account the more recent studies that have shown that the gradient of the interaction affinity plays an important role in driving isoform specificity in 14-3-3s rather than the isoforms themselves. The binding affinity of 14-3-3 isoforms to sucrose phosphate synthase (SPS) was investigated using quantitative yeast two-hybrid analysis, revealing significant differences in binding affinities between 14-3-3 isoforms and SPS (*87*). Whilst this study showed that specificity is not driving the interaction this is not necessarily applicable to all 14-3-3: client protein interactions. Pallucca *et al* showed that the proton pump, H^+/ATPase's activity is dependent on the 14-3-3 isoform it interacts with (*88*). Activity assays revealed that when bound to the non-epsilon isoforms, H^+/ATPase has higher activity than when interacting with epsilon isoforms. Nitrate reductase similarly shows 14-3-3 isoform specificity which in turn affects enzymatic activity (*89*). These experiments suggest that the functional redundancy and specificity is not binary – whether the 14-3-3 isoforms will bind or not to the client protein. The binding preference of the client protein is certainly, at least to a degree, dependent on the expression patterns and binding affinities of the individual isoforms.

Similar to the 14-3-3 proteins, the *Arabidopsis* nitrilases did not show any isoform specificity for 14-3-3 proteins when tested under BiFC conditions. In contrast to that, the AP-MS analysis indicated some degree of selectivity. It is unclear if the observed differences are due to the experimental approaches being different. Both systems as use highly over-expressed bait proteins (NIT-GFP or NIT-YC/YN). In BiFC, the prey protein (14-3-3-YN) is also highly over-expressed whilst in the AP-MS experiment; 14-3-3 proteins were expressed only by their endogenous promoters. Furthermore, the AP-MS experiment was performed in the native (*Arabidopsis*) background of these proteins whilst the BiFC studies relied on a different plant species. Maybe more significantly, AP-MS is less reliable in identifying weak and dynamic protein interactions which are

often stabilised in BiFC. This may explain some of the differences observed, *i.e.* 14-3-3 proteins that interact weakly in BiFC may be detected as interactors but lost in AP-MS. Due to the nature of BiFC, it was difficult to assess the affinity of the binding for the different isoforms to the *Arabidopsis* nitrilases to determine whether affinity is driving a level of specificity and thus explaining the discrepancies observed from the AP-MS and BiFC studies. This could be investigated *via* quantitative Y2H as done with SPS and 14-3-3 isoforms as mentioned previously. Fluorescence resonance energy transfer (FRET) is another quantitative method which could be used to assess binding affinities for the NIT:14-3-3 interactions. Finally, the AP-MS experiment was only conducted once and results may be incomplete whilst BiFC analysis was conducted several times with many cells studied in each experimental repeat and hence the result is statistically more significant.

3.3.6 Biological role of the NIT:14-3-3 interaction

The 14-3-3 proteins regulate client proteins through enzymatic activation/inactivation, alteration of localization, facilitate protein-protein interactions or alteration of the degradation state. Binding to 14-3-3 proteins is often associated with amino acid motifs in the target protein that contains phosphorylated serine or threonine residues (*70*). NIT1 has many serine and threonine residues with one predicted as a phosphorylation site. However, work done by Grisc *et al*, suggests that the enzymatic function of NIT is not affected by their phosphorylation status (*90*). Furthermore, expression of *Arabidopsis* nitrilases in *E. coli* and testing the activity of the expressed proteins showed that nitrilases maintained their expected enzymatic function despite lacking any post translational modifications such as phosphorylation (*18, 36*). The peptide fragment analysis presented here showed that the predicted phosphorylation site of NIT1 (serine 329) is not part of the 14-3-3 binding region. These reasons strongly argue against a phosphorylation dependent regulation of *Arabidopsis* nitrilases activity by 14-3-3s. It seems more likely that 14-3-3s play a role in sequestration of nitrilases in the cytosol. This was demonstrated by BiFC studies that showed that NIT:14-3-3 complexes are located only in the cytosol whilst nitrilase oligomers are also found in the nucleus. The BiFC experiments were in agreement with the NIT-GFP localisation studies that demonstrated nitrilases' presence in both the cytosol and the nucleus. As 14-3-3 proteins can also be found in the nucleus when studying 14-3-3 dimers using BiFC (*77*), it can be excluded that the NIT:14-3-3 complex found only in the cytosol is determined

by restriction of 14-3-3 protein localisation. Sequestration of proteins in the cytosol by 14-3-3 proteins is known for other proteins and can hence be used as a model for the sequestration of *Arabidopsis* nitrilases in the cytosol. For example, the tobacco Repressor of Shoot Growth 1 (RSG1) protein regulates gibberellic acid biosynthesis when in the nucleus. Binding to 14-3-3 proteins sequesters RSG1 to the cytosol, preventing its ability to activate GA biosynthesis genes (*91*). In the above example, binding to 14-3-3 proteins alters the localization of the client protein, thereby perturbing its function. Whilst *Arabidopsis* nitrilases have not been reported to have any transcriptional activation activity, some of the predicted interactors for *Arabidopsis* nitrilases (3.2.2) are localized to the nucleus or involved in RNA binding, pointing to a possible role of nitrilases in the nucleus. These activities could be associated with the known cell cycle and growth related functions of plant nitrilases (*25*). Therefore, it can be postulated that sequestration of *Arabidopsis* nitrilases to the cytosol by 14-3-3s prevents nitrilase activity in the nucleus.

Another reason for NIT:14-3-3 interaction could be the ability of 14-3-3s to mediate interaction between two target proteins. It is known that NIT1 can be ubiquitinated and it was predicted here that proteins which are involved in the ubiquitin-protease pathway interact with *Arabidopsis* nitrilases (*43*) (3.2.2). The homology between the *Arabidopsis* nitrilases suggests that all four isoforms could be ubiquitinated. Interaction of 14-3-3 proteins with nitrilase could be involved in the ubiquitination of the enzyme. This would be in analogy to 1-aminocyclopropane-1-carboxylate synthase (ACS) which upon binding to 14-3-3s is stabilised and then subsequently degraded *via* the ubiquitin-protease pathway (*92*).

3.3.7 Conclusion

This work has furthered the understanding of the biological function of *Arabidopsis* nitrilase by determining new interactors and characterizing one of those interactions in depth. Here, the possible involvement in abiotic and biotic stresses has been postulated due to the predicted and experimentally determined novel interactors of the *Arabidopsis* nitrilases. Further investigation into the potential of *Arabidopsis* nitrilases involved in stress responses is required. The homo- and heterodimerization ability of the *Arabidopsis* nitrilases shown here indicate the possibility of functional duality similar to other plant nitrilases. The in-depth characterization of the 14-3-3:NIT interaction has

provided some new insight into how the *Arabidopsis* nitrilases may function and regulated in the cell. Whilst the AP-MS study here has reported several novel interactors for NIT1 and 4; false positives are common, thus independent interaction assays are required to confirm the putative interactions. Regardless, a common theme can be observed from the predicted interactors and those determined experimentally which indicates that *Arabidopsis* nitrilases may have a direct role in mediating plant stress and interactions within the nucleus.

Here it was shown that all four NIT isoforms bind to the 14-3-3 isoforms, indicating a conservation of interaction. This implies that the interaction may extend to nitrilases of other plant species where 14-3-3s are found. Furthermore, there is a degree of redundancy for the 14-3-3 isoforms when interacting with the *Arabidopsis* nitrilases. The GFP localization studies and the interaction assays presented here led to the postulate that *Arabidopsis* nitrilases are sequestered to the cytosol by 14-3-3 proteins. The limitations of BiFC made it difficult to explore the dynamics of the interaction and discern how altering the localization of the *Arabidopsis* nitrilases affects the cellular biology.

Taken together, a combination of *in silico* and experimental analysis of interactors for the *Arabidopsis* nitrilases have initiated a new direction for assessing the biological roles these enzymes could have in addition to those already established through enzymatic characterization. An in depth characterization of one of those novel interactions, involving the 14-3-3 proteins, has already pointed out novel characteristics of the *Arabidopsis* nitrilases which could be extended to other plant nitrilases and could influence their function. Wider applicability of this could be tested by extending the work presented here to other plant nitrilases.

Chapter 4

Role of *Arabidopsis* nitrilases in JA signalling pathway

4.1 Introduction

4.1.1 Nitrilases and plant defence

Initially, *Arabidopsis* nitrilases were reported to be involved in only auxin synthesis. However, studies which looked into the involvement of nitrilases in auxin synthesis showed that *Arabidopsis* nitrilase knockout mutants lack the typical auxin starved phenotype (*58*). In addition, enzymatic studies indicated that IAN is a poor substrate for NIT1 homologues (*18*). This suggested alternative biological roles for the *Arabidopsis* nitrilases. Cyanogenic glycosides, glucosinolates and camalexins are defined as secondary metabolites which share common precursors and synthesis pathways (*5*, *93*). In maize and sorghum, nitrilases are involved in the breakdown of cyanogenic glycosides as part of cyanide production and detoxification (*93*). *Arabidopsis* plants like other cruciferous plants lack cyanogenic glycosides but rather have glucosinolates and camalexins (*94*). When catabolized by myrosinases, glucosinolates break down into nitrile products such as isothiocyanates and epithionitriles, all of which are possible substrates for nitrilases.

Glucosinolate catabolism occurs as a result of herbivory attacks or mechanical wounding; both defined as biotic stresses (*5*). Another form of biotic stress comes from pathogen attacks; together, herbivory and pathogen attacks form the majority of biotic stresses plants face in the environment. These two forms of biotic stress elicit two branches of defence responses in plants which are tightly controlled through signalling networks (*95*). Such networks are regulated by phytohormones which are signalling molecules released by plants in response to biotic and abiotic stresses. Phytohormones integrate the external stress into endogenous signals, allowing plants to respond. This works both in an antagonistic and synergistic manner in accordance to the type of stress, allowing a fine-tuned response (*96*). This fine tuning is beneficial to the plant as it could be dealing with a combination of both insect and pathogen attacks and by doing so it allows a more cost efficient manner to deal with the invader. Thus it can be said that the phytohormonal network is a complex mechanism which aids the plants' survival in changing environments. The three major phytohormones involved in plant defence are salicylic acid (SA), jasmonate acid (JA) and ethylene (ET). Abscisic acid (ABA), gibberellins (GA), auxin and cytokinins (CK) are also phytohormones involved in plant defence but to a lesser degree (*96*).

The AP-MS study presented in chapter three indicated that *Arabidopsis* nitrilases may interact with proteins such as AtMKK2 which are involved in biotic and abiotic stresses. This may indicate that plant nitrilases may have a broader biological role which goes beyond their ability to hydrolyse nitriles derived from the different pathways which are involved in plant defence.

4.1.2 JA signalling

When induced by herbivory or pathogen attack, JA is synthesised *via* the oxylipin biosynthesis pathway (*97*). JA in turn is converted in to the biologically active enantiomer jasmonoyl-isoleucine (JA-Ile). Transcription of JA-responsive genes is repressed by JASMONATE ZIM (JAZ) transcriptional repressor proteins along with several co-factors such as NINJA and HDA6. Activation of these genes requires the ubquitination and degradation of the JAZ proteins. JA-Ile binds to COI1, a F-box protein which acts as its receptor in the E3 ubiquitinin-ligase SKP1-Cullin-F-Box complex SCF^{COI1} (*98*). As illustrated in figure 4.1, upon binding to JA-Ile, SCF^{COI1} is activated which then ubiquitinates the JAZ protein leading to its degradation and allowing the transcription of the JA-responsive genes and thus activation of the signalling pathway (*98*). The JA signalling pathway can be divided into two branches of signalling – the MYC and ERF branch; with each branch regulated by different sets of transcription factors and downstream genes. The MYC branch is responsible for the wounding/herbivory defence response mediated by JA signalling. Conversely, along with ET, the ERF branch responds to necrotrophic pathogens.

The MYC branch is controlled by MYC2, a bHLH transcription factor. MYC2 activates wound responsive genes such as VSP2, TAT1, JR1 and LOX upon degradation of the JAZ proteins (*99*). The ERF branch is regulated by a set of ET signalling transcription factors and requires ET to act synergistically with JA. In the absence of JA, EIN3/EIL1 are repressed by the JAZ proteins and associated co-factors. Along with ET which stabilises EIN3/EIL1, JA signals the degradation of the JAZ proteins and thus the depression of EIN3/EIL1 (*100*). This allows EIN3/EIL1 to activate another set of transcription factors, ERF1 and ORA59 (*101*). The combination of these two transcription factors is then able to activate genes required for necrotrophic pathogen defence such as *PDF1.2*.

MYC2 itself is said to be a master regulator of the JA signalling and cross-talking pathways. The two branches converge through the regulation by MYC2 (*102*). An antagonistic relationship exists between the two branches which fine tunes the JA signalling response as dictated by the type of stress. MYC2 not only regulates the wound response but also regulates the ERF branch through the repression of EIN3/EIL1. Herbivory attacks not only initiate the expression of the MYC branch but also supresses the ERF branch. MYC2 interacts with EIN3/EIL1 to repress the transcription of ORA59 which is required for the necrotrophic pathogen response (*101, 103, 104*). The converse can also be said of EIN3/EIL1. It has been shown that EIN3/EIL1 are also able to repress the transcriptional activation function of MYC2 to supress the wound/herbivore response (*103*). Thus it could be said that JA and ET act both synergistically and antagonistically, modulating the response according to the type of stress the plant experiences.

4.1.3 SA-JA cross-talk and the plant MAPK cascade

SA signalling is another major component of plant defence; it initiates the systemic acquired response (SAR). Unlike JA signalling, SA signalling responds to biotrophic pathogens. In response to elicitors, SA is synthesised which leads to the dissociation of the NPR1 oligomer, allowing the monomers to translocate into the nucleus to activate a large set of defence-related genes such as PR-1 and the WRKY transcription factors (*95*).

The SA-JA cross-talk was first observed in tomato and was subsequently shown to be an antagonistic relationship (*105*). Leon-Reyes *et al* have further shown that the suppression does not occur at the level of JA biosynthesis but rather downstream of the JA signalling pathway (*106*). The suppression occurs *via* NPR1 in response to SA accumulation where it negatively regulates JA-responsive genes, *PDF1.2* and VSP2 (*107*). However, the cross-talking is not simply just antagonistic but also synergistic at low concentrations of SA and JA (*108*). Thus, the concentration, timing and sequence of the SA and JA signalling dictate the outcome of the cross-talking.

Plant MAPK (mitogen activated protein kinases) cascades have important roles regulating development and mediating plant signals in response to biotic and abiotic

stresses. The MAPK family in *Arabidopsis* consists of 20 MAPKs, 10 MAPKKs and 60 MAPKKKs (*109*). In plants, the MAPK cascade is one of the earliest signalling events in plant defence. In particular, the MPK4 cascade is involved in mediating plant defence through regulating both JA and SA signalling. Knockout mutants affecting the MPK4 cascade indicated that it negatively regulates the defence response. Mutants devoid of key components of the cascade resulted in constitutive activation of defence response genes (*110*). Peterson *et al* showed that *mpk4* mutants have increased SA levels and constitutive expression of PR1-1,2 and 5 and thus have constitutive SAR (*111*). Furthermore, in this mutant, *PDF1.2* is not constitutively expressed and neither can exogenous MeJA application induce *PDF1.2* expression (*111*). Together, this indicates that MPK4 positively regulates JA signalling whilst negatively regulating SA signalling. It also suggests that the MPK4 cascade specifically responds to necrotrophic and herbivory attacks which are regulated by JA signalling. The negative regulation of SA signalling by MPK4 further involves both EDS1 and PAD4, which are both part of the SAR (*112*). Thus it can be said that the MPK4 mediates the SA-JA antagonism through EDS1 and PAD4.

4.1.4 Glucosinolates and JA

Glucosinolates are a class of phytoanticipins which form a part of plant defence against insect herbivores and wounding. These secondary metabolites can be divided into either aliphatic or aromatic glucosinolates. When challenged with insect herbivory or physical wounding, glucosinolates are hydrolysed into toxic compounds which deter insects from further attacking the plant. In *Sinapis alba* and *Brassica napus*, MeJA increases levels of indolic glucosinolates (*113*). This was also observed in *Arabidopsis* (*114*). Thus, JA signalling is linked to glucosinolate accumulation and the expression of genes required for their biosynthesis. In addition, SA has also been shown to alter glucosinolate levels in *Brassica napus* (*115*).

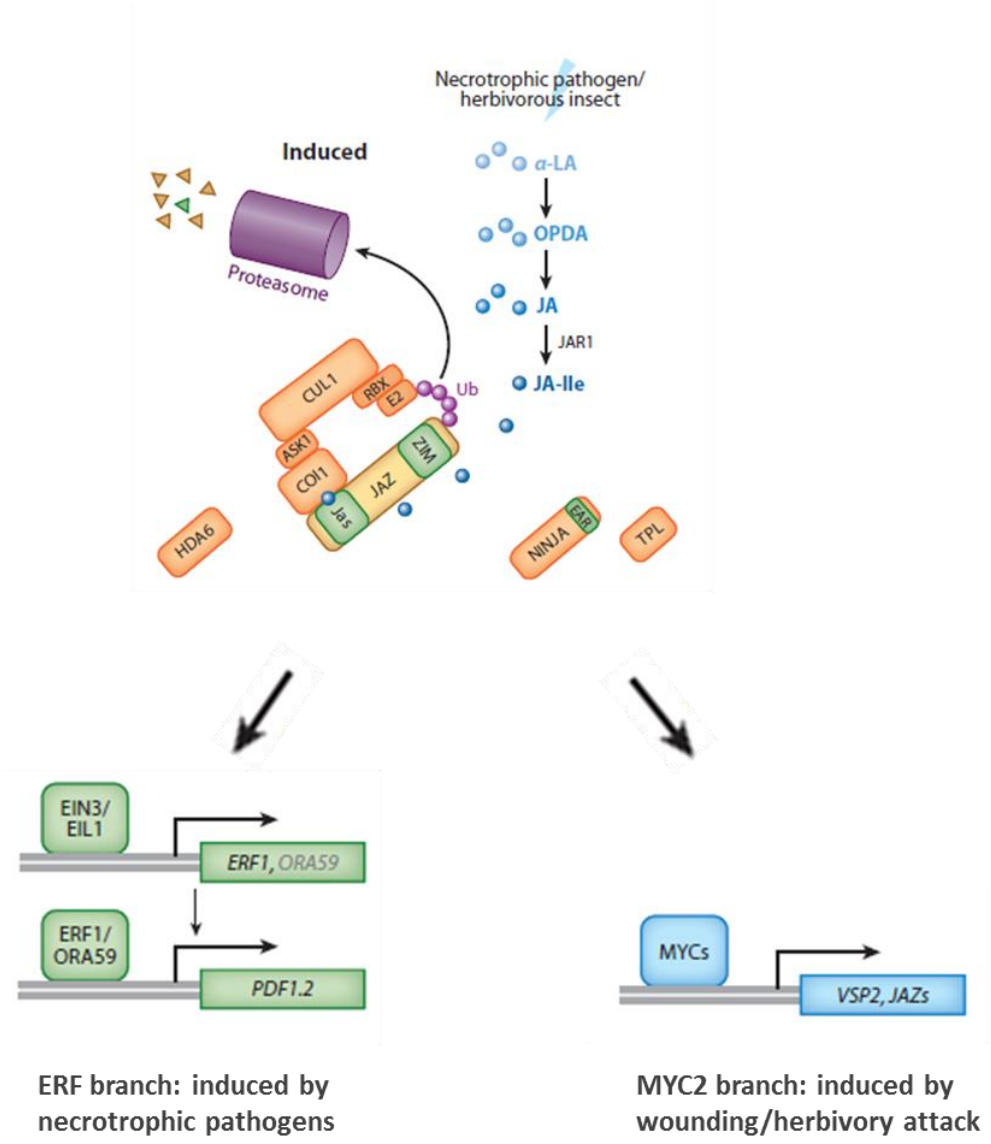


Figure 4.1 A schematic diagram illustrating the JA signalling pathway
Transcription of JA induced genes is suppressed by JAZ proteins and associated co-factors (HDA6 and NINJA). Necrotrophic pathogens, herbivores and wounding trigger a signalling cascade involving jasmonic acid that results in the formation of JA-Ile. JA-Ile binds to and activates the SCF^{COI1} ubiquitin ligase complex. The activated SCFCOI1 complex ubiquitinates JAZ proteins and associated factors which are then degraded via the proteasome. Degradation of the JAZ proteins then allows transcription of JA responsive genes. There are two branches of responses, each regulated by different transcription factors. The ERF branch is induced by necrotrophic pathogens whereas the MYC2 branch is induced by wounding or herbivores

Image adapted from Pieterse *et al (95)*

Out of the three major hormones involved in plant defence, JA is the most potent elicitor of glucosinolate biosynthesis. Mewis *et al* investigated the levels of glucosinolates in several mutant lines which are defective in JA, SA or ET signalling pathways – *coi1* (JA insensitive), *npr1* (SA insensitive) and *etr1* (ET insensitive) (*116*). They found that *coi1* mutants had decreased levels in glucosinolates. Furthermore, the *coi1* mutants have a lower resistance to most of the insect herbivores. In *etr1*, there was no change to the glucosinolate levels as compared to the wild type. Interestingly, in the *npr1* mutants, there was an increase in aliphatic glucosinolates in comparison to wild type *Arabidopsis*.

Proteins of the cytochrome P79 family catalyse the key steps in glucosinolate synthesis. Altering the expression profiles of these cytochromes will lead to a massive effect on the glucosinolate profile. For example, the synthesis of indolic glucosinolates requires CYP79B2/B3 whereas the CYP79F1/F2 are responsible for aliphatic glucosinolates. Mikkelson *et al* showed that both CYP79B2/B3 were highly induced in response to MeJA and that indolic glucosinolate levels had also increased. A similar effect was also observed for CYP79F1/F2 (*117*). MYC2, the master regulator of JA signalling, regulates both glucosinolate biosynthesis and accumulation. A triple knockout, myc2/3/4 was shown to have reduced expression of key biosynthesis genes such as CYP79B3 and SUR1 as compared to the wild type. This mutant was further shown to have reduced levels of glucosinolates (*118*). In addition, MYC transcription factors also interact with MYB transcription factors involved in glucosinolate biosynthesis, further linking JA signalling with glucosinolate biosynthesis (*118*, *119*).

4.1.5 Aims and hypotheses

The role(s) *Arabidopsis* nitrilases play in plant defence is not clearly defined. It has been suggested that the *Arabidopsis* nitrilases are involved in detoxifying the toxic compounds derived from hydrolysed glucosinolates in response to herbivory attacks. A link between nitrilases and JA seems to exist; microarray data showed that NIT1 and 2 are upregulated in response to MeJA (*34*). The understanding that JA signalling regulates glucosinolate levels and synthesis as well as responses to necrotrophic pathogens may link nitrilases to broader functions including in plant defence pathways controlled by JA signalling. The following three aims are directed towards an investigation of potential wider roles nitrilases have in plant defence responses:

1. Establish a potential connection between JA signalling and *Arabidopsis* nitrilases by identifying proteins involved in JA signalling which interact with the *Arabidopsis* nitrilases.

2. Investigate if expression of all four *NIT* genes change in response to MeJA treatment. Microarray data showed that both *NIT1* and *NIT2* are upregulated in response to MeJA treatment. Quantitative RT-PCR (qRT-PCR) will be used to determine the relative gene expression of all four nitrilases in Col-0 plants in response to MeJA treatment.

3. Define the impact of increasing nitrilases in plant defence gene expression. JA signalling can be divided into two branches as defined by marker genes *VSP1* and *PDF1.2*, qRT-PCR will be performed on NIT1 and 4 over-expressing lines to see whether there is a change in expression of these two marker genes and thus determine which part of the JA signalling pathway nitrilases are connected to.

4.2 Results

4.2.1 AtMKK2 interacts with *Arabidopsis* nitrilases

AtMKK2 was one of the potential NIT1 and 4 interacting proteins discovered in the AP-MS study presented in chapter three (3.2.4). AtMKK2 is part of the MAPK signalling pathway which controls myriad plant functions, ranging from cell cycle regulation to plant defence (*120*). MAPK kinases specifically target MAPKs, phosphorylating tyrosine residues. A highly specific kinase, one of the targets for AtMKK2 is MPK4, a kinase well characterised to be involved in regulating biotic and abiotic stress responses (*111*, *121*). Given that MPK4 regulates biotic and abiotic stress responses, confirming the interaction of AtMKK2 with NIT1 and 4 may link nitrilases to such stress responses. The interaction of AtMKK2 with NIT1 and 4, the representatives of the two branches of nitrilases, suggest that AtMKK2 may interact with all four *Arabidopsis* nitrilases. False positive interactions due to non-specific binding between prey and bait proteins is a common pitfall for AP-MS. Thus, before further studies can be conducted into the link between *Arabidopsis* nitrilases and stress

responses, a second, independent test (BiFC) was conducted to confirm the interaction of nitrilases with AtMKK2.

The *AtMKK2* ORF was cloned into the pG179NS-YN vector and transformed into *A.tumefaciens*. *Agrobacteria* strains harbouring NIT1 and 4 in the complementary BiFC vector pG179NS-YC were available due to the work described in chapter three. *N. benthamiana* epidermal leaf cells were transiently transformed with AtMKK2-YN and either NIT1-YC or NIT4-YC. Strong cytosolic fluorescence was observed in the YFP channel when AtMKK2 was tested with either NIT1 or NIT4 (figure 4.2). The absence of strong fluorescence in the RFP channel demonstrated that the YFP fluorescence was due to the reformation of the YFP protein, indicating interaction of AtMKK2 with the *Arabidopsis* nitrilases and not due to cellular autofluorescence (figure 4.2). The NIT:AtMKK2 interactions observed here using BiFC confirmed the AP-MS results described in chapter three.

4.2.2 *Arabidopsis* nitrilases interact with the transcription factor ERF1

Through Y2H analysis, Xu *et al* showed that a nitrilase from *Nicotiana tabacum*, TbNIT4A, interacts with two tobacco Ethylene Response Factors (ERFs) – EREBP2 and 3 (*49*). ERFs are transcription factors which are a part of the AP2/ERF superfamily. In *Arabidopsis*, this family has 147 members which are divided into five classes (*122*). These transcription factors regulate biotic and abiotic stresses and play a role in regulating plant defence. Xu *et al* further showed that the interaction between these ERFs and nitrilase require the DNA binding domain of the ERFs. They proposed that nitrilases sequester ERF proteins to the cytoplasm thereby regulating the transcription of genes related to pathogen defence such as the PR genes. TbNIT4A shares high sequence similarity with NIT4 (73% amino acid similarity) and to a lesser degree with the other *Arabidopsis* nitrilases (62-67%) (*49*). Thus, it can be hypothesised that *Arabidopsis* nitrilases are also able to interact with the *Arabidopsis* homologues of tobacco EREBP 2 and 3. GeneMANIA, a function prediction database (*123*), indicated that there was some functional relationship between *NIT4* and one of the AP2/ERF transcription factors – *ERF1* (At3g23240). Transcriptomic analysis further showed that NIT4 is upregulated fivefold in 35S:ERF1 transgenic plants (*124*).

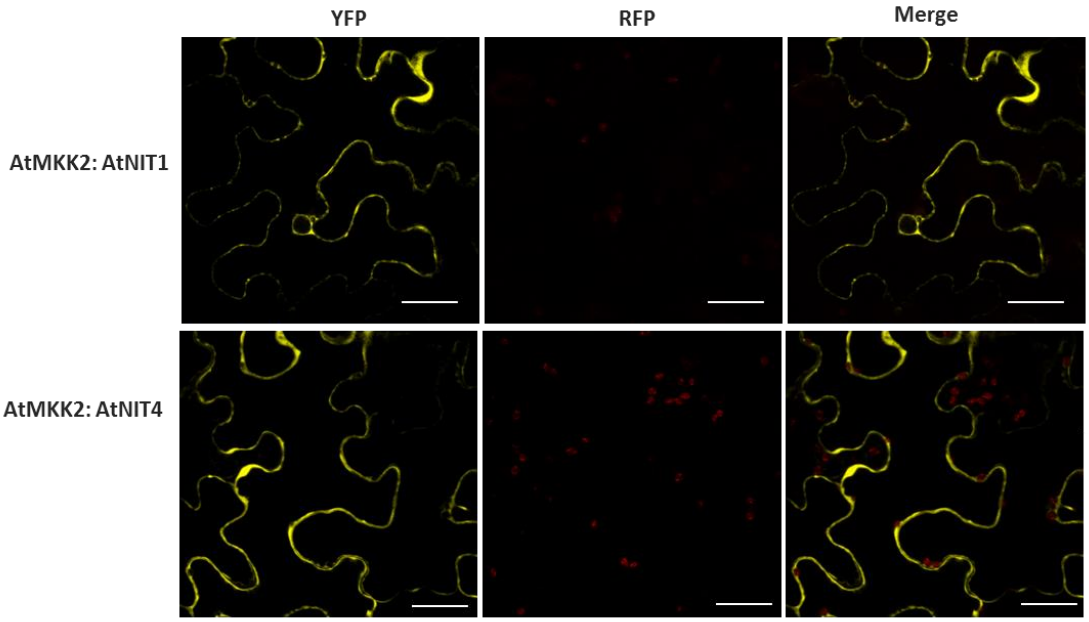


Figure 4.2 Interaction of *Arabidopsis* nitrilases with the MAPK kinase MKK2 as determined using BiFC

The putative interaction between AtNIT1 and AtMKK2 as determined from AP-MS was independently verified using BiFC. AtNIT1 was co-expressed in *N. benthamiana* leaf epidermal cells as a YC fusion protein with a AtMKK2 YN fusion protein. The expression and subsequent reformation of the split YFP as a consequence of AtNIT:AtMKK2 interaction was detected after three days post infection using confocal laser scanning microscopy. Autofluorescence due to organelles such as chloroplasts was detected using the RFP channel. Images from the YFP and RFP channel were merged to show that the fluorescence observed in the YFP channel is not due to autofluorescence.

Scale bars represent 35µm.

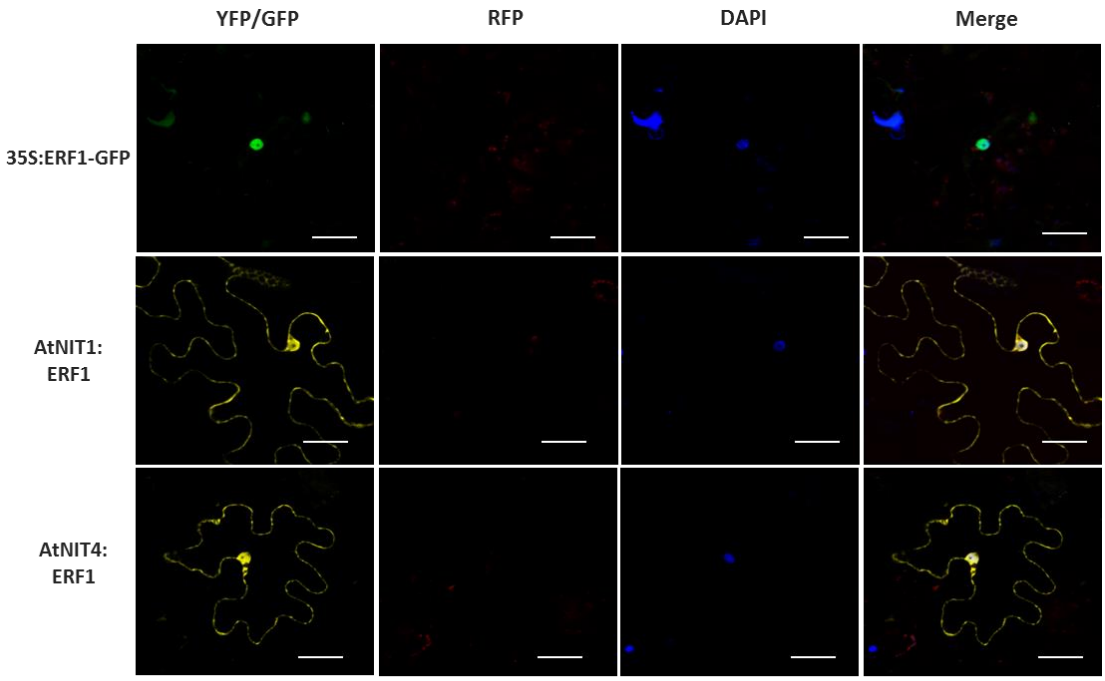


Figure 4.3 Investigating the interaction between ERF1 and the *Arabidopsis* nitrilases using BiFC.Interaction between ERF1 and AtNIT1 and 4 were investigated using BiFC. Fusions of ERF1-YN and AtNIT1-YC or AtNIT4-YC were transiently transformed into epidermal leaf cells of *N.benthamiana*. Leaf samples were analysed three days post transformtion. Green and yellow fluorescence was detected using either the GFP or YFP channels indicating expression (GFP constructs) and reformation of the split YFP (YC/YN constructs). Autofluorescence was detected using the RFP channel. Nuclei were shown with DAPI staining and detected with the DAPI channel. Images from the three channels were merged to show that the fluorescence observed in the YFP/GFP channels is not due to autofluorescence and to demonstrate the position of nuclei relative to the fluorescence

Scale bars represent 35µm

Furthermore, *ERF1* shows the highest homology of all the *Arabidopsis ERFs* with the tobacco *EREBP2* and *EREBP3* (*125*). The similarities of the *Arabidopsis* nitrilases and of ERF1 with the tobacco proteins and the predicted relationship between the *Arabidopsis* proteins makes ERF1 a suitable candidate for interaction with the *Arabidopsis* nitrilases. An interaction of *Arabidopsis* nitrilases with ERF1 would further manifest the role of nitrilases in stress response reactions. ERF1 is involved in JA and ET signalling *via* its interaction with ORA59 and EIN3/EIL1 in the nucleus (*101*). BiFC was employed to test the hypothesis that *Arabidopsis* nitrilases interact with ERF1. The experiment focused on the representatives of the two branches of *Arabidopsis* nitrilases: NIT1 and 4.

The *ERF1* ORF was cloned into the pG179NS-YN vector and transiently expressed with either NIT4-YC or NIT1-YC in the epidermal leaf cells of *N. benthamiana* plants as described in chapter three. Strong YFP fluorescence was observed in the nucleus and cytosol when ERF1 was tested with either NIT4 or NIT1 (figure 4.3). The absence of strong fluorescence in the RFP channel demonstrated that the YFP fluorescence was due to the reformation of the YFP protein, indicating interaction of ERF1 with the *Arabidopsis* nitrilases and not due to cellular autofluorescence (figure 4.3). DAPI staining allowed the visualization of the nuclei and overlays confirmed the nuclear and cytosolic localization of the interaction (figure 4.3). The localization of the ERF1 and NIT interacting complex did not agree with the localization of ERF1 in the literature. Previous work using protoplasts located ERF1-GFP fusion proteins exclusively to the nucleus (*126*). As work conducted in tobacco suggested that nitrilase sequesters the ERF protein in the cytosol, it was essential for the work presented here to compare the localization of ERF1 with that of the NIT:ERF1 complexes. Thus, the ERF1 ORF was cloned into the pG179NS-GFP vector and transiently expressed in epidermal leaf cells in *N. benthamiana* (2.3.1). Fluorescence indicating localization of the ERF1-GFP fusion protein was strong in the nucleus, agreeing with the report by Cheng *et al* (figure 4.3) (*126*). The difference in localization observed for ERF1-GFP and of the NIT:ERF1complexes suggested that interaction with the *Arabidopsis* nitrilases may impact on the localization of the ERF1 protein, in agreement with the tobacco model.

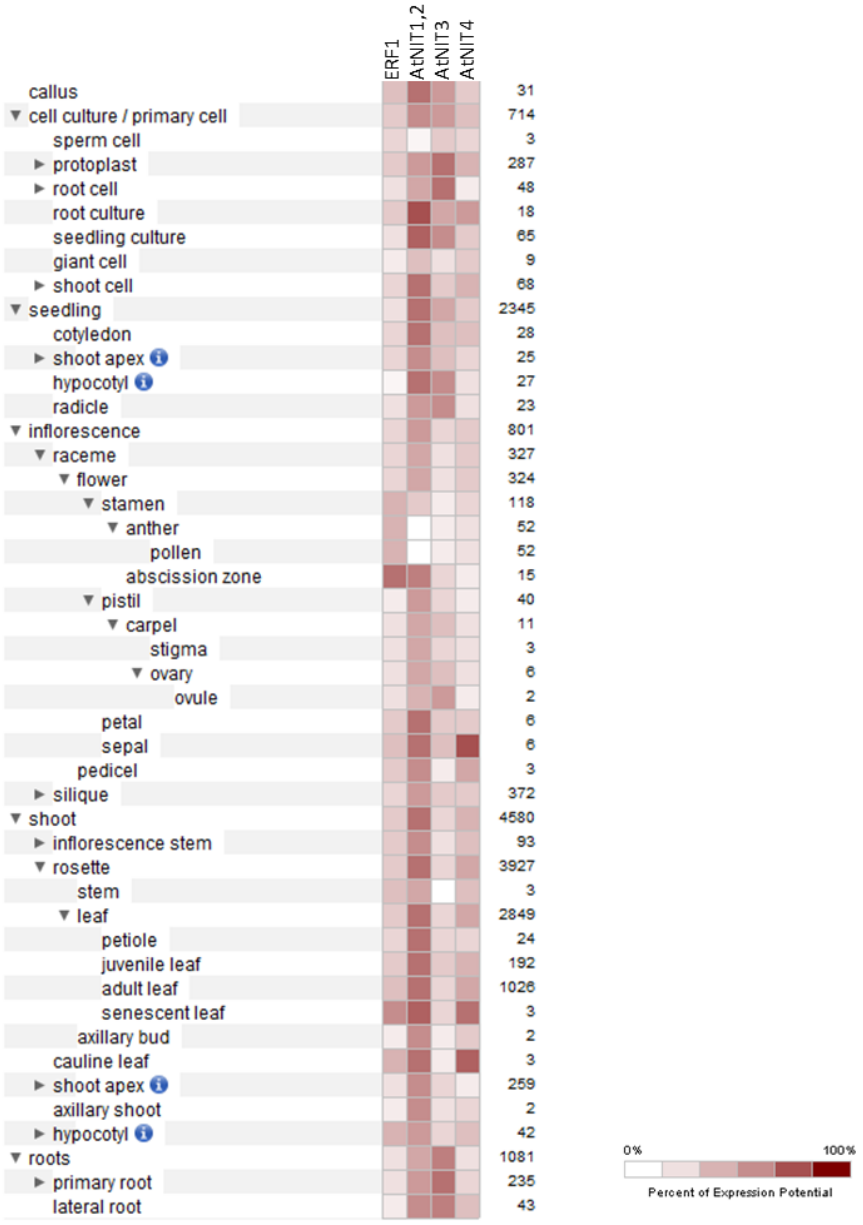


Figure 4.4 Tissue-specific co-expression for *ERF1* and the *Arabidopsis* nitrilases
Comparative analysis was done using data available from Genevestigator *(29)* to determine the expression for *ERF1* and the four *NIT* isoforms in *Arabidopsis* tissue. The Affymetrix *Arabidopsis* ATH1 Genome array platform was used to perform this analysis. The darkness of the red colour indicates the linear percentage of expression potential with white indicating zero expression.
Expression for the five genes is the highest in inflorescence and leaf tissue. In particular, expression is high in senescent leaves for *ERF1*, *NIT1*, *NIT2* and *NIT4*. Protoplast, seedlings and root tissue also show a medium level of expressing in the same tissues between the five genes.

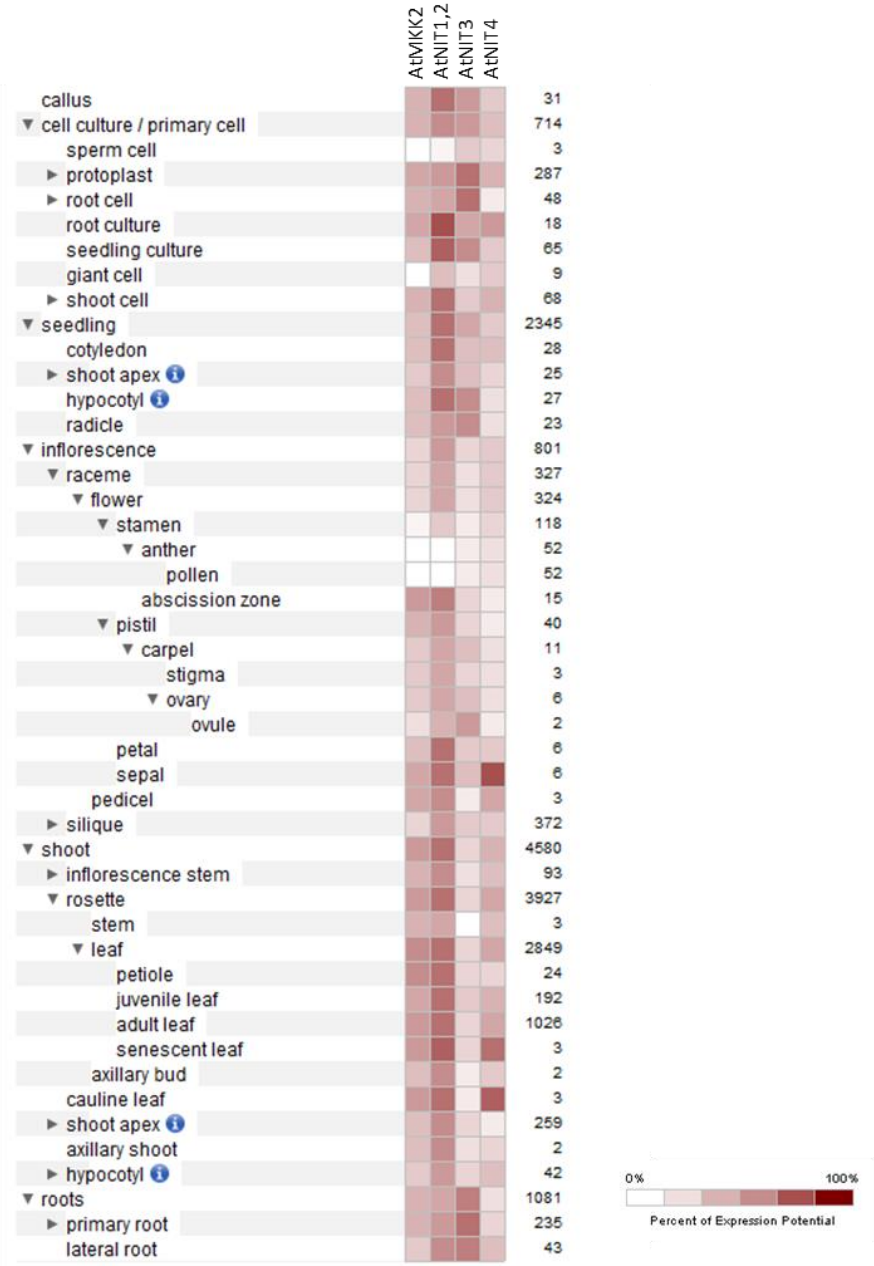


Figure 4.5 Comparative analysis of AtMKK2 and the four *Arabidopsis* nitrilases in *Arabidopsis* tissue

Comparative analysis was done using data available from Genevestigator *(29)* to determine the expression for *AtMKK2* and the four *NIT* isoforms in *Arabidopsis* tissue. The Affymetrix *Arabidopsis* ATH1 Genome array platform was used to perform this analysis. The analysis indicated that the five genes are co-expressed in most of the different types of Arabidopsis tissue. In particular, expression was strong in senescent leaves and in floral tissue such as abcission zone and carpel for the genes investigated.

4.2.3 Overlapping gene expression for *ERF1*, *AtMKK2* and the *Arabidopsis* nitrilases

The biological relevance of a protein-protein interaction is dependent on whether the interacting proteins are present in the same location, at the same time. When interacting with the *Arabidopsis* nitrilases, AtMKK2 and ERF1 are both found in the cytosol with ERF1 also interacting with the nitrilases in the nucleus. However, when not interacting with nitrilases, the localization of AtMKK2 and ERF1 is different with one being in the cytosol (AtMKK2) and the other exclusively in the nucleus (ERF1) (*127*). This may be explained by their different functions and targets, with AtMKK2 a kinase in the MAPK pathway and ERF1 being a transcription factor responsible for activating gene expression. Nevertheless, the localization patterns of the two interactors overlap with that of the nuclear and cytosolic *Arabidopsis* nitrilases. Hence it is feasible that *Arabidopsis* nitrilases and the two interactors come together in cells. This leaves the question whether the temporal and spatial expression patterns of these interactors overlap with that of the *Arabidopsis* nitrilases. As this question is difficult to answer *via* BiFC due to the constitutive expression conferred by the CaMV35S promoter used, microarray data was analysed for an answer.

The temporal and tissue-specific co-expression of these two interactors and the *Arabidopsis* nitrilases was determined using microarray data available from Genevestigator (*30*). Co-expression of *AtMKK2* and *ERF1* with the *Arabidopsis* nitrilases was observed in many tissues albeit at different levels. The expression for *ERF1* and the *Arabidopsis* nitrilases in *Arabidopsis* tissues was strongest in protoplasts, leaves and inflorescence (figure 4.4). In cauline leaves, *NIT1*, *NIT2* and *NIT4* had the highest expression with *ERF1* which was also observed in senescing leaves. Co-expression was also high in the abscission zone for *NIT1* and *NIT2*. Further co-expression in the stamen was observed for *NIT1* and *NIT2*. In sepals, there was some co-expression between the four *Arabidopsis* nitrilases and *ERF1*.

As for *AtMKK2*, co-expression with *Arabidopsis* nitrilases was highest in protoplasts, seedlings, the inflorescence and roots (figure 4.5). For seedlings, there was high co-expression in the hypocotyls and radicles of *AtMKK2* with *NIT1*, *NIT2* and *NIT3*. *NIT1*, *NIT2* and *NIT4* in flowers, in particular in the sepals. In the abscission zone, *NIT1* and *NIT2* were co-expressed with *AtMKK2*. High co-expression was observed in leaves for

NIT1, 2 and 4, in particular with *NIT4* in senescencing and cauline leaves. In roots, co-expression was seen for *NIT1*, *NIT2* and *NIT3* with *AtMKK2*.

The analysis suggests that the novel interactions described between ERF1 and AtMKK2 with the *Arabidopsis* nitrilases are biologically relevant given the shared subcellular localization and overlapping co-expression patterns. Furthermore, it indicated that the interactions for AtMKK2 and ERF1 with *Arabidopsis* nitrilases may be most relevant during the later stages of plant development in particular during floral development and senescence.

4.2.4 Gene expression of NIT alters in response to MeJA treatment

ERF1 and AtMKK2 are two different types of proteins with very specific functions. A converging point between the functions of the two is their involvement in JA signalling. ERF1 attenuates the ET/JA antagonism/synergism by interacting with ORA59 and MYC2 whereas AtMKK2 influences the JA/SA antagonism through the phosphorylation of MPK4 (*101*, *111*, *112*). As *Arabidopsis* nitrilases interact with both of these proteins, it can be hypothesised that they are involved in JA signalling, either affecting certain parts of the signalling pathway or as a form of plant defence mechanism. Thus, the next step was to see whether NIT gene expression was affected by MeJA and thus establishing another link with nitrilases and JA signalling. If this was indeed the case, it would establish another link between the interactors and nitrilases and place the *Arabidopsis* nitrilases in a novel context of JA signalling. Macroarray data on *Arabidopsis* seedlings responding to MeJA treatment showed that NIT1 and 2 do respond to MeJA, both are upregulated fourfold (*34*). Given the similarities of the *Arabidopsis* nitrilases in the context of defence responses it can be postulated that both NIT3 and 4 will have a similar response to MeJA.

qRT-PCR was used to investigate if *NIT3* and *NIT4* are also regulated by JA in wild type *Arabidopsis* seedlings. *NIT1* and *NIT2* were included in this analysis to enable a comparison between the experiment performed here and with that in the literature. In addition, the expression of two JA upregulated genes, Allene Oxide Synthase (*AOS*) and Oxophytodienoate-Reductase 3 *(OPR3),* were investigated to determine the efficacy of the JA treatment. Actin and ATPase were selected as reference genes to calculate the expression of the other genes. *Arabidopsis* wild type seeds (Col-0) were germinated and

grown for three weeks in Magenta vessels in ½ MS media (2.1.2). The seedlings were then treated with 100 μM MeJA or with the solvent (ethanol, 0.1% v/v) for 24 hours. Seedlings were then harvested and total RNA was extracted (2.4.10). The RNA was converted to cDNA which was used as a template for qRT-PCR analysis to determine the relative expression of the four *Arabidopsis* nitrilases in treated and non-treated seedlings (2.4.11). The experiments were repeated three times with more than 100 seedlings per treated or control sample. Expression of *AOS* and *OPR3* increased 28 and 12.7 fold respectively in response to MeJA treatment, indicating the efficacy of the treatment (figure 4.6). Expression of *NIT1* to *3* were upregulated in response to MeJA treatment, with *NIT3* showing the strongest upregulation (2.5, 2 and 4.3 fold increase respectively). These data confirmed the upregulation of *NIT1* and *NIT2*described by Sekimoto *et al* (*34*) but showed that *NIT3* was more affected by the treatment. Interestingly, in contrast to the *NIT1* homologues, expression of *NIT4* was downregulated by 5.6 fold (figure 4.6). Thus, JA has a contrasting impact on the two branches of the *Arabidopsis* nitrilases.

4.2.5 *VSP1* and *PDF1.2* expression in *NIT1* and *NIT4* over-expressing lines

The qRT-PCR analysis showed that all four NIT genes are regulated by JA, albeit with different consequences. The identification of ERF1 and AtMKK2 as NIT interaction partners supported a possible role for *Arabidopsis* nitrilases in JA signalling or response pathways. JA signalling itself is divided into two branches, each branch controlling a different type of defence response. One branch is referred to as the MYC branch which is responsible for wound responses. The other branch is the ERF branch which responds to necrotrophic pathogens. Each branch can be identified by marker genes which are involved in their respective branches. *VSP1*/2 are marker genes for the MYC branch whereas *PDF1.2* is considered as a marker gene for the ERF branch. The expression of these marker genes was investigated in *NIT1* and *NIT4* over-expression mutants and compared to the expression in wild type *Arabidopsis* to determine whether the disturbance of NIT expression and the expected increase in nitrilase activity impacts on JA signalling. If increasing NIT expression impacts on the expression of those markers, the experiment should further reveal which branch of JA signalling is affected.

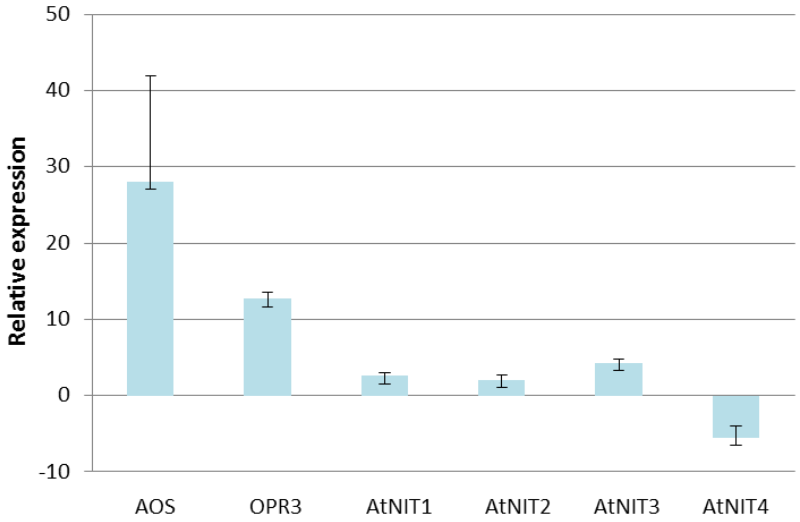


Figure 4.6 Quantitative RT-PCR analysis of the *Arabidopsis* nitrilases in response to methyl jasmonate

Arabidopsis seedlings (Col-0) were grown for three weeks on ½ MS liquid media in magenta vessels. Seedlings (n>100) were then treated with 100 µM of MeJA for 24 hours. Total RNA was extracted and cDNA was generated. The cDNA was used as template for qRT-PCR. *AOS* and *OPR3* were used as positive controls to determine whether there was a response to MeJA. To calculate the relative expression, actin (*ACT*) was selected as a reference gene to normalise the expression data. Relative gene expression of the *NIT*s compared to non-treated and treated cDNA samples. The relative expression was an average of three replicate experiments.

Error bars indicate the standard error.

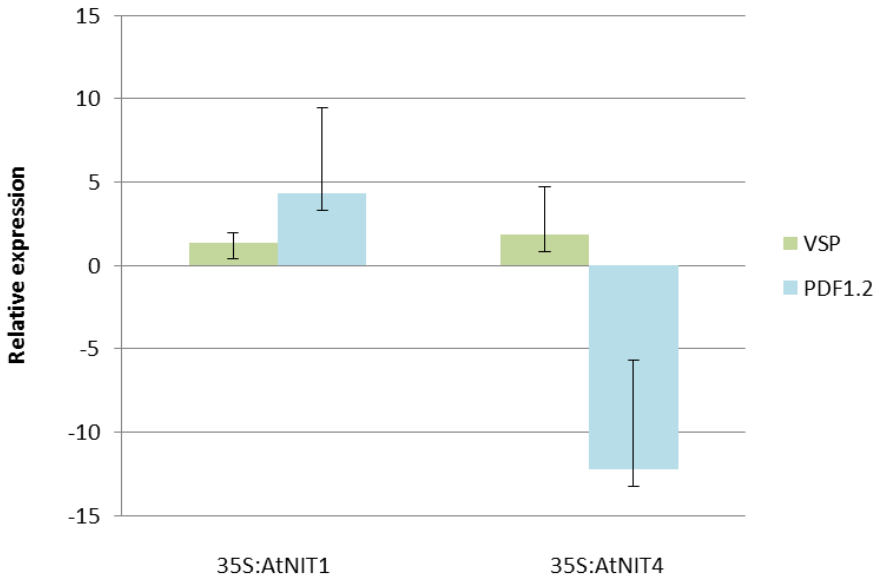


Figure 4.7 Quantitative RT-PCR analysis of JA signalling pathway markers, *VSP1* and *PDF1.2*, in *NIT1* and *NIT4* overexpressor lines
Arabidopsis seedlings were grown for three weeks on ½ MS agar plates. Seedlings (n>100) were harvested and total RNA was extracted. RNA was then converted into cDNA. The relative expression of *VSP1* and *PDF1.2* were then analysed using qRT-PCR. To calculate the relative expression, *ACT* was selected as a reference gene to normalise the expression data. Relative gene expression of *VSP1* and *PDF1.2* was compared to wild type Col-0 and the two over-expressor lines, *35S:NIT1* and *35S:NIT4*. The relative expression was an average of three replicate experiments.
Error bars indicate the standard error.

Arabidopsis lines Col-0, 35S:NIT1 and 35S:NIT4 were grown for three weeks on ½ MS agar plates. Seedlings were harvested and total RNA was extracted. The RNA was then converted into cDNA *via* reverse transcription and qRT-PCR was performed. Actin was selected as the reference gene for normalization and to calculate the relative expression of the marker genes, *VSP1* and *PDF1.2*. The experiment was repeated three times with more than 100 seedlings per sample. *VSP1* was upregulated in both NIT over-expressing lines with an observed 1.4 fold upregulation in the 35S:NIT1 line and a 1.8 fold increase in the 35S:NIT4 line (figure 4.7). *PDF1.2* was upregulated 4.3 fold in the 35S:NIT1 line (figure 4.7). However, in contrast to *VSP1*, *PDF1.2* was downregulated 12.2 fold in the 35S:NIT4 line (figure 4.7). Thus the qRT-PCR results demonstrated that over-expression of *Arabidopsis* nitrilases impacts on the expression of genes involved in JA signalling. This impact differs for *PDF1.2*, the marker of the ERF branch, depending on which NIT is over-expressed. This experiment further manifests a role for *Arabidopsis* nitrilases in JA-related biological roles by defining which branch of JA signalling *Arabidopsis* nitrilases may have an effect on.

4.3 Discussion

4.3.1 A new role for *Arabidopsis* nitrilases in JA signalling

The identification of AtMKK2 and ERF1 potential NIT interactors posed the hypothesis that *Arabidopsis* nitrilases may be involved, either directly *via* interaction or indirectly *via* their enzymatic functions in JA responses to biotic or abiotic stresses. Based on these interactions and further reports in the literature, this study investigated whether *Arabidopsis* nitrilases are involved in JA signalling and if so, what impact they have on the signalling pathways. AtMKK2 and ERF1 are involved in different aspects of the JA signalling response with AtMKK2 acting as a kinase and ERF1 acting as a transcriptional activator of gene expression. The study here provided evidence that both of these proteins interact with NIT1 and 4 *in planta*. It was further shown that the localization of NIT:AtMKK2 complexes match those of observed for AtMKK2-GFP and NIT-GFP fusion proteins. There were significant difference in localization of ERF1-GFP fusion proteins and of the NIT:ERF1 interaction complexes. Whilst ERF1-GFP fusion proteins were localized to the nucleus, ERF1 appeared in both the nucleus and cytosol when interacting with *Arabidopsis* nitrilases. The shift in localization observed for ERF1 when interacting with *Arabidopsis* nitrilases suggests a regulatory

role *via* cytosolic sequestration of ERF1 by the *Arabidopsis* nitrilases. The link between JA signalling and *Arabidopsis* nitrilases was further explored by examining the effect of exogenous MeJA on the expression of the *Arabidopsis* nitrilases. *NIT1* to *3* gene expression was upregulated several fold whereas the expression of *NIT4* was downregulated by MeJA. This demonstrated that the two nitrilase branches respond differently to the hormone and may point to different roles in stress responses. Next, the relative gene expression of marker genes of the two branches of JA signalling (*VSP1* and *PDF1.2*) was determined in *Arabidopsis NIT1* and *NIT4* over-expressing mutant lines. Whilst the expression of *VSP1* is only slightly perturbed by the over-expression of *Arabidopsis* nitrilases, *PDF1.2* was downregulated in *NIT4* seedlings whereas it was upregulated in the *NIT1* over-expressing line. As observed for the regulation of *Arabidopsis* nitrilases by JA, this showed a difference between the two nitrilase branches which may point to different biological functions and could be of evolutionary interest.

4.3.2 *Arabidopsis* nitrilases interact with AtMKK2

The AP-MS study presented in the previous chapter highlighted several putative interactors of interest for the *Arabidopsis* nitrilases. AtMKK2 was interesting as it was the only protein which was identified as a putative interactor for both NIT1 and 4. BiFC, an independent *in planta* interaction assay, confirmed that NIT1 and 4 interact with AtMKK2 and further showed that this interaction occurs in the cytosol

PhosPhAT, a phosphorylation proteome database for *Arabidopsis*, indicated one phosphorylated residue in the NIT1 protein (serine 329). With this in mind and given that AtMKK2 is a kinase, phosphorylation could be a functional role of the NIT:AtMKK2 interaction. However, this is most likely not the case as AtMKK2 is a very specific kinase. Y2H and biochemical studies have only identified MPKs as interactors and phosphorylation targets for AtMKK2 (*128*, *129*). Thus it specifically phosphorylates the MPKs which are part of the MAPK signalling cascade. Localization studies using AtMKK2-GFP fusion proteins showed that the kinase is localized to the cytosol (*127*). This reported AtMKK2 localization is identical to that observed for the NIT:AtMKK2 interaction which suggests that interaction with *Arabidopsis* nitrilases does not affect the localization of AtMKK2. Hence the interaction must have a different purpose. For example, *Arabidopsis* nitrilases could be involved in the regulation of

AtMKK2 enzymatic activity *via* structural interference or by regulating the interaction of AtMKK2 with other proteins. Determining where the *Arabidopsis* nitrilases binds to within the AtMKK2 and the dynamics of such interaction may provide further clues as to why and how *Arabidopsis* nitrilases interact with this kinase. A peptide fragment analysis where the fragments of the AtMKK2 protein is tested against *Arabidopsis* nitrilases could elucidate the binding site and thus determine the regulatory role. FRET could also be used to determine the dynamics of the peptide fragment analysis.

There are 10 members of the *Arabidopsis* MAPKK family of which AtMKK2 is a part of. The other members of this family should be tested in future experiments for interaction with the *Arabidopsis* nitrilases to investigate if nitrilase MAPKK interactions are generally conserved or restricted to one (AtMKK2) or several MAPKK members. Interaction with other MKK2 proteins could hint at biological functions not yet considered for the Arabidopsis nitrilases. AtMKK2 binds to not only NIT1 but also to NIT4, the primordial nitrilase in the family. This suggests that the interaction is not only conserved within *Arabidopsis* but may also exist in other plant families. The MAPK signalling cascade is found throughout plants; nitrilases similarly are found outside of the Brassicaceae family. A conserved interaction across plant species would suggest that the NIT:AtMKK2 interaction is generally important for plants.

4.3.3 *Arabidopsis* nitrilases interact with ERF1

Xu et al (1998) postulated that tobacco nitrilases are involved in regulating the transcriptional activity of the EREBPs. It was a logical extension to postulate that this is also observed in *Arabidopsis* given the homology between tobacco nitrilases and NIT4. Data gleaned from transcriptomes further suggests a functional link between ERF1 and NIT4. Aside from being involved in ET signalling, ERF1 is a major transcription factor which controls the ERF branch of JA signalling through its interaction with ORA59 and EIN3/EIL1. The work presented here revealed that not only NIT4 but also NIT1 interact with ERF1. The localization of ERF1 is nuclear as shown by ERF1-GFP fusion protein and protoplast studies reported in Cheng *et al* (*126*). The interaction between ERF1 and the *Arabidopsis* nitrilases was found to be mostly cytosolic with some of the interaction occurring in the nucleus. Based on this, it is feasible that one of the roles of *Arabidopsis* nitrilases is to sequester ERF1 to the cytosol. This conclusion is in agreement with what was found by Xu et al (*49*); they also found that TbNIT:EREBP interaction occurs in

the cytosol and proposed the sequestration model. Sequestration of ERF1 to the cytosol would prevent the protein from exerting its function in transcriptional regulation. Xu et al further described that the tobacco nitrilase binds to the DNA binding domain of EREBP (*49*). This further supports that the interaction of the two proteins relates to inhibiting the ability of ERF to activate transcription. ERFs within plant species are not very well conserved on the amino acid sequence level but the DNA binding domains are fairly conserved between the different ERFs. Thus it can be postulated that the *Arabidopsis* nitrilases bind to ERF1 *via* the DNA binding domain of the transcription factor. Peptide fragment analysis, testing the DNA binding domain for interaction with *Arabidopsis* nitrilases, would answer that question.

The interaction work presented in this study suggests conservation of interaction between the *Arabidopsis* nitrilases and proteins of interest. However, interpretation of the results should be taken with care as the *in planta* interaction assays, AP-MS and BiFC use constitutively expressed proteins and hence do not take into account the expression levels, temporal and spatial co-expression requirements which need to be assessed to validate whether the interactions are physiologically meaningful. Co-expression data for the *Arabidopsis* nitrilases *AtMKK2* and *ERF1* showed that they are co-expressed both in a developmental and spatial context, suggesting that the interaction could occur with the endogenous proteins and may be conserved between all four nitrilase isoforms on a physiological level.

4.3.4 *Arabidopsis* nitrilases regulating JA signalling responses

The novel interactors for *Arabidopsis* nitrilases, AtMKK2 and ERF1, are involved in mediating plant defence responses through JA signalling. Thus, it can be rationalized that there is a biological link between JA signalling and the *Arabidopsis* nitrilases. Indeed, transcriptomic data showed that *NIT1* and *NIT2* respond to exogenous application of MeJA (*34*). To further investigate this link, the relative expressions of *NIT3* and *NIT4*, as well as *NIT1* and *NIT2* were determined in Col-0 seedlings when exposed to 100 μM of MeJA. This experiment showed that the expression of the *NIT1* homologues increased two to four fold in response to MeJA. In contrast to the *NIT1* homologues, the expression of *NIT4* decreased fivefold. The differences observed here suggest a difference in their role in JA signalling and that this is a reflection of their differing biological roles. Based on their involvement in glucosinolate catabolism, the

NIT1 homologues biological role in JA signalling is most likely related to mediating herbivory responses. The downregulation for *NIT4* observed is an interesting finding as *NIT4* plays no role in glucosinolate catabolism and is mostly involved in cyanide detoxification. One indication of its biological role in JA signalling would be its interaction with ERF1 and the possibility that it may sequester ERF1 to the cytosol.

The second part of this study investigated which of the two branches of JA signalling the *Arabidopsis* nitrilases could be involved in. The relative expression of the markers of the two branches, *VSP1* and *PDF1.2,* were determined *via* qRT-PCR in *NIT1* and *NIT4* over-expressing *Arabidopsis* lines. Only small changes in *VSP1* expression were observed in the *NIT1* and *NIT4* over-expressing seedlings (1.4 to 1.8 fold, compared to wild type). In contrast, the changes in *PDF1.2* expression were more pronounced and differed between the two over-expressing lines. In *NIT1* over-expressing seedlings, *PDF1.2* expression was 4.3 fold upregulated whereas its expression is repressed 12.3 fold in *NIT4* over-expressing seedlings. An analysis of transcriptomic data that *NIT4* gene expression is downregulated when exposed to necrotrophic pathogens and in combination with the ERF1 branch of JA signalling regulating necrotrophic pathogen responses, it can be postulated that NIT4 negatively regulates JA signalling as a response to necrotrophic pathogens. Under ambient conditions *i.e.* not challenged by a pathogen or wounding, this proposal would suggest that NIT4 sequesters ERF1 to the cytosol, preventing it from interacting with ORA59 and EIN3/EIL1 and thus inactivating the signalling pathway. However, when the plant is challenged with necrotrophic pathogens, NIT4 could be degraded when the appropriate signalling pathway is activated. This degradation could be initiated *via* the ubiquitin-protease degradation pathway.

This model would logically mean that in the absence of NIT4, ERF1 would be able to localize to the nucleus where it would interact with ORA59 and EIN3/EIL1 thereby activating this JA signalling branch. The model is supported by the qRT-PCR results presented in this study; MeJA suppresses *NIT4* expression and *PDF1.2*, a marker for the ERF branch, is downregulated in the NIT4 over-expressing line. This model could be tested by determining the relative expression levels of ORA59, *EIN3/EIL1* in the *NIT4* over-expressing lines using qRT-PCR. If the model holds, then the relative expression of these two genes would be repressed as seen for the *PDF1.2* in the mutant lines as

ERF1 would be prevented by NIT4 to activate their transcription. A similar study could be done using *NIT4* knockout mutant lines to see if the converse also holds; *PDF1.2* expression would be either near wild type levels or higher. In a similar manner, the protein and gene levels of *NIT4* could also be determined in wild type *Arabidopsis* during a necrotrophic pathogen challenge. On a phenotypic level, *NIT4* over-expressing and *NIT4* knockout mutants could be challenged with necrotrophic pathogens and see whether they are more or less resistant to the pathogen.

ERF1 is an early ethylene response gene; there is rapid induction of *ERF1* in response to ET (*125*). By interacting with EIN3, ERF1 is able to induce secondary ethylene response genes such as those encoding for basic chitinase and *PDF1.2* (*130*). Thus, aside from JA signalling, *Arabidopsis* nitrilases could also be involved with ET signalling responses *via* the regulation of ERF1. Transcriptional profiling of the early ET response showed that NIT4 is upregulated when *Arabidopsis* is treated with ET (*35*). This suggests that the *Arabidopsis* nitrilases may also respond to ET and possibly be involved in ET signalling.

The studies presented here and in the previous chapter indicate a strong link between the *Arabidopsis* nitrilases and biotic stresses. This fits in well with the current view of the involvement of plant nitrilases in pathogen defence responses and in the detoxification of pathogen defence compounds and their metabolites. However, evidence obtained in these studies for an involvement of nitrilases in biotic stresses is rather indirect as it is based on connections between nitrilases and phytohormones known to be involved in biotic stress signalling. A direct connection between these phytohormones and *Arabidopsis* nitrilases is needed to address this problem. One way to achieve this is to see whether exogenous application of these phytohormones has a physiological effect on over-expressing *NIT* mutant lines. Altered responses of the over-expressing mutant lines as compared to wild type in response to the exogenous application would indicate that there is not just an effect on the genetic level but also on the physiological level.

4.3.5 A potential role in abiotic stress responses for *Arabidopsis* nitrilases

NIT1 and its homologues are likely to be involved in wound response and herbivory attacks. However the small changes in *VSP1* gene expression seems to refute such a role. There could be some knock down effect on *MYC2* expression which regulates the

wound/herbivory response branch. This would explain why *PDF1.2* was upregulated whilst small changes were observed for *VSP1*. In *jin1-1*, mutants where *MYC2* is knocked out, marker genes for the wound response were not expressed whereas genes such as *PDF1.2*, *PR-4* and *PR-1* had higher expression levels than wild type plants (*102*). Expression for *MYC2* in the *NIT1* over-expressor lines would need to be determined to see whether this could be the reason behind the increased expression of *PDF1.2*.

In the *NIT1* over-expressing line, *PDF1.2* expression was upregulated which suggests that the model proposed for NIT4 may not apply to NIT1 despite this protein also interacting with ERF1. The sequestration model could still hold up for NIT1 as well as for NIT4 but the genes it regulated by NIT1 action if the genes regulated are different from those regulated by NIT4. Another way to interpret this is that the NIT1:ERF1 interaction may not be related to JA signalling. A second point of convergence for AtMKK2 and ERF1 is that they both are involved in abiotic stresses. Analysis of AtMKK2 over-expressing and null mutant lines revealed opposing phenotypes to various abiotic stresses (*131*). ERF1 was similarly determined to be involved in regulating abiotic stress responses using over-expressing *ERF1* lines (*126*). In addition, the protein-protein interaction study presented in chapter three, identified putative interactors such as AHK2 which are involved in ABA signalling. This suggests that another role for the *Arabidopsis* nitrilases may lie in abiotic stress responses. Initial investigation into this can be performed on a phenotypic level. Physiological responses to abiotic stress such as germination and root growth can be determined for *NIT* over-expressing mutants.

4.3.6 Conclusion

The chief aim of this study was to develop a biological link between the *Arabidopsis* nitrilases and JA signalling. Both AtMKK2 and ERF1 are involved in plant defence responses through regulating JA signalling. The study provided such a link by showing that the *Arabidopsis* nitrilases responded to exogenous application of MeJA. The differential response to MeJa and the expression of the JA signalling marker genes provided evidence that the *Arabidopsis* nitrilases have different biological roles in plant defence responses. The experimental evidence presented here supports a model proposed by Xu *et al* where *Arabidopsis* nitrilases sequesters ERF1 to the cytosol to

prevent transcriptional activation (*49*). The biological context for the sequestration is clear for NIT4 where it negatively regulates the necrotrophic pathogen response which is regulated by JA signalling by regulating the localization of the transcription factor ERF1. However, this is not the case for NIT1 as the qRT-PCR studies suggested that the biological role for the sequestration of ERF1 may lie outside of JA signalling. The results from this study further suggests an involvement of NIT1 in abiotic stress responses. Further work is required to fully understand the regulatory role of *Arabidopsis* nitrilases in JA signalling and to investigate nitrilases' putative role(s) in abiotic stress responses.

Chapter 5

Investigating the impact of overexpressing *Arabidopsis* nitrilases on physiological stress responses

5.1 Introduction

5.1.1 Connecting *Arabidopsis* nitrilases with abiotic stress responses

The interactions of *Arabidopsis* nitrilases with AtMKK2 and ERF1 as described in chapter four may further support a role for nitrilases in abiotic stress responses. Both interactors are involved in regulating abiotic stresses such as cold, salinity and drought stresses. The AP2/ERF transcription factor family regulates genes involved in biotic and abiotic stress responses. It is well established that ERF1 is involved in plant defence responses *via* JA and ET cross-signalling. A link between nitrilases and biotic stress responses was determined through the interaction of ERF1 and *Arabidopsis* nitrilases as experimentally shown in chapter four. Aside from biotic stresses, ERF1 has been shown to be involved in abiotic stress responses. Over-expression of *ERF1* in *Arabidopsis* conferred tolerance to various abiotic stresses such as osmotic stress and salinity (*126*). Cheng *et al* also showed that these over-expressing lines had elevated levels of abscisic acid (ABA) as compared to the wild type. On a genetic level, *ERF1* was shown to not only bind to GCC elements but also to DRE elements with a higher affinity, further indicating its involvement in abiotic stress response (*126*). DRE elements are usually associated with abiotic stress genes that are regulated by the AP2/ERF family members such as *DREB* which are major regulators of abiotic stress responses (*132*). Similar to ERF1, AtMKK2 was shown to be involved in abiotic stress responses. Analysis of over-expressing and null mutant lines revealed opposing phenotypes to various abiotic stresses. Over-expressing *AtMKK2* conferred cold and salt tolerance whereas null mutants were hypersensitive to the two stresses (*131*). Marker genes for abiotic stress responses such as *RAV1* and *CBF2* were upregulated in over-expressing *AtMKK2* lines (*131*). Further supporting evidence for a role of AtMKK2 in abiotic stress responses came from the finding that MPK4 and 6, phosphorylation targets of AtMKK2, are activated by low temperatures and hyperosmolarity (*121*).

In addition to the data presented in chapters three and four which suggested a link between *Arabidopsis* nitrilases and stress responses, other research groups also have recent experimental evidence linking them with abiotic stress responses. For example, transcriptomic analysis of *Arabidopsis* plants subjected to high salinity or cold stress revealed that the expression of *Arabidopsis* nitrilases is altered in response to these stresses (*32, 133*). Furthermore, the expression of *NIT2* is regulated by ATAF2, a NAC

transcription factor (*52*), known to respond to high salinity as well as to pathogens (*51, 133*). When Bohmer *et al* performed a comparative analysis of *Arabidopsis* suspension cells in response to ABA, they found that the expression of *NIT1* and *NIT2* are upregulated in response to ABA on the transcriptomic and proteomic level (*30).*

Thus it appears that *Arabidopsis* nitrilases may have roles in both biotic and abiotic stress responses. This is not surprising as signalling pathways, including the biotic and abiotic stress pathways, are frequently cross-talking with each other, fine tuning the responses to stresses. Synergistic and antagonistic cross-talking mechanisms within the signalling pathways regulating biotic and abiotic stresses are well established (*95*).

5.1.2 ABA signalling in abiotic stresses

ABA is extensively involved in abiotic stress signalling whilst also playing a role in disease resistance (*134*). During vegetative growth, an important function of ABA signalling is to optimise growth during environmental stresses by maintaining osmotic homeostasis. The various abiotic stresses such as drought and salinity affect this homeostasis. ABA maintains homeostasis by regulating transpiration rate through controlling stomatal aperture. Stomatal apertures are controlled by the turgor pressure of guard cells which is responsive to ABA levels; increased levels of ABA promote the closure of stomatal pores by reducing the turgor pressure (*135*). By promoting the closure of stomatal pores, ABA regulates the transpirational loss of water during stresses such as drought and high salinity. Aside from maintaining stomatal closure, ABA signalling leads to the activation of genes which are involved in cold and dehydration responses such as CBF2, DREB1D and DREB1E (*136*).

5.1.3 Phytohormones affect plant growth and development

The previous chapter showed that expression of the *Arabidopsis* nitrilases alters in response to exogenous application of MeJA. Aside from JA, other phytohormones which are involved in defence and stress responses may affect NIT expression. ABA is known to alter NIT levels on both a transcriptomic and proteomic level. Furthermore, ethylene has a similar effect on the expression of NIT4 in *Arabidopsis* (*35*). The genetic responses to phytohormones such as JA, ABA and ET raises the question of the phenotypic manifestation of these expression changes.

In plants, there is a trade-off between growing and defending as a way to optimise the allocation of resources available to the plant when exposed to various biotic and abiotic stresses. As a result, the growth of a plant is often significantly altered, either delayed or accelerated, as part of the response to the environment. JA signalling is well known to delay plant growth when activated (*97, 137*). Root growth inhibition is one of the first described physiological effects of JA treatments (*138*). JA inhibits root growth through an antagonistic relationship with the phytohormones involved in promoting root growth such as auxin (*97*). For example, JA inhibits lateral root formation due to its antagonistic relationship with auxin which promotes lateral root formation. Seed germination is also inhibited by JA though it seems it is the JA precursor OPDA not JA itself which is involved in inhibiting seed germination (*139*). Senescence is also induced by JA as key transcription factors involved in senescence such as WRKY53 are activated in response to JA signalling (*140*). Cotyledons accumulate anthocyanins in response to exogenous JA due to genes regulating anthocyanin production being induced by JA signalling (*137, 141*).

As with JA, ABA signalling governs certain aspects of plant development, particularly seed development, germination and dormancy (*142*). Well characterized is the effect ABA has on embryo and seed development. ABA is essential to induce seed dormancy with *de novo* synthesis of ABA maintaining dormancy (*143*). Seed desiccation tolerance is promoted and germination of mature seeds is also prevented in response to exogenous ABA application further demonstrating the role of ABA in seed dormancy (*144*). In addition, seedling establishment and vegetative development is also affected by ABA. For example, ABA inhibits root elongation, impacts on lateral root formation and emergence and, together with auxin; ABA regulates primary root growth (*145*).

ET also has various morphogenic effects on plants. Like JA, it induces senescence in plant organs; most notably it induces fruit ripening as part of the senescence process. ET and auxin have a synergistic relationship in regards to root growth and development; auxin promotes root growth and development whereas ET inhibits it. ET does this by blocking auxin signalling which causes an inhibition of root cell elongation and also of lateral root development (*146*).

5.1.3 Nitrilases and herbicides

Bromoxynil is a nitrile herbicide which is commercially used to spray crops. It works by binding to the D2 and D1 proteins of photosystem II (PSII), disrupting the electron transport chain (ETC) and thus preventing photosynthesis. Inhibition of photosynthesis causes the formation of reactive oxygen species (ROS). There are two ways ROS affect plants; either acting as signalling molecules for abiotic and biotic stress responses or reacting with biomolecules and inducing cell death through tissue necrosis (*147*).

Bacterial nitrilases are able to break down bromoxynil. Furthermore, transgenic *N. benthamiana* plants which are over-expressing a bacterial bromoxynil specific nitrilase are resistant to bromoxynil (*148*). It is not known whether bromoxynil is a substrate for the NIT1 homologues. Enzymatic characterization done separately by Osswald *et al* and Vorwerk *et al* showed that the NIT1 homologues are able to use a variety of nitriles as substrates (*18, 36*). They found that aliphatic nitriles such as phenylproprionitrile (PPN) were the best substrates whilst the aromatic nitriles were the least preferred substrates. Thus, given the broad variety of nitrile substrates used by NIT1, it can be hypothesised that the enzyme may provide resistance to nitrile herbicides such as bromoxynil, similar to bacterial nitrilases.

5.1.4 Aims and hypotheses

The molecular and proteomic studies presented in this thesis so far has led to the postulation that the *Arabidopsis* nitrilases are involved in JA signalling. How this is manifested on a phenotypical level has yet to be determined. The studies further suggested that the *Arabidopsis* nitrilases may be involved in ET signalling given the cross talking between JA and ET signalling in biotic stress responses and the interaction with ERF1. Establishing a link between *Arabidopsis* nitrilases and ET on a physiological level will also determine whether *Arabidopsis* nitrilases are involved in ET signalling responses. The work presented here in conjunction with recent research indicate that nitrilases may be involved in abiotic stress responses. The two novel interactors of *Arabidopsis* nitrilases, AtMKK2 and ERF1, are involved in abiotic stress responses. This suggests that *Arabidopsis* nitrilases may also impact on ABA signalling responses. Given the synergistic and antagonistic cross-talking between the phytohormones, it can be hypothesised that *Arabidopsis* nitrilases are regulating both biotic and abiotic stress signalling responses. To further investigate the link between

Arabidopsis nitrilases and the physiological impact on biotic and abiotic stress responses with an emphasis on abiotic stresses, the aims of this chapter are to:

1. Determine whether plant nitrilases, like bacterial nitrilases, confer resistance to bromoxynil. Germination of wild type and *NIT1* over-expressing seeds on bromoxynil will be investigated to see if over-expressing *NIT1* confers tolerance. This will further show if the over-expression of *NIT1* leads to increased nitrilase activity.

2. Determine whether *NIT* over-expressing mutants have a different physiological manifestation of response to MeJA. Root length assays will be used to test for JA sensitivity for the *NIT1* and *NIT4* over-expressing mutants in comparison to wild type Col-0 plants.

3. Establish if a link between ET signalling and *Arabidopsis* nitrilases exists by assessing physiological responses to 1-amino-cyclopropane-1-carboxylic acid (ACC) in the NIT1 and 4 over-expressing mutants.

4. Investigate whether there is a physiological response to abiotic stress in the *NIT* over-expressing mutants. Germination frequencies of *NIT1* and *NIT4* over-expressing lines will be determined when exposed to salt (150 μM) or ABA (1 μM) and compared with wild type Col-0 to see whether there is a sensitive/insensitive phenotype in the over-expressing mutants .

5.2 Results

5.2.1 Over-expression of *NIT1* confers specific resistance to bromoxynil

N.tabacum plants expressing a bacterial nitrilase are resistant to bromoxynil (*148*). It is not currently known if expression of plant nitrilases such as NIT1 and its homologues can also confer resistance to bromoxynil. To determine whether NIT1 has a similar as bacterial nitrilase in mediating bromoxynil resistance, germination studies were performed. Pilot studies involving wild type Col-0 seeds showed that bromoxynil concentrations of more than 50 μM completely inhibited germination (data not shown). Thus a bromoxynil concentration range from 0 μM to 50 μM was used for the

germination assays. Col-0 and 35S:NIT1 seeds were plated on ½ MS plates (control) or ½ MS plates supplemented with 0 μM, 10 μM, 25 μM or 50 μM of bromoxynil (2.1.2).

Germination was scored every 24 hours until 96 hours, using the emergence of the radicle from the seed coat as a criterion (figure 5.1). Under control conditions, germination of Col-0 and 35S:NIT1 reached 81% and 93% respectively at 48 hours (95% and 97% at 96 hours). Thus, under control conditions, the two lines behaved similarly. The germination rate of both lines decreased with increasing concentrations of bromoxynil with 35S:NIT1 germinating faster than Col-0 (figure 5.1). In the presence of 10 μM bromoxynil, the germination rates for Col-0 and 35S:NIT1 were 28% and 81% respectively at 48 hours. However, Col-0 recovered and showed a germination rate close to that seen under control conditions at 96 hours (90%). 35S:NIT1 reached germination rates comparable to control conditions earlier than Col-0, at 72 hours (94%). A two way ANOVA test was performed which showed that there was a significant difference in the proportion of seeds germinated at 48 hours (P-value<0.05) between the two lines yet at 96 hours there was no significant difference (P-value>0.05). At 25 μM, the inhibitory effect of bromoxynil was much stronger on the germination rate of Col-0 than on that for 35S:NIT1. The strong difference in germination rate became obvious at 48 hours (Col-0: 8.7% and 35S:NIT1: 52.6%) and further exemplified at 96 hours where the 35S:NIT1 mutant showed a near control germination rate (89%) but Col-0 barely reached 20%. At the highest concentration (50 μM), only 2.85% of seeds germinated for Col-0 whereas the 35S:NIT1 seeds germinated more than six times better, at 18.1%. ANOVAs performed showed that there was significant difference between Col-0 and 35S:NIT1 regardless of time point for the 25 μM and 50μM concentrations tested (P-values<0.05). The results presented here showed that 35S:NIT1 seeds were less sensitive (thus more resistant) to bromoxynil than Col-0 especially at concentrations of 25 μM and above.

Bromoxynil inhibits PSII *via* binding to the D2 protein thereby inhibiting photosynthesis. In theory, nitrilases could act by detoxifying bromoxynil or activating the photosynthetic system *via* an unknown mechanism. To differentiate between these possibilities, two other photosynthetic inhibitors Atrazine (AT) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were tested for their effects on the germination of Col-0 and 35S:NIT1 seeds. These two herbicides act downstream of

PSII by inhibiting plastoquinone (PQ) and thus, similar to bromoxynil, inhibiting photosynthesis. Pilot studies using Col-0 seeds determined the concentrations of AT and DCMU to use for this experiment as 100 μM and 150 μM respectively (data not shown). Col-0 and 35S:NIT1 seeds were germinated on either ½ MS plates (control) or ½ MS plates supplemented with AT or DCMU at the concentrations stated s above.

Germination was scored as described for bromoxynil, every 24 hours for a 96 hour period. Germination for Col-0 and 35S:NIT1 was 72.3% and 83% respectively at 48 hours (95% and 86% at 96 hours) under control conditions (figure 5.2). Again, the two lines behaved similarly. In the presence of 100 μM AT, Col-0 and 35S:NIT1 had similar germination at 48 hours (13.7% and 14% respectively). This was also observed at 96 hours, with 66% germination for Col-0 and 58% for 35S:NIT1. This was similarly observed for the DCMU treatment (figure 5.2). At 48 hours, germination for Col-0 and 35S:NIT1 was 4.1% and 7.3% respectively (28.1% and 44.4% respectively at 96 hours). Two-way ANOVAs were performed for the germination rates at 48 and 96 hour time point for the two treatments. The ANOVA test showed that regardless of treatment and time point, there was no statistical significance ($P>0.05$) despite the graphs showing that wild type seeds seem to be more sensitive than NIT1 transgenic seeds to the two treatments. Student's t-tests were also done to determine the statistical significant difference for the individual treatments on the lines. For AT, there was no statistical difference between the two lines at both 48 and 96 hour time points, with the P-values being 0.94 and 0.23 respectively. This was similarly observed for the DCMU treatment, with P-values of 0.40 and 0.18 at 48 and 96 hours respectively. Taken together, the results showed that Col-0 and 35S:NIT1 had the same sensitivity for both AT and DCMU.

5.2.2 *Arabidopsis* nitrilases exhibit the triple response phenotype

As discussed above, *Arabidopsis* nitrilases may be involved in stress responses mediated by ET signalling. A simple way to investigate this is to focus on a well characterized ethylene response during the germination process and early post-germinative growth. During the germination and seedling emergence process, ET is produced in localized parts of the shoot axis. ET signalling then leads to the formation of the apical hook, a protective mechanism for the apical tissues during its emergence from soil. When etiolated seedlings are germinated in the presence of ET or its

precursors such as 1-amino-cyclopropane-1-carboxylic acid (ACC), the triple response phenotype can be observed during germination (*149*). The triple response is a phenotype which occurs when plants are exposed to ET or its precursors; plants exhibiting this are described as having exaggerated apical hook curvature, inhibition of root growth, and hypocotyl elongation and swelling of the hypocotyl (*150*). Disruption to ET perception or its signalling pathway will result in plants which are insensitive to the hormone and do not display the triple response phenotype.

To investigate if nitrilase over-expression causes a change in ethylene responses, the observation of the triple response phenotype (or lack of) was to be determined in 35S:NIT1 and 35S:NIT4 mutant lines and compared with a wild type (Col-0) line. These lines were germinated in the dark on ½ MS plates supplemented with 10 μM of the ET precursor ACC and 1% (w/v) sucrose.

Seedlings were assessed after 72 hours of germination and growth and appearance visually scored for the presence or absence of the triple response phenotype (figure 5.3). Etiolated wild type seedlings germinated on 10 μM of ACC exhibited stunted root and hypocotyl growth, exaggerated curvature of the apical hook and swollen hypocotyls, indicating the triple response to the treatment. Conversely, wild type seedlings germinated under control conditions did not show the triple response phenotype. The two NIT over-expressing lines behaved just as the wild type did under both the control and treatment conditions. Using only visual observations, the severity of the response to ACC was indistinguishable between the three lines, indicating that there was no constitutive synthesis of ET or activation of the signalling pathway in these mutant lines.

5.2.3 NIT1 and 4: different responses when exposed to exogenous MeJA

The work presented in chapter four indicated that *Arabidopsis* nitrilases may be linked to JA signalling responses. The relative expression study presented in the previous chapter showed that NIT1 and 4 gene expression responded differently to exogenous application of MeJA. A simple assay to determine the effect of JA on plants is based on root growth inhibition. Root growth of *Arabidopsis* plants is inhibited when plants are exposed to MeJA. To test whether the difference observed on the genetic level is translated to the phenotypic level, root growth inhibition in response to MeJA was assessed for the 35S:NIT1 and 35S:NIT4 over-expressing lines and the wild type (Col-

0) line. Seeds of the three lines were germinated and grown vertically on ½ MS plates till their primary root reached 1 cm in length. The seedlings were then transferred to either ½ MS plates (control) or ½ MS plates supplemented with 50 μM of MeJA. The increase in root length was then monitored between day 0 (day of transfer) and day 4 following the transfer. This was done by marking the position of the root tip for each seedling at day 0 and day 4 on the plates. After four days of treatment, the plates were scanned and root lengths were measured using the scans. Measurements were done using the Image J software and the measure and label plugin (*151*).

Under control conditions, the lengths of the roots of all three lines had increased by 66% between day 0 and 4 (figure 5.4). In the presence of MeJA, root growth was inhibited in all three lines. In addition to root growth inhibition, seedlings had also accumulated anthocyanin as a result of the MeJA treatment (figure 5.5). The wild type and the NIT1 over-expressing lines displayed a similar degree of inhibition, both had a 39% increase in root length. In contrast, the increase in root length for the NIT4 line was reduced from 66% to 30%. The difference observed here in root length increase for the 35S:NIT4 and the Col-0 lines was statistically significant ($P<0.05$) as determined by using the two-way Analysis of Variance (ANOVA). Conversely, there was no statistical significant difference between Col-0 and the 35S:NIT1 lines. The data suggested that there is a phenotypic difference manifested as a change in root growth in response to MeJA between the NIT4 and 1, and NIT4 and Col-0. This indicated an increased sensitivity of NIT4 over-expressing plants to MeJA. Furthermore, as NIT1 showed a similar inhibition of root growth to the wild type, it was concluded that NIT1's response to MeJA was unchanged as compared to the wild type.

5.2.4 The effect of salt stress and ABA treatment on NIT1 and 4

Seed germination and dormancy are defined as mechanisms in which the morphological and physiological alterations result in the activation or inactivation of the embryo (*152*). Both mechanisms are regulated on hormonal and environmental levels. Seed dormancy is primarily controlled by ABA; induction of dormancy requires endogenous ABA and the *de novo* synthesis of ABA in seeds maintains dormancy during imbibition. A small amount of ABA (<3 μM) is enough to supress the germination of mature seeds (*143*).

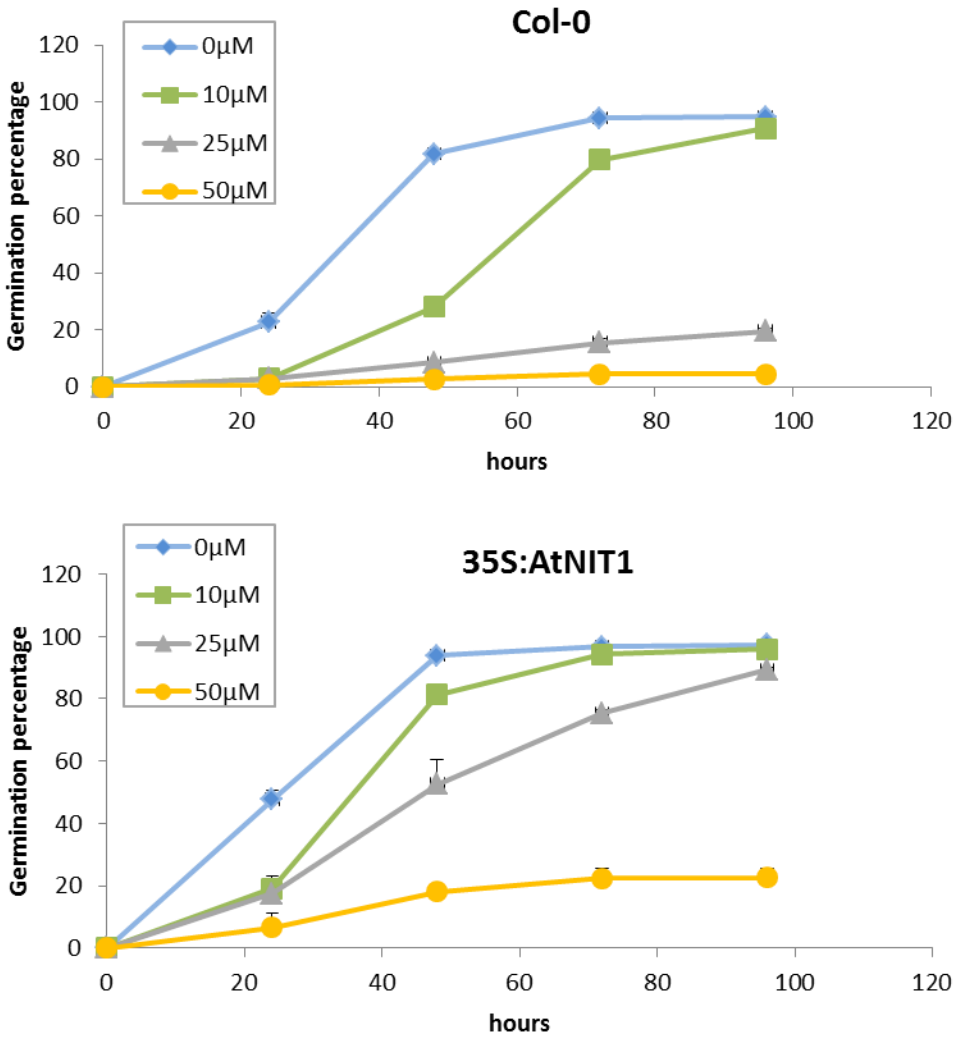


Figure 5.1 The germination of Col-0 and 35S:AtNIT1 over-expressing lines tested on different concentrations of bromoxynil

Seeds were germinated on ½ MS plates supplemented with bromoxynil at different concentrations to assess its effect on germination (n>100). Germination was measured every 24 hours until close to 100% germination was reached. Plotted results are averages of three replicates with n>100 seeds per replica. Error bars indicate standard error.

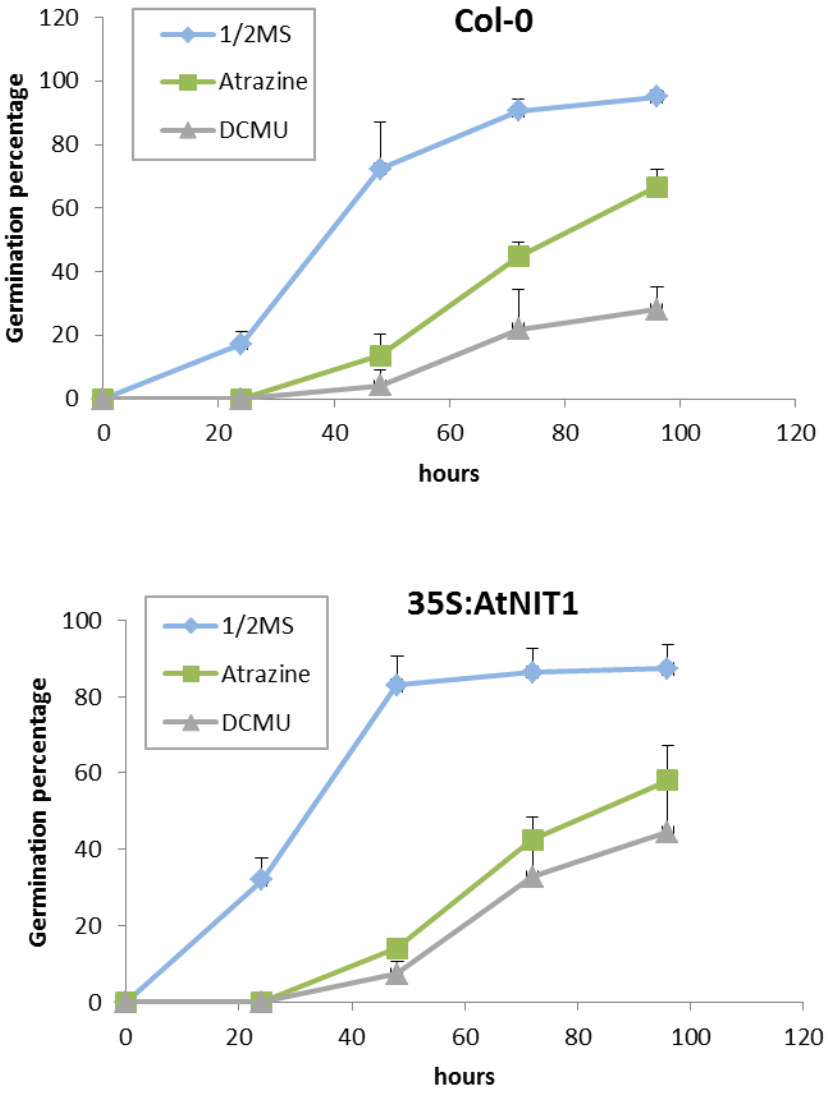


Figure 5.2 The germination of Col-0 and 35S:AtNIT1 over-expressing lines tested on Atrazine and DCMU

Seeds were germinated on ½ MS plates supplemented with either Atrazine (100 µM or DCMU (150 µM) (n>100). Germination was measured every 24 hours until the wild type seeds reached close to 100% germination. The plotted results are an average of three replicates with n>100 seeds per replica. Error bars indicate the standard error

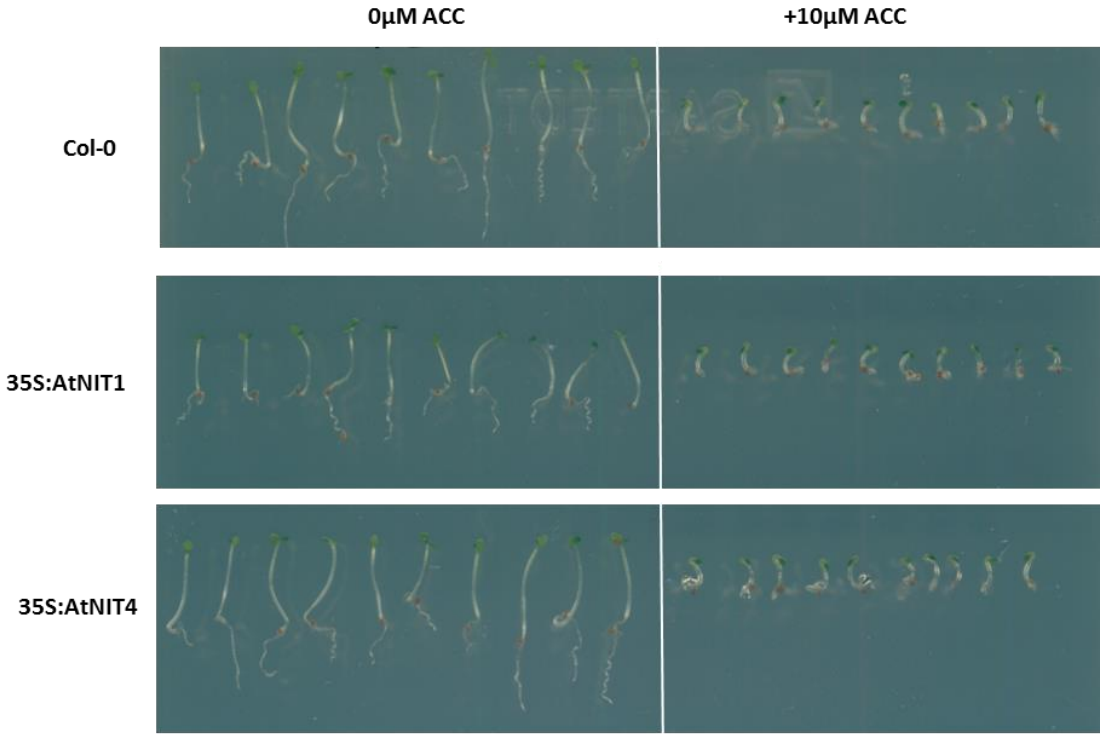


Figure 5.3 ***Arabidopsis*** **Col-0, AtNIT1 and AtNIT4 overexpressing lines exhibit the triple response phenotype when germinated on 10 µM ACC**
Seeds were germinated in either 0 µM or 10 µM ACC ½ MS plates supplemented with 1% (w/v) sucrose in the dark for 72 hours (n= 10). Seedlings were scored on whether they showed the triple response phenotype indicating sensitivity to ACC. The experiment was repeated three times with n = 10 seedlings per replica.

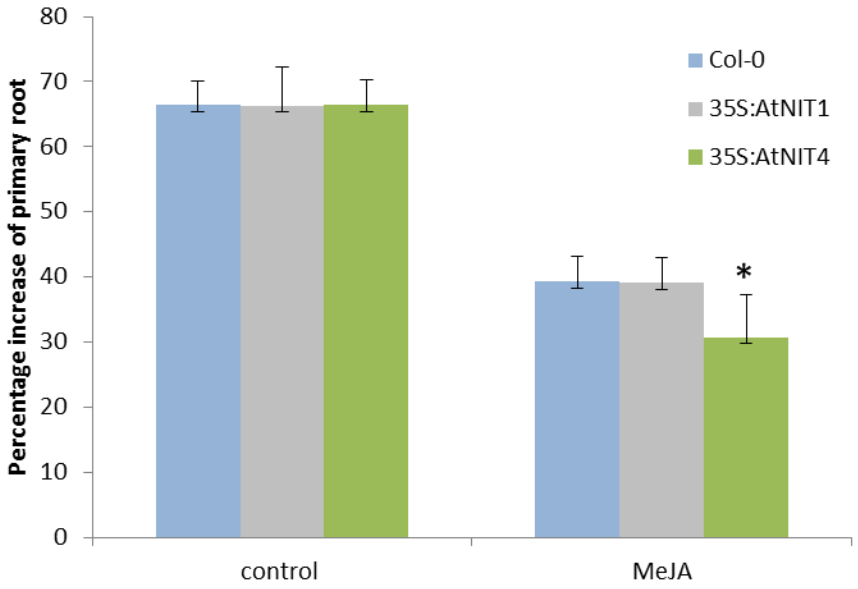


Figure 5.4 Inhibition of root growth for AtNIT1 and 4 over-expressing lines when grown on 50 µM MeJA
Seeds were germinated on vertical ½ MS media until the primary root was 1 cm in length. Seedlings (n = 10) were transferred to vertical plates containing ½ MS or ½ MS supplemented with 50 µM of MeJA. The percentage increase in root length was compared between the wildtype (Col-0) and the over-expressor lines by measuring the root length at day 0 and then again at day 4using the Image J software and the measure and label plugin software *(151)*. Root growth was inhibited by MeJA in all three lines. The inhibition was statistically significant for the 35S:AtNIT4 line compared to the wildtype and the AtNIT1 line as shown by ANOVA analysis (*P-value <0.05). Results are averages of three replicates with n=10 per replica. Error bars indicate the standard errors.

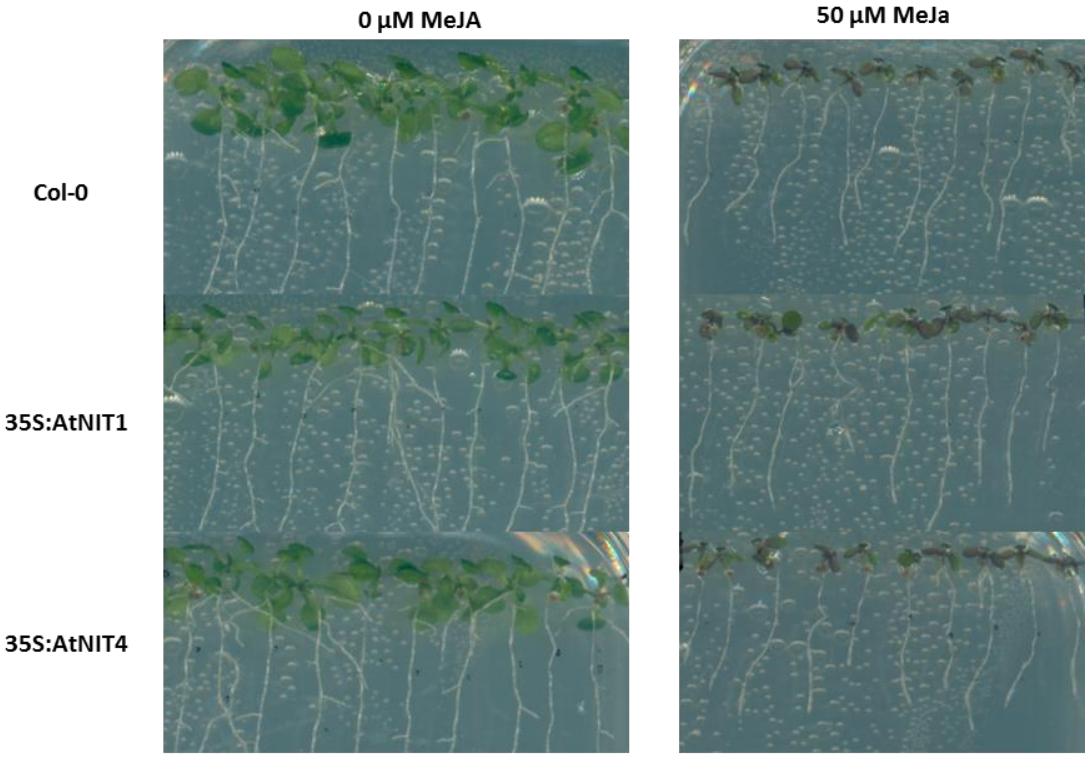


Figure 5.5 Anthocyanin accumulation in response to MeJA treatment in AtNIT1 and AtNIT4 overexpressing lines and in Col-0 wild type.
Seeds were germinated on vertical ½ MS plates until the primary root was 1 cm in length. Seedlings (n = 10) were then transferred onto 0 µM or 50 µM MeJA vertical ½ MS plates and grown for another four days. The lines were compared for anthocyanin production as indicated by the appearance of a red/purple colouring.

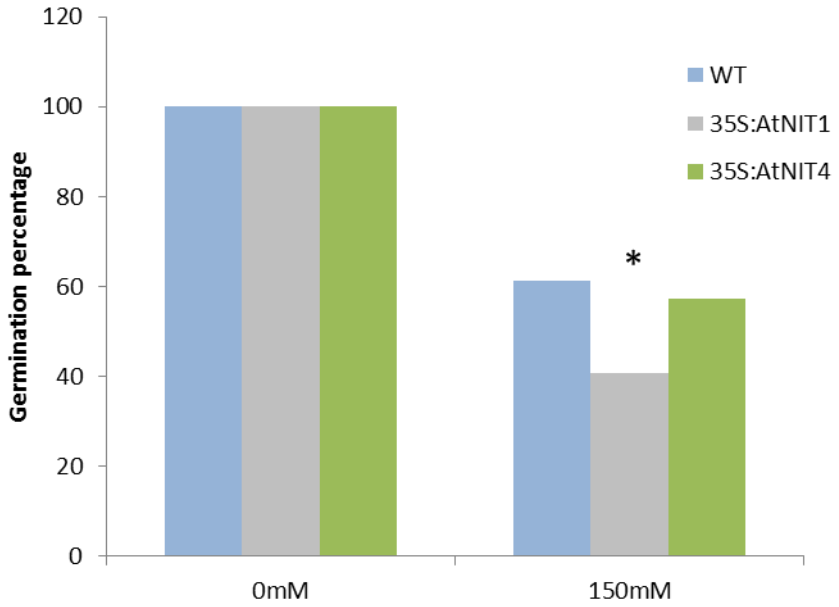


Figure 5.6 Germination of *Arabidopsis* wildtype and the AtNIT1 and AtNIT4 over-expressing lines during salt stress
Seeds (n >100) were germinated on either ½ MS plates or plates supplemented with 150 mM NaCl. Germination was scored based on the emergence of the radicle from the seed after five days of growth. The 35S:AtNIT1 line did not have 100% germination under control conditions. Thus, the relative germination rates are shown. The relative germination rates were calculated by dividing the germination frequencies under treatment conditions for each line by their respective control germination rates. Statistical analysis (ANOVA) showed that the inhibition of germination by salt stress compared to wild type was significant for 35S:AtNIT1 (*P-value<0.05).

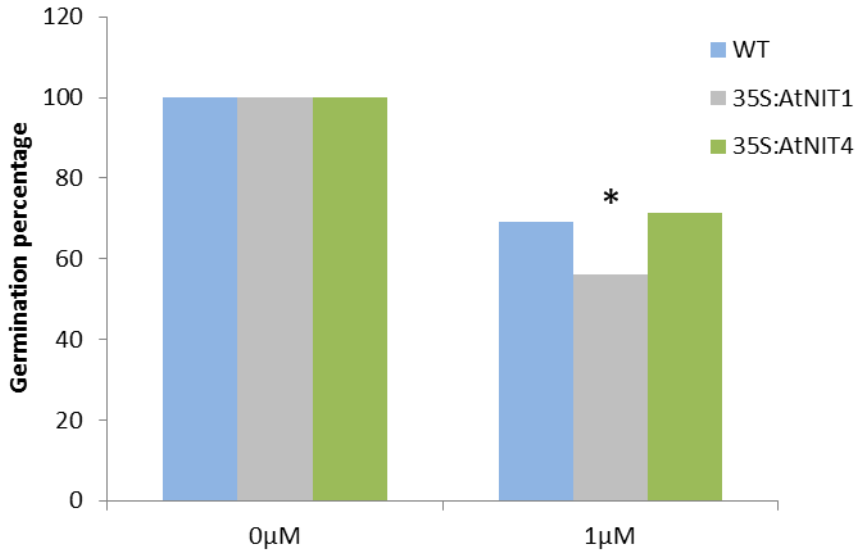


Figure 5.7 The effect of ABA on the germination of wildtype *Arabidopsis* and the AtNIT1 and 4 overexpressing lines
Seeds (n>100) were germinated on ½ MS plates supplemented with 1µM ABA and the germination was scored after five days of growth (n>100). The 35S:AtNIT1 line did not have 100% germination under control conditions. Thus, the relative germination rates are shown. The relative germination rates were calculated by dividing the germination frequencies under treatment conditions for each line by their respective control germination rates. Statistically significant inhibition for the 35S:AtNIT1 line compared to wild type Col-0 as shown by ANOVA analysis (*P-value<0.05).

Salinity also inhibits germination by affecting nitrogen levels in seeds, enhancing ABA levels and altering membrane permeability and water behaviours in seeds (*152*). Earlier, it has been shown that over-expressing NIT1 or 4 does not have an impact on germination rates under control conditions (5.2.1). However a link between ABA and *Arabidopsis* nitrilases suggests that over-expressing *Arabidopsis* nitrilases may influence germination under abiotic stress. Germination assays were performed here to determine whether NIT1 and 4 over-expression impact on germination during abiotic stress conditions. Col-0, 35S:NIT1 and 35S:NIT4 lines were germinated on ½ MS plates without supplementation or supplemented with either 150 mM NaCl or 1 μM ABA. Seeds were monitored for germination at day 0 and 5. Germination was scored based on the presence of the radicle emerging from the seed coat. The calculated germination frequencies were then used to compare between treatments (NaCl or ABA supplemented ½ MS plates) or control (½ MS plates) and between the lines.

Under control conditions, Col-0 and 35S:NIT4 seeds had germinated to 100% by day 5. In contrast, the germination of the 35S:NIT1 seeds under control conditions was usually near 80%, never reaching 100% for both treatments (appendix figure 3). As such, the relative germination frequency based on control conditions was calculated and used for the statistical analysis. In the presence of 150 mM NaCl, germination of all three lines was inhibited (figure 5.6). There was no significant difference in inhibition between Col-0 (61%) and 35S:NIT4 (57%) lines (P-value>0.05). However, only 40.7% of seeds germinated for 35S:NIT1 line. There was significant difference in inhibition between Col-0 (P-value<0.05) and 35S:NIT1 line which was similarly observed between the 35S:NIT1 and 35S:NIT4 lines (P-value<0.05).

A similar trend was observed when seeds were germinated in the presence of 1 μM ABA. All lines were inhibited but the extent of the inhibition differed. As for NaCl, Col-0 and 35S:NIT4 seeds were inhibited to a similar extent (69% and 71% respectively) with no significant difference (P>0.05) (figure 5.7). As seen for NaCl treatment, 35S:NIT1 germination was inhibited to a greater extent (56%) which was significantly different to Col-0 and 35S:NIT4 (P>0.05). Together, these results indicated that NIT1 over-expression causes reduced germination rates when exposed to abiotic stresses like high salinity or to the mediator of abiotic stresses, ABA. In contrast, over-expressing NIT4 is inconsequential for germination rates under the tested conditions.

Thus, the two *Arabidopsis* nitrilases belonging to the different nitrilase branches behave differently under the abiotic stress conditions tested here.

5.3 Discussion

5.3.1 Summary

Potential links between several defence-related phytohormones and the *Arabidopsis* nitrilases were investigated on a phenotypic level. Sensitivity to ET was tested by using the well-established triple response assay. When exposed to ACC, an ET precursor, NIT1 and 4 over-expressing seedlings, showed no significant difference to the wild type, *i.e.* all three lines exhibited the characteristic features of the triple response. Root growth of NIT4 over-expressing seedlings was more sensitive to MeJA than that of NIT1 over-expressing mutant and Col-0 seedlings. The germination of 35S:NIT1 seeds was significantly more inhibited when exposed to high salinity or ABA. In contrast to salt and ABA, NIT1 over-expressing seeds were better able to germinate in the presence of various concentrations of bromoxynil. In the presence of other types of photosynthetic inhibitors, AT and DCMU, there was no significant difference in germination for 35S:NIT1 and Col-0 lines.

5.3.2 Plants over-expressing 35S:NIT1 or 4 show sensitivity to ACC

The interaction between *Arabidopsis* nitrilases and ERF1 suggested possible involvement in ET signalling. This was investigated by testing 35S:NIT1 and 4 over-expressing seedlings for the triple response phenotype when exposed to ACC. During the process of seed germination and emergence, the terminal part of the shoot axis produces the apical hook which protects the apical tissues from damage when emerging from the soil. In germinating seedlings, ET is produced in the apical hook region to induce curvature of the hook. Exposing germinating seeds to exogenous ET or its precursor ACC will exaggerate the formation of the hook in etiolated seedlings and thus exhibit the triple response phenotype. Once exposed to white light, the production of and sensitivity to ET decreasing as the seedlings become more photosynthetically competent. Thus the best time point to test for ET sensitivity is during early germination and before seedlings are exposed to light.

ACC treatment resulted in formation of the triple response phenotype for the NIT1 and NIT4 over-expressing seedlings, indistinguishable to that of the Col-0 seedlings (figure 5.1). This demonstrated that the *Arabidopsis* nitrilases have no role in the main ET signalling or perception pathways during early germination. NIT1 and 4 also did not show a constitutive activation of ET signalling in the absence of ACC as can be observed in etiolated ERF-1 over-expressing seedlings which exhibit a partial triple response; lacking only the exaggerated curvature of the apical hook (*125*). The partial response was found to be due to constitutive activation of the ET signalling pathway rather than increased levels of ethylene (*125*). However, NIT4 gene expression does respond to ET treatment (*35*). An explanation for this discrepancy between gene expression changes and the lack of a visible phenotype may reside in the role *Arabidopsis* nitrilases potentially have in ET signalling. ERF1 is a mediator between JA and ET signalling through interacting with EIN3/EIL1. Like ERF1, the *Arabidopsis* nitrilases may also act as mediators between the two signalling pathways hence the response to ET is observed on a genetic level but not on a phenotypic level.

Here, the response to ET was tested during the germination process. However, NIT4's gene expression in response to ET occurs relatively late; with the highest expression during leaf senescence and during the flowering stages (figure 1.1). Given that ET signalling also affects these two late stages, it could be hypothesised that a phenotype in response to ET may be detectable in NIT over-expressing lines when older plants are tested. Older NIT1 and 4 over-expressing seedlings could be tested for root growth inhibition in the presence of ACC as ET is also known to inhibit root growth. This could also be complemented on the genetic level by determining whether there is a temporal expression of genes involved in ET signalling such as ETR1 or ERF1 in NIT1 and 4 over-expressing plants.

5.3.3 NIT4 over-expression causes hypersensitivity to MeJA

Together, JA and auxin regulate root growth through an antagonistic relationship. The NIT1 homologues are said to be involved in auxin synthesis through the hydrolysis of indole-3-acetonitrile (IAN), the precursor for auxin. On a molecular level, there is a difference in response to exogenous MeJA for the NIT1 homologues and NIT4 (chapter four). Here it was further explored if this difference is manifested on a phenotypic level.

A simple assay, based on the impact JA has on root growth, was used to test for altered sensitivities of NIT1 and 4 transgenic lines to the presence of JA. The effect of auxin on root growth was also investigated for the NIT1 over-expressing line. If NIT1 is promoting auxin synthesis, one would expect increased root growth for the over-expressing line when compared to the wild type. Yet, under control conditions, both the wild type and 35S:NIT1 had a growth increase of 66% (figure 5.5). Thus, the root growth studies suggested that NIT1 is not involved in synthesising the majority of auxin in *Arabidopsis* during early seedling growth. When exposed to MeJA, root growth of the 35S:NIT4 seedlings was significantly more inhibited than that of Col-0 and 35S:NIT1 seedlings. This suggested that over-expressing NIT4 makes plants more sensitive to MeJA, correlating with the gene expression data where NIT4 is downregulated when exposed to MeJA. Root growth of 35S:NIT1 seedlings was similarly affected as Col-0, suggesting that 35S:NIT1 is not hypersensitive to the hormone.

The contrast in what is observed on a phenotypic and genetic level for NIT1 suggests that NIT1 may not have a direct role in JA signalling responses. The upregulation of NIT1 gene expression observed for the gene expression study in chapter 4 could be a knock-on effect due to NIT1 and its homologues involvement in glucosinolate catabolism. Genes such as CYP79B2/B3 involved in glucosinolate synthesis are known to be upregulated in response to MeJA (*117*). Interestingly, *VSP1* gene expression did not alter much in the 35S:NIT1 transgenic line. As glucosinolate catabolism is a response to wounding and herbivory attacks and the purported involvement of NIT1 in such responses, it was expected that *VSP1* expression would be increased. This result, and the lack of sensitivity to MeJA observed in the root growth inhibition assay further supports a lack of involvement in JA signalling response of NIT1, particularly during wounding or herbivory attack. The hypersensitivity observed in the 35S:NIT4 plants, along with the downregulation of NIT4 gene expression in response to MeJA further supports the hypothesis that NIT4 could be providing a negative regulatory role in JA signalling responses. The downregulation of *PDF1.2* in NIT4 over-expression mutants also indicates a possible negative regulatory role for *Arabidopsis* nitrilases.

5.3.4 High salinity and ABA treatment have an effect on 35S:NIT1 plants but not on 35S:NIT4 plants

Both AtMKK2 and ERF1 are involved in regulating abiotic stresses, in particular salt stress which is under the control of ABA signalling. Several transcriptomic and proteomic experiments have also linked the *Arabidopsis* nitrilases to abiotic stress response. At the onset of this analysis, it was unknown whether the *Arabidopsis* nitrilases are involved in ABA signalling which regulates a majority of abiotic stresses. To answer whether there is a link between *Arabidopsis* nitrilases, ABA and abiotic stresses such as high salinity, germination assays were performed.

Germination is defined as the process during which the plant embryo transitions from dormancy to growth. This process is regulated by signalling pathways which respond to environmental cues such as light, temperature, and humidity. It begins with the uptake of water by the embryo and is completed when the radicle (the embryonic root) penetrates the seed coat (*153*). Seed dormancy is the opposing process whereby the imbibition process is prevented and the initial uptake of water by the embryo is stopped. Gibberellins (GAs) and ABA are the two main phytohormones regulating the germination process. ABA maintains dormancy whereas GA synthesis and signalling initiates germination.

Germination data showed that NIT1 transgenic seedlings are hypersensitive to both salt and ABA treatments as compared to Col-0 (figure 5.6 and 5.7). This is not observed with NIT4 over-expressing seedlings which behaved like the wild type. The difference in response is interesting given that both NIT1 and 4 interact with AtMKK2 and ERF1 (chapter four, 5.2.4). One has to keep in mind that the nitrilase interactors were identified using transgenic plants expressing NIT bait proteins under the control of the strong and constitutive CaMV 35S promoter. The bait proteins were expressed at high levels and in some tissues likely to be out of their usual spatial and temporal context (chapter four). Indeed, an important difference between NIT1 and 4 are their different expression patterns. Whilst NIT1 in wild type is highly expressed in the shoot apex and radicle, there is little expression of NIT4 in the same regions (figure 1.2). This may be one explanation for the apparent contradiction of the identical interaction with AtMKK2 and ERF1 and the different behaviours of the NIT1 and 4 over-expressing plants in response to ABA and high salinity. This suggests that developmental stages and timing

may come into play for nitrilases interacting with AtMKK2 and ERF1 and their overall effect on abiotic and biotic stress responses. The results here suggest that NIT1 may have a direct role in abiotic stress responses whilst NIT4 does not. This poses the question: how does NIT1 directly affect abiotic stress responses? The hypersensitivity observed suggest that NIT1 over-expression somehow either causes increased ABA synthesis or constitutive activation of the signalling pathway thereby increasing the dormancy of the seeds which explains the reduced germination rate observed for the NIT1 transgenic seeds. However, it can also be hypothesised that for the NIT4 over-expressing seeds, the response to high salinity or ABA treatment may be dependent on the spatial and temporal contexts. Instead of looking at germination ability, the root growth inhibition could be looked at in NIT4 over-expressing mutants. As seedlings are used for such experiments, it may be possible to see whether the developmental stage and timing plays a role in how NIT4 responds to ABA or abiotic stress treatments. One could explore this further by investigating expression levels for markers of ABA response and thus for abiotic stress responses. Expression of genes such as the ABI genes could be determined in the NIT4 over-expressing lines during different developmental stages and tissues. This would also provide clues into whether NIT4 is also involved in abiotic stress responses.

An interesting observation was made during the germination experiements with the NIT1 over-expressing seeds under control, bromoxynil, ABA and salt treatments. The NIT1 transgenic seeds reached closed to 100% germination and germinated faster than the Col-0 seeds under control conditions for the bromoxynil experiments (figure 5.1). However, for the ABA and salt treatment experiments, NIT1 transgenic seeds only germinated to 80% under control conditions after five days (appendix figure 3). Seeds used for the bromoxynil experiment were relatively new; two to three months post-harvest. However, the seeds used for the ABA and salt treatments had been kept in storage for six months. The storage process had not affected Col-0 nor NIT4 transgenic seeds; they reached 100% germination after five days in both experiments (appendix figure 3). Thus, it appears that older NIT1 transgenic seeds have increased dormancy. This suggests that the over-expression of NIT1 has an effect on ABA signalling in seeds, increasing dormancy of seeds over time. It could also be due to an increase in ABA levels over time which would also lead to the same result. Confirmation of this observation could be achieved with germination studies using seeds of different storage

ages; dormancy can then be compared with how long the seeds have been stored. In addition, ABA levels in the ANIT1 transgenic seeds stored at different lengths of time could also be determined to see whether it supports the dormancy hypothesis.

Therefore, it can be postulated that NIT1 has some role in ABA signalling, particularly in seed dormancy based on the results presented here and previous chapters. Furthermore, the difference in response to the treatments suggest the notion of a divergence of roles for the four *Arabidopsis* nitrilases with NIT1 involved in ABA signalling responses.

5.3.5 NIT1 confers specific resistance to the PSII inhibitor bromoxynil

Bromoxynil, Atrazine and DCMU are herbicides which disrupt photosynthesis by binding to various components of the photosynthetic machinery. Specifically, bromoxynil binds to the D2 protein of PSII and both Atrazine and DCMU bind to plastoquinone (PQ). Whilst bromoxynil is a potential substrate for the NIT1 homologues, both Atrazine and DCMU are not substrates for nitrilases based on their chemical structures. This allowed a differentiation between functions related to the enzymatic activity of the nitrilases and other potential impacts nitrilases may have in protecting photosynthesis. Thus, this study investigated the potential for NIT1 transgenic plants to be tolerant to bromoxynil whilst expecting that they would still be sensitive to the herbicides Atrazine and DCMU.

NIT1 over-expressing seeds showed an altered response compared to wild type when germinated on bromoxynil containing ½ MS media. The testing of bromoxynil at 10, 25 and 50 μM led to results in which a tolerant phenotype observed for the 35S:NIT1 plants as compared to Col-0. The tolerant phenotype observed here for the NIT1 is not dissimilar to that observed for the tobacco plants stably transformed with a bacterial nitrilase. Stalker *et al* reported that *N.tabacum* leaves over-expressing a bacterial nitrilase had less chlorosis than the wild type leaves at up to 100 μM bromoxynil (*148*). Whilst chlorosis in leaves was not investigated here but rather germination ability, it was still observed that at a high concentration (50 μM), tolerance to bromoxynil was observed in the 35S:NIT1 seeds. At concentrations 50 μM and higher, wild type seeds failed to germinate. However, in this study it was not determine whether this is also the case for the 35:NIT1 seeds. Thus, germination assays using high concentrations (100

μM or higher) could be performed to see whether 35:NIT1 can still germinate. Chlorosis of the Col-0 and 35:NIT1 leaves could also be tested at the same concentrations to complement the study by Stalker *et al* did and the germination study presented here. Ioxynil and chloroxynil are herbicides which are part of the same family of nitrile herbicides as bromoxynil. Out of the three, ioxynil is the most toxic as the heavier the halogen, the more toxic the herbicide is (*154*). Further studies could test the tolerance to these nitrile herbicides to demonstrate that over-expression of NIT1 has an effect not just limited to one type of nitrile herbicides.

ANOVA analysis indicated that over-expressing NIT1 conferred no tolerance to both Atrazine and DCMU as there was no significant difference between Col-0 and 35:NIT1. Atrazine and DCMU are similar to bromoxynil in their mode of action in that all three inhibit photosynthesis by blocking electron transfer albeit at different positions in the photosynthetic electron transport chain. The lack of tolerance to Atrazine and DCMU suggests that the tolerance of 35:NIT1 is specific to nitrile herbicides as bromoxynil is a substrate for NIT1 as it is for bacterial nitrilases. Tolerance is most likely achieved in the NIT1 transgenic plants through the breakdown of bromoxynil by the nitrilase thereby preventing the binding of the herbicide to the D2 protein of PSII.

5.3.6 Conclusion

A significant finding in this study are the differences NIT1 and NIT4 when exposed to MeJA, ABA and high salinity. Particularly, that expression differences in response to MeJA are also reflected on the phenotypic level. The response to high salinity and ABA differed between the two nitrilase isoforms when over-expressed. However, the timing of the expression was not investigated here but may come into play for NIT4. In the previous chapter, it was hypothesised that NIT1 may be regulating a different set of genes when compared to NIT4. The differences in response to ABA and salinity treatments seem to agree with that hypothesis. The insensitivity to MeJA treatment in NIT1 over-expressing seedlings further supports this hypothesis. Taken together, the results presented in this chapter provide further evidence that there is diversification of functional roles within the NIT family particularly in relation to abiotic and biotic stress responses. The specific resistance to photosynthetic stress observed due to the over-expression of NIT1 in plants shows that *Arabidopsis* nitrilases do not confer general

resistance to photosynthetic stress. The resistance observed is related to the enzymatic ability of nitrilases to degrade nitrile substrates such as bromoxynil.

Chapter 6

General Discussion

6.1 Summary

Hitherto, the biological role of the *Arabidopsis* nitrilases has been defined by their enzymatic capabilities. Originally thought to be involved in auxin synthesis, the role for the *Arabidopsis* nitrilases shifted towards glucosinolate catabolism. Recent research has indicated that these enzymes may have additional roles not necessarily defined by their enzymatic activity alone. For example, mammalian nitrilases are now known to be involved in cell cycle regulation and progression. The involvement of mammalian nitrilases in these processes is independent of their enzymatic function. Similar roles in cell cycle regulation have been found for the NIT1. Interactions of plant nitrilases with transcription factors such as ATAF and EREBP further suggest novel roles beyond nitrile degradation. Thus, nitrilases may be more than just nitrile degrading enzymes and their biological role may not be solely definable by their enzymatic function.

The work presented in this thesis aimed to expand our knowledge of the potential biological roles of *Arabidopsis* nitrilases and determine if diversification exists by probing on several levels. In addition, it aimed to investigate how much the enzymatic function defines these biological roles of the *Arabidopsis* nitrilases. Several approaches were taken to achieve this. Firstly, the subcellular localization of the *Arabidopsis* nitrilases and proteins which interact with *Arabidopsis* nitrilases were determined using fluorescent protein methods. Secondly, *in silico* predictions and AP-MS were used to identify *Arabidopsis* nitrilase interacting proteins to increase our knowledge of the potential biological context of nitrilase activity. Thirdly, gene expression studies using nitrilase over-expressing mutants and wild type were used to investigate the impact *Arabidopsis* nitrilases have in the context of hormone signalling pathways. Finally, physiological characterizations of these mutants were used to interrogate findings of the aforementioned approaches.

The NIT1 to 4 GFP fusion proteins were determined to be localized to both the nucleus and cytosol. The ability to form dimers was subsequently investigated; all four isoforms were able to form homo-and heterodimers which were found to also localize to the nucleus and cytosol. Thus, the two fluorescent protein approaches supported each other and revealed a previously unknown subcellular localisation for the *Arabidopsis* nitrilases, the nucleus.

Novel interactors were identified for NIT1 and 4 using a combination of *in silico* and experimental analysis using PPI databases and AP-MS. The *in silico* analysis predicted nuclear proteins, serine/threonine kinases and ubiquitin proteases as potential interactors. Proteins involved in the auxin/glucosinolate synthesis pathway were also predicted. The AP-MS performed for NIT1 and 4 over-expressing *Arabidopsis* plants identified the *Arabidopsis* nitrilases themselves as interactors, confirming the dimerization studies and thereby validating the AP-MS approach. Furthermore, the use of AP-MS and BiFC lead to the identification of 14-3-3 proteins as interactors of the *Arabidopsis* nitrilases. It was found that the interaction between 14-3-3s and the four NIT isoforms was not discriminative, *i.e.* all *Arabidopsis* nitrilases are able to interact with both epsilon and non-epsilon 14-3-3 isoforms. Interestingly, the NIT:14-3-3 interactions were only observed in the cytosol and not in the nucleus. This was surprising as NIT-GFP fusion proteins, NIT dimers and 14-3-3 dimers can be observed in both the cytosol and the nucleus (*77*). Protein fragment deletion analysis showed that the interaction of NIT1 and 14-3-3 proteins required the first 69 amino acids within the NIT1 protein. This validated the interaction of nitrilases and 14-3-3 proteins as it demonstrated that these interactions require certain nitrilase domains and are not non-specific interactions.

AtMKK2 and ERF1, two proteins involved in the JA signalling pathway, were identified as interactors of NIT1 and 4. Upon interaction with the *Arabidopsis* nitrilases, the localization of ERF1 shifted from purely nuclear to both cytosolic and nuclear, indicating that the interaction between the two proteins is meaningful in a biological context. This prompted investigation into the possible involvement of *Arabidopsis* nitrilases in JA signalling. First of all, it was established that the gene expression of NIT1, 2 and 3 was upregulated in seedlings in response to the MeJA treatment whereas gene expression of NIT4 was downregulated. Further investigation using two marker genes for the two branches of JA signalling (*VSP1* and *PDF1.2*) (figure 4.1) showed that NIT4 over-expression impacts negatively on the ERF branch whilst NIT1 over-expression does not influence the two branches in a significant way.

Phenotypic studies revealed that NIT4 over-expression caused MeJA hypersensitivity manifested as an inhibition of root growth. This indicated a possible connection between the gene expression changes seen in NIT4 over-expressing seedlings and its phenotype. NIT1 over-expressing plants did not show a pronounced MeJA sensitivity.

This was not due to a lack of biological activity as over-expression of NIT1 mediated an expected resistance to the herbicide bromoxynil. The biological activity of NIT1 over-expression was further investigated using germination assays. These assays revealed that NIT1 over-expression causes hypersensitivity to abiotic stresses such as high salinity and ABA, a phytohormone linked to abiotic stress signalling.

Taken together, NIT1 and 4 appear to be connected to different types of stress responses; whilst NIT4 appears to be linked to biotic stresses *via* JA, NIT1 may be involved in abiotic stress responses involving ABA. Hence, NIT4, the *Arabidopsis* nitrilase representative of nitrilases found in other plants, appears to be connected to the assumed nitrilase function of biotic stress response. Conversely, the biological functions of NIT1 may be deviating from this.

6.2 A regulatory model for *Arabidopsis* nitrilases

6.2.1 Sequestration by 14-3-3s

GFP fusion and dimerization studies for the four nitrilase isoforms show that these proteins have both nuclear and cytosolic localization (figure 3.1, 3.2). On the other hand interaction with 14-3-3 proteins occurred exclusively in the cytosol independent of the nitrilase isoform (figure 3.4). It is well documented that 14-3-3 proteins regulate client protein activity, localization pattern, stability and their ability to bind to other proteins (*155*). It was initially thought that the 14-3-3s regulate the enzymatic activity of nitrilases. Usually, 14-3-3 binding requires phosphorylation of a client protein's 14-3-3 binding motif (*70*). However, enzymatic function of the *Arabidopsis* nitrilases is independent of their phosphorylation status (*90*). The disconnection between enzymatic function and phosphorylation is seen as a point that argues against the regulation of nitrilase activity by 14-3-3s. The literature suggests other interpretations of the interaction of *Arabidopsis* nitrilases with 14-3-3s. The 14-3-3 proteins can impact on the biological activity of a client protein by sequestration, tobacco RSG1 being an example of this. Tobacco RSG1 is a transcription factor required in gibberellic acid (GA) biosynthesis; interaction with 14-3-3 proteins will sequester RSG1 to the cytosol, inhibiting transcription of genes required in GA biosynthesis (*91, 156*). It is possible that 14-3-3 proteins regulate the biological function of nitrilases in a way analogous to

the RSG1:14-3-3 model, *i.e.* sequestration to the cytosol may prevent a nitrilase function in the nucleus. Another possibility is that the interaction with 14-3-3 proteins affects the stability of the *Arabidopsis* nitrilases. Interaction with 14-3-3 proteins could mark nitrilases for degradation *via* the ubiquitin-proteosomal pathway. The discovery of proteins related to the ubiquitin-proteosomal pathway such as RAD23 and AtUBP3 as nitrilase interactors support this idea (*43*).

6.2.2 A proposed model for the nuclear localization of *Arabidopsis* nitrilases

The nuclear localization of NIT-GFP fusion proteins and of the NIT dimers was an interesting observation given its enzymatic role is most likely to have no nuclear function and that the proteins themselves lack typical nuclear localization signals (NLSs). This raises the question: why the *Arabidopsis* nitrilases are found in the nucleus? The predictive and experimental interaction studies presented in this thesis may explain the nuclear localization (3.2.2 and 3.2.4). Furthermore, the *Arabidopsis* nitrilases may have atypical NLSs. The *in silico* nitrilase interactome studies predicted several nuclear proteins (P21CIP1 binding related, Gar1 RNA-binding) as nitrilase interacting proteins (3.2.2). Thus, *Arabidopsis* nitrilases may play a role in the function of these nuclear proteins. The experimental evidence obtained here suggests that *Arabidopsis* nitrilases may bind to nuclear proteins and sequester them to the cytosol (4.2.2). This hypothesis is supported by the interaction of the *Arabidopsis* nitrilases with the transcription factor ERF1 in the cytosol and nucleus (figure 4.2). ERF1 on its own was shown to localize exclusively to the nucleus which is in agreement with its function as a transcriptional regulator (figure 4.2)(*126*). The subcellular localisation of the NIT:ERF1 interaction may indicate that *Arabidopsis* nitrilases impact on the localisation of ERF1 by sequestration to the cytosol (figure 4.2) thereby interfering with the ability of ERF1 to activate transcription. If this hypothesis holds true, it may also be applicable to the interaction of *Arabidopsis* nitrilases with other nuclear proteins.

Based on the experimental data obtained for this thesis, a regulatory model based on that proposed by Xu *et al* can be proposed describing the biological context of the NIT:14-3-3 and NIT:ERF1 interactions (*49*) (figure 6.1). In the absence of a stress signal, the *Arabidopsis* nitrilases interact with ERF1 which results in the sequestration of ERF1 to the cytosol. This may require 14-3-3 proteins to act as an adaptor to allow the interaction to occur which may or may not require phosphorylation. The

sequestration means that the initiation of transcription of defence related genes such as *PDF1.2* is prevented. Under stress conditions, the interaction with 14-3-3 proteins retain the *Arabidopsis* nitrilases in the cytosol and/or mark them for degradation which prevents interaction with nuclear proteins. This results in the release of ERF1 from the *Arabidopsis* nitrilase complex, allowing relocalization of ERF1 (or newly synthesized ERF1) into the nucleus to initiate transcription. Such a model would be in agreement with our current idea of how 14-3-3 proteins exert their influence on client proteins (*155*). In the nucleus, *Arabidopsis* nitrilases could interact with nuclear proteins such as ERF1 and recruit those to the cytosol thereby preventing the nuclear activity of ERF1 and other nuclear proteins. If this proposed model or a modification thereof is correct, it would suggest a role for nitrilases beyond their enzymatic functions. The concept that enzymes have secondary roles is not new; such proteins can be defined as "moonlighting" proteins (*157*). Within plants, the most well studied example is hexokinase which not only acts as a sugar phosphorylating enzyme but also regulates sugar sensing and signalling (*158*). Enolase, another metabolic enzyme regulated by 14-3-3s, also has a secondary role in mediating abiotic stress responses through regulating the expression of genes such as Zat10 (*61*).

The regulatory model proposed in this thesis has not been rigorously tested. How the regulation by 14-3-3s is involved in the whole plant defence pathway remains to be determined. One of the main hypotheses presented here is that *Arabidopsis* nitrilases regulate the localization of nuclear proteins such as ERF1 possibly involving the 14-3-3 proteins. A follow-up experiment would seek to identify the 14-3-3 binding site in the *Arabidopsis* nitrilases and generate *NIT* mutants which can no longer bind to 14-3-3s. Such mutants could be used to determine whether the interaction of *Arabidopsis* nitrilases with ERF1 and the sequestration of ERF1 in the cytosol require 14-3-3s. In addition, an interactomic profile of these mutant nitrilases could be determined to see how the loss of 14-3-3 interaction will affect other observed interactions of the *Arabidopsis* nitrilases. On a genetic level, the expression of plant defence related genes in these mutants can also be determined to ascertain if and how the NIT:14-3-3 interaction is impacting on defence responses.

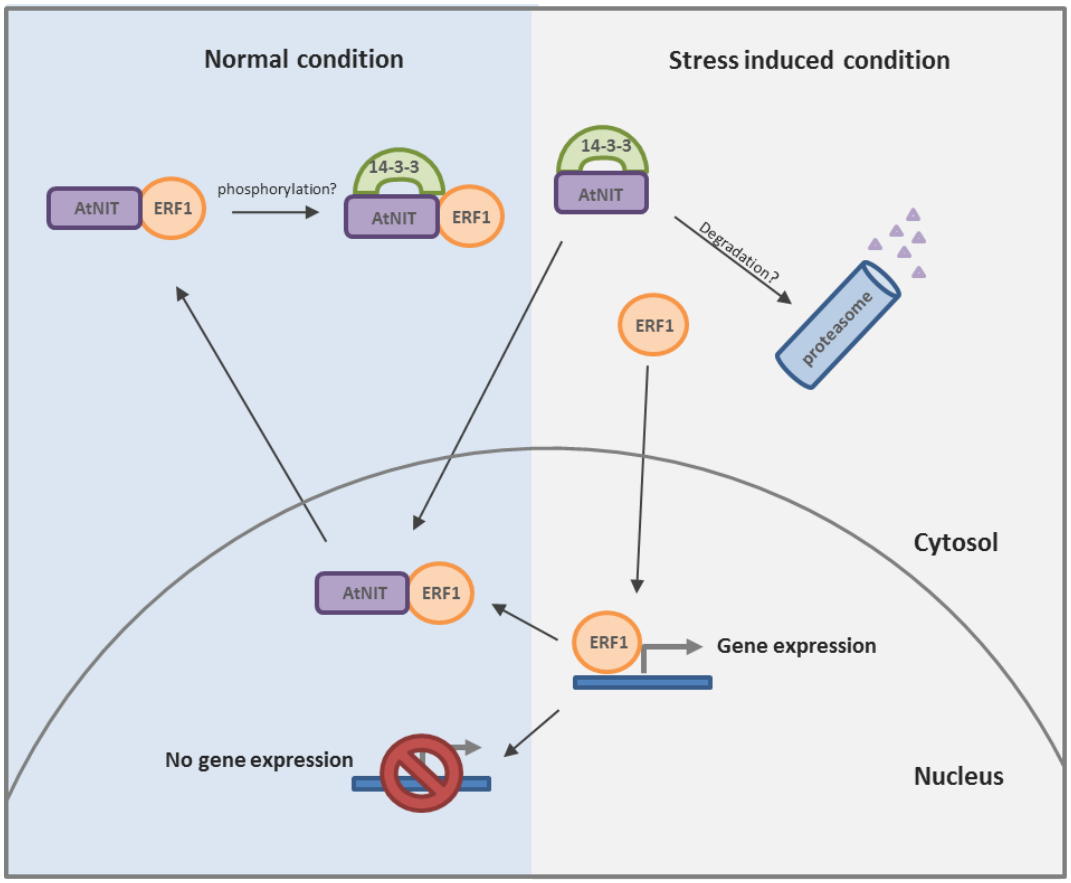


Figure 6.1 Proposed regulatory model of nuclear proteins by *Arabidopsis* nitrilases
Arabidopsis nitrilases are localized to both the nucleus and cytosol. In the cytosol, nitrilases interact with 14-3-3 proteins. Under normal conditions, *Arabidopsis* nitrilases bind to the nuclear proteins (such as ERF1 here), preventing transcription of genes by sequestering them to the cytosol. 14-3-3s may act as a bridge between *Arabidopsis* nitrilases and the nuclear protein (ERF1) preventing relocalisation to the nucleus. This may involve phosphorylation of nitrilase to enable 14-3-3 binding. When induced by a stress signal the nuclear protein (ERF1) is released and can localize to the nucleus to initiate transcription. *Arabidopsis* nitrilases may continue to interact with 14-3-3s and remain in the cytosol or be degraded via the ubiquitin/proteasome pathway.

6.3 Novel roles for *Arabidopsis* nitrilases in stress responses

The lack of a detrimental phenotype for NIT mutants (knockouts or over-expressor lines) appears to indicate that their importance to the overall fitness of the plant is small under normal growth conditions. However, plants rarely face 'normal lab' growth conditions in nature. Plants are constantly exposed to changing environments with challenges leading to abiotic and biotic stress conditions. *Arabidopsis* nitrilases' role in plant defence has always been defined by their ability to detoxify nitrile compounds such as those produced as a result of glucosinolate catabolism. However, there is little biochemical and genetic evidence directly linking *Arabidopsis* nitrilases with plant defence specifically in the context of glucosinolate catabolism. With transcriptomic data indicating that *NIT1* and *NIT2* are upregulated in response to MeJA treatment, a link between JA signalling and *Arabidopsis* nitrilases appears to be possible.

6.3.1 Roles in biotic and abiotic stress responses

A part of this thesis focused on investigating whether a link between JA signalling and *Arabidopsis* nitrilases exists which would provide further directions towards our understanding of the *Arabidopsis* nitrilases roles in plant defence. Protein interaction studies showed that two proteins involved in JA signalling (AtMKK2 and ERF1) interact with *Arabidopsis* nitrilases. Furthermore, gene expression studies showed that the expression of the four *Arabidopsis* nitrilases altered in response to exogenous MeJA. This was further explored by determining that over-expressing *NIT4* led to a downregulation of *PDF1.2*, a marker gene of the ERF branch of JA signalling. This downregulation was not observed when *NIT1* was over-expressed. The study of root growth in response to MeJA also showed that *NIT4* over-expressing mutants are hypersensitive to MeJA and responded differently to the hormone than *NIT1* over-expressors. This indirectly suggests a split in the biological roles within the NIT family, reflecting the diversification observed on both enzymatic and functional levels.

A contradiction to this is that ERF1 interacts with both NIT1 and NIT4, suggesting no diversification within the *Arabidopsis* nitrilases. However, this apparent contradiction may not be valid due to the methodologies used to study protein-protein interactions in this thesis. BiFC and AP-MS use constitutively over-expressed *Arabidopsis* nitrilases, systems which do not take into account the native temporal and spatial expressing

conditions. Thus the interaction studies solely showed that the interaction is possible *in vivo* but do not prove that it occurs under physiological conditions. It could be that NIT1 and ERF1 may not be expressed under the same circumstances, in the same location or at the same time. Furthermore, the amino acid sequences of the four *Arabidopsis* nitrilases are very similar, with homologies up to 60% which may result in ERF1 interacting with either NIT1 or NIT4 when these nitrilases are artificially co-expressed with ERF1. Adding to the complication is the fact that BiFC cannot assess the dynamics of the interactions. Due to the reformation process, BiFC cannot determine whether an interaction is weak or not; just whether interaction occurs or not. This means it is difficult to determine the strength of the interaction between NIT1 and ERF1 which could further determine how physiologically likely the interaction is. Using methods such as FRET or quantitative Y2H would be able to bypass these limitations and assess the strength of the interaction to determine the plausibility of the interaction under physiological conditions.

The localization of the NIT:ERF1 is both nuclear and cytosolic, suggesting that ERF1 is not fully retained in the cytosol. This means that there is a possibility that transcriptional activation would not be fully repressed by the interaction (figure 4.2). Under what conditions would lead to the full sequestration of ERF1 to the cytosol by the *Arabidopsis* nitrilases? Given that ERF1 is responsible for regulating JA signalling elicited by necrotrophic pathogens, ERF1-intiated transcription should be inhibited by wounding signals. This could be analysed by generating stable transformants of the NIT:ERF1 complexes in *Arabidopsis* and subjecting such plants to both wounding and necrotrophic pathogen treatment. The localization of the complexes may alter in response to these treatments and provide further clues for the biological context of this interaction.

The possible involvement of *Arabidopsis* nitrilases in abiotic stress responses as suggested by interactomic studies presented in this thesis directed phenotypic analysis under non-standard growth conditions. Such analysis revealed phenotypes associated with NIT over-expression. Germination assays under stress conditions induced by ABA or high salinity showed that *NIT1* over-expression conferred ABA and high salinity sensitivity which is not observed when *NIT4* is over-expressed. The response further highlights the difference in response between *NIT1* and *NIT4* observed during gene

expression studies. The sensitivity of *NIT1* over-expressing mutants provides a starting point for further analysis. For example, it is not known if the other NIT1 homologues, NIT2 and 3, show similar responses to ABA and high salinity. Determining if the hypersensitivity is NIT1 specific or conserved within the NIT1 homologues will further our understanding of the diversification of the *Arabidopsis* nitrilases. Other abiotic stresses such as drought are also regulated by ABA signalling. It would be interesting to see whether *NIT1* over-expression confers sensitivity to salinity only or provides a ubiquitous sensitive response to abiotic stresses which are mediated by ABA. The results obtained here are not definitive; whilst there is an observable phenotype, the underlying molecular pathways affected need to be determined. Over-expression of *NIT1* results in a heightened response to abiotic stresses which suggests NIT1 negatively regulates this response particularly during germination. Its involvement in biotic stresses (as mediated by JA signalling) seems to be minimal according to the physiological and gene expression studies presented in this thesis. NIT4 appears to be acting in different stress responses pathways to NIT1. The experimental evidence suggests that NIT4 is involved in biotic stress responses where it negatively regulates the response to determine which of the two branches of JA signalling should be switched on. On a holistic scale, the different roles of the *Arabidopsis* nitrilases can be envisioned as mediating a balance of responses. Plants are exposed to a gradient of environmental stresses; abiotic and biotic stresses are not discrete. More often than not, plants are faced with both at the same time perhaps in varying degrees of severity. Thus, pathways which mediate these responses or enable cross talk between them, require proteins to bridge between the pathways. The data presented here suggests that *Arabidopsis* nitrilases could be such bridges mediating between abiotic and biotic stress responses. To obtain more clarity, the next steps would involve the identification of ABA signalling pathways nitrilases are involved in. The approaches could make use of a genetics approach similar to that performed in this thesis for the JA signalling pathway. In addition, the putative interactors involved in ABA signalling, AHK2 and AtMYB52, could also be tested for interaction using the BiFC assay to link *Arabidopsis* nitrilases more strongly with ABA signalling.

6.3.2 Plant nitrilases confer resistance to bromoxynil

Germination on various photosynthesis inhibiting herbicides showed that *NIT1* expression does not confer general resistance to photosynthetic stress but only to nitrile

herbicides that impact on PSII such as bromoxynil. The resistance conferred by NIT1 could potentially be applied to any nitrile herbicide as nitriles are known substrates for nitrilases. Furthermore, the activity of nitrilases was shown to be specific as NIT1 over-expression did not confer tolerance to herbicides which lack the nitrile C≡N group such as Atrazine and DCMU. Here it is established that plant nitrilases act in the same manner as bacterial nitrilases in response to nitrile herbicides. This could be used in agricultural applications. Nitrilases can already be found in crop species such as maize, rice and sorghum; manipulation of their endogenous levels would allow optimized use of nitrile herbicides without the introduction of foreign genes such as bacterial nitrilase genes. This could be achieved using novel molecular systems such as the CRISPR systems to genetically manipulate either the transcriptional regulation or promoter regions so that over-expression of nitrilase can occur. Another potential lies in introducing a plant nitrilase into crop plants which lack nitrilases. Introducing *Arabidopsis* nitrilases into such crop plants would provide these plants with resistance allowing the use of these herbicides at higher concentrations or the use of nitrile herbicides with a higher level of potency without damaging the plants.

6.4 Future perspectives and conclusion

In the past, much of the research into *Arabidopsis* nitrilases focused heavily on their enzymatic capabilities thereby defining their biological roles solely on this parameter. This thesis set out to elucidate new biological roles for nitrilases in *Arabidopsis* which may be independent of their enzymatic roles. In doing so, this thesis has demonstrated that *Arabidopsis* nitrilases may have biological roles that cannot be defined by their enzymatic activity alone. Instead, it appears that these nitrilases can be defined as a family of moonlighting proteins. The results presented in this thesis suggest novel roles in transcriptional regulation in biotic stress response signalling and the possible involvement of *Arabidopsis* nitrilases in abiotic stress responses.

The work has utilized small scale studies to shed light on what specific biological pathways the *Arabidopsis* nitrilases are involved in. Given that biotic and abiotic pathways share a high degree of cross-talking, such approaches make it difficult to assess how *Arabidopsis* nitrilases, or any protein, affect plant biology on a holistic level. The method (qRT-PCR) used to screen for genes which alter in response to NIT over-

expression or hormone treatment is useful for small scale studies where specific genes are targeted. Given that the effects which over-expressing *Arabidopsis* nitrilases in plants can have may span both abiotic and biotic stresses, to look at gene expressions *via* qRT-PCR is difficult and time consuming. For a holistic characterization, microarrays and RNA sequencing are better approaches in that they allow comparison between samples across the whole transcriptome and without bias or prior knowledge of genes affected. Such large scale approaches were successfully applied to plants over-expressing *ERF1* in the context of responses to MeJA and ET (*124*). Using such techniques in a comparative way, *i.e.* by comparing biotic and abiotic stress responses or with *NIT1* and *NIT4* over-expressors, one can also identify overlaps in responses between the nitrilases or between pathways. Thus, future work should take advantage of such large scale approaches to answer the question if individual nitrilase isoforms are involved in two discrete responses (abiotic or biotic) or somehow involved in a complex cross-talking network.

In most other plant species, there are mostly two isoforms of nitrilases. With the exception of the Brassicaceae family, these isoforms are similar to NIT4 in terms of amino acid sequence. Some of these NIT4 homologues, such as those in maize and sorghum are defined as dual enzymes, able to use IAN and β-cyanoalanine as substrates which is not observed in *Arabidopsis* nitrilases. Such duality is not described in the literature for the *Arabidopsis* nitrilases indicating that there may be significant differences between plant species. These differences between *Arabidopsis* and other plants justify a comparative analysis of isoform specific functionality across plant species in the context of abiotic and biotic stresses, in an extension of the project described in this thesis. Such an analysis would add valuable information to the hypothesis posed here that suggests discrete biological roles for nitrilase isoforms. It would further show if the division of biological roles suggested for the *Arabidopsis* nitrilases, which belong to different homologue groups, is common to plants or if it is a novel evolutionary feature only present in Brassicaceae plants. If one should find that nitrilases outside of the Brassicaceae do not have discrete roles in abiotic and biotic stress responses, it would pose the interesting question of how non-Brassicaceae plants deal with the roles observed for *Arabidopsis* nitrilases. However, should a comparative analysis show that a diversification similar to the one suggested here for the *Arabidopsis*

nitrilases is also found in non-Brassicaceae it would imply that the stress response role may be ancient.

Both nitrilases and 14-3-3 proteins are found across plant species. One interesting point to determine is whether the nitrilase:14-3-3 interaction is conserved across plant species. If this interaction is conserved across plants, it would suggest that the interaction is important to the biology of plants. Furthermore, if a similar type of regulation is observed in other plant species, it strengthens the legitimacy of the sequestration model in the context of nuclear proteins regulation presented in 6.2. Similarly, the sequestration hypothesis applied to the NIT:ERF1 interaction should be further investigated and include other plant species The regulatory role of *Arabidopsis* nitrilases may exist outside of *Arabidopsis* as the ERF1:nitrilase interaction has been observed in *Nicotiana tabacum* and that NIT4 homologues are found in other plant such as maize, rice and sorghum.

The results presented here contributed new knowledge to our understanding of *Arabidopsis* nitrilases especially in the context of abiotic and biotic stress signalling responses and proposed a novel model of nitrilase regulation by 14-3-3 proteins. It was further hypothesised that *Arabidopsis* nitrilases regulate the activity or biological function of nuclear proteins by sequestration to the cytosol. This suggests that *Arabidopsis* nitrilases are “moonlighting” proteins which aside from their enzymatic functions have the secondary role of regulating activities of nuclear proteins. The thesis has also shown that ultimately the enzymatic function may not reflect all of the biological roles of these proteins. A more holistic approach involving genetic and physiological approaches like those used here is required to understand how a protein behaves in a biological context. Ideally, these approaches should be complemented by large scale, non-biased transcriptome analysis and by species and isoform specific comparative analysis to show validity of the snapshots obtained.

References

1. Jones, J. D., and Dangl, J. L. (2006) The plant immune system, *Nature 444*, 323-329.
2. Mithöfer, A., and Boland, W. (2008) Recognition of herbivory-associated molecular patterns, *Plant physiology 146*, 825-831.
3. Piasecka, A., Jedrzejczak‐Rey, N., and Bednarek, P. (2015) Secondary metabolites in plant innate immunity: conserved function of divergent chemicals, *New Phytologist 206*, 948-964.
4. Howe, G. A., and Jander, G. (2008) Plant immunity to insect herbivores, *Annu. Rev. Plant Biol. 59*, 41-66.
5. Halkier, B. A., and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates, *Annual review of plant biology 57*, 303.
6. Falk, K. L., Tokuhisa, J. G., and J, G. (2007) The effect of sulfur nutrition on plant glucosinolate content: physiology and molecular mechanisms, *Plant biology 9*, 573.
7. Petersen, B., Chen, S., Hansen, C., Olsen, C., and Halkier, B. (2002) Composition and content of glucosinolates in developing Arabidopsis thaliana, *Planta 214*, 562-571.
8. Wittstock, U., and J, G. (2002) Constitutive plant toxins and their role in defense against herbivores and pathogens, *Current Opinion in Plant Biology 5*, 300.
9. Kliebenstein, D. J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., and Mitchell-Olds, T. (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation, *Plant physiology 126*, 811-825.
10. Koroleva, O. A., Davies, A., Deeken, R., Thorpe, M. R., Tomos, A. D., and Hedrich, R. (2000) Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk, *Plant physiology 124*, 599-608.
11. Winde, I., and Wittstock, U. (2011) Insect herbivore counteradaptations to the plant glucosinolate–myrosinase system, *Phytochemistry 72*, 1566-1575.
12. Bednarek, P., Pi lewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., and Sanchez-Vallet, A. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense, *Science 323*, 101.
13. Clay, N. K., Adio, A. M., Denoux, C., Jander, G., and Ausubel, F. M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response, *Science 323*, 95-101.
14. Hirai, M. Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M., and Saito, K. (2003) Global expression profiling of sulfur‐starved Arabidopsis by DNA macroarray reveals the role of O‐acetyl‐l‐serine as a general regulator of gene expression in response to sulfur nutrition, *The Plant Journal 33*, 651-663.
15. Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H., and Hoefgen, R. (2003) Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: interlacing of biosynthetic pathways provides response specificity, *The Plant Journal 33*, 633-650.
16. Maruyama-Nakashita, A., Inoue, E., Watanabe-Takahashi, A., Yamaya, T., and Takahashi, H. (2003) Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways, *Plant physiology 132*, 597-605.
17. Pace, H. C., and Brenner, C. (2001) The nitrilase superfamily: classification, structure and function, *Genome Biol 2*, 1-9.
18. Vorwerk, S., Biernacki, S., Hillebrand, H., Janzik, I., Muller, A., Weiler, E. W., and Piotrowski, M. (2001) Enzymatic characterization of the recombinant Arabidopsis thaliana nitrilase subfamily encoded by the NIT 2/NIT 1/NIT 3-gene cluster, *Planta 212*, 508.
19. Raczynska, J. E., Vorgias, C. E., Antranikian, G., and Rypniewski, W. (2011) Crystallographic analysis of a thermoactive nitrilase, *Journal of structural biology 173*, 294-302.

20. Zhang, L., Yin, B., Wang, C., Jiang, S., Wang, H., Yuan, Y. A., and Wei, D. (2014) Structural insights into enzymatic activity and substrate specificity determination by a single amino acid in nitrilase from Syechocystis sp. PCC6803, *Journal of structural biology 188*, 93-101.
21. Thuku, R., Brady, D., Benedik, M., and Sewell, B. (2009) Microbial nitrilases: versatile, spiral forming, industrial enzymes, *Journal of applied microbiology 106*, 703-727.
22. O'Reilly, C., and Turner, P. D. (2003) The nitrilase family of CN hydrolysing enzymesâ□□a comparative study, *Journal of applied microbiology 95*, 1161.
23. Kaplan, O., Bezouška, K., Plíhal, O., Ettrich, R., Kulik, N., Vaněk, O., Kavan, D., Benada, O., Malandra, A., and Šveda, O. (2011) Heterologous expression, purification and characterization of nitrilase from Aspergillus niger K10, *BMC biotechnology 11*, 2.
24. Harper, D. B. (1985) Characterization of a nitrilase from Nocardia sp.(Rhodochrous group) NCIB 11215, using p-hydroxybenzonitrile as sole carbon source, *International Journal of Biochemistry 17*, 677-683.
25. Doskočilová, A., Kohoutová, L., Volc, J., Kourová, H., Benada, O., Chumová, J., Plíhal, O., Petrovská, B., Halada, P., and Bögre, L. (2013) NITRILASE1 regulates the exit from proliferation, genome stability and plant development, *New Phytologist 198*, 685-698.
26. Thimann, K. V., and Mahadevan, S. (1964) Nitrilase* 1,* 2:: I. Occurrence, preparation, and general properties of the enzyme, *Archives of biochemistry and biophysics 105*, 133-141.
27. Piotrowski, M. (2008) Primary or secondary? Versatile nitrilases in plant metabolism, *Phytochemistry 69*, 2655.
28. Park, W. J., Kriechbaumer, V., Müller, A., Piotrowski, M., Meeley, R. B., Gierl, A., and Glawischnig, E. (2003) The nitrilase ZmNIT2 converts indole-3-acetonitrile to indole-3-acetic acid, *Plant physiology 133*, 794-802.
29. Jenrich, R., Trompetter, I., Bak, S., Olsen, C. E., Møller, B. L., and Piotrowski, M. (2007) Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism, *Proceedings of the National Academy of Sciences 104*, 18848.
30. Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes, *Advances in bioinformatics 2008*.
31. Böhmer, M., and Schroeder, J. I. (2011) Quantitative transcriptomic analysis of abscisic acid‐induced and reactive oxygen species‐dependent expression changes and proteomic profiling in Arabidopsis suspension cells, *The Plant Journal 67*, 105-118.
32. Oono, Y., Seki, M., Satou, M., Iida, K., Akiyama, K., Sakurai, T., Fujita, M., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Monitoring expression profiles of Arabidopsis genes during cold acclimation and deacclimation using DNA microarrays, *Functional & integrative genomics 6*, 212-234.
33. Van Der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flügge, U.-I., and Kunze, R. (2006) Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence, *Plant physiology 141*, 776-792.
34. Sasaki‐Sekimoto, Y., Taki, N., Obayashi, T., Aono, M., Matsumoto, F., Sakurai, N., Suzuki, H., Hirai, M. Y., Noji, M., and Saito, K. (2005) Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis, *The Plant Journal 44*, 653-668.
35. De Paepe, A., Vuylsteke, M., Van Hummelen, P., Zabeau, M., and Van Der Straeten, D. (2004) Transcriptional profiling by cDNA‐AFLP and microarray analysis reveals

novel insights into the early response to ethylene in Arabidopsis, *The Plant Journal 39*, 537-559.

36. Osswald, S., Wajant, H., and Effenberger, F. (2002) Characterization and synthetic applications of recombinant AtNIT1 from Arabidopsis thaliana, *European Journal of Biochemistry 269*, 680-687.
37. Ishikawa, T., Okazaki, K., Kuroda, H., Itoh, K., Mitsui, T., and Hori, H. (2007) Molecular cloning of Brassica rapa nitrilases and their expression during clubroot development, *Molecular plant pathology 8*, 623-637.
38. Janowitz, T., Trompetter, I., and Piotrowski, M. (2009) Evolution of nitrilases in glucosinolate-containing plants, *Phytochemistry 70*, 1680-1686.
39. Piotrowski, M., Schönfelder, S., and Weiler, E. W. (2001) The Arabidopsis thaliana Isogene NIT4 and Its Orthologs in Tobacco Encode β-Cyano-l-alanine Hydratase/Nitrilase, *Journal of biological chemistry 276*, 2616-2621.
40. Machingura, M., and Ebbs, S. D. (2014) Functional redundancies in cyanide tolerance provided by β-cyanoalanine pathway genes in Arabidopsis thaliana, *International Journal of Plant Sciences 175*, 346-358.
41. Kriechbaumer, V., Park, W. J., Piotrowski, M., Meeley, R. B., Gierl, A., and Glawischnig, E. (2007) Maize nitrilases have a dual role in auxin homeostasis and β-cyanoalanine hydrolysis, *Journal of experimental botany 58*, 4225-4233.
42. Semba, S., Han, S.-Y., Qin, H. R., McCorkell, K. A., Iliopoulos, D., Pekarsky, Y., Druck, T., Trapasso, F., Croce, C. M., and Huebner, K. (2006) Biological Functions of Mammalian Nit1, the Counterpart of the Invertebrate NitFhit Rosetta Stone Protein, a Possible Tumor Suppressor, *Journal of biological chemistry 281*, 28244-28253.
43. Igawa, T., Fujiwara, M., Takahashi, H., Sawasaki, T., Endo, Y., Seki, M., Shinozaki, K., Fukao, Y., and Yanagawa, Y. (2009) Isolation and identification of ubiquitin-related proteins from Arabidopsis seedlings, *Journal of experimental botany 60*, 3067-3073.
44. Saracco, S. A., Hansson, M., Scalf, M., Walker, J. M., Smith, L. M., and Vierstra, R. D. (2009) Tandem affinity purification and mass spectrometric analysis of ubiquitylated proteins in Arabidopsis, *The Plant Journal 59*, 344-358.
45. Cutler, S. R., and Somerville, C. R. (2005) Imaging plant cell death: GFP-Nit1 aggregation marks an early step of wound and herbicide induced cell death, *BMC Plant Biology 5*, 4-15.
46. Quirino, B. F., Normanly, J., and Amasino, R. M. (1999) Diverse range of gene activity during Arabidopsis thaliana leaf senescence includes pathogen-independent induction of defense-related genes, *Plant molecular biology 40*, 267-278.
47. Grassl, J., Pružinská, A., Hörtensteiner, S., Taylor, N. L., and Millar, A. H. (2012) Early events in plastid protein degradation in stay-green Arabidopsis reveal differential regulation beyond the retention of LHCII and chlorophyll, *Journal of proteome research 11*, 5443-5452.
48. Hörtensteiner, S. (2009) Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence, *Trends in plant science 14*, 155-162.
49. Xu, P., Narasimhan, M. L., Samson, T., Coca, M. A., Huh, G.-H., Zhou, J., Martin, G. B., Hasegawa, P. M., and Bressan, R. A. (1998) A nitrilase-like protein interacts with GCC box DNA-binding proteins involved in ethylene and defense responses, *Plant physiology 118*, 867-874.
50. Jensen, M. K., and Skriver, K. (2014) NAC transcription factor gene regulatory and protein–protein interaction networks in plant stress responses and senescence, *IUBMB life 66*, 156-166.
51. Delessert, C., Kazan, K., Wilson, I. W., Straeten, D. V. D., Manners, J., Dennis, E. S., and Dolferus, R. (2005) The transcription factor ATAF2 represses the expression of pathogenesis‐related genes in Arabidopsis, *The Plant Journal 43*, 745-757.

52. Huh, S. U., Lee, S.-B., Kim, H. H., and Paek, K.-H. (2012) ATAF2, a NAC transcription factor, binds to the promoter and regulates NIT2 gene expression involved in auxin biosynthesis, *Molecules and cells 34*, 305-313.
53. Liang, W.-S. (2003) Drought stress increases both cyanogenesis and β-cyanoalanine synthase activity in tobacco, *Plant Science 165*, 1109-1115.
54. Machingura, M., Sidibe, A., Wood, A. J., and Ebbs, S. D. (2013) The β-cyanoalanine pathway is involved in the response to water deficit in Arabidopsis thaliana, *Plant Physiology and Biochemistry 63*, 159-169.
55. Johansen, L. K., and Carrington, J. C. (2001) Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system, *Plant physiology 126*, 930.
56. Nelson, C. J., Alexova, R., Jacoby, R. P., and Millar, A. H. (2014) Proteins with high turnover rate in barley leaves estimated by proteome analysis combined with in planta isotope labeling, *Plant physiology 166*, 91-108.
57. Lamesch, P., Berardini, T. Z., Li, D., Swarbreck, D., Wilks, C., Sasidharan, R., Muller, R., Dreher, K., Alexander, D. L., and Garcia-Hernandez, M. (2012) The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools, *Nucleic acids research 40*, D1202-D1210.
58. Normanly, J., Grisafi, P., Fink, G. R., and Bartel, B. (1997) Arabidopsis mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the NIT1 gene, *The Plant Cell Online 9*, 1781-1790.
59. Farmer, L. M., Book, A. J., Lee, K.-H., Lin, Y.-L., Fu, H., and Vierstra, R. D. (2010) The RAD23 family provides an essential connection between the 26S proteasome and ubiquitylated proteins in Arabidopsis, *The Plant Cell 22*, 124-142.
60. Paul, A. L., Liu, L., McClung, S., Laughner, B., Chen, S., and Ferl, R. J. (2009) Comparative interactomics: analysis of arabidopsis 14-3-3 complexes reveals highly conserved 14-3-3 interactions between humans and plants, *Journal of proteome research 8*, 1913-1924.
61. Lee, H., Guo, Y., Ohta, M., Xiong, L., Stevenson, B., and Zhu, J. K. (2002) LOS2, a genetic locus required for cold‐responsive gene transcription encodes a bi‐functional enolase, *The EMBO journal 21*, 2692-2702.
62. Tanz, S. K., Castleden, I., Hooper, C. M., Vacher, M., Small, I., and Millar, H. A. (2012) SUBA3: a database for integrating experimentation and prediction to define the SUBcellular location of proteins in Arabidopsis, *Nucleic acids research*, gks1151.
63. Braun, P., Aubourg, S., Van Leene, J., De Jaeger, G., and Lurin, C. (2013) Plant protein interactomes, *Annual review of plant biology 64*, 161-187.
64. Pellegrini, M., Marcotte, E. M., Thompson, M. J., Eisenberg, D., and Yeates, T. O. (1999) Assigning protein functions by comparative genome analysis: protein phylogenetic profiles, *Proceedings of the National Academy of Sciences 96*, 4285-4288.
65. Zhang, Y., Gao, P., and Yuan, J. S. (2010) Plant protein-protein interaction network and interactome, *Current genomics 11*, 40-46.
66. Rubio, M. P., Geraghty, K. M., Wong, B. H., Wood, N. T., Campbell, D. G., Morrice, N., and Mackintosh, C. (2004) 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking, *Biochemical Journal 379*, 395.
67. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine, *Cell 84*, 889-897.
68. Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H., and Liddington, R. C. (1998) 14-3-3ζ binds a phosphorylated Raf peptide and an unphosphorylated

peptide via its conserved amphipathic groove, *Journal of Biological Chemistry 273*, 16305-16310.

69. Bridges, D., and Moorhead, G. B. G. (2004) 14-3-3 proteins: a number of functions for a numbered protein, *Science's signal transduction knowledge environment 2004*, re10-re10.

70. Yaffe, M. B. (2002) How do 14-3-3 proteins work?-Gatekeeper phosphorylation and the molecular anvil hypothesis, *FEBS letters 513*, 53.

71. van Heusden, G. P. H., van der Zanden, A. L., Ferl, R. J., and Steensma, H. Y. (1996) Four Arabidopsis thaliana 14-3-3 protein isoforms can complement the lethal yeast bmh1 bmh2 double disruption, *FEBS letters 391*, 252-256.

72. DeLille, J. M., Sehnke, P. C., and Ferl, R. J. (2001) The Arabidopsis 14-3-3 family of signaling regulators, *Plant physiology 126*, 35.

73. Brandão, M. M., Dantas, L. L., and Silva-Filho, M. C. (2009) AtPIN: Arabidopsis thaliana protein interaction network, *BMC bioinformatics 10*, 454.

74. Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., and Tsafou, K. P. (2014) STRING v10: protein–protein interaction networks, integrated over the tree of life, *Nucleic acids research*, gku1003.

75. Lin, M., Shen, X., and Chen, X. (2011) PAIR: the predicted Arabidopsis interactome resource, *Nucleic acids research 39*, D1134-D1140.

76. Van Leene, J., Eeckhout, D., Cannoot, B., De Winne, N., Persiau, G., Van De Slijke, E., Vercruysse, L., Dedecker, M., Verkest, A., and Vandepoele, K. (2015) An improved toolbox to unravel the plant cellular machinery by tandem affinity purification of Arabidopsis protein complexes, *Nature protocols 10*, 169-187.

77. Liu, H.-C. (2010) A Systematic in planta Survey of 14-3-3 Protein Interactions by Bimolecular Fluorescence Complementation, In *Biochemistry and Molecular Biology*, The University of Western Australia.

78. Wu, K., Lu, G., Sehnke, P., and Ferl, R. J. (1997) The heterologous interactions among plant 14-3-3 proteins and identification of regions that are important for dimerization, *Archives of biochemistry and biophysics 339*, 2-8.

79. Heazlewood, J. L., Durek, P., Hummel, J., Selbig, J., Weckwerth, W., Walther, D., and Schulze, W. X. (2008) PhosPhAt: a database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor, *Nucleic acids research 36*, D1015-D1021.

80. Merkle, T. (2001) Nuclear import and export of proteins in plants: a tool for the regulation of signalling, *Planta 213*, 499-517.

81. Kosugi, S., Hasebe, M., Matsumura, N., Takashima, H., Miyamoto-Sato, E., Tomita, M., and Yanagawa, H. (2009) Six classes of nuclear localization signals specific to different binding grooves of importin α, *Journal of Biological Chemistry 284*, 478-485.

82. Grebenok, R. J., Pierson, E., Lambert, G. M., Gong, F. C., Afonso, C. L., Haldeman‐Cahill, R., Carrington, J. C., and Galbraith, D. W. (1997) Green‐fluorescent protein fusions for efficient characterization of nuclear targeting, *The Plant Journal 11*, 573-586.

83. Kutz, A., Müller, A., Hennig, P., Kaiser, W. M., Piotrowski, M., and Weiler, E. W. (2002) A role for nitrilase 3 in the regulation of root morphology in sulphur starving Arabidopsis thaliana, *The Plant Journal 30*, 95-106.

84. Zheng, Z.-L., Nafisi, M., Tam, A., Li, H., Crowell, D. N., Chary, S. N., Schroeder, J. I., Shen, J., and Yang, Z. (2002) Plasma membrane–associated ROP10 small GTPase is a specific negative regulator of abscisic acid responses in Arabidopsis, *The Plant Cell 14*, 2787-2797.

85. Paul, A.-L., Sehnke, P. C., and Ferl, R. J. (2005) Isoform-specific subcellular localization among 14-3-3 proteins in Arabidopsis seems to be driven by client interactions, *Molecular biology of the cell 16*, 1735-1743.
86. Paul, A.-L., Denison, F. C., Schultz, E. R., Zupanska, A. K., and Ferl, R. J. (2012) 14-3-3 phosphoprotein interaction networks-does isoform diversity present functional interaction specification, *Front. Plant Sci 3*, 10.3389.
87. Börnke, F. (2005) The variable C-terminus of 14-3-3 proteins mediates isoform-specific interaction with sucrose-phosphate synthase in the yeast two-hybrid system, *Journal of plant physiology 162*, 161-168.
88. Pallucca, R., Visconti, S., Camoni, L., Cesareni, G., Melino, S., Panni, S., Torreri, P., and Aducci, P. (2014) Specificity of ε and non-ε isoforms of arabidopsis 14-3-3 proteins towards the H+-ATPase and other targets, *PloS one 9*, e90764.
89. Bachmann, M., Huber, J. L., Athwal, G. S., Wu, K., Ferl, R. J., and Huber, S. C. (1996) 14‐3‐3 proteins associate with the regulatory phosphorylation site of spinach leaf nitrate reductase in an isoform‐specific manner and reduce dephosphorylation of Ser‐543 by endogenous protein phosphatases, *FEBS letters 398*, 26-30.
90. Grsic, S., Kirchheim, B., Pieper, K., Fritsch, M., Hilgenberg, W., and Ludwig‐Müller, J. (1999) Induction of auxin biosynthetic enzymes by jasmonic acid and in clubroot diseased Chinese cabbage plants, *Physiologia plantarum 105*, 521-531.
91. Igarashi, D., Ishida, S., Fukazawa, J., and Takahashi, Y. (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG, *The Plant Cell 13*, 2483-2497.
92. Yoon, G. M., and Kieber, J. J. (2013) 14-3-3 regulates 1-aminocyclopropane-1-carboxylate synthase protein turnover in Arabidopsis, *The Plant Cell 25*, 1016-1028.
93. Gleadow, R. M., and Møller, B. L. (2014) Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity, *Annual review of plant biology 65*, 155-185.
94. Tattersall, D. B., Bak, S., Jones, P. R., Olsen, C. E., Nielsen, J. K., Hansen, M. L., Høj, P. B., and Møller, B. L. (2001) Resistance to an herbivore through engineered cyanogenic glucoside synthesis, *Science 293*, 1826-1828.
95. Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012) Hormonal modulation of plant immunity, *Annual review of cell and developmental biology 28*, 489-521.
96. Bari, R., and Jones, J. D. (2009) Role of plant hormones in plant defence responses, *Plant molecular biology 69*, 473-488.
97. Wasternack, C., and Hause, B. (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany, *Annals of Botany 111*, 1021-1058.
98. Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S. Y., Howe, G. A., and Browse, J. (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling, *Nature 448*, 661-665.
99. Dombrecht, B., Xue, G. P., Sprague, S. J., Kirkegaard, J. A., Ross, J. J., Reid, J. B., Fitt, G. P., Sewelam, N., Schenk, P. M., and Manners, J. M. (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis, *The Plant Cell 19*, 2225-2245.
100. Zhu, Z., An, F., Feng, Y., Li, P., Xue, L., Mu, A., Jiang, Z., Kim, J.-M., To, T. K., and Li, W. (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in Arabidopsis, *Proceedings of the National Academy of Sciences 108*, 12539-12544.
101. Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C. M., and Memelink, J. (2008) The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense, *Plant physiology 147*, 1347-1357.

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

103. **Song, S., Huang, H., Gao, H., Wang, J., Wu, D., Liu, X., Yang, S., Zhai, Q., Li, C., and Qi, T. (2014) Interaction between MYC2 and ETHYLENE INSENSITIVE3 modulates antagonism between jasmonate and ethylene signaling in Arabidopsis, *The Plant Cell 26*, 263-279.**

104. **Verhage, A., Vlaardingerbroek, I., Raaijmakers, C., Van Dam, N. M., Dicke, M., Van Wees, S. C., and Pieterse, C. M. (2011) Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory, *Frontiers in plant science 2*, 1-12.**

105. **Doherty, H. M., Selvendran, R. R., and Bowles, D. J. (1988) The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids, *Physiological and Molecular Plant Pathology 33*, 377-384.**

106. **Leon-Reyes, A., Van der Does, D., De Lange, E. S., Delker, C., Wasternack, C., Van Wees, S. C., Ritsema, T., and Pieterse, C. M. (2010) Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway, *Planta 232*, 1423-1432.**

107. **Spoel, S. H., Koornneef, A., Claessens, S. M., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., Buchala, A. J., Métraux, J.-P., Brown, R., and Kazan, K. (2003) NPR1 modulates cross-talk between salicylate-and jasmonate-dependent defense pathways through a novel function in the cytosol, *The Plant Cell 15*, 760-770.**

108. **Mur, L. A., Kenton, P., Atzorn, R., Miersch, O., and Wasternack, C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death, *Plant physiology 140*, 249-262.**

109. **Meng, X., and Zhang, S. (2013) MAPK cascades in plant disease resistance signaling, *Annual Review of Phytopathology 51*, 245-266.**

110. **Su, S.-H., Bush, S. M., Zaman, N., Stecker, K., Sussman, M. R., and Krysan, P. (2013) Deletion of a tandem gene family in Arabidopsis: increased MEKK2 abundance triggers autoimmunity when the MEKK1-MKK1/2-MPK4 signaling cascade is disrupted, *The Plant Cell 25*, 1895-1910.**

111. **Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J., and Parker, J. E. (2000) Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance, *Cell 103*, 1111-1120.**

112. **Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M. A., Shokat, K. M., Rietz, S., Parker, J., and Mundy, J. (2006) Arabidopsis MAP kinase 4 regulates salicylic acid‐ and jasmonic acid/ethylene‐dependent responses via EDS1 and PAD4, *The Plant Journal 47*, 532-546.**

113. **Bodnaryk, R. P. (1992) Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard, *Phytochemistry 31*, 2671-2677.**

114. **Brader, G., Tas, E., and Palva, E. T. (2001) Jasmonate-dependent induction of indole glucosinolates in arabidopsis by culture filtrates of the nonspecific pathogenerwinia carotovora, *Plant physiology 126*, 849-860.**

115. **Kiddle, G. A., Doughty, K. J., and Wallsgrove, R. M. (1994) Salicylic acid-induced accumulation of glucosinolates in oilseed rape (Brassica napus L.) leaves, *Journal of experimental botany 45*, 1343.**

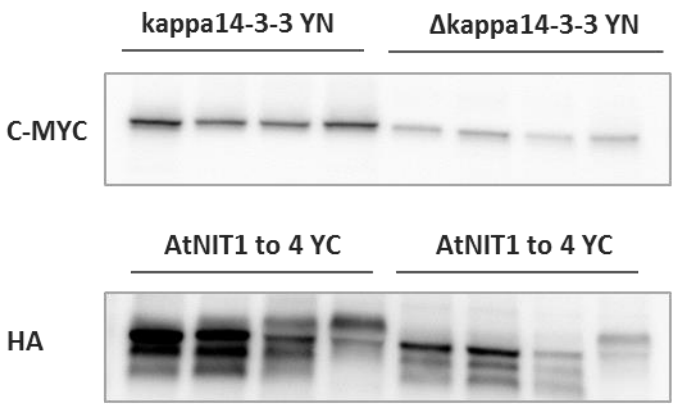
116. **Mewis, I., Tokuhisa, J. G., Schultz, J. C., Appel, H. M., Ulrichs, C., and Gershenzon, J. (2006) Gene expression and glucosinolate accumulation in Arabidopsis thaliana in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways, *Phytochemistry 67*, 2450-2462.**

117. Mikkelsen, M. D., Petersen, B. L., Glawischnig, E., Jensen, A. B., Andreasson, E., and Halkier, B. A. (2003) Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways, *Plant physiology 131*, 298-308.
118. Schweizer, F., Fernández-Calvo, P., Zander, M., Diez-Diaz, M., Fonseca, S., Glauser, G., Lewsey, M. G., Ecker, J. R., Solano, R., and Reymond, P. (2013) Arabidopsis basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior, *The Plant Cell 25*, 3117-3132.
119. Frerigmann, H., Berger, B., and Gigolashvili, T. (2014) bHLH05 is an interaction partner of MYB51 and a novel regulator of glucosinolate biosynthesis in Arabidopsis, *Plant physiology 166*, 349-369.
120. Cristina, M. S., Petersen, M., and Mundy, J. (2010) Mitogen-activated protein kinase signaling in plants, *Annual review of plant biology 61*, 621-649.
121. Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K. (2000) Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6, *The Plant Journal 24*, 655-665.
122. Müller, M., and Munné-Bosch, S. (2015) Ethylene response factors: a key regulatory hub in hormone and stress signaling, *Plant physiology 169*, 32-41.
123. Warde-Farley, D., Donaldson, S. L., Comes, O., Zuberi, K., Badrawi, R., Chao, P., Franz, M., Grouios, C., Kazi, F., and Lopes, C. T. (2010) The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function, *Nucleic acids research 38*, W214-W220.
124. Lorenzo, O., Piqueras, R., Sánchez-Serrano, J. J., and Solano, R. (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense, *The Plant Cell 15*, 165-178.
125. Solano, R., Stepanova, A., Chao, Q., and Ecker, J. R. (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1, *Genes & development 12*, 3703-3714.
126. Cheng, M.-C., Liao, P.-M., Kuo, W.-W., and Lin, T.-P. (2013) The Arabidopsis ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals, *Plant physiology 162*, 1566-1582.
127. Koroleva, O. A., Tomlinson, M. L., Leader, D., Shaw, P., and Doonan, J. H. (2005) High‐throughput protein localization in Arabidopsis using Agrobacterium‐mediated transient expression of GFP‐ORF fusions, *The Plant Journal 41*, 162-174.
128. Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998) Isolation of ATMEKK1 (a MAP Kinase Kinase Kinase)-interacting proteins and analysis of a MAP kinase cascade inArabidopsis, *Biochemical and biophysical research communications 253*, 532-543.
129. Matsuoka, D., Hadiarto, T., and Nanmori, T. Cell Signaling and Response via Mitogen-Activated Protein Kinase (MAPK) Cascade in Arabidopsis, *Plant Stress 1*, 113-117.
130. Stepanova, A. N., and Ecker, J. R. (2000) Ethylene signaling: from mutants to molecules, *Current opinion in plant biology 3*, 353-360.
131. Teige, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., Dangl, J. L., and Hirt, H. (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis, *Molecular cell 15*, 141-152.
132. Mizoi, J., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2012) AP2/ERF family transcription factors in plant abiotic stress responses, *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1819*, 86-96.
133. Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., and Sakurai, T. (2002) Monitoring the expression profiles of

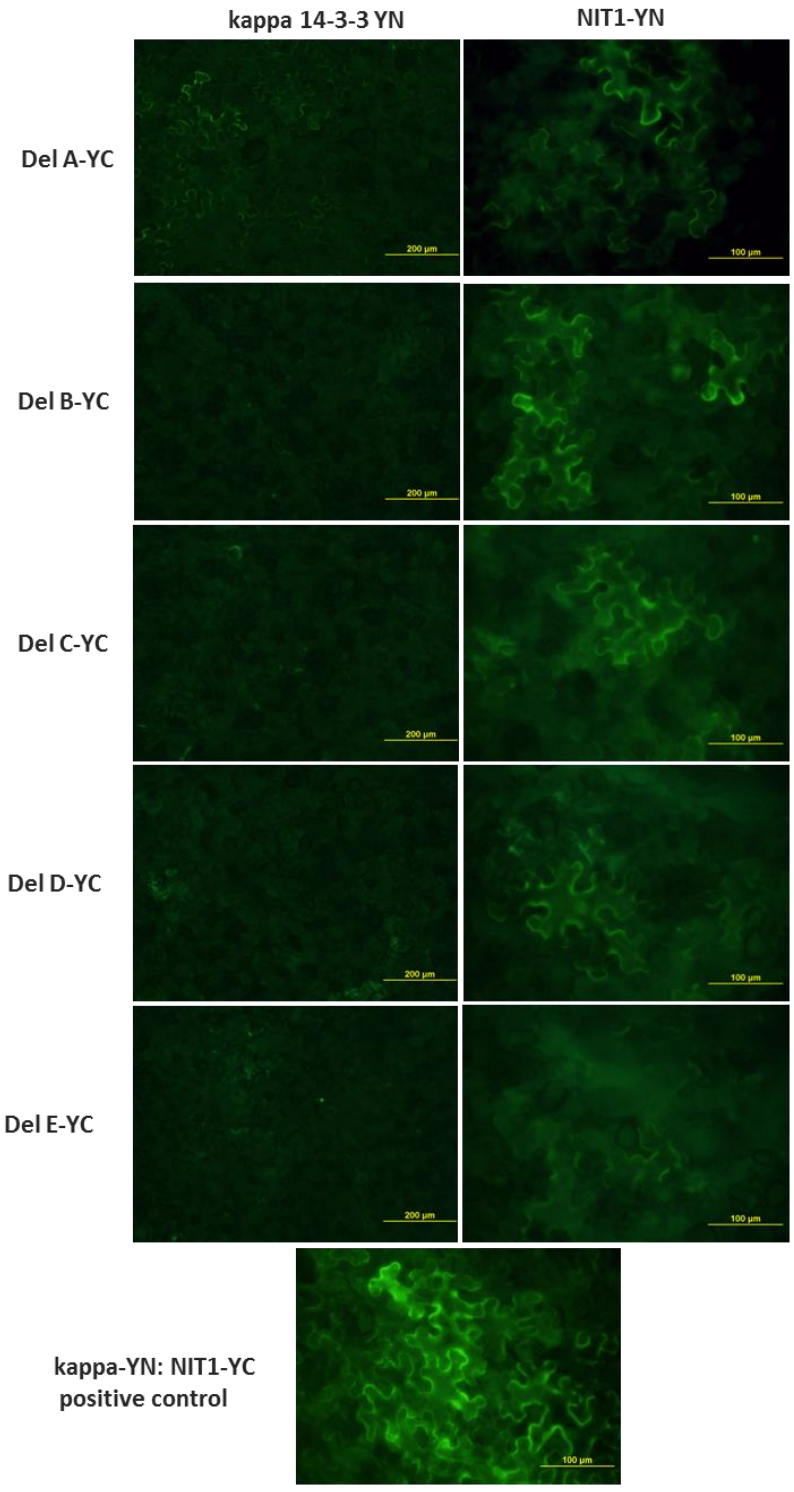
7000 Arabidopsis genes under drought, cold and high‐salinity stresses using a full‐length cDNA microarray, *The Plant Journal 31*, 279-292.
134. Mauch-Mani, B., and Mauch, F. (2005) The role of abscisic acid in plant–pathogen interactions, *Current opinion in plant biology 8*, 409-414.
135. Leung, J., and Giraudat, J. (1998) Abscisic acid signal transduction, *Annual review of plant biology 49*, 199-222.
136. Fujita, Y., Fujita, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants, *Journal of plant research 124*, 509-525.
137. Acosta, I. F., and Farmer, E. E. (2009) Jasmonates, *The Arabidopsis Book*, e0129.
138. Staswick, P. E., Su, W., and Howell, S. H. (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an Arabidopsis thaliana mutant, *Proceedings of the National Academy of Sciences 89*, 6837-6840.
139. Dave, A., Hernández, M. L., He, Z., Andriotis, V. M., Vaistij, F. E., Larson, T. R., and Graham, I. A. (2011) 12-Oxo-phytodienoic acid accumulation during seed development represses seed germination in Arabidopsis, *The Plant Cell 23*, 583-599.
140. Miao, Y., and Zentgraf, U. (2007) The antagonist function of Arabidopsis WRKY53 and ESR/ESP in leaf senescence is modulated by the jasmonic and salicylic acid equilibrium, *The Plant Cell 19*, 819-830.
141. Shan, X., Zhang, Y., Peng, W., Wang, Z., and Xie, D. (2009) Molecular mechanism for jasmonate-induction of anthocyanin accumulation in Arabidopsis, *Journal of experimental botany 60*, 3849-3860.
142. Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006) Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks, *Current opinion in plant biology 9*, 436-442.
143. Finkelstein, R. R., and Rock, C. D. (2002) Abscisic acid biosynthesis and response, *The Arabidopsis Book*, e0058.
144. Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., and Abrams, S. R. (2010) Abscisic acid: emergence of a core signaling network, *Annual Reviews Plant Biology 61*, 651-679.
145. Hong, J. H., Seah, S. W., and Xu, J. (2013) The root of ABA action in environmental stress response, *Plant cell reports 32*, 971-983.
146. Wang, F., Cui, X., Sun, Y., and Dong, C.-H. (2013) Ethylene signaling and regulation in plant growth and stress responses, *Plant cell reports 32*, 1099-1109.
147. Apel, K., and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction, *Annu. Rev. Plant Biol. 55*, 373-399.
148. Stalker, D. M., McBride, K. E., and Malyj, L. D. (1988) Herbicide resistance in transgenic plants expressing a bacterial detoxification gene, *Science 242*, 419-423.
149. Kieber, J. J. (1997) The ethylene response pathway in Arabidopsis, *Annual review of plant biology 48*, 277-296.
150. Guzman, P., and Ecker, J. R. (1990) Exploiting the triple response of Arabidopsis to identify ethylene-related mutants, *The Plant Cell 2*, 513-523.
151. Abràmoff, M. D., Magalhães, P. J., and Ram, S. J. (2004) Image processing with ImageJ, *Biophotonics international 11*, 36-42.
152. Miransari, M., and Smith, D. (2014) Plant hormones and seed germination, *Environmental and Experimental Botany 99*, 110-121.
153. Bewley, J. D. (1997) Seed germination and dormancy, *The Plant Cell 9*, 1055.
154. Zaki, M., Taylor, H., and Wain, R. (1967) Studies with 3, 5‐diiodo‐4‐hydroxybenzonitrile (ioxynil) and related compounds in soils and plants, *Annals of applied biology 59*, 481-491.

155. Fu, H., Subramanian, R. R., and Masters, S. C. (2000) 14-3-3 proteins: structure, function, and regulation, *Annual Review of Pharmacology and Toxicology 40*, 617.
156. Ishida, S., Fukazawa, J., Yuasa, T., and Takahashi, Y. (2004) Involvement of 14-3-3 signaling protein binding in the functional regulation of the transcriptional activator REPRESSION OF SHOOT GROWTH by gibberellins, *The Plant Cell 16*, 2641-2651.
157. Jeffery, C. J. (1999) Moonlighting proteins, *Trends in biochemical sciences 24*, 8-11.
158. Harrington, G. N., and Bush, D. R. (2003) The bifunctional role of hexokinase in metabolism and glucose signaling, *The Plant Cell 15*, 2493-2496.

Appendix

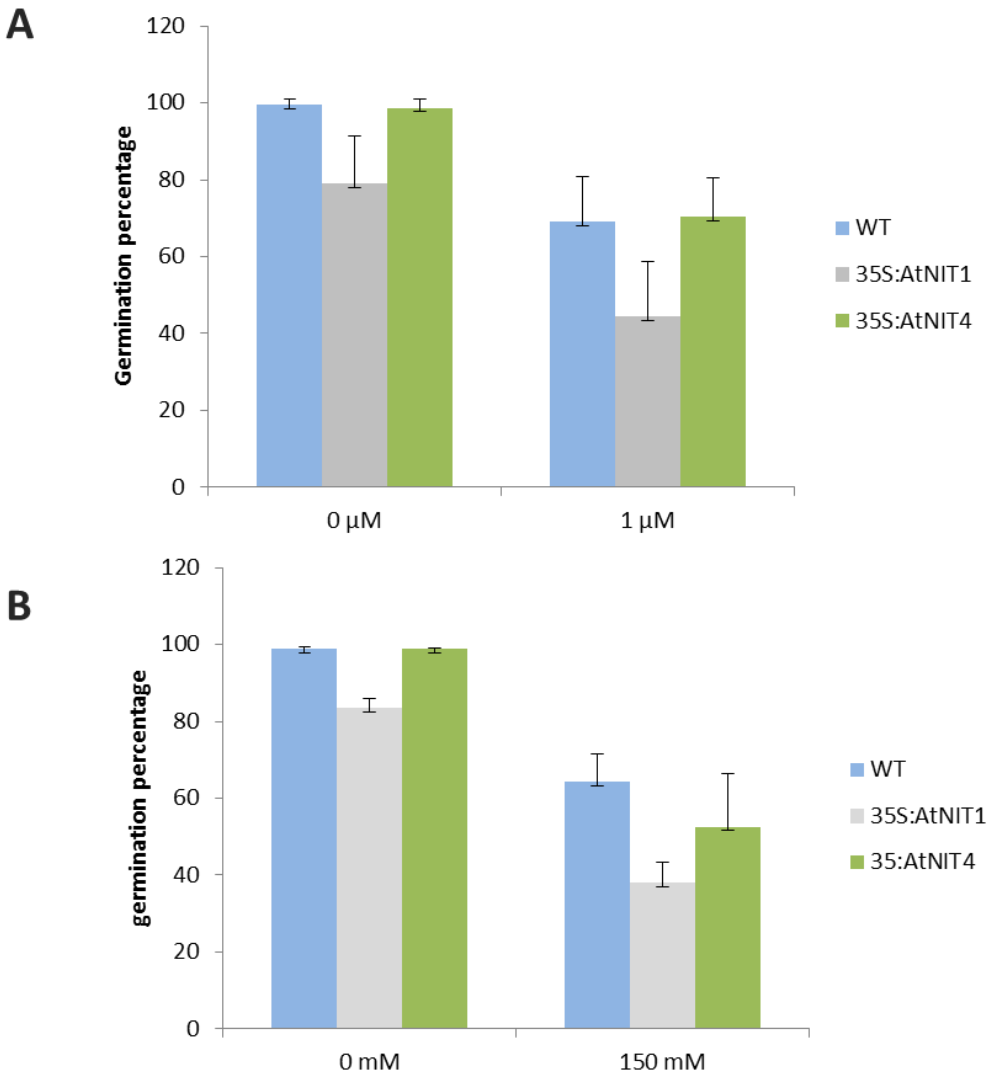


Appendix figure 1: Western blot analysis demonstrating the expression of interacting and non-interacting 14-3-3 and AtNIT proteins.
Expression of AtNIT-YC, kappa 14-3-3 YN and Δkappa 14-3-3 YN constructs was tested via western blots. Fusion proteins were detected using anti c-MYC (YN constructs) and anti HA (YC constructs) antibodies. In each case, a band corresponding to the expected sizes of the *Arabidopsis* nitrilases, 14-3-3 or N-terminal shortened (Δ) 14-3-3 proteins was observed. Different sized bands were observed for *Arabidopsis* nitrilases; this may be due to the phosphorylation state increasing the size of the *Arabidopsis* nitrilases .



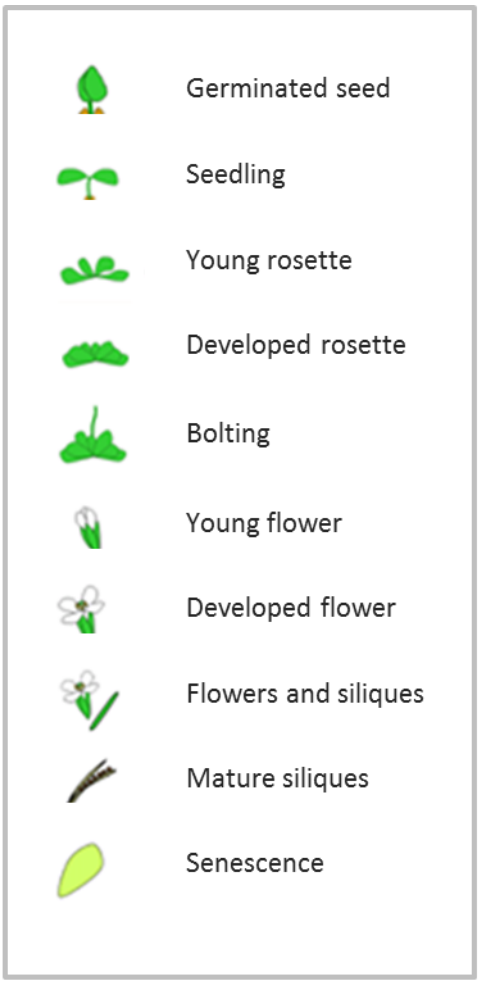
Appendix figure 2: Testing for 14-3-3 interaction using peptide fragments of AtNIT1 protein

The *NIT1* gene was divided into five fragments (Del A to E) and cloned into the pG179NS-YC vectors. The five constructs were then tested against kappa 14-3-3 YN for interaction using BiFC in *N.benthamiana* leaf cells. Expression and reformation of split YFP ws analysed three days post infection was determined using epifluorescent microscopy. The GFP filter was used to detect the fluorescence. The positive control represents the expected level of fluorescence when interaction has occurred.



Appendix figure 3: The germination of Col-0, 35S:AtNIT1 and 35S:AtNIT4 lines in the presence of ABA or salt

Seeds (n>100) of Col-0, 35S:AtNIT1 and 35S:AtNIT4 were germinated in the absence (control) or presence of either 1 µM ABA (A) or 150 µM NaCl (B). The absolute germination frequencies after five days of treatment was calculated and plotted for both control and treatment conditions. The plotted results are averages of three replicates. Error bars indicate the standard error.



Appendix figure 4: The key developmental stages of *Arabidopsis thaliana*
The various developmental stages of the Arabidopsis thaliana as defined by Genevestigator *(29)* and used to determine the various gene expression levels at each developmental stage.

Appendix table 1: Functional partners of the Arabidopsis nitrilases as determined using the STRING database

Functional partners for the four nitrilase isoforms were determined using the STRING database (*74*), selecting a maximum of 50 interactors and other settings were kept as the default settings.

NIT1		NIT2		NIT3		NIT4	
AAO1	3702.AT5G20960.1	AAO1	AT5G20960.1	AAO1	AT5G20960.1	AAO1	AT5G20960.1
ALDH2B4	3702.AT3G48000.1	GLN1-1	AT5G37600.1	YUC6	AT5G25620.2	ACC1	AT1G36160.1
GLN1;4	3702.AT5G16570.1	GS2	AT5G35630.1	YUC5	AT5G43890.1	ACC2	AT1G36180.1
GS2	3702.AT5G35630.1	GLN1;4	AT5G16570.1	TGG2	AT5G25980.2	ACL5	AT5G19530.1
GLN1-1	3702.AT5G37600.1	AT4G37560	AT4G37560.1	YUC2	AT4G13260.1	AMI1	AT1G08980.1
AT4G37560	3702.AT4G37560.1	GDH2	AT5G07440.1	YUC8	AT4G28720.1	AT1G63250	AT1G63250.1
ALDH3F1	3702.AT4G36250.1	ALDH3I1	AT4G34240.1	GS2	AT5G35630.1	AT1G63660	AT1G63660.1
ALDH3I1	3702.AT4G34240.1	ALDH2B4	AT3G48000.1	AT4G37560	AT4G37560.1	AT2G15240	AT2G15240.1
TGG1	3702.AT5G26000.1	ALDH3F1	AT4G36250.1	GLN1-1	AT5G37600.1	AT2G22530	AT2G22530.1
YUC5	3702.AT5G43890.1	YUC1	AT4G32540.1	GLN1;4	AT5G16570.1	AT3G01380	AT3G01380.1
YUC6	3702.AT5G25620.2	TGG1	AT5G26000.1	TGG1	AT5G26000.1	AT3G10400	AT3G10400.1
GDH2	3702.AT5G07440.1	YUC6	AT5G25620.2	ALDH3F1	AT4G36250.1	AT4G29680	AT4G29680.1
YUC2	3702.AT4G13260.1	TGG2	AT5G25980.2	ALDH2B4	AT3G48000.1	AT4G29690	AT4G29690.1
YUC1	3702.AT4G32540.1	GDH1	AT5G18170.1	ALDH3I1	AT4G34240.1	AT4G29700	AT4G29700.1
TGG3	3702.AT5G48375.1	TGG3	AT5G48375.1	YUC4	AT5G11320.1	AT4G29710	AT4G29710.1
TGG2	3702.AT5G25980.2	YUC2	AT4G13260.1	YUC1	AT4G32540.1	AT5G17250	AT5G17250.1
GDH1	3702.AT5G18170.1	YUC4	AT5G11320.1	GDH1	AT5G18170.1	AT5G22270	AT5G22270.1
YUC4	3702.AT5G11320.1	YUC5	AT5G43890.1	GDH2	AT5G07440.1	AT5G22280	AT5G22280.3
YUC8	3702.AT4G28720.1	YUC8	AT4G28720.1	TGG3	AT5G48375.1	B73	AT5G20990.1
NMNAT	3702.AT5G55810.2	NMNAT	AT5G55810.2	NMNAT	AT5G55810.2	CYP71A13	.AT2G30770.1

NIT1		NIT2		NIT3		NIT4	
AO	AT5G14760.1	ASP3	AT5G11520.1	AO	AT5G14760.1	CYP79C1	AT1G79370.1
GLT1	AT5G53460.1	AO	AT5G14760.1	GLT1	AT5G53460.1	CYP83B1	AT4G31500.1
EMB1873	AT5G08170.1	ASN1	AT3G47340.1	EMB1873	AT5G08170.1	CYSC1	AT3G61440.1
ADSS	AT3G57610.1	GLT1	AT5G53460.1	ADSS	AT3G57610.1	D22	AT4G39640.1
AT4G24830	AT4G24830.1	EMB1873	AT5G08170.1	AT4G24830	AT4G24830.1	EMB1873	AT5G08170.1
ASN3	AT5G10240.1	ADSS	AT3G57610.1	ASN1	AT3G47340.1	GAD	AT5G17330.1
ASP3	AT5G11520.1	AT4G24830	AT4G24830.1	GAD	AT5G17330.1	GAD2	AT1G65960.2
GAD	AT5G17330.1	GAD	AT5G17330.1	ALDH12A1	AT5G62530.1	GAD3	AT2G02000.1
ASN2	AT5G65010.2	ASN2	AT5G65010.2	ASP2	AT5G19550.1	GAD4	AT2G02010.1
ASP2	AT5G19550.1	ALDH12A1	AT5G62530.1	ASN3	AT5G10240.1	GAD5	AT3G17760.1
ALDH12A1	AT5G62530.1	ASP2	AT5G19550.1	ASN2	AT5G65010.2	GGT2	AT4G39650.1
ASP5	AT4G31990.3	ASP3	AT5G11520.1	ASP3	AT5G11520.1	GGT3	AT1G69820.1
						GGT4	AT4G29210.1
						NAPRT1	AT4G36940.1
						NAPRT2	AT2G23420.1
						NUDX1	AT1G68760.1
						NUDX23	AT2G42070.1
						PAD3	AT3G26830.1
						SPDS1	AT1G23820.1
						SPDS2	AT1G70310.1
						SUR1	AT2G20610.1
						TOC64-III	AT3G17970.1
						TOC64-V	AT5G09420.1
						URE	AT1G67550.1