Examination of the whole genome response in *Arabidopsis thaliana* to auxinic herbicide 2,4-dichlorophenoxyacetic acid coupled with adjuvant NUL1026

A thesis submitted in fulfillment of the requirements for the degree Doctor of Philosophy

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 Thesis abstract

Since the beginning of agriculture around 10,000 BC, humans have been waging a constant battle against weeds. Over the years, numerous strategies have been developed to keep weeds under control and the use of chemicals such as auxinic herbicides have proven to be amongst the most successful. Nowadays, most foliage-applied herbicide formulations also consist of compounds known as surfactants that promote the uptake of herbicide molecules through leaf surfaces.

Most surfactants work by increasing cuticle wettability and therefore, permeability by dissolving the waxy layer covering leaf surfaces. Several lines of research have demonstrated that the efficacy of most herbicides relies on the amount of herbicide molecules successfully entering the plant tissues and reaching the target sites, rather than on increasing the dosage of the herbicides. Therefore, combining the right surfactant to a compatible herbicide formulation may lead to a decrease in the amount of active ingredient being used in weed control.

In recent years, there have been increasing concerns about the detrimental environmental effects of herbicides and numerous cases of herbicide resistance have also been reported. Hence in order to comply with the growing pressures to reduce agrochemical usage, agrochemical companies are now investing in the development of new surfactant products, with the aim of developing novel herbicide-surfactant formulations which are effective at lower active ingredients.

Hence with the aim of developing more effective herbicides, this study took a genomic approach using microarray technology to elucidate the mode of action of an etheramine surfactant, called NUL1026. Knowledge gained from this study will aid in the identification of metabolic and
physiological processes that may contribute to enhanced herbicide activity. The same gene expression profiling approach was also undertaken to investigate the synergistic effect of combining surfactant NUL1026 to the auxinic herbicide 2,4-dichlorophenoxyacetic acid.

*Arabidopsis thaliana* plants (14 days) grown *in vitro* were separately sprayed using the Potter spray tower with either of the following solutions: water, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. The total RNA in response to each of the sprayed solutions was extracted and quantified at 1 h and 24 h post-application. The whole genome oligonucleotide microarray (ATH1-121501) manufactured by Affymetrix® was used to measure gene expression levels of approximately 24,000 genes. This is the first publicly available study that characterises the molecular changes in the entire transcriptome of *Arabidopsis* after the application of a surfactant. This is also the first report that investigates the transcriptomic response in *Arabidopsis* after being treated with an auxinic herbicide formulation in combination with a surfactant.

Expression profiling results in response to surfactant NUL1026 treatment revealed that at both 1 h and 24 h, a number of gene transcripts involved in transcription factor activity, signal transduction pathways, cell wall organisation and biogenesis, disease response, detoxification, senescence and hormone signalling showed altered expression. Of note was the significant regulation of genes involved in jasmonic acid (JA) biosynthesis found to be exclusively regulated upon exposure to the surfactant. Also showing increased expression were the *ACS6* coding for the key enzyme involved in ethylene production and the transcription factor *ERFI*. This transcription factor represents the converging point for JA and ethylene and may be playing an important role in the overall mode of action of surfactant NUL1026.
Foliar-application of 1.0 mM 2,4-D resulted in the differential expression of 45 genes, 1 h post-treatment. Functional annotation of these genes showed that they were known auxin-response genes such as *IAA1, SAUR* and *GH3*. Induced expressions of these auxin-inducible genes were also regulated in common with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment. The low level of gene response to 1.0 mM 2,4-D may be due to the small amount of active ingredient penetrating the leaf cuticle. In contrast, in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, there was approximately 10 times more genes being regulated for the same time point. This suggests that the surfactant may have promoted the uptake of the herbicide 2,4-D. A large proportion of genes (356 genes) that showed altered expression as a result of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, at both 1 h and 24 h, were also regulated in response to the surfactant. Presence of these overlapping genes implies their roles in general plant stress responses.

Apart from the expression of these overlapping genes, 248 and 354 genes were also exclusive differentially expressed after spraying with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h respectively. More in-depth analysis of these exclusively regulated genes showed the increased expression of additional auxin-inducible genes at both time points. Also showing significant up-regulation were genes involved in detoxification and senescence while those transcripts associated with cell wall expansion and photosynthesis were down-regulated. The phenotypic changes recorded correlate with the gene expression results since extensive leaf epinasty and eventual plant death were recorded after treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. Such responses have also been observed in plants undergoing natural plant death. The microarray results of this study highlighted the synergistic influence of surfactant NUL1026 in enhancing 2,4-D toxicity.
Having identified the genes that were responsive to the herbicide and surfactant formulation, the next step was to look at the expression of these genes in herbicide resistant and susceptible biotypes of wild radish (*Raphanus raphanistrum L*). Any differences in gene expression between the two biotypes may imply their involvement in the herbicide resistance mechanism. However, wild radish is an outbreeder and as such it was important to verify that the genetic framework of the resistant and susceptible populations were the same before monitoring gene expressions in the susceptible and resistant plants. This will ascertain that any differences in gene expression recorded may most likely be due to the resistance mechanism. Hence RAPD-PCR was performed on three populations of wild radish from Western Australian, two of which (WARR 5 and WARR 6) have been shown to be resistant to 2,4-D while WARR 7 was susceptible to this herbicide. RAPD-PCR showed that the underlying genetic structure of these 3 populations were similar.

Results from this study have shown that surfactant NUL1026 is not an inert compound and it has its own intrinsic biological activity and this deserves to be further investigated in order to develop more efficient herbicide portfolios. In addition, surfactant NUL1026 is compatible with the herbicide 2,4-D and it may be enhancing the overall herbicidal effect of 2,4-D. This study also highlights the use of microarray technology as a novel way of assessing the performance of herbicide-surfactant formulations.
Declaration

I declare that this thesis contains my original work. Information from published or unpublished sources has been clearly acknowledged within the thesis. None of the work contained in this thesis has been submitted to qualify for any other academic award. The content of the thesis is a result of the work, which has been carried out during the enrolled period of the program. I declare that this thesis is less than 100,000 words in length.

Priyadharshini Madhou

June 2006
Publications pertaining to this thesis

Journal Publications:


Other publication:


Conference:


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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ABI</td>
<td>abscisic acid-insensitive</td>
</tr>
<tr>
<td>ACA1</td>
<td>calcium - transporting ATPase 1</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ACCCase</td>
<td>acetyl-Coenzyme A carboxylase</td>
</tr>
<tr>
<td>acifluorfen</td>
<td>5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>ACO</td>
<td>1-aminocyclopropane-1-carboxylic acid oxidase</td>
</tr>
<tr>
<td>ACS</td>
<td>1-aminocyclopropane-1-carboxylic acid synthase</td>
</tr>
<tr>
<td>ACTIN</td>
<td>actin</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
<tr>
<td>AIR</td>
<td>auxin-induced in root cultures</td>
</tr>
<tr>
<td>ALS</td>
<td>acetolactate synthase</td>
</tr>
<tr>
<td>AMOVA</td>
<td>analysis of molecular variance</td>
</tr>
<tr>
<td>AOC</td>
<td>allele oxide cyclases</td>
</tr>
<tr>
<td>AOS</td>
<td>allele oxide synthase</td>
</tr>
<tr>
<td>AP2</td>
<td>APETALA2</td>
</tr>
<tr>
<td>ARF</td>
<td>auxin response factor</td>
</tr>
<tr>
<td>ARR4</td>
<td>response regulator 4</td>
</tr>
<tr>
<td>ASA</td>
<td>anthranilate synthase alpha subunit</td>
</tr>
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</table>
ATH1-121501  whole genome oligonucleotide array manufactured by Affymetrix®

ATHAL   Arabidopsis

ATHB-12  homeobox-leucine zipper protein 12

atrazine  6-chloro-N-ethyl-N’-(1-methylethyl)-1,3,5-triazine-2,4-diamine

AUX1     auxin-herbicide resistant 1

Avg      average

bHLH     basic helix-loop-helix

bioB     Escherichia coli biotin synthase

bioC     E. coli bioC protein

bioD     E. coli dethiobiotin

bp       base pairs

bromoxynil  3,5-dibromo-4-hydroxybenzonitrile

BSA      bovine serum albumin

butylate S-ethyl bis (2-methylpropyl) carbamothioate

CAO      chlorophyll a oxygenase

CBP      calcium binding proteins

CDF      Dof-type zinc finger domain-containing

cDNA     complementary DNA

CDPK     calcium dependent protein kinase

CH1      chlorophyll B synthase

CHL12    magnesium chelatase 12

CHS      chalcone synthase

clethodim  (E,E)-(±)-2-[1-[(3-chloro-2-propenyl)oxy] imino]propyl]-5-[2-(3-ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CNGC2</td>
<td>cyclic nucleotide-gated channel</td>
</tr>
<tr>
<td>cre</td>
<td>bacteriophage P1 cre recombinase protein</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
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<tr>
<td>CSL</td>
<td>cellulose synthase-like</td>
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<td>CTAB</td>
<td>cetylmethyl ammonium bromide</td>
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<tr>
<td>CTR1</td>
<td>constitutive triple response</td>
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<tr>
<td>DAG</td>
<td>Directed Acyclic Graph</td>
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<td>dap</td>
<td>Bacillus subtilis diaminopropionic</td>
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<td>dazomet</td>
<td>tetrahydro-3,5-dimethyl-1,3,5-thiadiazine-2-thione</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>dicamba</td>
<td>3, 6-dichloro-2-methoxybenzoic acid</td>
</tr>
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<td>dichlobenil</td>
<td>2,6-dichlorobenzonitrile</td>
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<td>diclofop-methyl</td>
<td>(±)-2-[4-(2,4-dichlorophenoxy)phenoxy] propanoic acid</td>
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<td>DIN11</td>
<td>dark inducible 11</td>
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<td>diquat</td>
<td>9,10-dihydro-8a,10a-diazeniapthenanthrene</td>
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<tr>
<td>diuron</td>
<td>N’-(3,4-dichlorophenyl)-N,N-dimethylurea</td>
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<td>DMT</td>
<td>Data Mining Tool (a part of the MAS 5.0 software)</td>
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<td>DNOC</td>
<td>2-methyl-4,6-dinitrophenol</td>
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<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EBF</td>
<td>EIN3- Binding F-Box Protein</td>
</tr>
<tr>
<td>EDGP</td>
<td>extracellular dermal glycoprotein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIN</td>
<td>ethylene insensitive</td>
</tr>
<tr>
<td>EPSP</td>
<td>enolpyruvylshikimate-3-phosphate synthase</td>
</tr>
<tr>
<td>ERD</td>
<td>early responsive to dehydration</td>
</tr>
<tr>
<td>EREBP</td>
<td>ethylene response element binding proteins</td>
</tr>
<tr>
<td>ERF</td>
<td>ethylene response factor</td>
</tr>
<tr>
<td>ERS</td>
<td>ethylene response sensor</td>
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<td>EST</td>
<td>expressed sequence tags</td>
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<tr>
<td>ETR</td>
<td>ethylene response</td>
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<td>EXGT</td>
<td>endo-xyloglucan transferase</td>
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<td>EXP</td>
<td>expansin</td>
</tr>
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<td>EXT</td>
<td>extension</td>
</tr>
<tr>
<td>FGQ1</td>
<td>flavodoxin-like quinine reductase 1</td>
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<tr>
<td>FLA</td>
<td>fasciclin-like arabinogalactan</td>
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<td>flufenacet</td>
<td>1,3,4-Thiadiazol-2(3H)-one, 5-(trifluoromethyl)</td>
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<td>flumetsulam</td>
<td>N-(2,6-difluorophenyl)-5-methyl [1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonamide</td>
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<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrase</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
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<td>glufosinate</td>
<td>2-amino-4-(hydroxymethylphosphinyl) butanoic acid</td>
</tr>
<tr>
<td>glyphosate</td>
<td>N-(phosphonomethyl) glycine</td>
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<td>GO</td>
<td>gene ontology</td>
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<td>GS</td>
<td>galactinol synthase</td>
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<td>GSH2</td>
<td>glutathione synthetase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<td>HAT22</td>
<td>homeobox-leucine zipper protein 22</td>
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<tr>
<td>HFR1</td>
<td>long hypocotyl in far-red 1</td>
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<td>HRAC</td>
<td>Herbicide Resistance Action Committee</td>
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<tr>
<td>HVA22</td>
<td>ABA-responsive protein</td>
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<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
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<td>IAOX</td>
<td>indole-3-acetaldoxime</td>
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<td>IGPS</td>
<td>indole-3-glycerol phosphate synthase</td>
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<td>imazapic</td>
<td>(±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1 h-imidazol-2-yl]-5-methyl-3-pyridinecarboxylic acid</td>
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<tr>
<td>IVT</td>
<td><em>in vitro</em> transcription</td>
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<tr>
<td>JA</td>
<td>jasmonic acid</td>
</tr>
<tr>
<td>LA</td>
<td>linolenic acid</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>LPO</td>
<td>lipid peroxidation</td>
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<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LUT2</td>
<td>lycopene epsilon cyclase</td>
</tr>
<tr>
<td>lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MAS 5.0</td>
<td>Microarray Suite version 5.0 (developed by Affymetrix)</td>
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<td>MATDB</td>
<td>MIPS Arabidopsis thaliana database</td>
</tr>
<tr>
<td>MCPA</td>
<td>4-chloro-2-methylphenoxyacetic acid</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug-resistance</td>
</tr>
<tr>
<td>MES</td>
<td>1´ 2-[N-Morpholino]ethansulfonic acid</td>
</tr>
</tbody>
</table>
Met  Met  Methionine
MicroDB Micro Database (a part of MAS 5.0 software)
MIPS Munich Information of Protein Sequence
MKK9 MAPK kinase 9
MKS1 MAP kinase 4 (MPK4) substrate 1
MM Mismatched
M-MLV Moloney Murine Leukemia Virus Reverse Transcriptase
MPK3 MAP kinase 3
NAA naphthalene acetic acid
NAC no apical meristem (NAM)
NBS nucleotide-binding sites
NCBI National Centre of Biotechnology Information
NCED3 9-cis-epoxycarotenoid dioxygenase
NDPK1A nucleotide diphosphate kinase 1A
NetAffx Database maintained by Affymetrix
norflurazon 4-chloro-5-(methylamino)-2-(3-(trifluoromethyl)phenyl)-3(2H)-pyridazinone
OD optical density
OPDA 12-oxo-phytadienoic acid
OPR 12-oxo-phytodienoic acid reductases
P5CS delta-1-pyrroline-5-carboxylate synthase
PCA principal component analysis
PDF protodermal factor
PDR12 pleiotropic drug resistance
PGP4 p-glycoprotein 4
phe Phenylalanine
picloram 4-amino-3,6,6-trichloropicolinic acid
PINOID protein kinase/pinoid (PID) binding protein 1
PIP2C plasma membrane intrinsic protein 2C
PM perfectly matched
PORC protochlorophyllide oxidoreductase
POT oligopeptide transport
PP2C protein phosphatase 2C
PPO Polyphenoloxidase
PR4 pathogenesis-related 4
Protox protoporphyrinogen oxidised
PRP2 proline-rich protein 2
PSII photosystem II
quinclorac 3,7-dichloro-8-quinolinecarboxylic acid
quinmerac 7-chloro-3-methyl-8-quinolinecarboxylic acid
RALF rapid alkalinization factor
RAPD random amplified polymorphic DNA
rbohD respiratory burst oxidase protein D
RFLP restriction fragment length polymorphisms
ROS reactive oxygen species
rpm  revolutions per minute
RT   reverse transcription
RT-PCR real time PCR
SAG  senescence-associated gene
SAM  s-adenosylmethionine
SAPE streptavidin linked phycoerytherin
SAUR small auxin up-regulated
SCAR sequence amplification region
Sen1 dark inducible 1
SEX1 starch excess protein
SLR  signal log ratio
TAIR The *Arabidopsis* Information Resource
TBE  tris-borate ethylene diaminetetra-acetic acid
TCH  calmodulin-related touch gene
TGT  target value
thr  Threonine
TIR  toll and interleukin-1 receptor
triasulfuron 2-(2-chloroethoxy)-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl–benzenesulfonamide
trifluralin 2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine
TRIS tris (hydroxymethyl) aminomethane
Trp  Tryptophan
Trp1 anthranilate phosphoribosyltransferase
TSA1 tryptophan synthase alpha chain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>UBQ</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted Paired Group Method with Arithmetic Averages</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphates</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>VC</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>WA</td>
<td>Western Australia</td>
</tr>
<tr>
<td>WSSA</td>
<td>Weed Science Society of America</td>
</tr>
<tr>
<td>XET</td>
<td>xyloglucan endotransglycosylase</td>
</tr>
<tr>
<td>XTH</td>
<td>xyloglucan endotransglycosylase/hydrolase</td>
</tr>
<tr>
<td>XTR</td>
<td>xyloglucan endotransglycosylase-related</td>
</tr>
<tr>
<td>YLS</td>
<td>yellow-leaf- specific</td>
</tr>
<tr>
<td>ZAT</td>
<td>zinc finger (C2H2 type)</td>
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Folder name: Chapter 3

Subfolder name: Data mining
File name: Significantly regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment

This file consists of four worksheets that contain the list of probe sets that were significantly up- and down-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.

Subfolder name: Gene Ontology
File name: Common and Exclusively regulated Surf 1 h and 24 h

This file consists of five worksheets that contain the annotation of genes that commonly up-regulated in response to both 1 h and 24 h application of 0.2% (v/v) surfactant NUL1026. The annotations of genes that were exclusively up- and down-regulated in response to 0.2% (v/v) surfactant NUL1026 at each of the time points (1 h or 24 h) are also included. The name of the work sheet specifies the treatment details.

Folder name: Chapter 4

Subfolder name: Data mining
File name: Sig. reg in response to 1.0 mM 2,4-D, 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-treatment
This file consists of six worksheets that contain the list of probe sets that were significantly up- and down-regulated in response to 1.0 mM 2,4-D, 1 h post-application and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.

**Subfolder name: Gene Ontology**

File name: Sig. reg in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h

This file consist of six worksheets that contain the annotation of genes that commonly up- and down-regulated in response to 1.0 mM 2,4-D, 1 h post-application and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.

File name: Exc. Up-regulated genes

This file consists of five worksheets that contain the annotation of genes that were specifically up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-application, 1.0 mM 2,4-D, 1 h post-application and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.

File name: Exc. Down-regulated genes

This file consists of five worksheets that contain the annotation of genes that were specifically down-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-application, 1.0 mM 2,4-D, 1 h post-application and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.

File name: Exc. Common Up- and Down-regulated genes
This file consists of 2 worksheets that contain the annotation of genes that were specifically and commonly up- and down-regulated in response to 1.0 mM 2,4-D, 1 h post-application and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The name of the work sheet specifies the treatment details.
Chapter 1: Review of Literature

1.1 Introduction

Human beings have been fighting a constant battle against the detrimental effects of weeds since the start of agriculture around 10,000 B.C. (Hay, 1974). Over the years, various techniques to control weeds have been developed and these include hand-weeding to primitive hoes (6,000 B.C.), animal-powered implements (1000 B.C.), mechanically-powered implements (1920 A.D.), biological control (1930 A.D.) and herbicidal control (1947 A.D.) (Hay, 1974). The introduction of the first selective herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), in 1947, revolutionised agriculture and considerably improved world agricultural production (Freed, 1980). Herbicides are today considered as the cheapest and most reliable way to control weeds. The sustainability and abundance of food production, important for feeding a growing world population has been attributed in part, to the successful use of herbicides (Avery, 1995). Hence, today, the developed world relies heavily on herbicides.

However, in recent years, there have been major concerns about herbicide residues and associated food safety issues, their negative impacts on the environment and the increasing occurrence of herbicide resistance in weed populations. Therefore, there is an urgent need to reduce the amount of active ingredient of herbicides being applied (the term “active ingredient” has been defined by the Weed Science Society of America (WSSA) (1994) as “the chemical in an herbicide formulation primarily responsible for its phytotoxicity and which is identified as the active ingredient on the product label”).
Studies have shown that the efficacy of most herbicides depends on the amount of herbicide molecules being able to successfully penetrate the plant tissues and reach the target sites (Underwood, 2000). Increasing the dosage or concentration of the herbicides has little or no proportional effects on herbicidal activity (Underwood, 2000). In modern agriculture, compounds known as adjuvants that aid in promoting herbicide uptake are usually added to herbicide formulations. By combining an adjuvant that is highly effective with specific herbicides, the amount of active ingredient used in weed control can sometimes be reduced. Most of the studies carried out on adjuvants to test their ability to promote herbicidal efficacy have concentrated on the effect of a particular adjuvant on the uptake of specific herbicides using radiolabelled chemicals (Kirkwood, 1993) and more recently using confocal laser scanning microscopy (Liu, 2004a, b; Liu et al., 2004). Factors such as the concentration and structure of adjuvants, the physicochemical properties of the active ingredients penetrating into the target species and the leaf surface structure of the plant species have also been studied (Zabkiewicz, 2000).

These investigations have been successfully used to identify a 'good' adjuvant for a particular herbicide. However in order to comply with the increasing environmental pressures to reduce the amount of active ingredients of herbicides, manufacturers and researchers are looking towards improved adjuvant formulations and as a result, there is a need for sophisticated approaches to study the complex herbicide-surfactant-plant interaction system along with the current empirical spray and rank methodologies. In order to develop herbicide application regimes effective at lower active ingredients, it is therefore important to understand both the mode of action of the herbicide and the adjuvant.
This review documents among others, the mode of action of herbicide 2,4-D and our current understanding of adjuvants with emphasis on surfactants and the use of microarrays to elucidate the mode of action of surfactants at the molecular level.

1.2 Herbicides

Herbicides have revolutionised weed control. Despite the enormous improvement agrochemicals have brought to agriculture, not everyone considers herbicides to be beneficial. However, despite widespread concern and controversy over the potential contamination of the environment, there has been a steady increase in the number and type of agrochemicals on the market. Indeed, these compounds are now considered as “prescription tools” for modern agriculture and are believed to be crucial for food production for the ever-expanding world population (Avery, 1995).

Before the introduction of herbicides, measures used to control and eradicate weeds included manual weeding, crop rotation, ploughing and a range of methods to prevent weed seeds from being dispersed in crop seeds (Brian, 1976). The 1980s represented an exciting phase for both industry and academia as modern herbicides were developed (Boger et al., 2002). Herbicides belonging to the following classes, acetolactate synthase (ALS) inhibitors, acetyl-CoA carboxylase (ACCase) and protoporphyrinogen oxidised (Protox) inhibitors were introduced and these new chemistries, which combine lower dosage, crop safety, specific and known mechanism of action, have been quickly adopted worldwide.

Traditionally, herbicides were discovered by randomly visually assessing the effect of a large number of compounds on small populations of weeds and this approach has been termed as the “spray and pray” technique whereby, essentially selection of herbicides has been by trial and
error (Cole et al., 2000; Lein et al., 2004). As such, the majority of the herbicides marketed today have been selected after random screening of chemicals for herbicidal activity.

1.2.1 Herbicide application

Herbicides may be classified either as pre-emergence or post-emergence, that is, they are applied either before or after the weeds emerge from the soil and start to grow. Pre-emergence herbicides eradicate weeds shortly after they germinate or emerge from the ground. Post-emergence herbicides in contrast, control weeds that are already growing and easily visible. Some herbicides are applied to the soil and are then passively taken up by the seedlings roots or shoots and are therefore said to have soil activity while foliage applied herbicides are sprayed to the leaves, stems or shoots of plants (Baumann et al., 2002). Post-emergence herbicides are effective as a foliar spray and through root application while pre-emergence herbicides are primarily taken up by the root system or by coleoptile uptake (Grossmann, 1998).

1.2.2 Herbicide mode of action

In order to be effective, an herbicide must be able to reach target plants, be able to penetrate the plant cuticle, cell wall and cell membrane and move to the site of action without being metabolised and must also reach the site of action at concentrations which are toxic to the plants (Lingenfelter and Hartwig, 2003) (Figure 1.1). This sequence of events from the time the plant takes in the herbicide to the plant death, how the herbicide kills the plant, is termed the mode or mechanism of action (Lingenfelter and Hartwig, 2003). Understanding the mode of action of herbicides is important since this will indicate which groups of weeds are likely to be affected, the herbicide application technique to be utilised, indication of herbicide injury problems and prevent and/or delay the evolution of herbicide-resistant weeds.
Herbicides are usually grouped according to their mode of action. Though there are a number of herbicides on the market, most of them share the same chemical properties and herbicidal activity. Thus herbicides with similar mode of action are grouped into “families” and if two or more “families” may have the same mode of action, they are grouped into “classes” (Table 1.1) (Lingenfelter and Hartwig, 2003).
In order to be effective, foliage-applied herbicides must be able to reach the plants effectively, be able to penetrate the plant cuticle and membrane and move to the site of action.
Table 1.1 Examples of some important classes of herbicides. Adapted from Lingenfelter and Hartwig (2003).

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Site of action</th>
<th>Family</th>
<th>Active ingredients</th>
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<tbody>
<tr>
<td>Plant growth regulators (PGR)</td>
<td>IAA-like</td>
<td>phenoxy</td>
<td>2,4-D</td>
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<td></td>
<td></td>
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<td>MCPA</td>
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<td>benzoic acid</td>
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<td></td>
<td>Dicamba</td>
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<tr>
<td>Amino acid biosynthesis inhibitor</td>
<td>ALS enzyme</td>
<td>imidazolinone</td>
<td>Imazapic</td>
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<td></td>
<td>sulfonyleurea</td>
<td>Triasulfuron</td>
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<td>triazolopyrimidine</td>
<td>Flumetsulam</td>
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<tr>
<td></td>
<td></td>
<td>EPSP enzyme</td>
<td>amino acid derivatives</td>
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<td></td>
<td></td>
<td>Glyphosate</td>
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<tr>
<td>Fatty acid (lipid) biosynthesis inhibitors</td>
<td>ACCase enzyme</td>
<td>aryloxyphenoxy</td>
<td>Dichofop</td>
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<tr>
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<td>cyclohexanediones</td>
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<td>dinitroanilines</td>
<td>Trifluralin</td>
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<td>Seedling growth inhibitors (root and shoot)</td>
<td>Microtubule</td>
<td>nitriles</td>
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<td>Seedling growth inhibitors (root and shoot)</td>
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<td>oxyacetamides</td>
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<td>photosystem II</td>
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<td>Photosynthesis inhibitors (mobile 1)</td>
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<td>Classification</td>
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<td>Photosynthesis inhibitors</td>
<td>photosystem II</td>
<td>ureas</td>
<td>Diuron</td>
</tr>
<tr>
<td>Photosynthesis inhibitors (nonmobile)</td>
<td>photosystem II</td>
<td>nitriles</td>
<td>Bromoxynil</td>
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<td>Cell membrane disrupters</td>
<td>PPO enzyme</td>
<td>diphenyl ethers</td>
<td>Acifluorfen</td>
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<tr>
<td>Cell membrane disrupters</td>
<td>photosystem I</td>
<td>bypyridyliums</td>
<td>Diquat</td>
</tr>
<tr>
<td>Pigment inhibitors</td>
<td>Diterpenes</td>
<td>pyridazinones</td>
<td>Norflurazon</td>
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<tr>
<td>Phosphorylated amino acid</td>
<td>GS enzyme</td>
<td>amino acid derivatives</td>
<td>Glufosinate</td>
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<tr>
<td>Unknown</td>
<td>?</td>
<td>?</td>
<td>Dazomet</td>
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</tbody>
</table>

Abbreviations: IAA - indole-3-acetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; MCPA - 2-methyl-4-chlorophenoxyacetic acid; ALS - acetolactate synthase; EPSP - enolpyruvylshikimate-3-phosphate synthase; ACCase - acetyl-Coenzyme A carboxylase; PPO - polyphenoloxidase; GS - galactinol synthase.
1.2.3 Herbicide absorption, translocation and metabolism

Plants take up herbicides mainly through leaf surfaces, fruits and roots and the active ingredient need to be able to move across the plasma membrane to reach the target site (Figure 1.1). Plasma membrane proteins may be facilitating this entry and transport (Gerst, 1999). Most herbicides possess an ionisable group that become highly lipophilic at low pH and this facilitates diffusion across the plasma membrane (Gerst, 1999). Presence of ATPase pumps in the plasma membrane create a pH gradient across the membrane thereby enabling the active uptake of the herbicide ions (Harper et al., 1990). The degree and manner in which an herbicide is distributed within the plant relies on the physical and chemical properties of the herbicide. Contact herbicides, as the name suggests, kill or injure foliage upon contact and do not move after they enter the plants. Systemic herbicides are taken up via the roots or leaves and translocated throughout the plant (Figure 1.2) (Grossmann, 1998; Hansen and Grossmann, 2000).
Herbicides are taken up by roots and leaves and are then translocated to all parts of the plants.

Generally, plants have mechanisms for the degradation and sequestration of foreign compounds. Plant metabolism of these xenobiotics can be divided into three phases. In Phase I metabolism, generally catalysed by the cytochrome P450 superfamily, a xenobiotic compound may be oxidised, reduced or hydrolysed to reveal or introduce a functional group, thereby rendering the parent compound more water soluble, and usually less toxic than the parent (Van Eerd et al., 2003). In Phase II of detoxification, the activated metabolite is deactivated by conjugation with endogenous hydrophilic
compounds such as malonate, glucose or glutathione to form more hydrophilic and even less toxic compounds compared with the parent compound. These Phase II reactions are catalysed by glycosyl-, malonyl- or glutathione S-transferases. In the final step of detoxification (Phase III), the S-conjugated metabolites are exported from the cytosol by transport proteins and sequestered into the vacuole (Coleman et al., 1997; Morant et al., 2003). The transport proteins performing the sequestration belong to the ATP binding cassette (ABC) transporter family and the multidrug-resistance (MDR) associated proteins.

1.2.4 Herbicide classification, target sites and selectivity

Ideally, an herbicide should be effective at low rates, economical to the manufacturer, have a broad spectrum of use, be safe and easy to handle and have little or no adverse effect on the environment. Currently there are more than 400 herbicides that have been registered or are in the process of undergoing registration (Boger et al., 2002). To date 269 kinds of herbicides with known mode of action are in use around the world (Boger et al., 2002). Categorisation of such herbicides is carried out by the Herbicide Resistance Action Committee (HRAC) in collaboration with WSSA (Boger et al., 2002).

Approximately 269 herbicides interact with one of only three targets within the plant and these targets include photosystem II (PSII), acetolactate synthase (ALS) and protoporphyrinogen (Protox) (Cole et al., 2000). Herbicides interact with target/s and induce a primary response, which then in turn triggers a secondary effect, thereby leading to the death of the plant (Cole et al., 2000). Herbicides such as sulfonylureas target the ALS and inhibit the biosynthesis of branched chain amino acids valine, leucine and isoleucine (Cole et al., 2000; Boger et al., 2002). Herbicides (phenylureas) targeting the
PSII, prevent electron transport which eventually leads to photoxidation within the plant cells and death while diphenylethers, which target the Protox inhibit chlorophyll biosynthesis (Duke, 1990; Cole et al., 2000).

Selectivity is the process by which an herbicide successfully controls and kills certain plants but does not harm others (Baumann et al., 2002). Selectivity may be as simple as controlling dicot plants in a field of monocots or differences in the waxy covering on leaves on certain plants which render them more susceptible to herbicides than other plant species. A high level of herbicide selectivity can be achieved by identifying and targeting unique processes, proteins or enzymes within the plants. An ideal herbicide acts on a target that not only inhibits an essential metabolic process within the plant cells but also affects the metabolic events up- and down-stream of the target, thereby triggering a cascade of biochemical responses within the plant eventually leading to plant death (Cole et al., 2000).

To date, no distinct mode(s) of action and/or target(s) have been identified for one of the most successful classes of herbicide chemicals that have had a major impact on agriculture worldwide, the plant growth regulators. The next few paragraphs will detail our current knowledge of these plant hormones used as herbicides.

1.2.5 Auxin and auxin-like herbicides

Indole-3-acetic acid (IAA) is the main natural auxin found in most plants and along with other natural auxins, is said to possess phytohormonal activity (Davies, 1995). Auxin in association with other plant hormones such as abscisic acid (ABA), cytokinins, ethylene, gibberellins and other plant regulators regulate important growth and development
processes such as cell elongation and division, differentiation of vascular tissues, root initiation, flowering, fruit setting and growth and control of apical dominance, tropic responses and senescence (Davies, 1995; Goda et al., 2004).

Increasing concentrations of auxins leads to a number of growth abnormalities are induced within 24 h treatment (Grossmann, 2000). These include downward curvature of leaves (leaf epinasty), stem tissue proliferation, formation of adventitious root and to a greater extent of shoot with decreased internode elongation and leaf area and intensified leaf pigmentation (Grossmann, 2000). Reduction in stomatal aperture, transpiration and carbon assimilation also occur (Sterling and Hall, 1997; Goda et al., 2004). These phenomena are then followed by accelerated foliar senescence with chloroplast destruction and progressive chlorosis, damage of membrane and vascular system integrity, desiccation and eventual plant death (Sterling and Hall, 1997; Grossmann, 2000; Zheng and Hall, 2001; Goda et al., 2004).

These effects are often described as an ‘auxin overdose’ (Sterling and Hall, 1997) and has allowed the use of auxin analogs as bioregulators and as selective herbicides in agriculture (Gianfagna, 1995; Grossmann, 1998; Grossmann, 2000; Grossmann et al., 2001). Auxinic herbicides were discovered independently by British and American scientists during the 1940’s (Library of Crop Technology Lesson Modules, 2006). Auxin-like herbicides mimic the biphasic effects of IAA and induce the same deformative and growth limiting effects as those elicited by IAA at higher concentrations. Auxinic herbicides are applied to the foliage but are also effective in the soil (Gerst, 1999) and they act in a systemic manner (Hansen and Grossmann, 2000). The herbicide 2,4-D is the most widely used herbicide in the world and is highly selective in crop
production systems (Filkowski et al., 2003; Grabinska-Sota et al., 2003). It is used for to control broad-leaved weeds in cereal crops such as wheat, sorghum, rice and pastures and has low toxic effects on humans and animals (Grabinska-Sota et al., 2003)

Auxinic herbicides include a number of different chemical classes. They all have auxin-like effects but they can differ markedly in their selectivity. Classes of auxin-like herbicides can be differentiated into four major classes based on the location of the carboxylic acid moiety and the type of aromatic compounds (Figure 1.3) (Zheng and Hall, 2001). The four classes are: phenoxyalkanoic acids (e.g. 2,4-D, MCPA and MCPP), benzoic acid (e.g. dicamba), pyridines (e.g. picloram) and quinoline carboxylic acid (e.g. quinclorac and quimerac) (Figure 1.3) (Grossmann, 2000).
IAA and NAA are natural plant hormones. The four different classes are the phenoxyacetic acids (e.g. 2,4-D and MCPA); benzoic acid (e.g. dicamba); pyridinecarboxylic acids (e.g. pichloram) and quinolinecarboxylic acids (e.g. quinclorac and quinmerac). (adapted from Grossmann, 2000).
1.2.6 Elucidation of the mode of action of auxinic herbicides

While the effects of natural auxins on plants are well characterised and auxinic herbicides have been used for decades, the mode of action of either natural or synthetic auxins has yet to be completely elucidated. Over the years, intensive research mainly based on the response of the model plant *Arabidopsis* to auxins has been undertaken and such studies have greatly improved our knowledge of the mechanisms of action of these auxins. Since auxinic herbicides are said to mimic the action of plant auxins, a vast majority of researchers have assumed that insights gained from the literature on the natural auxins would also provide insight into the mode of action of auxinic herbicides.

However, recent studies on early auxin signalling using tobacco c.v. Virginia Bright Italia-0 cells have revealed that processes such as cell division and cell elongation are distinct and differentially regulated by different auxin species (Campanoni and Nick, 2005). The two types of exogenously applied auxin analogs in that study were 2,4-D and 1- naphthaleneacetic acid (NAA) and it was hypothesised that these two different auxins activate different signal transduction pathways for the control of cell division and cell elongation (Campanoni and Nick, 2005). Hence, caution should be applied with this “copy-cat” approach of trying to understand the mode of auxinic herbicides action and signal transduction pathways based on knowledge about natural auxins. The following paragraphs detail the current and overall status of our understanding of the mode of action of auxinic herbicides and the approaches employed by researchers to elucidate this mystery.
1.2.7 Biochemical approach

Research carried out over the years based on the effects of herbicides on a number of plant species have revealed that herbicides stimulate ethylene production (Sterling and Hall, 1997). Auxinic herbicides in particular were found to highly stimulate ethylene biosynthesis. Therefore leaf and stem epinasty and leaf abscission that occurred as a result of auxinic herbicide treatments were attributed to ethylene (Grossmann, 1998; Wei et al., 2000).

Pioneering biochemical studies in cleavers (Galium aparine) based on the measurements of the activity of several enzymes and levels of products and hormone intermediates after root-treatment with 0.5 mM IAA and auxinic herbicides have shown that auxin-induced ethylene promoted abscisic acid biosynthesis (ABA). This ABA synthesis eventually caused a number of abnormalities such as stomatal closure, significant reduction in photosynthesis and transpiration leading to growth inhibition and plant senescence (Grossmann, 2000; Hansen and Grossmann, 2000; Grossmann and Hansen, 2001).

Grossmann (2000) has proposed a model for the mode of action of auxinic herbicides (Figure 1.4). This model suggests that auxin and auxin analog at high concentrations and auxinic herbicides promote the increased activity of a key regulatory enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which results in increased ethylene biosynthesis (Sato and Theologis, 1989). Ethylene in turn triggers xanthophyll cleavage which subsequently leads to ABA biosynthesis. Additional experiments with the tomato mutant never ripe which is defective in ethylene perception, further supported the theory that it is ethylene induced by high concentration of auxin or auxinic herbicides, that stimulates ABA biosynthesis (Hansen and Grossmann, 2000).
During the biosynthesis of ethylene, cyanide is also produced as a coproduct and is present at physiologically damaging levels (Grossmann and Kwiatkowski, 2000). Production of cyanide has been shown to be detrimental to sensitive grasses (Grossmann and Kwiatkowski, 2000) though the early growth inhibiting effects of auxins in dicots have not been attributed to cyanide (Grossmann, 1998). Auxinic herbicides were also found to induce the overproduction of hydrogen peroxide (H$_2$O$_2$) which contributed to plant death in cleavers and it has been hypothesised that the increase in production of H$_2$O$_2$ was due to ABA- mediated stomatal closure (Grossmann et al., 2001).
Methionine
Leaf epinasty and senescence
Ethylene
Horizontal stem curvature, necrosis
IAA, Auxinic herbicides

Growth inhibition
Inhibition of CO₂ assimilation
Inhibition of transpiration

Induction of stomatal closure

ABA
Senescence
Growth inhibition of root and shoot

ABA

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Xanthoxin

ACC synthase

SAM

ABA

Growth inhibition

Induction of stomatal closure

Inhibition of transpiration

ABA

Growth inhibition of root and shoot

IAA, Auxinic herbicides

Horizontal stem curvature, necrosis

Leaf epinasty and senescence

Ethylene

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Figure 1.4 Phytotoxic effects induced by high concentrations of auxin and auxinic herbicides.

At high concentrations, auxin (IAA) and auxinic herbicides induce growth inhibition and senescence in dicotyledonous plant species, as illustrated for cleavers (*Galium aparine*). SAM - s-adenosylmethionine, ACC - 1-aminocyclopropane-1-carboxylic acid, ABA - abscisic acid. (adapted from Grossmann, 2000).

### 1.2.8 Genomic approach

Historically, genetic and molecular studies based on mutant plants and microarray studies in response to a plethora of abiotic and biotic factors have dissected and isolated components involved in complex networks of interactions between auxin, ethylene and ABA. Such studies have considerably increased our knowledge of various plant hormonal pathways and contributed to our understanding of the mechanism of action of auxinic herbicides.

#### 1.2.8.1 Auxin

A number of genes induced in response to auxins have been isolated, characterised and assigned functions. Such genes include the early response genes (e.g. small auxin up-regulated genes (*SAUR*s), growth hormone (*GH3*s) and *Aux/IAA* genes), which are induced within minutes of auxin application and the transcription factors auxin response factors (ARFs) (Abel and Theologis, 1996; Hagen and Guilfoyle, 2002). The C-terminal domain of ARF proteins is homologous to domain III and IV of the Aux/IAA proteins (Leyser, 2001) and the promoters of auxin-responsive genes *Aux/IAA* consist of an auxin-response element (AuxRE) (Hagen and Guilfoyle, 2002) involved in auxin-induced transcription.

At low auxin concentrations, the Aux/IAA proteins are stable and form dimers with ARF proteins, thereby inhibiting the function of ARF proteins. However, at high auxin levels, the
Aux/IAA proteins are destabilised resulting in dimer formation between ARF proteins themselves, leading to auxin induced transcription (Kepinski and Leyser, 2002; Leyser, 2002) (Figure 1.5). The ARF proteins contain a non-conserved middle region (MR) and those ARF proteins with a glutamine-rich (Q-rich) MR function as activators of auxin response genes, whereas the others function as repressors of auxin responsive genes (Tiwari et al., 2003).

Auxin signalling, which involves ubiquitin-mediated degradation of auxin response proteins (SAURs, GH3 and Aux/IAA) via the 26S proteosome, is essential for auxin mediated growth and development (Leyser, 2001) (Figure 1.5). The three main enzymes that regulate auxin signalling are the ubiquitin-activating enzyme-E1; ubiquitin-conjugating enzyme-E2; and the ubiquitin-protein ligase-E3 (Leyser, 2001; Kepinski and Leyser, 2002; Leyser, 2002). These enzymes are involved in covalently binding ubiquitin to target proteins thus marking them for degradation by the 26S proteosome (Hellmann and Estelle, 2002).
Figure 1.5 Transcriptional regulation of auxin response by ARFs and degradation of Aux/IAA proteins via the 26S proteosome.

At low auxin concentrations, the Aux/IAA proteins dimerise with ARF while at high concentrations of auxin, the Aux/IAA proteins are destabilized resulting in ARF-ARF dimer formation. Dimerisation of ARFs triggers auxin induced transcription of auxin responsive genes. Glutamine rich (Q-rich) ARFs are activators of auxin response while other ARFs are repressors of the auxin response. Auxins induce the degradation of Aux/IAA via the 26S proteosome and this degradation of auxin-induced proteins is required for proper auxin signalling. ARF - auxin response factor; ARF-Q - ARF with a glutamine-rich middle region; AuxRE - auxin response element (Kepinski and Leyser, 2002).
1.2.8.2 Ethylene

Ethylene gas is involved in plant developmental processes such as germination, fruit ripening and root development (Alonso and Ecker, 2001; Schaller and Kieber, 2002; Alonso and Stepanova, 2004). Research has also shown its involvement in response to abiotic and biotic stresses (Solano and Ecker, 1998; Wang et al., 2002). The precursor of ethylene is the amino acid methionine. Methionine is converted to s-adenosylmethionine (AdoMet) by the enzyme AdoMet synthase (Schaller and Kieber, 2002) and AdoMet in turn is converted to ACC by the enzyme ACC synthase. The conversion of ACC to ethylene occurs in the presence of ACC oxidase (Zarembinski and Theologis, 1994; Schaller and Kieber, 2002; Wang et al., 2002; Chae and Kieber, 2005) (Figure 1.6).

Figure 1.6 Ethylene biosynthesis pathways.

The enzymes catalyzing each step are shown in red above the arrows. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; AdoMet, S-adenyl-methionine; Met, methionine. (Chae and Kieber, 2005).

A protein family of five members (ETR1, ETR2, ERS1, ERS2 and EIN4) has been identified as ethylene receptors in Arabidopsis and these receptors play a key role in regulating down-stream signalling of ethylene (Figure 1.7) (Chang and Shockey, 1999; Schaller and Kieber, 2002). In
the absence of ethylene, these receptors repress ethylene related responses via activation of the constitutive triple response gene (*CTR1*) (Chang and Shockey, 1999) (Figure 1.7). Studies have shown that the kinase domain of ETR1 and ERS1 can interact with CTR1 (Wang et al., 2002) and this interaction is required to switch off the ethylene-signalling pathway (Huang et al., 2003).

Disruption of the interaction between CTR1 and ETR1 in the presence of ethylene results in ethylene signalling (Figure 1.7) (Guo and Ecker, 2004). Down-stream of EIN2 are two types of transcription factors EIN3/EIL (Guo and Ecker, 2004). *EIN3* positively regulates ethylene signalling and both EIN3 and EIL bind to the promoter region of the ethylene response factor (*ERF1*) gene (Schaller and Kieber, 2002). ERF1 is also a transcription factor (Fujimoto et al., 2000) which is induced by EIN3/EIL and this shows the sequential involvement of transcription factors in ethylene signal transduction (Schaller and Kieber, 2002) (Figure 1.7). *ERF1* is a member of the ethylene response element binding proteins (EREBP) family of transcription factors (Riechmann and Meyerowitz, 1998).
Figure 1.7 Ethylene signalling and response pathway.

Ethylene is perceived by ethylene receptors (ETR1, ETR2, ERS1, ERS2 and EIN4). CTR1 is a negative regulator of ethylene response and occurs down-stream of ethylene receptors while EIN2 is a positive regulator of ethylene signalling. Transcription factors involved in mediating ethylene signalling are EIN3/EIL and ERF1. In the presence of ethylene CTR1 is detached from the ethylene receptors and ethylene signalling continues while in the absence of ethylene, CTR1 binds to ethylene receptors and inhibits ethylene signalling. (Schaller and Kieber, 2002).
1.2.8.3 Abscisic acid

The phytohormone ABA plays key roles in growth inhibition, seed development and several abiotic and biotic stresses (Finkelstein and Rock, 2002). ABA biosynthesis involves the key regulatory enzyme, 9-cis-epoxycarotenoid dioxygenase which catalyses xanthophylls cleavage (Grossmann, 2000; Qin and Zeevaart, 2002). ABA is said to be important for plant survival as this hormone modulates changes at the cellular and whole plant level in order to protect the plant from external stress (Xiong and Zhu, 2003). Gene expression studies carried out by various research groups have isolated genes involved in ABA biosynthesis, transcription factors and signal transduction pathways. Stress conditions such as water deficit, senescence, salinity conditions and low temperature have also been identified as triggering its biosynthesis (Leung et al., 1997; Qin and Zeevaart, 1999; Choi et al., 2000; Farras et al., 2001; Chen et al., 2002; Hoth et al., 2002; Himmelbach et al., 2003; Lopez-Molina et al., 2003; Schwartz et al., 2003).

1.2.9 Holistic approach

Recently, Raghavan et al. (2005, 2006) took a holistic approach using microarray technology to look at the genome-wide response to root-applied auxinic herbicide 2,4-D in Arabidopsis. Based on the tremendous amount of genetic information now available on auxin, ethylene ABA processes and stress responses within plants, these scientists (Raghavan et al., 2005, 2006) were able to associate functions to most of the genes that were regulated in response to the herbicidal treatment. Genes previously identified as being regulated in response to auxins, showed enhanced transcript expressions, thereby, confirming the auxinic nature of 2,4-D. Also up-regulated in response to the herbicidal treatment were those genes involved in the different aspects of ethylene and ABA biosynthesis, signalling and transcription factors (Raghavan et al.,
2005, 2006). These data were found to be consistent with the pathway proposed by Hansen and Grossmann (2000) (Figure 1.4), in that genes involved in ethylene and ABA biosynthesis were induced when treated with 1.0 mM 2,4-D.

However, most importantly, their results indicated the regulation of a number of other genes, either as a direct response or secondary response to 2,4-D, that have not previously been associated with auxinic herbicides (Raghavan et al., 2005, 2006). These included the regulation of genes known to be involved in jasmonic acid and salicylic acid biosynthesis, transcription factors and senescence. Hence, these data highlight the fact that response to auxinic herbicides is not a linear sequence of events. Rather the mode of action and the signal transduction pathways of auxinic herbicides seem to entail a complex network of interactions that consists of genes involved in perception, signalling, defence, stress, senescence and transcription factor activity.
Figure 1.8 A schematic diagram of genes that were regulated in response to root-applied 1.0 mM 2,4-D, 1 h treatment.

Thick arrows indicate up-regulated genes while thin arrows indicate down-regulated genes in the boxes. Arabidopsis ethylene response factor (AtERF) 4 and AtERF8 are repressors of the GCC-motif containing stress response. Up-regulated genes (+); Down-regulated genes (-) (Raghavan et al., 2005).
1.3 Adjuvants- what are they?

Foliage-applied herbicides are usually applied through hydraulic spray nozzles with water as the carrier. Losses occurring due to droplet-reflection, run-off, drift and evaporation can occur using this method of delivery, substantially reducing the proportion of the herbicide dose actually reaching the target plants (Leaper and Holloway, 2000). Moreover, leaf surface characteristics such as the presence of trichomes and epicuticular waxes can also inhibit droplet spread and leaf contact (Hess and Falk, 1990). Such losses cannot be compensated by the addition of more active ingredients but can however, be significantly reduced by the inclusion of the right adjuvants (Underwood, 2000).

WSSA describes an adjuvant as “any substance in an herbicide formulation or added to the spray tank to modify herbicidal activity or application characteristics” (WSSA, 1994). Adjuvants can be broadly divided into two types: (i) formulation adjuvants, in which case adjuvants are part of the formulation and (ii) spray adjuvants whereby the adjuvants are added along with the formulated product to the water in the tank of the spray apparatus before being applied to the fields (Krogh et al., 2003). Adjuvants are added to improve the effectiveness of agrochemicals by enhancing the solubility and compatibility of the active ingredients. Other roles include facilitating absorption, penetration and translocation of the active ingredients into the target species, augmenting rain fastness and modulating selectivity of the active ingredients in various plants (Foy, 1993, 1996).

The adjuvant market is closely associated with the pesticide market and it is estimated that the worldwide economic value of adjuvants is about $1 billion (U.S.) (Underwood, 2000).
There are currently two fundamental types of adjuvants: (i) those that modify the physical characteristics of the spray mixture and (ii) those that enhance the biological effectiveness of the crop production chemical (American Society for Testing and Materials, 1999). This project will concentrate on the second types of adjuvants, more commonly termed as activators. They are the most difficult to classify as their modes of action are not mutually exclusive. Activator adjuvants include surfactants, more precisely wetter-spreader adjuvants, sticker adjuvants, humectants, penetrating agents and herbicide modifiers (Hazen, 2000). These surfactants function by improving the properties of a liquid by changing its surface characteristics. Surfactants make up the largest group of adjuvants and are the best-known class of adjuvants. In the next sections, more emphasis will be placed on surfactants as they are by far, the most commonly used adjuvant for post-emergence herbicides.

1.3.1 Surfactants

The WSSA herbicide handbook (1994) defines a surfactant as “a material that improves the emulsifying, dispersing, spreading, wetting or other properties of a liquid by modifying its surface characteristics”. Hence, the primary role of a surfactant is to enhance the penetration of a chemical through the cuticle or the leaf surface and into the plant.

1.3.2 Sites and modes of action of surfactants

The herbicide-plant-surfactant interaction is complex and a surfactant can act at several stages of herbicide application including, deposition and retention, penetration and translocation from the site of application to the site(s) of action (O'Donovan et al., 1985; Schonherr and Reiderer, 1989; Feng et al., 1999). In the presence of surfactants, herbicidal effectiveness depends on at least four factors: the physiochemical properties of the active ingredients, the structure and
concentration of surfactants, the environmental conditions at the time of spray and the type of
leaf surface of individual target plants (Liu, 2004b). Not all surfactants are able to promote the
uptake of herbicides and there is currently no practical formula or model that can quantitatively
predict the effect of a specific surfactant on a particular herbicide (Liu, 2004b).

Although more is known concerning the behaviour of herbicides, relatively little is known about
the uptake, distribution and fate of surfactants, per se, and their role(s) in enhancing the
performance of specific herbicides. Recently, attention has shifted to the probable mode(s) and
site(s) of action of surfactants in promoting herbicidal effects following foliar herbicide
application (Zabkiewicz, 2000). Understanding the roles of surfactants in improving herbicide
efficacy is important for the optimum use of surfactants in herbicide application.

There are four main sites whereby the rate of herbicidal uptake into a leaf may be enhanced: (i)
on the surface of the cuticle, (ii) within the cuticle itself, (iii) underneath the cuticle in the outer
epidermal wall, (iv) at the cell membrane of internal tissues (Stock and Holloway, 1993). After
spraying, surfactants are deposited along with herbicide molecules on the cuticle of the leaf.
Numerous studies have, however, shown that certain surfactants may also penetrate the cuticle
and hence may act in more than one location, resulting in multiple biochemical and
physiological mechanisms operating simultaneously during the herbicidal uptake. Thus, some
surfactants influence herbicide penetration via their effect inside the cuticle (Baur et al., 1997)
while others mainly act by influencing the driving forces that promote the uptake of the
herbicide molecules (Baur, 1999). Therefore, the net effects of surfactant interactions at these
locations result in an increase in the uptake of the spray molecules from the leaf surface to the
internal tissues of the plants.
1.3.2.1 Probable mode of action on the leaf surface

The mechanisms of action of surfactants on the leaf surface have been extensively reviewed (Kirkwood, 1991). Surfactants are able to increase the contact area of deposits especially on waxy and hairy plant species by reducing the surface tension and contact angle of the spray droplets (Singh et al., 1984; Kirkwood, 1991; Kocher and Kocur, 1993) (Figure 1.9).

Figure 1.9 θ, the contact angle is measured as the tangent from the point of droplet contact with the surfactant.

A lower contact angle results when the substrate free energy is reduced by a surfactant. The contact angle therefore decreases as the droplet flattens on the substrate, indicating better wetting (Hazen, 2000). Figure 1.9 was taken from Hazen (2000)

Theoretically, increasing the contact area of a spray droplet would be expected to increase the magnitude of diffusion of the spray molecule through the cuticle and this has been effectively shown by an increase of uptake of the lipophilic compound permethrin with the addition of a surfactant (Stock et al., 1993). However, on leaves of wettable species, the same mechanism has little effect on the deposit area of spray droplets. Studies have shown that the interaction of surfactants with the epicuticular waxes, while common, is not uniform (Kuzych and Meggitt, 1983; Noga et al., 1987; Stevens and Baker, 1987; Stevens and Bukovac, 1987; de Ruiter et al., 1988; Knoche et al., 1992). Though some researchers found considerable change in epicuticular wax characteristics with surfactants (Kuzych and Meggitt, 1983; Noga et al., 1987; Knoche et
al., 1992; Feng et al., 1999), others have observed no change (Stevens and Baker, 1987; Stevens and Bukovac, 1987; de Ruiter et al., 1988).

Based on the documented detergent properties of surfactants (Hazen, 2000), it is thought that surfactants are able to increase cuticle wettability and therefore permeability to spray molecules by disrupting or dissolving the waxy covering on leaf surfaces (Feng et al., 1999). This phenomenon has only been observed with high surfactant concentrations or when large droplets were used and it is also believed that under field conditions, any damage to the epicuticular wax is minimal (Stock and Holloway, 1993). In addition, the solubilising effects of certain surfactants on spray droplets have also been mentioned as probable means by which uptake of the agrochemicals is promoted (Feng et al., 1999). Foliar deposit solubility has been shown to increase significantly in aqueous surfactant solutions (Nassetta et al., 1991).

Surfactants such as humectants do not dry out quickly after the aqueous portion of the spray has evaporated since they are able to draw moisture from the environment (Hazen, 2000). This maintains the agrochemical in a hydrated and solubilised state longer, extending the contact and penetration time. The most common humectants are glycerine and propylene glycol. However, these humectants are not important in aiding penetration of non-water soluble molecules.

It has also been shown that spray molecules can be made to penetrate directly into the leaves through the stomata provided certain conditions are met (Schonherr and Bukovac, 1972). These conditions include low solution surface tension and the physical dimensions of the stomatal apertures, which also need to be open. Activation of the stomatal pathway is achieved by the use of highly surface-active organosilicone surfactants such as Silwet L-77 and stomatal infiltration results in rapid uptake of the agrochemical within 10 minutes of application (Stevens
and Zabkiewicz, 1988; Stock and Holloway, 1993; Knoche, 1994). There have also been reports indicating that surfactants can penetrate through cracks and faults in the leaf cuticle thereby causing the leaf cuticle to swell. This swelling is thought to have resulted from wetting which produces an enlargement of the surface defects of the leaf, thus allowing easier penetration of the agrochemical molecules (Fumidge, 1959; Foy and Smith, 1969).

1.3.2.2 Probable mode of action within the leaf

Research examining surfactant action effects on transport processes within tissues of the leaf has been extensively reviewed by Holloway and Stock (1990) and Schonherr et al. (1991). Most of these investigations have been concentrated on the passage of solute through the cuticle and several mechanisms of action within the leaf have been suggested (Holloway and Stock, 1990; Schonherr et al., 1991). Rates of entry of any xenobiote will depend on how permeable the cuticle is to that xenobiote and will vary according to the principal driving force, which is the concentration gradient between its outer and inner boundaries. The permeances of cuticles in turn depend on their partition coefficient, which control movement in and out of the cuticles, and their diffusion coefficients (Stock and Holloway, 1993; Schonherr and Baur, 1994). It is believed that sufficient quantities of surfactants under the appropriate conditions are able to increase the permeability of the cuticle by increasing the solubility of the agrochemicals in the lipophilic cuticle. This causes the partitioning system to change from water-cuticle to water + surfactant-cuticle + surfactant (Figure 1.10) (Stock and Holloway, 1993; Schonherr and Baur, 1994).
Figure 1.10 Changes that take place in the composition of the partitioning phases between an agrochemical and an immiscible lipid in the presence of a surfactant (adapted from Stock and Holloway, 1993).

Parameters such as the composition and amount of surfactants will obviously influence the quantity and proportion of surfactants present in both phases. Additionally, the presence or /and passage of a surfactant through a cuticle may also decrease the resistance to diffusion of a herbicide molecule, therefore increasing its mobility (Stock and Holloway, 1993; Baur et al., 1999). The ability to penetrate the cuticle also implies that a surfactant can modify the diffusive route of a co-applied herbicide (Stock and Briggs, 2000). Agrochemicals may be able to penetrate the cuticle via various routes or pathways (polar/aqueous pathways and apolar/lipophilic pathways) and the passage taken will depend on their physicochemical properties and on the modifying properties of the surfactant. It has been speculated that polysaccharide fibrils in the underlying epidermal cell wall might most likely supply the transport link between the cuticle and the apoplast, therefore allowing solutes to pass into the leaf (Stock and Holloway, 1993).
The co-penetration theory suggests that surfactants act as a ‘co-solvent’ for particular agrochemicals. This hypothesis stems from the observation that in a formulation containing both the active ingredients and the surfactant, the enhanced rate of uptake of the active ingredients will be similar to the rate of entry of the surfactant. Studies with both surfactant and agrochemicals have shown that there might be pre-penetration and co-penetration by the surfactant, in relation to the active ingredients (Holloway and Stock, 1989). Radiotracer work with difenzoquat and octaethylene glycol monododecyl ether C$_{12}$E$_{8}$ as the surfactant, however, has revealed that uptake of the surfactant occurred at a slower rate than that in the absence of the difenzoquat (Silcox and Holloway, 1989).

The mechanisms of herbicide uptake are still not fully understood and studies on surfactant uptake into leaves are limited. Nevertheless, these studies have highlighted the very large variations in behaviour between plant species and surfactant compositions (Holloway and Stock, 1989; Roggenbuck et al., 1993; Sharma and Singh, 2000; Singh et al., 2002).

### 1.3.2.3 Surfactant phytotoxic and stimulatory effects

Phytotoxicity or contact phytotoxicity is the term used to describe the damage caused by a formulation to plant tissues thereby resulting in general or localised cell death respectively (Zabkiewicz, 2000). This term is more commonly associated to the herbicidal activity of a chemical to the target plant or to a non-target plant after herbicide application and less commonly to surfactant formulations. Over the years, research studies have shown that surfactants themselves are potentially phytotoxic and these characteristics may influence herbicide performance.
The inhibitory and phytotoxic effects of different chemical classes of surfactant have been well-documented by Parr (1982) and the toxicity is generally in the order cationic surfactant > anionic surfactant > non-ionic surfactant (Parr, 1982). Besides the nature of the ionic charge, phytotoxicity of surfactants depends on the physical size of the surfactant molecule (Fumidge, 1959), the differences in wax composition of the plant species (Falk et al., 1994) and surfactant concentrations (Jansen et al., 1961; Jansen, 1965; Smith et al., 1966). Studies have shown that most surfactants reduce surface tension at concentrations ranging from 0.01 to 0.1% (w/v) while phytotoxic effects are induced at concentration greater than 0.1% (v/v) (Temple and Hilton, 1963; Parr, 1982). Surfactant phytotoxicity is an indirect indication of penetrations into the plants. Stevens and Bukovac (1987) have shown that the phytotoxic response to certain surfactants is mainly due to their differential penetration rather than different innate surfactant toxicity at the cellular level.

Initially, phytotoxicity occurs after cuticular penetration and movement to the plasmalemma (Haapala, 1970). Thereafter, depending on the concentration and chemical structure of the surfactant, the plasmalemma may become disrupted allowing the surfactant molecules into the cytosol (Parr, 1982). Based on the extent of the surfactant penetration and concentration, a number of stimulatory or inhibitory effects are induced by the surfactants. At low surfactant concentrations (< 0.1% v/v), enhanced growth, enzyme activation, auxin synergism, increased respiration and electron transport have been recorded but plants become “normal” as the surfactant molecules are dissipated (Czeczuga et al., 1960; Stowe, 1960; Westwood and Batjer, 1960; Jansen et al., 1961; Stowe and Obreiter, 1962; MacDowall, 1963). In contrast, at high surfactant concentrations (> 0.1% v/v), a number of inhibitory activities have been observed (Table 1.2) (Parr, 1982).
Tween 20 at 0.01% (v/v) was found to cause leakage in enzymatically isolated and purified oat mesophyll protoplasts (Watson et al., 1980). Several studies have also revealed that certain surfactants can cause a significant decrease in growth, germination and organ development of several plant species (Spurrier and Jackobs, 1955; Stowe, 1960; Jansen et al., 1961; Schopmeyer, 1961; Temple and Hilton, 1963; Parr and Norman, 1964; Buchanan, 1965; Vieitez et al., 1965). Surfactants were also found to inhibit mitosis in pea (*Pisum Sativum* L.) and disruption was found at several stages in the mitotic cycle (Nethery, 1967). There are also reports whereby (Buchanan, 1965) surfactants have been found to affect ion absorption and translocation of nutrients by plants (Koontz and Biddulph, 1957; Parr and Norman, 1964). For instance, Parr and Norman (1964) found that Tween 20 and Tween 80 repressed potassium uptake by excised barley roots at concentration of 0.01% (v/v).

Moreover, St. John et al. (1974) used isolated plant cells from soybean and wild onion (*Allium canadense* L.) to study the effects of four surfactants on cell permeability as measured by loss of intracellular material and on photosynthesis $^{14}$CO$_2$ fixation. These scientists (St. John et al., 1974) found that while the surfactants did induce cell permeability, they inhibited photosynthesis fixation of $^{14}$CO$_2$ at a concentration of 0.01% (w/v). Rate of photosynthesis was found to decrease drastically and $^{14}$C-leakage from cells increased when surfactant concentrations were increased. These results clearly indicate that surfactants have biological activity and their effects are not restricted to the plasmalemma but also influence other plant processes (Table 1.2).
Table 1.2 Phytotoxic and inhibitory effects of 4 different surfactants on corn, oat and soybean test systems (Buchanan, 1965).

<table>
<thead>
<tr>
<th>Systems</th>
<th>Response</th>
<th>Surfactants trade name</th>
<th>Concentration (w/v%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>Reduced germination</td>
<td>Triton X-100</td>
<td>0.05</td>
</tr>
<tr>
<td>Oat</td>
<td>Reduced germination</td>
<td>Triton X-100</td>
<td>0.01</td>
</tr>
<tr>
<td>Soybean</td>
<td>Leaf injury</td>
<td>Triton X-100</td>
<td>0.10</td>
</tr>
<tr>
<td>Corn</td>
<td>Decreased root growth</td>
<td>Triton X-100</td>
<td>0.05</td>
</tr>
<tr>
<td>Corn</td>
<td>Reduced germination</td>
<td>Tetronic 707</td>
<td>more than 5</td>
</tr>
<tr>
<td>Oat</td>
<td>Reduced germination</td>
<td>Tetronic 707</td>
<td>0.10</td>
</tr>
<tr>
<td>Soybean</td>
<td>No injury observed</td>
<td>Tetronic 707</td>
<td>1.00</td>
</tr>
<tr>
<td>Corn</td>
<td>Decreased root growth</td>
<td>Tetronic 707</td>
<td>0.10</td>
</tr>
<tr>
<td>Corn</td>
<td>Reduced germination</td>
<td>Ultrawet DS</td>
<td>0.10</td>
</tr>
<tr>
<td>Oat</td>
<td>Reduced germination</td>
<td>Ultrawet DS</td>
<td>0.01</td>
</tr>
<tr>
<td>Soybean</td>
<td>Leaf injury</td>
<td>Ultrawet DS</td>
<td>0.10</td>
</tr>
<tr>
<td>Corn</td>
<td>Decreased root growth</td>
<td>Ultrawet DS</td>
<td>0.01</td>
</tr>
<tr>
<td>Corn</td>
<td>Decreased root growth</td>
<td>Hyamine 2389</td>
<td>more than 0.01</td>
</tr>
<tr>
<td>Soybean</td>
<td>Leaf injury</td>
<td>Hyamine 2389</td>
<td>0.10</td>
</tr>
</tbody>
</table>

1.3.2.4 Approaches to study the mode of action of surfactants

Various approaches have been used to study the mechanisms of foliar uptake of foreign compounds. These methods include basic observation of biological responses such as stem curvature, leaf necrosis, growth inhibition following droplet application of the active ingredients and the surfactant to directly following the movement of radiolabelled molecules across intact plants (Sharma and Singh, 2000) and isolated cuticles (Bukovac and Petracek, 1993; Schonherr and Baur, 1994). The effects of surfactants on foliar uptake are in most cases examined using radiolabelled chemicals (Stock et al., 1992; Stock and Holloway, 1993) and intact or detached
leaves (Stock et al., 1992; Foy, 1993; Feng et al., 1999) and isolated cuticles. Studies using intact plants or excised leaves and leaf discs closely mimic surfactant applications under field conditions.

However, these intact plants or tissues are complex and are exposed to the physical and physiological influences of the living tissue (Schreiber and Schonherr, 1993). Thus, data from these systems are not easily interpretable and in most cases cannot be related to other studies. To overcome such limitations, cuticles isolated from leaves and fruits have been used as models. Isolated cuticles enable the close and detailed monitoring of the kinetics of the effects of radiolabelled surfactants. Hence, interactions between surfactants and leaf surface can be identified and quantified under highly controlled and well-defined conditions (Bukovac and Petracek, 1993).

Nevertheless, there are a number of drawbacks to the use of isolated cuticles: (i) only thick, astomatous cuticles can bear the mechanical strain of isolation, thereby excluding important crops and weeds from being studied; (ii), while being prepared, isolated cuticles are shaken for a long time in liquid solutions and thus damage to the epicuticular wax may falsely influence the behaviour and effect of surfactants (Kirkwood, 1999). Besides, after radioactive compounds have been applied to the leaf surface, uptake is usually calculated on the basis of the amount of radioactivity present in the wash and in the leaf (Gauvrit, 1994). Radioactive surfactants are not always available and not convenient to handle.

Recently, confocal laser scanning microscopy (CLSM) has been used to visualize the effect of a surfactant on the uptake of xenobiotics (Liu, 2004a; Liu et al., 2004). This non-destructive technique enables the in vivo study of uptake and diffusion pathways of fluorescent chemicals
from the leaf surface layer to the inner plant tissues, thereby providing an estimate of the relative proportion of absorbed molecules that can move from the leaf surface to the internal tissues (Liu, 2004a; Liu et al., 2004). CLSM has also been used to visualize stomatal infiltration of surfactants and other compounds into various plant systems (Gaskin et al., 1998; Hutzler et al., 1998; Veraverbeke et al., 2001). However, the major limiting factor of CLSM is the unavailability of fluorescent agrochemicals. Instead, researchers have been using fluorescent dyes to simulate the diffusion features of the agrochemicals which clearly have limitations with regard to interpretation of the results.

1.3.2.5 Approaches to study the “toxic” mode of action of surfactants

Membrane solubilisation and interactions with proteins are considered as primary factors causing cell necrosis and tissue damage following surfactant application (Helenius and Simons, 1975). Consequently, several approaches have been developed using both in vivo and in vitro techniques to study surfactant toxicity in order to gain a better understanding of the “toxic” mode of action of the surfactants. One of the common procedures used is the visual ratings of lesions following spray applications (Lownds and Bukovac, 1988; Falk et al., 1994) whereby any discoloration or damage to the entire droplet area is assessed and given a rating.

Scanning electron microscopy has also been used to visualise the damage caused by treatments with phytotoxic levels of surfactants (Falk et al., 1994). Moreover, the ion efflux method has been used to verify surfactant toxicity based on the measurement of membrane integrity in plants (Silcox and Holloway, 1986). In vitro tests using this assay include incubating detached leaves in surfactant solution and measuring the conductivity of these solutions at several time points. In vivo tests involve the application of spray droplets to the leaf surface and then
detaching the treated leaves and measuring conductivity following their immersion in distilled water. This ion leakage study provides an estimation of the damage caused to the cell membrane based on ions released by the plant cells after surfactant exposure (Coupland et al., 1989).

Toxic effects on plants have also been measured by quantifying the amount of ethylene released after surfactant treatments using gas chromatography (Yang and Hoffman, 1984; Knoche et al., 1992). The amount of ethylene released at a specific surfactant concentration is assumed to be proportional to the amount of tissue damage, provided the tissue damage is not reducing ethylene production (Lownds and Bukovac, 1989). Knoche et al. (1992) found that ethylene production increased linearly as surfactant concentrations were increased, though in some other studies scientists have reported high phytotoxic ratings but low ethylene production due to complete tissue damage and no further ethylene synthesis (Shafer et al., 1989).

**1.3.2.6 Molecular mode of action of surfactants?**

No research has yet been conducted to understand the mechanisms of action of surfactants following foliar application. Current evidence suggests that surfactants are able to penetrate leaf tissues and based on results obtained on the phytotoxicity and stimulatory effects of surfactants, it is clear that surfactants have their own intrinsic biological functions. Little information is available on the molecular effects of a particular surfactant on a plant species and as such, data are needed to more fully understand the biological effects on plants induced by spray components other than the active ingredients.

Most adjuvant studies have dealt with identifying and correlating physical properties of agrochemicals and sprays. However, a new trend is emerging that takes into account more
science and technology for the development and better understanding of adjuvants. Improved formulations of adjuvants are primarily required to reduce off-target deposition, improve spray retention on target, enhance uptake and translocation of systemic herbicides and work in synergism with the active ingredients so as to ultimately allow for lower usage rates of herbicides (Underwood, 2000; Zabkiewicz, 2000). Such approach will therefore require, along with the current empirical spray and rank methodology, a better understanding of the mode(s) of action of adjuvants in agrochemical formulations.

Studies directed at identifying the molecular mode of action safeners (which reduce the phytotoxicity of herbicides to crop plants) using microarrays are already emerging (Rishi et al., 2004). It is anticipated that future formulations will be conceived in the laboratory, evaluated under controlled conditions and analysed for efficacy using computer-based models (Zabkiewicz, 2000). Hence, strategies for the development of improved agrochemical formulations are shifting from the testing of chemicals for efficacy on whole plants towards the analysis of the whole genome response using expression arrays.

1.4 Microarrays

During the last half of the 20th century, the analysis of the regulation and function of genes and gene products have been mainly carried out by step-by-step studies of individual gene and proteins (Harrington et al., 2000). However, over the past decade, there have been major improvements in the field of functional genomics and a large number of genes can now be analysed in a simultaneous and serialised manner (Harrington et al., 2000; Aharoni et al., 2002; Aharoni and Vorst, 2002). An important tool in this process has been the development of DNA microarrays. Microarray technologies can be considered as hybridisation techniques originating
from the principles of northern and Southern hybridisation techniques (Lemieux, 1998; Schena, 1998; Aharoni et al., 2002).

DNA microarray technology consists of an ordered matrix of different DNA sequences, which enable the monitoring of gene expression over a large number of genes simultaneously. The number of published studies using microarray technology to study plant response to abiotic and biotic stresses is increasing substantially (Kothapalli et al., 2002; Goda et al., 2004; Leonhardt et al., 2004; Marshall, 2004; Raghavan et al., 2005, 2006). Of significance to this review is the use of microarray technology to identify the molecular mode of action, mechanisms of resistance and identification of potential target sites of action for agrochemical compounds and their additives (Cole et al., 2000; Weller, 2001; Gutterson and Zhang, 2004; Lein et al., 2004; Rishi et al., 2004; Raghavan et al., 2005, 2006). Gene expression studies have identified plant genes that are responsive to agrochemicals and adjuvants and further functional analysis of those regulated genes may help to elucidate the molecular mechanism of action of particular active ingredients and adjuvants. Further genetic and biochemical studies of those related genes and proteins may also be used to identify potential target proteins for the development of new herbicides (Cole et al., 2000; Lein et al., 2004).

1.4.1 Brief overview of DNA microarray

DNA microarrays provide a platform for the measurements of the expression levels of thousands of genes in parallel in a single hybridisation assay (Clarke and Zhu, 2006). Each array (often also described as chip or GeneChip) contain reproducible patterns of thousands of different DNA sequences (either PCR products or oligonucleotides) attached to a solid glass support or matrix (Aharoni and Vorst, 2002; Nguyen et al., 2002). RNA or DNA prepared and
fluorescently labelled from messenger RNA (mRNA) is hybridised to the complementary DNA segment on the array and fluorescence is detected by laser scanning (Harrington et al., 2000; Aharoni and Vorst, 2002; Nguyen et al., 2002). The hybridisation intensities for each DNA sequence present on the array are calculated using an automated process and provide quantitative data of the individual gene expression level (Aharoni et al., 2002; Nguyen et al., 2002; Hardiman, 2004; Clarke and Zhu, 2006). Such data may then be further processed to identify expression patterns and variation in response to a specific treatment.

1.4.2 Microarray platforms

The two most common types of array technologies will be described in this review: spotted microarrays and oligonucleotide arrays (Figure 1.11), though more emphasis will be placed on oligonucleotide chips since they were used in this project.

1.4.2.1 cDNA microarrays

Gene expression studies using complementary DNA (cDNA) microarray is useful when there is little information on gene sequences available for the organism to be studied. Such arrays, referred to as the “target” in this assay, consist of single or double stranded DNAs generated from either direct amplification of genomic DNA or by amplification of inserts from cDNA libraries (Harrington et al., 2000; Aharoni and Vorst, 2002; Nguyen et al., 2002). Using highly mechanised robots, the PCR products are bound or “printed” onto the array, which is usually made up of glass (Figure 1.11). The nucleic acid samples, the “probe” hybridised to the arrays are most commonly, labelled by incorporating fluorescently labelled (tagged) nucleotides during the reverse transcription of total RNA to cDNA by oligo-dT or random primers. Fluorophores, generally Cyanine 3- (Cy 3) and Cyanine 5- (Cy 5) uridine triphosphates (UTP) are used to label
the cDNA from control (reference) and experimental (test) RNAs respectively (Harrington et al., 2000; Aharoni and Vorst, 2002; Nguyen et al., 2002).

Labelled cDNAs are then mixed together prior to hybridisation onto the array. The amount of a particular gene transcript in each of the two samples is quantified by measuring the signal intensities detected for each of the fluorophores and then calculating the signal ratios. cDNA microarrays have been applied to a large number of studies ranging from drug discovery, cancer research to the analysis of gene expression profiles in plants in response to stress, hormones and cell death (Debouck and Goodfellow, 1999; Schenk et al., 2000; Desikan et al., 2001; Seki et al., 2001; Seki et al., 2002; Swidzinski et al., 2002; Kimura et al., 2003).
Figure 1.11 Diagrams showing array preparation and expression assay for cDNA microarray and oligonucleotide arrays.

(a) Spotted arrays are made by printing amplified DNA prepared from genomic or cDNA onto the glass slides. Each spot on the slide represents a gene of few hundred base pairs. Oligonucleotide arrays are mainly manufactured *in situ* on glass wafers using photolithographic manufacturing procedures. Genes are presented by 15-20 oligomer pairs (PM, perfectly matched and MM, mismatched) on the array. (b) In the expression assay for the cDNA microarray, mRNAs from the test (sample 1) and treatment (sample 2) are labelled with fluorescence dyes, hybridised to DNA and scanned. Dots at the bottom, labelled X, Y and Z represent hypothetical genes with increased expression in X, sample 1, in Y (sample 2) and no change or similar expression in Z. In the GeneChip array, RNA is converted to biotinylated cRNA, stained and scanned by laser scanning. Sets of paired oligonucleotides for hypothetical genes present at high levels in X (sample 1), increased expression in Y (sample 2) and similar levels in X and Y. Figure 1.11 is taken from Harrington et al. (2000).

### 1.4.2.2 Oligonucleotide microarrays

Instead of PCR products, oligonucleotide arrays consist of large synthetic single stranded base sequences, which are either printed onto glass surface or synthesised *in situ* on the slide (Figure 1.11). In this assay, the array is called the “probe” and these probes are designed based on sequences publicly available on databases such as the one maintained by the National Centre of Biotechnology Information (NCBI) (Lipshutz et al., 1999). Hence, production of oligonucleotide arrays is restricted to organisms for which gene sequences are available.

Oligonucleotide arrays are of two types based on the length of the sequence used as a probe. Companies such as Agilent and NimbleGen supply long oligonucleotide arrays in which oligonucleotides are 40-80 bases in length (Barrett and Kawasaki, 2003). The advantages of such long arrays include ease of production and design compared with cDNA arrays and arrays
can be readily modified as more genomic information become available about organisms (Barrett and Kawasaki, 2003). Affymetrix® is the first company to produce short oligonucleotide arrays, also commonly called GeneChip array, in which oligonucleotides are 25 bases in length. This company has also introduced a now vastly popular technique of synthesizing oligonucleotides *in situ* on glass wafers using photolithographic manufacturing procedures.

Typically on a GeneChip array, a given gene is represented by 11-25 different 25-mer oligonucleotides designed from a non-conserved region of the gene (Figure 1.12) (Harrington et al., 2000; Aharoni and Vorst, 2002). This probe serves as a unique sequence-specific interrogator of the cRNA to be hybridised. On the Affymetrix® *Arabidopsis* whole genome Array (ATH1 – 121501), a given gene is represented by 11 oligonucleotide probe pairs, that is 11 perfect match (PM) and 11 additional mismatch oligonucleotides (MM). Those 11 probe pairs make up a probe set (Figure 1.12). The PM oligonucleotide represents a perfectly complementary match to a specific gene whereas the MM includes a single base difference to the perfect match in the middle of the oligonucleotide sequence (Mei et al., 2003; Hardiman, 2004). In the presence of a transcript, the assumption is that the PM probe will hybridise more strongly than the MM partners (Aharoni and Vorst, 2002).
The probe set is made up 11 probe pairs. Each probe pair consists of a perfect match (PM) and a mismatch (MM). The signal intensity of perfect match and mismatch is used to calculate the signal value for the probe set, indicating the mRNA transcript level for this specific gene.

Thus, the presence of the mismatched oligonucleotides enables the estimation of cross-hybridisation and background (Hardiman, 2004). Such data are then subtracted from the PM signal to give specific target abundance. Signals generated from PM and MM indicate the presence or absence of a transcript. The ATH1 – 121501 array consists of 22,810 probe sets representing 24,000 genes. These probe sets are designed in such a way that they span and hybridise to different regions of the same mRNA and are spatially separated on the GeneChip in order to overcome any variation due to chip surface (Aharoni and Vorst, 2002).

In a GeneChip assay, target is generated from the RNA extracted from a biological sample. Total RNA is reverse-transcribed to first generate a single stranded cDNA and then a double stranded cDNA. This double stranded cDNA is then in vitro transcribed in the presence of biotin labelled nucleotide to generate biotin labelled cRNA. This target is subsequently hybridised to the probe (Figure 1.12). After hybridisation and washing, the biotin labelled target...
on the array is stained using fluorescent stain, streptavidin-linked phycoerytherin (SAPE) and the fluorescence is then read by a scanner, which generates an image. This image is then used for further analysis (Aharoni and Vorst, 2002). More details about the probe, target, procedures, softwares and databases used for image analysis will be provided in the next Chapters of this thesis.

1.5 *Arabidopsis thaliana* - the model weed

*Arabidopsis thaliana*, hereafter refered to as *Arabidopsis*, is a model plant system and for many years now has been used for studying all aspects of plant structure and function (Meyerowitz et al., 1991). This plant is an angiosperm that belongs to the *Cruciferae* (family Brassicaceae) or Mustard family. It is considered as a “model” eukaryote for genetic plant research because it is easy to grow in the laboratory, it has a short life cycle (6 weeks), produces a lot of seeds and foremost has a small genome compared to other higher plants. The entire genome of *Arabidopsis* was sequenced in 2000 (The *Arabidopsis* Genome Initiative, 2000) and about 26,000 nuclear, 80 chloroplast and 60 mitochondrial genes encoding proteins are present in this plant. The nuclear genome contains 125 Mb of DNA.

Over the years, considerable effort has been directed towards developing genetic and molecular tools for this species and as such, today, tagged mutagenised seed stocks, extensive expressed sequence tags (EST) and gene expression databases and commercially produced cDNA and oligonucleotide arrays are available (Horvath et al., 2003b). The Affymetrix® *Arabidopsis* array (ATH1 – 121501) has been extensively used to study the genome-wide response of the *Arabidopsis* plants to various abiotic and biotic stresses (Wang et al., 2003). These resources have provided excellent tools for the study of plant physiology and development.
1.6 Comparative genomics

Given the economic impacts of weeds, it is interesting to note that little is known about the genomics of weeds (Horvath et al., 2003a; Basu et al., 2004) and an increased understanding of the genomics of weed species is required to devise better weed control strategies which may subsequently prevent the emergence of herbicide-resistant biotypes (Basu et al., 2004). To date, in the plant community, extensive sequence information is available for *Arabidopsis* and rice (*Oryza sativa*) (Ausubel, 2000; Yu et al., 2002) and cross-species comparison using the available resources will enable gene discovery in plant species with limited known genomic sequence information and resources.

Numerous studies have shown relationships at the genome sequence level within the Poaceae, Solanaceae, Brassicaceae, Fabaceae as well as between families (e.g. *Arabidopsis*/rice, *Arabidopsis*/moss, *Arabidopsis*/tomato) (Livingstone et al., 1999; Ku et al., 2000; Fulton et al., 2002; Babula et al., 2003; Nishiyama et al., 2003; Rensink and Buell, 2004). These studies have all revealed that although different levels of gene restructuring are observed (e.g. inversion, rearrangements), some degree of gene presence and order can be identified. Therefore, knowledge gained from one species can be productively applied to the other. As a Brassicaceae, *Arabidopsis* is an excellent model for major weed species such as wild radish (*Raphanus raphanistrum* L.) which is a member of the same family. Previous comparative mapping of *Brassica* and *Arabidopsis* using molecular markers have revealed extensive synteny between the two species (Babula et al., 2003).

Although Gressel (2000) has critisised *Arabidopsis* as a model weed since it is not a competitive plant and does not affect agriculture, other scientists have, however, shown that though this plant...
lacks some of the complex traits of weeds, it can be exploited to find genes of interest and transcriptional regulation in weedy species (Yamaguchi-Shinozaki and Shinozaki, 1993; Jones et al., 1997; Horvath and Olson, 1998; Horvath et al., 2003b; Horvath et al., 2003a). Recently, an Arabidopsis microarrays was successfully used in weed science to study the differential gene expression involved in dormancy in underground buds of leafy spurge (Euphorbia escula) (Horvath and Anderson, 2002). The tillering of wild oat (Avena fatua) has also been investigated using Arabidopsis microarrays (Horvath et al., 2003a) and Horvath et al. (2003b) have analysed the transcriptional regulation in both wild oat and leafy spurge using the same approach.

1.7 Aims of present study

Limited information is available on the mode(s) of action of surfactants and therefore, the main objective of this study was to elucidate the mode(s) of action of an etheramine surfactant at the molecular level. This study used microarray technology to measure the gene expression levels in response to 0.2% (v/v) foliar-application of an etheramine surfactant termed NUL1026, 1 h and 24 h post-treatments in Arabidopsis plants. It is hoped that this study will aid in the identification of pathways and processes that are regulated by surfactant NUL1026 with the ultimate aim of developing more effective herbicides formulations. This is the first kind of study undertaken to examine the effect of a surfactant at the molecular levels over the entire genome of Arabidopsis.

The present investigation also examined the synergistic molecular effect of combining surfactant NUL1026 to auxinic herbicide 2,4-D (1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026) using the same microarray approach. Studies have shown that not all surfactants are able to promote
the action of herbicides (Gaskin and Holloway, 1992; Liu, 2004b) and the use of these genomic tools may prove to be a novel in vitro way of assessing whether surfactant chemistries complement those of herbicides.

Microarray results obtained in this study identified a list of genes, which were specifically regulated by foliar-application of 2,4-D + surfactant NUL1026 formulation in Arabidopsis. These genes provide insights into the mode of action of herbicide 2,4-D and offer a starting point in understanding and targeting genes involved in the mechanisms of herbicide resistance in weeds. As mentioned in this review, Arabidopsis microarray may be applicable to species with uncharacterised genome sequence. As such, it would be interesting to investigate the differential gene expression in herbicide resistant and susceptible populations of the troublesome weed, wild radish. However, wild radish is an outbreeder and therefore it is important to verify that the resistant and susceptible biotypes have similar genetic frameworks. This will ensure that any possible difference in gene expression recorded between the two biotypes is likely to be linked to the resistance mechanisms.

1.8 Thesis structure

The following five chapters have been structured based on a sequence of the various aspects of the study. Chapter 2 introduces the stepwise microarray procedures and the absolute analyses, required for the development of the initial microarray datasets for each of the various treatments (0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026), at different time points under investigation. This chapter also draws attention to the quality of array results and to the use of alternate techniques to validate microarray results.
Chapter 3 develops on the initial data obtained from absolute analysis of the microarray experiments in response to 0.2% (v/v) surfactant NUL1026 by introducing comparison analysis and data mining. This chapter focuses on extraction of surfactant-responsive genes that were significantly regulated. Comparison analysis and data mining using the MAS 5.0 software is also discussed in detail in this chapter. Moreover, this chapter considers functional genomics and discusses the functional classification of significantly surfactant-regulated genes at molecular and biological levels.

Chapter 4 also includes comparison analysis and data mining of the herbicide and the herbicide + surfactant formulations. Genes that are significantly up- and down-regulated in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026) are identified. This chapter further focuses on the genes that were specifically regulated in response to the surfactant, the herbicide and the herbicide + surfactant formulations. The functional classifications of genes exclusively regulated in response to the three different treatments are also discussed.

Chapter 5 is a preliminary study carried out to examine the underlying genetic variability between two herbicide resistant and one susceptible populations of wild radish using RAPD - PCR. Results from this study showed that the three populations were overall genetically similar. This has now paved the way for the use of genomic tools like microarray technology to target the specific genes that may potentially be responsible for conferring herbicide resistance in the less characterised wild radish species.

Chapter 6 of the thesis is based on the results derived from the previous chapters. This study has highlighted that the jasmonic acid and ethylene pathways may be involved in the mode of action
of surfactant NUL1026 in *Arabidopsis*. Furthermore, this is the first study to examine the genome-wide changes in the transcriptome of *Arabidopsis* in response to a surfactant and an herbicide + surfactant formulation. The thesis concludes by suggesting possible areas of future research and development.
Chapter 2: Analysis of initial microarray data in response to herbicide 2,4-D and surfactant NUL1026

2.1 Introduction

Affymetrix® oligonucleotide arrays are now a widely used technique that enable the simultaneous examination of the abundance of thousands of individual mRNA molecules within a biological sample (Lockhart et al., 1996) and as an extension, different samples can then be quantitatively compared. Microarray experiments include a number of multi-step procedures (Chen et al., 2004). Firstly the scientist extracts total and/or polyadenylated RNA from cells and tissues, generates corresponding complimentary DNA (cDNA), transcribes complementary RNA (cRNA) from the cDNA template and hybridises the cRNA (the target) onto the probes (oligonucleotide sequences) on the array. Hybridisation is then followed by fluorescent staining and scanning of the array (Finkelstein et al., 2002). The scanner measures the fluorescence emitted as a result of the hybridisation of a target molecule to a specific probe on the array and specifically designed analysis softwares such as Microarray Suite version 5.0 (MAS 5.0) is then used to capture the scanned image for subsequent analysis.

2.1.1 Absolute analysis

Though each microarray experiment measures the expression of thousands of genes simultaneously and produces a huge amount of biological data, only a small percentage of genes present on any given array is identified as being differentially regulated. Hence the challenge is to then process this vast amount of information objectively and efficiently so as to obtain biologically relevant, meaningful and reliable information and thereafter to compare information gained across multiple experiments.
The first analysis performed using MAS 5.0 prior to any other more mature analysis of microarray data is called absolute analysis. Absolute analysis is the analysis of hybridisation between the targets (cRNA derived from mRNA) and the probes on a chip. Hence absolute analysis is also used to indicate the array quality and to verify the overall hybridisation performance on a chip. Absolute analysis converts the measured fluorescent intensity of every probe cell into a signal value and then allocates a detection call (present or absent) for every probe set on the entire array.

Signal values assign a relative measure of abundance to transcripts and is calculated for each probe set (Rajagopalan, 2003). Calculation of overall signal intensity for each probe set/gene (11 probe pairs per probe set in case of Affymetrix® chip ATH1-121501) is based on the signal intensity from each probe pair (match and mismatch). Signal intensities generated by mismatches due to cross hybridisation or non-specific binding are used to calculate the actual signal intensity of a probe set that is due to specific binding (Liu et al., 2002).

Absolute analysis also generates a Detection $p$-value by using probe pair intensities. This $p$-value is assessed against user-definable cut-offs and based on the detection $p$-values; a transcript is reliably assigned as being “present”, “absent” or “marginally present”. The lower the $p$-value, the stronger is the certainty of the call. MAS 5.0 software categorises genes as present up to a transcript concentration of 2 pM (Liu et al., 2002).

### 2.1.2 Pre-analysis

However, before running any kind of analysis using the MAS 5.0 software, array data generated needs to be monitored for quality control. As in any kind of experimental procedures, technical and biological variations (McClintick et al., 2003) are invariably introduced and prior to
drawing meaningful and unbiased conclusions about data derived from the array experiments, these variations need to be identified and removed (Coombes et al., 2002).

2.1.2.1 Probe array inspection

After scanning the probe array, the resulting image data is stored as a .dat file with the name of the scanned experiment and this .dat file is then inspected for the presence of image artefacts. Artefacts may arise due to problems on the array itself (i.e. scratches on the array) or due to non-uniform spread of the hybridization cocktails, for instance due to presence of air-bubbles while the target is being loaded onto the array. Drying of the array between washes or improper washing and staining may also be responsible for such artefacts. Probe cells exhibiting an artefact are not included and are masked prior to further analysis.

2.1.2.2 B2 Oligo control

MAS 5.0 places a grid over the image data or the .dat file and this grid is used to determine the size of each probe cell and to align the grid such that each probe cell is centred. This ensures that the probes on the array and the measured signals are within limits of the grid. B2 Oligo probes border the GeneChip array and serve as a positive hybridisation control. Oligo B2 is included in the target hybridisation cocktail and positive hybridisation of B2 is shown on the image by the presence of alternating pattern of B2 hybridisation intensities on the border and the checkerboard pattern at each corner of the array.

2.1.2.3 Normalisation

Initial readouts from oligonucleotide arrays after hybridisation of cRNA to the array generate a large amount of assay noise (Affymetrix Technical Note, 2002a). Noise is defined as the
variation (pixel to pixel) in signals from a probe cell on an array (Affymetrix® Technical Note, 2002a). The two main sources of this array noise are: between cRNA variation (independently prepared cRNAs have variable purity) and intrinsic noise level associated with technology (inconsistencies in array fabrication, staining and scanning). Because these factors significantly affect array readouts, array data needs to be re-scaled to enable unbiased comparison. This step is crucial in any analysis of gene expression data. This correction method, called normalisation, does not necessarily mean transforming data to the normal distribution but rather normalising array intensity to a common standard (Hill et al., 2001). The MAS 5.0 software includes a normalisation method for oligonucleotide arrays. This method, also called global scaling involves scaling probe sets on each array to an arbitrary fixed level (which is user defined) such that the signal intensities of all probes on the array are scaled to this specified value. MAS 5.0 software also gives the user the option of scaling two sets of arrays (the experimental and the baseline or control array) before conducting a comparison analysis.

The multiplication factor by which the average signal is adjusted in an array is called the scaling factor and a scaling factor of 1.0 means that the average array intensity is equivalent to the target intensity. Scaling factors are also often used to check comparability between replicates (Affymetrix® Technical Note, 2002a). Scaling factors tend to vary across different samples and though there are no particular guidelines for any specific sample type, a scaling factor of more than 3-fold within a set of experiments suggests that data should be treated with caution (Affymetrix® Technical Note, 2002a). Other ways of normalisation include spiking with a foreign known concentration of mRNA species that has been deliberately included on the array and the use of housekeeping genes.
2.1.2.4 Spike controls

Another approach that complements normalisation involves the use of spike controls. Spike controls (transcripts of genes such as bioB, bioC and bioD involved in the biotin synthesis pathway of *Escherichia coli* and *cre*, a recombinase gene of P1 bacteriophage) are spiked at a concentration of 1.5 pM to 100 pM) into the hybridisation cocktail, independent of the RNA sample preparation. Probes of genetically distant test organisms are also represented on the *Arabidopsis* chip (Finkelstein et al., 2002). Data from these controls are then used to monitor sample hybridisation efficiency on the arrays. Signal values of the spike control should be relative to the concentration of the transcripts that were spiked with the target. This array sensitivity is important since it adjusts signals from low-level transcripts. However, although this adjustment introduces a small systematic error in data, it retains low level transcript readings, which would otherwise not be taken into consideration even though they might be biologically significant. Moreover, hybridisation of the spike controls to the respective probes on the array shows successful hybridisation of target to probe.

2.1.2.5 Poly-A controls

The *Arabidopsis* GeneChip array used in this study contains probe sets for several *Bacillus subtilis* genes that are absent in eukaryotic samples. These Poly-A controls are included to monitor the entire target labelling procedure. *B. subtilis* genes such as lys, phe thr and dap that have been modified by the addition of poly-A tails are spiked into the RNA sample. Microarray array procedures are carried out with spiked RNA sample and these spiked genes are assessed as internal control genes. The final concentrations of the controls relative to the RNA sample are: 1: 100,000; 1:50,000; 1: 25,000; 1:7,500 respectively. Hence these controls should be called...
“Present” and their signal values should reflect their respective concentrations (Chudin et al., 2002).

### 2.1.2.6 Housekeeping genes

Housekeeping genes such as actin, glyceraldehyde 3-phosphate dehydrase (*GAPDH*) and ubiquitin are also included in the microarray process as they monitor the quality of target RNA. Housekeeping genes are defined as genes that are expressed in every plant and animal tissue to maintain fundamental cellular function (Watson et al., 1965; Zhang and Li, 2004). These genes are believed to be constitutively expressed under any condition and in each cell, however, there are recent reports indicating some regulation (Pfaffl et al., 2004). Uniform expression levels of these housekeeping genes are taken to indicate successful reverse transcription, *in vitro* transcription and biotin labelling. In addition, Affymetrix® arrays are designed to assess the hybridisation of the transcript to the probes at the 3’ and 5’ end.

### 2.1.3 Replication

Microarray analysis includes a number of procedures and each step has the potential to introduce a number of variations (Chen et al., 2004). One of the daunting difficulties that makes microarray analysis so challenging is the presence of a high variability between individual arrays (even for the same tissue sample) (Szabo et al., 2002). This variability makes it harder to monitor biologically significant changes in gene expression. Variability between replicates arises either from probe labelling procedures (technical variation) and the instability of experimental conditions such as humidity, hybridisation patterns and temperature (Chen et al., 2004) and/or due to biological variation (Yang and Speed, 2002). Two samples grown, maintained and prepared under identical conditions would still yield mRNA samples that would
not be truly identical. This inherent variability in gene expression reflects host characteristics, which arise due to genetic factors (Chen et al., 2004). According to Zhu and Wang (2000), it is more likely that variation arises as a result of biological rather than technical variation. Hence to discriminate between true differences in gene expression and random fluctuation, biological replication is advisable (Zhu and Wang, 2000; Chen et al., 2004). Usually, replicate probes showing signal intensities with minimum variations are considered reliable.

2.1.4 Verification

Despite the high variability of microarray data, most of the studies using microarray analysis have included limited or no repeats at all (Watson et al., 2000). The prohibitive cost of commercial oligonucleotide arrays and the further costs involved in the completion of the entire procedure make it difficult for many laboratories to afford replicate microarray experiments such that reliable conclusions can be drawn (Lee et al., 2000). Hence, results from microarray experiments are alternatively verified by more conventional validation methods. These mRNA quantification methods include Northern blotting, real-time PCR (RT-PCR) and other semi-quantitative PCR assays (Aharoni et al., 2002; Bovet et al., 2003; Wang et al., 2003) to enable a comparison of relative mRNA levels for a number of specific genes. These techniques have all been shown to be well correlated with data derived from microarray analysis (Wang et al., 2003; Zhong and Burns, 2003; Leonhardt et al., 2004) though some investigators (Donson et al., 2002) have questioned the reliability of these techniques.
2.1.5 Aims

In this study, preliminary analyses of the gene expressions in *Arabidopsis* in response to foliar application of (1) herbicide 1.0 mM 2,4-D; (2) 0.2% (v/v) of an etheramine surfactant, termed as NUL1026 and (3) 1.0 mM 2,4-D combined with 0.2% (v/v) surfactant NUL1026, one hour post-spraying were carried out.

Transcriptomic response of *Arabidopsis*, 24 h post-spraying, with (4) 0.2% (v/v) NUL1026 and (5) 1.0 mM 2,4-D coupled with 0.2% (v/v) NUL1026, were also studied. The aims of the experiments carried out in this chapter were to develop the initial datasets for each of the microarray experiments. These datasets will be further interrogated in the following chapters. The extent of experimental variations between targets (RNA samples) using absolute analysis was also assessed and microarray results were verified using the technique of semi-quantitative PCR.
2.2 Materials and methods

2.2.1 Seed germination

Seeds of *Arabidopsis*, ecotype Columbia were surface sterilised by soaking in 1 mL of 100% ethanol for 5 minutes in a microcentrifuge tube. Ethanol was decanted after centrifuging at minimum speed (1,000 rpm) for one minute and the seeds were then soaked in 1% sodium hypochlorite solution for 20 minutes. Sodium hypochlorite was decanted after centrifuging at 1000 rpm for one minute. The seeds were then washed twice by pipetting with sterile distilled water.

Surface sterilised seeds were sown on 30 mL half strength Murashige and Skoog medium (Appendix I). Murashige and Skoog basal salt mix (Sigma #M5524) was supplemented with 1.5% (w/v) sucrose and vitamins (pH 5.7) and solidified with 1% phytigel (w/v) in deep Petri dishes (35 mm x 10 mm). Fourteen seeds were sown per plate, kept in the dark at 4°C for 48 h and were then transferred to culture rooms at 22°C under a photoperiod of 16 h. The plants were grown for 14 days from date of sowing. At this stage the plants had developed four rosette leaves measuring > 1.0 mm, which represented the principal growth stage 1.04 (Boyes et al., 2001).

2.2.2 Experimental treatments and spraying

Gene expressions in response to spray applications of *Arabidopsis* seedlings with (1) 1.0 mM 2,4-D; (2) 0.2% (v/v) surfactant NUL1026 and (3) 1.0 mM 2,4-D combined with 0.2% (v/v) surfactant NUL1026 at two different time points, 1 hour and 24 hours were investigated. Gene expression after one hour of spraying was investigated for the three above-mentioned treatments.
while genomic response to treatments with 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 was also investigated after 24 h of spraying.

Previously published studies carried out in our laboratory (Raghavan et al., 2005, 2006) examined the gene expression and phenotypic changes in Arabidopsis in response to root-uptake of varying concentrations (0.001 mM, 0.01 mM, 0.1 mM and 1.0 mM) of 2,4-D for a period of one hour. Herbicide 2,4-D at 1.0 mM was shown to be herbicidal while lower concentrations acted in an auxinic manner. Hence a concentration of 1.0 mM 2,4-D was tested in this study. Most surfactants are used at concentrations ranging from 0.01 to 0.1% (w/v) though the efficacy of these surfactants also depends on their chemical structure and dosage used (Buchanan, 1965). A concentration of 0.2% (v/v) was used to treat plants as per recommendation from Nufarm Australia Limited. The acid formulation of 2,4-D and surfactant NUL1026 used in this project were supplied by Nufarm Australia Limited. Stock solutions of 1.0 mM 2,4-D were made by dissolving in water and the pH adjusted to 7.0. An aqueous solution of 0.2% (v/v) NUL1026 was added to form the treatment formulations consisting of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.

Petri dishes were covered with vermiculite to prevent root-uptake of spray and were then sprayed with either 1.0 mM 2,4-D; 0.2% (v/v) NUL1026 or 1.0 mM 2,4-D combined with 0.2% (v/v) NUL1026 at a rate of 96 mL across 12 plates using the Potter spray tower (Burkard Scientific, Uxbridge, UK) at 40 kPa (Herron et al., 1995) (Figure 2.1). Control plants were sprayed with water.
Figure 2.1 Potter spray tower used for spraying.

(a) The Potter spray tower was used to maximise uniformity of spraying of *Arabidopsis* plants; (b) Petri dish consisting of 14 days old seedlings was placed on the spray tower platform beneath the spray tube; (c) the spray tower was switched on, resulting in the platform moving upwards, “sandwiching” the Petri dish between the platform and the spray tube and the sprayed solution was applied, ensuring uniform spraying of the different treatments.
Foliage applied herbicides are usually delivered through a hydraulic spray nozzle with water as the carrier. This method of application is, however, inherently inefficient with losses occurring from droplet reflection, run-off, drift, and in-flight evaporation. The net result is that only a small proportion of the herbicide applied actually reaches the intended target. The Potter spray tower is internationally recognised as the standard of reference for chemical spraying techniques in the laboratory and has been used mainly for studying the biological effects of contact poisons on organisms (Herron et al., 1995; Bernard et al., 2004). The Potter tower was used to spray the *Arabidopsis* plants as this instrument has been designed to apply precise and uniform deposits of spray and therefore is able to maximise uniformity of spraying of these plants.

In order to prevent contamination between treatments, the Potter tower was firstly rinsed three times with a solution consisting of half-acetone and half water followed by three rinses with distilled water. Four sets of plant (consisting of 12 Petri dishes per set) for each treatment (1.0 mM 2,4-D, 0.2% (v/v) NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026) were separately treated in order to get biological replicates. Once sprayed, the Petri dishes were again maintained at 22°C in the culture rooms for a period of 1 h and 24 h. Plants were then harvested and immediately frozen in liquid nitrogen and tissue stored at -70°C.

The phenotypic changes in response to the different treatments were recorded. Plants were photographed 1 h, 24 h and 48 h after being sprayed with either water, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D or 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.
2.2.3 RNA extraction

Total RNA was extracted from 100 mg of plant material (from approximately 10 plates containing 14 plants each) using the RNeasy plant mini kit (Qiagen # 74904). The total RNA was further purified using the RNeasy mini-columns including the on-column DNase digestion as described in Appendix D of the Qiagen RNeasy plant Mini Handbook (3rd edition; Qiagen). Since both technical and biological variations play a big role in determining the meaningfulness of microarray expression data, large pools of plants grown independently were used for RNA isolation and target synthesis. In order to obtain sufficient RNA yield that would also diminish biological variability, samples from individual plants are usually pooled (Zhu and Wang, 2000). Peng et al. (2003) have suggested two methods of pooling RNA: complete pooling and sub-pooling. During complete pooling, samples from one treatment group is pooled and hybridised to one array with no replication. In this case, expression data cannot be analysed statistically. In contrast, during sub-pooling, sub-sets of the samples are selected and pooled and a number of chips are then used for each sub-group (Peng et al., 2003). In this study, it was not possible to obtain sufficient RNA from a single Arabidopsis seedling. Therefore a variation of the complete pooling method was used. One hundred mg of plant tissues for each RNA extraction were obtained from 10 Petri dishes each consisting of 14 Arabidopsis seedlings. Each set of 10 Petri dishes was separately exposed to the various treatments being investigated and RNA was independently extracted.

Over 3000 plants and around 30 RNA extractions were performed to generate samples with biological replicates in order to verify reliability of the expression results generated from microarray experiments and to validate microarray results using semi-quantitative PCR. Thus per treatment (1.0 mM 2,4-D; 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v)
surfactant NUL1026, 1 h post spraying; and 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post spraying), two sets of plants were treated separately and RNA extracted after each treatment was independently used for target preparation for subsequent microarray experiments. Results from both duplicates were used to confirm the reliability of the microarray results. Only one set of control plants each for the 1 and 24 h time intervals was used for the microarray procedures.

The quantity and quality of total RNA extracted were checked by spectrophotometry and gel electrophoresis. Total RNA was quantified by measuring absorbance at 260 nm and 280 nm using a spectrophotometer. An optical density (OD) of 1 at 260 nm equals 40 µg RNA per mL and the A260/A280 ratio of RNA samples between 1.8 and 2.1 indicates high purity of RNA. Ratios below 1.8 indicate possible contamination of RNA samples while ratios above 2.1 imply the possible presence of degraded RNA and/or excess free nucleotides. The quality of 1 µl total RNA (~0.5 µg) was checked on a 1% agarose gel pre-stained with 1 µl ethidium bromide (10 mg/mL) alongside a 1 kb (GeneRuler™) molecular marker. The gels were run in 1x TBE buffer (Appendix II) at 90V and the ribosomal RNA bands were examined. Non-distinct ribosomal bands indicate RNA degradation which can result in poor dsDNA synthesis and cRNA yield.

2.2.4 Target (cRNA) synthesis for microarray experiments

This section outlines the experimental procedures recommended by Affymetrix® (Affymetrix® Note, 2002a) for target preparation and labelling for subsequent hybridisation onto the GeneChip expression arrays. The steps involved in target preparation are briefly outlined in Figure 2.2. The reagents and protocols have been developed and optimised by Affymetrix® and are described in the sections below.
Approximately 10 µg of total RNA was reverse-transcribed to first strand cDNA and then to second strand cDNA using the enzymes reverse transcriptase and T4 DNA polymerase respectively. The double-stranded cDNA was cleaned up and in vitro transcribed in the presence of T7 RNA polymerase and biotin labelled ribonucleotides, to produce biotin labelled cRNA. The labelled cRNA (the target) was cleaned, fragmented and added to a hybridisation cocktail. RT - reverse transcription; cDNA - complementary DNA; IVT - in vitro transcription; cRNA - complementary RNA.

2.2.4.1 Poly - A controls

Prior to initiating the steps for target preparation and labelling, the extracted RNA was spiked with a determined amount of poly-A labelling controls in a proportion to reflect the total weight
of RNA to be labelled. The poly-A controls are from a kit designed by Affymetrix® (Cat. No.900433) and consists of in vitro synthesised polyadenylated transcripts for genes from *B. subtilis*. The four genes (*lys, phe, thr, dap*) are represented in staggered final concentrations of: 1:100,000, 1: 50,000, 1: 25,000 and 1: 7,500. These controls were then amplified and labelled together with the RNA sample. Examination of the hybridization intensities of these controls on the GeneChip array helped to monitor the labelling process independently from the quality of the starting RNA samples.

### 2.2.4.2 First strand cDNA synthesis

Approximately 5 µg total RNA was incubated with 100 µM HPLC purified T7 - (oligo dT) 24 primer (100 pmol/µL = 100 µM) (Genset Corp.) at 70°C in a thermal cycler (PerkinElmer) for 10 min, then immediately placed on ice. Further reactions were carried out in 5 x First strand buffer containing 0.1 M dithiothreitol (DTT) and 10 mM deoxyribonucleoside triphosphate (dNTP) at 42°C for 2 min, following which 200 units (U) of Superscript II RT (Invitrogen Cat. # 18090-019) was added. The samples were then incubated at 42°C for 1 h during which time the RNA was reverse transcribed to first strand cDNA. Two first strand reactions were carried out simultaneously to obtain an optimum yield.

### 2.2.4.3 Second strand cDNA synthesis

Second strand cDNA synthesis was separately carried out on each of the first strand cDNA reactions. The second strand synthesis reaction was performed in 5 x Second strand buffer containing 10 mM dNTP mix, 10 U *E. coli* DNA ligase, 10 U *E. coli* DNA polymerase and 2 U *E. coli* RNase H. The final volume of the total reaction for each of the tubes was made up to 150 µL with diethyl pyrocarbonate (DEPC) treated water (RNase-free water). The total volume
or content of each tube was divided into two PCR tubes and incubated at 16°C for 2 h in the thermal cycler (PerkinElmer). Five U of T4 DNA polymerase were added to each of the above reactions and the PCR tubes were incubated at 16°C for 5 minutes, following which 0.5 M ethylenediaminetetraacetic acid (EDTA) pH 8.0 was added to end the reactions. Contents of all the four reactions were then pooled and the volume measured.

2.2.4.4 Clean-up of double-stranded cDNA

The double stranded cDNA was purified using the phase lock gel (Eppendorf-5 Prime, Inc., Cat. # pl-188233). The phase lock gel was centrifuged at 12,000 g for 30 seconds. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris-HCl pH 8.0 / 1 mM EDTA (Ambion, # 9732) was added to the double stranded cDNA and briefly vortexed. This was then loaded on to the phase lock gel and centrifuged at 12,000 g for 2 minutes. The upper layer was then transferred into a fresh tube and the volume was measured. This was followed by the addition of a half volume of 7.5 M ammonium acetate and 2.5 × volume ice-cold absolute ethanol. This mixture was then centrifuged at 12,000 g for 20 minutes and supernatant was subsequently discarded. The pellet was washed with 500 µL of 80% ice-cold ethanol, centrifuged at 12,000 g for 5 minutes and supernatant was discarded. The above step was repeated. Finally the pellet was air-dried, suspended in 12 µL of RNase-free water and stored at -20°C.

2.2.4.5 In vitro transcription (IVT) of cDNA and biotin labelling

The purified double stranded cDNA was in vitro transcribed into complementary RNA (cRNA) using the BioArray High Yield RNA Transcript Labelling Kit, Affymetrix® (Millennium Science, Australia, Cat. # 900182). During this in vitro transcription procedure, biotin- labelled
ribonucleotides were incorporated. For *in vitro* transcription 10 µL of the double stranded cDNA was used as a template and reaction was carried out in 10 X hybridisation buffer containing 10 X biotin labelled ribonucleotides, 10 X DTT, 10 X RNase inhibitor mix and 20 X T7 RNA polymerase. The total volume of the reaction was made up to 40 µL with RNase-free water and incubated at 37°C for 5 h in a thermal cycler (PerkinElmer). During this incubation step, contents of the tube were mixed by gentle shaking every half-hour for efficient transcription. The quality of cRNA was checked on a 1% agarose gel pre-stained with 1 µl ethidium bromide (10 mg/mL) alongside a 1 Kb ladder (GeneRuler™).

The cRNA was cleaned using the RNeasy plant mini kit (Qiagen Cat # 74904) and the quality of cleaned cRNA was checked on a 1% agarose gel pre-stained with 1 µl ethidium bromide (10 mg/mL). The cRNA was also quantified spectrophotometrically by reading the absorbance at 260 nm and 280 nm and using the following equation: adjusted cRNA yield = measured cRNA - (total RNA) (cDNA fraction used in IVT).

### 2.2.4.6 Fragmentation

Twenty micrograms of cRNA were used for fragmentation. The cRNA was added to the 5 × Fragmentation buffer (Appendix III) in a 1:4 ratio and the final concentration of cRNA was 0.5 µg/µL. The mix was incubated at 94°C for 35 min in thermal cycler (PerkinElmer) and then immediately placed on ice after incubation. The size of fragmented cRNA was checked on a 1% agarose gel pre-stained with 1 µl ethidium bromide (10 mg/mL) alongside a 1 Kb ladder (GeneRuler™).
### 2.2.4.7 Hybridisation cocktail + target preparation

A hybridisation cocktail consisting of the fragmented cRNA at a final concentration of 0.05 µg/µL was prepared. This hybridisation cocktail was prepared in 1× MES hybridisation buffer (Appendix III) consisting of 0.1 mg/mL herring sperm DNA and 0.5 mg/mL bovine serum albumin (BSA). The cocktail also included an alignment control, Oligo B2 at a final concentration of 50 pM. Spike controls, bioB, bioC, bioD, cre were added to the cocktail at a final concentration of 1.5 pM, 5.0 pM, 25 pM, 100 pM respectively. The final volume of hybridisation cocktail was 300 µL. The cocktail was mixed and then divided into three tubes of 100 µL each for incubation at 99°C for 5 minutes and then at 45°C for 5 min in the thermal cycler (PerkinElmer). The contents were subsequently pooled.

### 2.2.5 Hybridisation

The *Arabidopsis* array ATHI-121501 (Affymetrix®) was used as the probe. Details of the array are provided in Table 2.1. In all, 12 GeneChip arrays were used for this study. The probe array was equilibrated at room temperature and then pre-hybridised with 200 µL 1× 2-[N-Morpholino]ethansulfonic acid (MES) buffer (Appendix III) for 10 min at 45°C in the hybridisation oven. The pre-hybridisation buffer was removed and replaced with 200 µL of the hybridisation cocktail. The hybridisation process was carried out for 16 h at 45°C in the hybridisation oven (GeneChip hybridisation oven 640 Affymetrix®).
Table 2.1 ATH1 genome array specification for the Arabidopsis Affymetrix® chip ATHI-121501.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences represented</td>
<td>&gt; 24,000 gene sequences</td>
</tr>
<tr>
<td>Feature size</td>
<td>18 um</td>
</tr>
<tr>
<td>Oligo length</td>
<td>25 mers</td>
</tr>
<tr>
<td>Probe pairs/Sequences</td>
<td>11</td>
</tr>
<tr>
<td>Control Sequences</td>
<td>B2 Oligo; <em>E. coli</em> genes <em>bioB, bioC, bioD</em>, Phage P1 <em>cre</em> gene; Poly-A Controls: <em>B. subtilis</em> genes – <em>lys, phe, thr</em> and <em>dap</em>; <em>Arabidopsis</em> housekeeping genes <em>GAPDH, ubiquitin</em> and <em>actin</em></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1:100,000</td>
</tr>
</tbody>
</table>

Abbreviations: *bioB* - *Escherichia coli* biotin synthase; *bioC* - *E. coli* bioC protein; *bioD* - *E. coli* dethiobiotin; *cre* - Bacteriophage P1 cre recombinase protein; *lys* - *Bacillus subtilis* lysine, *phe* - *B. subtilis* phenylalanine, *thr* - *B. subtilis* threonine, *dap* - *B. subtilis* diaminopropionic; *GAPDH* - glyceraldehyde 3-phosphate dehydrogenase.

### 2.2.6 Washing and scanning

After 16 h of hybridisation, the hybridisation cocktail was removed from the probe array and replaced with 200 µL of non-stringent buffer (Appendix IV). The GeneChip fluidics station 400 Affymetrix® was used to wash and stain the probe array and it was operated by the MAS 5.0 software. An experimental file was created and saved as .EXP file. The hybridised probe was loaded on to the fluidics station for staining and washing. The fluidics station followed a series of steps to stain and wash the array. The array was exposed to streptavidin linked phycoerytherin (SAPE), biotinylated anti-streptavidin antibody and then again to SAPE, along with intermittent washes with non-stringent buffer and stringent buffer. Figure 2.3 shows the principle of staining and amplification. The stain was prepared in 1× stain buffer (Appendix V),
which contained 2 mg/mL bovine serum albumin (BSA) and 10 μg/mL SAPE (Molecular Probes Cat. # S-866). An antibody solution was prepared in 1× stain buffer, which contained 2 mg/mL BSA, 0.1 mg/mL normal goat IgG (Sigma Chemical # 15256) and 3 μg/mL biotinylated antibody (Vector Laboratories Cat. # BA-0500).

```
Streptavidin linked phycoerytherin (SAPE)

Anti-steptavidin antibody, biotinylated

Biotin labeled cRNA
```

Figure 2.3 Schematic drawing of the principle of staining and amplification.

cRNA generated during the IVT step was biotin labelled by incorporation of biotin labelled ribonucleotides. The biotin on the cRNA is coupled with streptavidin. This streptavidin has a phycoerytherin moiety (SAPE) attached to it, which has fluorescent property. To amplify the signals generated, a biotin-labelled anti-streptavidin antibody was included in the staining procedure. Presence of abundant biotin attracts more SAPE, resulting in the amplification of the signals. - biotin molecule; - streptavidin; - phycoerytherin.
2.2.7 Scanning

Scanning of the stained array was carried out using the GeneArray scanner Affymetrix® and the MAS 5.0 software was used to control the scanner. Two laser scans were run per array at a wavelength of 570 nm and a pixel value of 3 µm and the scanned image was stored as a .dat file. The software then computes cell intensity data (.cel file) from the image file. During scanning, laser beams are passed through the phase between the array and the target. The fluorescence emitted is then caught by a lens and passed thorough optical filters to the detector (Fodor et al., 1991; Fodor et al., 1993; Lipshutz et al., 1999). Figure 2.4 is a schematic explanation of the whole microarray procedure.

2.2.8 Image analysis

After scanning the array in the first step of analysis, a grid was placed by MAS 5.0 over the .dat file in order to verify the alignment of the probes in the array. The hybridisation of the B2 Oligo along the border with alternating intensities and corners with checkerboard pattern was also checked for proper alignment and positive hybridisation. The image file was also visually scanned for the possible presence of artefacts such as specks and scratches on the array.
Eukaryotic Target Labeling for GeneChip® Probe Arrays

1. Primer hybridization
   - Primer binds to 5' end of RNA
   - Total RNA: AAAAA 3'
   - Primer: TTTTT 5'
   - Experiment Time: 15 minutes

2. Reverse transcription
   - First strand cDNA synthesis
   - Total RNA: AAAAA 3'
   - Primer: TTTTT 5'
   - Experiment Time: 1 hours 15 minutes

3. Second strand cDNA synthesis
   - cDNA: AAAAA 3'
   - Primer: TTTTT 5'
   - Experiment Time: 2 hours 30 minutes

4. Cleanup of double-strand cDNA
   - Experiment Time: 30 minutes

5. Amplification and biotin labeling of antisense cRNA
   - Biotinylated Ribonucleotides
   - U - Biotin
   - C
   - Experiment Time: 4 hours

6. Cleanup of biotinylated cRNA
   - Experiment Time: 30 minutes

7. Fragmentation
   - Experiment Time: 45 minutes

8. Hybridization
   - Streptavidin-phycocerythrin
   - Biotinylated anti-streptavidin antibody
   - Experiment Time: 18 hours

9. Washing/Staining
   - Experiment Time: 75 minutes

10. Scanning
    - Experiment Time: < 10 minutes

Legend:
- AAAAA: RNA
- TTTTT: DNA
- Black: T7 Primer
- Blue: Biotin
Figure 2.4 Schematic drawing of the whole microarray procedures undertaken in this study.

Total RNA is first reverse-transcribed to single-stranded cDNA and then double-stranded cDNA. Following these procedures, the double strand cDNA is purified and used as a template for the in vitro transcription (IVT) reaction. During the IVT, biotin-labelled ribonucleotides are incorporated resulting in biotinylated cRNA. This cRNA is cleaned-up, fragmented and hybridised to the GeneChip expression array. After a series of staining and washing, the array is scanned and expression data was recorded for further analysis.

2.2.9 Absolute analysis

After completing the pre-analysis of the image, absolute analysis was conducted for each array. This analysis generated a signal value, a detection call and a detection $p$-value for all probe sets. The fluorescent intensity of each cell was converted into a signal value. Signal values represent transcript levels and are generated by the One-Step Tukey’s Biweight Estimate for each probe set (Affymetrix Technical Note, 2002a). The overall signal of a probe set relies on the intensity of each probe pair. Mismatch intensities highlight stray signal due to non-specific hybridisation and signal values calculated by MAS 5.0 do not generate negative signal values (Affymetrix Technical Note, 2002a).

The detection algorithm used by MAS 5.0 also uses probe pair intensities to generate the detection $p$-value and assign a Present, Marginal and Absent call. Each probe pair in a probe set plays a significant role in determining whether the transcript is Present or Absent. For each probe pair, a value called as the Discrimination score [$R$] is calculated using the following equation:

$R = \frac{PM-MM}{PM + MM}$, where $R$-discrimination score; $PM$-perfect match intensity; $MM$-mismatch intensity
This R score of a probe pair measures the target-specific intensity difference of the probe pair relative to its overall hybridisation intensity. This score was then compared to a predefined threshold Tau. In this study a default value for Tau was used, though users can adjust this parameter. Probe pairs with scores higher than Tau indicate presence of the transcript while scores lower than Tau mean absence of the transcript. The overall scoring results of a probe set is summarised as the \( p \)-value. The higher the R score as compared with the Tau value, the lower is the \( p \)-value and the higher the likelihood that a transcript is Present in a sample. The One-sided Wilcoxon’s Signed Rank test is used to generate a detection \( p \)-value (Liu et al., 2002). Based on the \( p \)-value cut-offs Alpha 1 (\( \alpha_1 \)) and Alpha 2 (\( \alpha_2 \)), the probes are assigned a detection call. Alpha 1 and 2 values can be changed to increase or decrease levels of stringency (Affymetrix Technical Note, 2002a). Default values have been used for all analyses carried out in this study. For the default parameter settings refer to Appendix VI.

2.2.10 Biological replication

Biological replicates of the different treatments, 1 h and 24 h, post – spraying were used to check the reproducibility of the microarray experiments. Prior to verifying reproducibility of the microarray results, the mean hybridisation intensities of all probe sets on all arrays and for all samples were scaled to an arbitrary fixed level (Affymetrix Technical Note, 2002a). The output of every experiment was multiplied by a scaling factor to adjust its average intensity to a target intensity of 50. This enabled comparisons between the replicates and between the different treatments.

The reproducibility of arrays was investigated using scatter plots. The Affymetrix® Data Mining Tool (DMT) version 3 (DMT 3.0) was used to generate the scatter plots. Expression levels of
each gene, measured by calculating the average difference of hybridisation signal intensity between perfect match and mismatch probes from duplicate arrays, were plotted.

Furthermore, a comparison of array results was carried out for both duplicates for each treatment and the coefficients of variations were estimated for those probe sets consistently called “Present” in both duplicate arrays for each treatment. The variation coefficients (%) were estimated by calculating the ratios of the standard deviation to the means of those genes having a “Present” call, multiplied by 100 (Mussig et al., 2002a).

2.2.11 Verification

The expression profiles obtained from the chip hybridisation were validated by semi-quantitative reverse transcriptase (RT) PCR using the first-strand cDNA synthesised from independently isolated RNA samples from each of the treatments, 1 h post-spraying. Two micrograms of total RNA were reversed transcribed into cDNA according to the manufacturer’s instructions (Promega) and used as template for the PCR reactions. In brief, total RNA (2 µg) was annealed with 6 µM of oligo (dT) (17-mer) primer. Reaction was incubated for 5 minutes at 70°C.

Reverse transcription was then performed by adding a mixture of 5 X Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) buffer, 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP, 25 units of Recombinant RNAsin® Ribonuclease Inhibitor and 200 units of M-MLV- Reverse Transcriptase (RT) enzyme in a final volume of 25 µl. Reaction was again incubated for 1 h at 37°C and inactivated by placing the reaction mixtures on ice. Control reverse - transcription reactions were also performed using RNA samples from each treatment. However, in the control reactions, the reverse - transcriptase enzyme, M-MLV-RT, was not included. Amplification of any bands in PCR reactions using such control templates would
indicate the possible presence of any carry-over genomic DNA that may be present in the RNA samples.

RT-PCR amplifications were performed with randomly selected 15 genes that showed transcript level induction or repression or no significant change in response to the different treatments (1.0 mM 2,4-D; 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D coupled with 0.2% (v/v) surfactant NUL1026), 1 hour post-spraying (Table 2.2). The RT-PCR reactions were conducted twice with two independently isolated total RNA samples from each treatment.

Specific primers for the 15 genes (Table 2.2) were designed based on probe sequences on the Affymetrix® array (ATH1-121501). Designing probe based on the target sequences provided by Affymetrix® ensures that the same RNA section is assayed in both microarray and RT-PCR techniques. Primer pairs were designed by accessing the BioNavigator™ portal and pasting the probe sequence of each selected gene. These individual gene sequences were subjected to the “Prime” program (Genetics Computer Group Inc. 1998) of BioNavigator™, which is able to design primer pairs (19-25 mers) for the chosen sequence. Primers were synthesised by GeneWorks (Australia), provided in a lyophilised form and resuspended in sterile MilliQ water at a final concentration of 100 µM. Primers were further diluted to a 10 µM working stock and stored at -20°C.

The housekeeping gene, GAPDH was used as the control for RT-PCR experiments and was amplified for a number of cycles (15, 20, 25 and 30) in preliminary PCR reactions to determine the linear phase. The amplified products were run on a 1% agarose gel pre-stained with 1% ethidium bromide (10 mg/mL) and visualised using the Gel Doc® (BioRad) system to indicate the linear phase of the PCR products. It was determined that 25 cycles represented the linear
phase, which is the phase at which the control gene is detectable and the PCR reactions components are still in excess and PCR products are accumulating at a constant rate.

All the PCR amplifications were carried out for 25 cycles and conducted in a reaction volume of 25 μl containing 1 μl of the RT reaction, 1 Unit Taq DNA Polymerase, 50 mM MgCl₂, 10 x PCR buffer (500 mM KCl, 100 mM Tris-HCL pH 9.0, 1% Triton X-100), 10 mM dNTP, 10 μM primers and sterile water. RT-PCR was performed via the Thermohyaid PCR machine: 1 cycle of 10 minutes at 94°C, 25 cycles of 30 seconds at 94°C, 1 minute at 55°C - 60°C and 72°C for 1 minute, and 1 cycle of final extension at 72°C for 5 minutes. Negative controls with the RT-reaction mix, minus the reverse-trancriptase enzyme were also included in the PCR reactions. The amplification products were separated on 1.5% (w/v) prestained (50 ng/mL ethidium bromide) agarose gel, in 1 x TBE buffer, at 90 V for an hour and a half. Gels were then viewed under an ultra violet (UV) transilluminator and amplification profiles were captured using the Biorad Gel Doc® system. The brightness of the bands was used to indicate level of gene expression.
Table 2.2 The forward and reverse primers used for the semi-quantitative PCR, 1 h post-spraying and the description of the corresponding probe set present on the ATH1-121501 chip.

<table>
<thead>
<tr>
<th>Description</th>
<th>Primer sequence</th>
<th>Probe set_ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTU20</td>
<td>5' GTT CTG GTC CAC AAC GGT AA 3' 5' CAT AGG CTT GGA ACC AAC TC 3'</td>
<td>At1g78370</td>
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<tr>
<td>SAUR</td>
<td>5' GCC ACT CTC ATA CTT GAA CC 3' 5' GGA GCC GAG AAG TCA CAT TG 3'</td>
<td>At5g18060</td>
</tr>
<tr>
<td>IAA1</td>
<td>5' CGC AAG AAC AAC GAC TCA AC 3' 5' CAT CAC CGA CCA ACA TCC AA 3'</td>
<td>At4g14560</td>
</tr>
<tr>
<td>TCH4</td>
<td>5' CGT CGA TGG AAC TCC GAT CA 3' 5' TGA CAA CCA CGA GCC AGT AG 3'</td>
<td>AT5g57560</td>
</tr>
<tr>
<td>PINOID</td>
<td>5' AAC TCG GTG GAG AAG GTC TG 3' 5' CTC GAT CAC CTC CGT CAC AA 3'</td>
<td>AT5g54490</td>
</tr>
<tr>
<td>POT</td>
<td>5' TTC CGC AGG AAT CGT AGA GA 3' 5' GAA TCC AGC CAT GCA CAT CA 3'</td>
<td>AT4g21680</td>
</tr>
<tr>
<td>HAT22</td>
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<td>AT4g37790</td>
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<tr>
<td>CAO</td>
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<td>AT1g44446</td>
</tr>
<tr>
<td>NCED3</td>
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<td>5' ACT AGA ACC AGG CGG TTA CT 3' 5' CGG TCG AAT GGA CAT GAA CA 3'</td>
<td>AT5g15410</td>
</tr>
</tbody>
</table>
Abbreviations: GSTU20 - glutathione S-transferase; SAUR- small auxin up-regulated; IAA1- indole-3-acetic acid 1; TCH4 - calmodulin-related touch 4; PINOID - protein kinase/pinoid (PID) binding protein 1; POT - oligopeptide transport; HAT22 - homeobox-leucine zipper protein 22; CAO - chlorophyll a oxygenase; NCED3 - 9-cis-epoxycarotenoid dioxygenase; PP2C - protein phosphatase 2C; EXP1 - expansin 1; CNGC2 - cyclic nucleotide-gated channel; GH3 - growth hormone; XTR6 - xyloglucan endotransglycosylase-related 6; IAA19 - indole-3-acetic acid; GAPDH - glyceraldehyde 3-phosphate dehydrogenase.

2.3 Results

2.3.1 Phenotypic changes on exposure to the various spray solutions

Similar phenotypic changes were recorded in all plants treated with the same spray solution. Control plants showed proper growth and development while plants treated with 1.0 mM 2,4-D + 0.2% surfactant NUL1026 exhibited significant leaf curling (epinasty) 24 h post-treatment and the effect was more pronounced after 48 h (Figure 2.5). Complete plant senescence was observed one week after plants were sprayed with the herbicide and surfactant combination. In response to 1.0 mM 2,4-D, injury symptoms typical of auxinic herbicides such as leaf epinasty and petiole elongation (Sterling and Hall, 1997; Grossmann, 2000) were recorded 24 h and 48 h post-spraying (Figure 2.5). Nonetheless, plant death was not observed after treatment with 2,4-
D only, though proper plant growth were perturbed and plants appeared stunted. Plants sprayed with 0.2% surfactant NUL1026 appeared stressed and parts of the older leaves looked chlorotic 24 h and 48 h post-treatment (Figure 2.5). However, surfactant-treated plants continued to grow as shown by the development of two additional new rosette leaves 24 h and 48 h (Figure 2.5) and subsequently proper plants growth and development were restored.
1 h post-treatment

Control

0.2% (v/v) NUL1026

1.0 mM 2,4-D

1.0 mM 2,4-D + 0.2% NUL1026

24 h post-treatment

Control

0.2% (v/v) NUL1026

1.0 mM 2,4-D

1.0 mM 2,4-D + 0.2% NUL1026

48 h post-treatment

Control

0.2% (v/v) NUL1026

1.0 mM 2,4-D

1.0 mM 2,4-D + 0.2% NUL1026
Figure 2.5 Phenotype of 14 days old Arabidopsis seedlings, 1 h, 24 h and 48 h post-treatment.

Control plants exhibited proper growth and development. Herbicidal effects were observed in plants treated with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 after 24 h and 48 h, eventually leading to plant death. Plants treated with 1.0 mM 2,4-D showed leaf curling and petiole elongation after 24 h and 48 h. Although growth appeared stunted, there was no plant mortality. Surfactant treated plants continued to grow as shown by the development of new rosette leaves after 24 h and 48 h though parts of the older leaves appeared chlorotic.

2.3.2 RNA quality

The concentration of the total RNA samples ranged from 0.5 - 0.6 µg/µl and the presence of clear ribosomal bands indicated that the RNA was not degraded (Figure 2.6). The A260/A280 ratio of the RNA samples ranged between 1.8 and 2.1, thereby indicating purity of the RNA samples.

![Figure 2.6 Checking the quality of RNA samples prior to target preparation.](image)

RNA samples (1 µl) were visualised on a 1% agarose gel. Presence of clear ribosomal bands (25S, 23S, 18S and 16S) indicated that the RNA was not degraded. *M-GeneRuler™ 1 Kb ladder (Fermentas).
2.3.3 Target preparation, hybridization and image analysis

Biotin labelled targets were successfully processed from total RNA and hybridised on the GeneChip arrays. The scanned image of every array was carefully scrutinised for white specks, scratches or uneven hybridisation. There were no artefacts in any of the array images generated in this study (Supplementary data). Hence none of the probe sets was masked and all the probes present on the ATHI-121501 chips were used for further analysis. Positive hybridisation of Oligo B2 along the boundaries of the probe area with the expected alternating pattern of intensities on the border and presence of the checkerboard pattern at each corner indicated efficient hybridisation of the targets to the probes.

2.3.3.1 Noise and average background values

These reports (Tables 2.3 and 2.4) highlight the fact that overall, the 12 arrays have comparable Noise values (Raw Q) and the average background values are within the Affymetrix’s recommended range of 20 - 100 for arrays scanned using the GeneArray Scanner. Hence these reports generated for the 12 arrays indicate low or inconsequential background.
Table 2.3 Summary report of absolute analysis of array results, 1 h post-spraying.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>0.2% NUL1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM NUL1026 (Array 1)</th>
<th>1.0 mM NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noise (RawQ)*</td>
<td>2.23</td>
<td>2.57</td>
<td>2.14</td>
<td>2.33</td>
<td>0.79</td>
<td>2.15</td>
<td>2.19</td>
</tr>
<tr>
<td>Background Avg</td>
<td>53.62</td>
<td>63.99</td>
<td>52.64</td>
<td>60.37</td>
<td>27.28</td>
<td>54.80</td>
<td>54.09</td>
</tr>
<tr>
<td>% present</td>
<td>69.10</td>
<td>69.10</td>
<td>67.10</td>
<td>67.60</td>
<td>63.40</td>
<td>66.80</td>
<td>67.70</td>
</tr>
<tr>
<td>% absent</td>
<td>29.90</td>
<td>30.10</td>
<td>32.00</td>
<td>31.40</td>
<td>35.70</td>
<td>32.20</td>
<td>31.50</td>
</tr>
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</table>

Signal log ratio (3'5') of housekeeping genes

<table>
<thead>
<tr>
<th>Housekeeping genes</th>
<th>Control</th>
<th>0.2% NUL1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM NUL1026 (Array 1)</th>
<th>1.0 mM NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATHAL-ACTIN</td>
<td>25.44</td>
<td>27.02</td>
<td>26.68</td>
<td>20.29</td>
<td>12.44</td>
<td>37.11</td>
<td>40.76</td>
</tr>
<tr>
<td>ATHAL-GAPDH</td>
<td>1.77</td>
<td>1.57</td>
<td>2.19</td>
<td>1.97</td>
<td>1.11</td>
<td>2.11</td>
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</tr>
<tr>
<td>ATHAL-UBQ</td>
<td>2.30</td>
<td>2.17</td>
<td>2.36</td>
<td>2.29</td>
<td>2.00</td>
<td>2.66</td>
<td>2.34</td>
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</table>

Signal values

<table>
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<tr>
<th>Spike controls</th>
<th>Control</th>
<th>0.2% NUL1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM NUL1026 (Array 1)</th>
<th>1.0 mM NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2% NUL1026 (Array 2)</th>
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<tr>
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<td>43.97</td>
<td>39.05</td>
<td>45.90</td>
<td>56.35</td>
<td>32.79</td>
<td>37.61</td>
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<tr>
<td>BIOC</td>
<td>159.89</td>
<td>136.07</td>
<td>107.65</td>
<td>80.40</td>
<td>96.07</td>
<td>106.02</td>
<td>129.39</td>
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<tr>
<td>BIODN</td>
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<td>294.24</td>
<td>274.87</td>
<td>330.26</td>
<td>253.71</td>
<td>314.60</td>
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<td>1481.80</td>
<td>1388.75</td>
<td>1063.19</td>
<td>1099.45</td>
<td>1303.29</td>
<td>955.82</td>
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</tr>
<tr>
<td>DAPX</td>
<td>137.54</td>
<td>135.54</td>
<td>103.50</td>
<td>86.35</td>
<td>135.71</td>
<td>109.31</td>
<td>116.00</td>
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<tr>
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<td>20.56</td>
<td>14.60</td>
<td>14.23</td>
<td>20.90</td>
<td>14.65</td>
<td>17.06</td>
</tr>
<tr>
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<td>40.27</td>
<td>22.49</td>
<td>25.05</td>
<td>40.56</td>
<td>23.17</td>
<td>27.76</td>
</tr>
</tbody>
</table>

Scaling factor @ TGT50 | 0.35 | 0.27 | 0.40 | 0.35 | 1.68 | 0.45 | 0.37 |
Abbreviations: Avg - Average; ATHAL - Arabidopsis; ACTIN - actin; GAPDH - glyceraldehydes 3-phosphate dehydrogenase; UBQ - ubiquitin; bioB - Escherichia coli biotin synthase; bioC - E. coli bioC protein; bioD - E. coli dethiobiotin; cre - Bacteriophage P1 cre recombinase protein; dap - Bacillus subtilis diaminopropionic; lys - B. subtilis lysine; phe - B. subtilis phenylalanine; thr - B. subtilis threonine; TGT - target value. *Noise (RAWQ) – this value indicates the degree of pixel-to-pixel variation among the probe cells.
Table 2.4 Summary report of absolute analysis for each treatment, 24 h post-spraying.

<table>
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<th>Treatments</th>
<th>Control (Array 1)</th>
<th>0.2% NUL 1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 2)</th>
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</thead>
<tbody>
<tr>
<td>Noise (RawQ)</td>
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<td>3.05</td>
<td>4.26</td>
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<tr>
<td>Background Avg</td>
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<td>54.96</td>
<td>89.98</td>
<td>128.06</td>
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<tr>
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<td>68.1</td>
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<td>63.2</td>
</tr>
<tr>
<td>% absent</td>
<td>35.80</td>
<td>30.2</td>
<td>30.9</td>
<td>31.2</td>
<td>35.6</td>
</tr>
</tbody>
</table>

**Signal log ratio (3'/5') of housekeeping genes**

<table>
<thead>
<tr>
<th>Housekeeping genes</th>
<th>Control (Array 1)</th>
<th>0.2% NUL 1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATHAL-ACTIN</td>
<td>15.10</td>
<td>15.08</td>
<td>27.5</td>
<td>18.51</td>
<td>20.64</td>
</tr>
<tr>
<td>ATHAL-GAPDH</td>
<td>1.21</td>
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<td>1.58</td>
<td>1.15</td>
<td>1.37</td>
</tr>
<tr>
<td>ATHAL-UBQ</td>
<td>2.60</td>
<td>2.36</td>
<td>2.53</td>
<td>1.99</td>
<td>2.34</td>
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</table>

**Signal**

<table>
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<tr>
<th>Spike controls</th>
<th>Control (Array 1)</th>
<th>0.2% NUL 1026 (Array 1)</th>
<th>0.2% NUL1026 (Array 2)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 1)</th>
<th>1.0 mM + 0.2 % NUL1026 (Array 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOB</td>
<td>17.29</td>
<td>16.44</td>
<td>28.77</td>
<td>11.61</td>
<td>16.59</td>
</tr>
<tr>
<td>BIOC</td>
<td>60.99</td>
<td>65.5</td>
<td>108.16</td>
<td>48.6</td>
<td>54.41</td>
</tr>
<tr>
<td>BIODN</td>
<td>180.46</td>
<td>178.39</td>
<td>301.52</td>
<td>125.91</td>
<td>147.82</td>
</tr>
<tr>
<td>CREX</td>
<td>790.5</td>
<td>959.25</td>
<td>1575.4</td>
<td>605.82</td>
<td>758.21</td>
</tr>
<tr>
<td>DAPX</td>
<td>136.19</td>
<td>54.92</td>
<td>47.56</td>
<td>169.45</td>
<td>121.29</td>
</tr>
<tr>
<td>LYSX</td>
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<td>7.63</td>
<td>22.95</td>
<td>19.08</td>
</tr>
<tr>
<td>PHEX</td>
<td>19.17</td>
<td>7.44</td>
<td>6.44</td>
<td>24.61</td>
<td>18.59</td>
</tr>
<tr>
<td>THRX</td>
<td>36.93</td>
<td>16.85</td>
<td>14.7</td>
<td>43.81</td>
<td>28.02</td>
</tr>
</tbody>
</table>

**Scaling factor @ TGT 50**

| Scaling factor @ TGT 50 | 0.32 | 0.32 | 0.39 | 0.22 | 0.2 |
Abbreviations: Avg - Average; ATHAL – *Arabidopsis*; ACTIN – actin; GAPDH – glyceraldehydes 3-phosphate dehydrogenase; UBQ- ubiquitin; *bioB* - *Escherichia coli* biotin synthase; *bioC* - *E. coli* bioC protein; *bioD* - *E. coli* dethiobiotin; cre - Bacteriophage P1 cre recombinase protein; *dap* - *Bacillus subtilis* diaminopropionic; lys - *B. subtilis* lysine; *phe* - *B. subtilis* phenylalanine; thr - *B. subtilis* threonine; TGT – target value. *Noise (RAWQ)* – this value indicates the degree of pixel-to-pixel variation among the probe cells.

### 2.3.3.2 Housekeeping genes

The ratio of the signal values for the 3’ probe set to the 5’ probe set for the 3 housekeeping genes should in general be no more than 3. A value of less than 3 indicates good RNA sample and assay quality. However, in this study, as shown in Tables 2.3 and 2.4, the signal log ratio (3’/5’) for the housekeeping gene *actin* was found to be very high while the signal log ratios for *GAPDH* and *ubiquitin* was less than 3.

### 2.3.3.3 Spike controls

The entire labelling process was successful as indicated by the presence of the Poly-A RNA controls which showed increasing signal values in the order of lys, *phe*, thr and *dap* (Tables 2.3 and 2.4). The hybridisation controls (*bioB*, *bioC*, *bioD* and cre) were also detected and the signal values reflected their relative concentrations that were spiked in the hybridisation cocktail. This further shows that hybridisation was successful in all cases. Besides, the hybridisation controls were added to each replicate sample equally and the signal intensities of the *bioB*, *bioC*, *bioD* and cre probe sets were found to be approximately equal (Tables 2.3 and 2.4).
2.3.4 Biological replication

Biological replicates for all treatments (except for the control treatments with water) at 1 h and 24 h, post-spraying were used to check the reproducibility of the microarray experiments. Global scaling was applied on all arrays to a target intensity (TGT) of 50 and the scaling factor for all the arrays (Tables 2.3 and 2.4) were found to be below 3-fold. This indicates that there was no significant variability in the assays and the level of reproducibility was high.

The reproducibility of the duplicate arrays for the different treatments at the two time intervals were also analysed using scatter plots. Scatter plots of the raw signal intensity values between the duplicate arrays showed that the majority (> 80%) of the significantly expressed genes are aligned along a central line within the twofold relative intensity, thereby showing that most genes exhibit less than two-fold variation in signal intensity between two independent hybridisation (Figure 2.7 (a)-(e).

This degree of reproducibility may be attributed to tightly controlled growth and experimental conditions. Those genes with low raw signal intensities showed more variability and were situated between the 3-, 10- and 30- fold lines and this may be attributed to either noise or presence of low abundance total RNAs.
Figure 2.7 (a)  

Figure 2.7 (b)
Figure 2.7 (c)  Figure 2.7 (d)
Figure 2.7 Scatter plots from biological replicates from the various treatments, 1 h and 24 h post-spraying.

Scatter plots show the alignment of the signals along a central line within the low fold change thus highlighting the reproducibility of microarray experiments. Each gene is represented by one dot. For each gene, the raw RNA expression level in one experiment is given on the x-axis and the expression level of the same gene in the other experiment is provided on the y-axis. The diagonal lines show the relative intensity differences by a factor of 2, 3, 10 and 30 between the two independent hybridisation for visual reference. Significantly expressed genes detected as Present or Marginally Present in the two samples are indicated by the red and blue dots respectively while genes for which the expression levels were not significant in the two samples are shown in yellow.

Two sets of plants were independently separately treated with either 1.0 mM 2,4-D, 0.2% (v/v) surfactant NUL1026 or 1.0 mM + 0.2% (v/v) NUL1026 and RNA extracted 1 h post-spraying.
RNA was also extracted from two sets of plants that were independently sprayed with 0.2% (v/v) NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-spraying. The two cRNA samples from each treatment at both intervals of time were individually hybridised to two different arrays (Array 1 and Array 2). The diagonal lines represent the fold changes (two, three, ten and thirty) in expression level between samples.

(a) Scatter plot of signal values from Array 1 and Array 2, 0.2% (v/v) NUL1026, 1 h post-spraying.

(b) Scatter plot of signal values from Array 1 and Array 2, 1.0 mM 2,4-D, 1 h post-spraying.

(c) Scatter plot of signal values from Array 1 and Array 2, 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying.

(d) Scatter plot of signal values from Array 1 and Array 2, 0.2% (v/v), 24 h post-spraying.

(e) Scatter plot of signal values from Array 1 and Array 2, 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying.
To further evaluate the reproducibility of the biological duplicate arrays in response to the different treatments (1.0 mM 2, 4-D, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026) at 1 h and 24 h post-spraying, the coefficient of variation (VC) was calculated for all the genes called Present in the duplicate arrays for each treatment. This type of analysis, often used in whole genome transcript analysis studies (Mussig et al., 2002a; Raghavan et al., 2005) indicated that the duplicates used in this study showed a low level of biological variability. Comparison of array results for each treatment, 1.0 mM 2, 4-D, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026 at 1 h post-application indicated that 98%, 94% and 99% of the genes that were called present in both duplicates (14,086, 14,755 and 14,580 respectively) showed a variation coefficient of less than 50% (Figure 2.8). Ninety-nine % and 98% of the genes that were called present (15,002 and 14,092 genes were called present, respectively) in both duplicate arrays in response to 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-spraying, showed a VC of less than 50% respectively (Figure 2.8).
Variation coefficient classes:

1: VC ≤ 10%
2: 10% < VC ≤ 20%
3: 20% < VC ≤ 50%
4: 50% < VC ≤ 100%
5: VC > 100%
Figure 2.8 Histogram showing reproducibility of biological replicates in response to the various treatments.

The histogram represents the variation coefficients (VC) of genes called Present in the biological replicates as a result of spray application of the different treatments ((1.0 mM 2,4-D, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026), 1 h and 24 h post-application. Variation coefficients (in %) were calculated as follows: standard deviation/mean signal x 100 (Mussig et al., 2002a):

(a) 1.0 mM 2,4-D, 1 h post-treatment: 14,086 genes were called present in Array 1 and Array 2 and 98% of the genes had a VC < 50%.

(b) 0.2% (v/v) surfactant NUL1026, 1 h post-treatment: 14,755 genes were called present in Array 1 and Array 2 and 94% of the genes had a VC < 50%.

(c) 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h post-treatment: 14,580 genes were called present in Array 1 and Array 2 and 99% of the genes had a VC < 50%.

(d) 0.2% (v/v) surfactant NUL1026, 24 h post-treatment: 15,002 genes were called present in Array 1 and Array 2 and 99% of the genes had a VC < 50%.

(e) 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-treatment: 14,091 genes were called present in Array 1 and Array 2 and 98% of the genes had a VC < 50%.
2.3.5 Semi-quantitative RT-PCR

Semi-quantitative reverse-transcriptase (RT)-PCR was used to verify the consistency of the results obtained from the microarray experiments. Amplification of the different RT reaction with the GAPDH primers resulted in bands with similar intensities (Figure 2.9) suggesting that the RT reaction operated at a comparable efficiency on RNAs extracted from *Arabidopsis* exposed to the different treatments. Absence of bands (data not shown) in the negative control PCR reactions showed absence of contamination due to genomic DNA.

All other genes (*IAA 19, XTR6, GH3, CNGC2, EXP1, PP2C, NCED3, CAO, HAT22, POT, PINOID, TCH4, IAA1, SAUR, GSTU20*) showed differential expression in response to the various treatments (Figure 2.9). As shown in Figure 2.9, the relative intensity of the indole-3-acetic acid (IAA) *IAA 19, IAA1*, the early-auxin inducible gene, *GH3* and the small auxin up-regulated RNA gene (*SAUR*) bands were considerably higher in the RT-PCR from the 1.0 mM 2,4-D and the 1.0 mM 2,4-D + 0.2 % (v/v) surfactant NUL1026 RNA samples than from the control and surfactant treated plants. The expression levels of the cyclic nucleotide-gated ion channel (*CNGC2*) and xyloglucan endotransglycosylase-related proteins 6 (*XTR6*) genes showed a high level of expression compared to the control, in response to 1.0 mM 2,4-D + 0.2 % (v/v) surfactant NUL1026 treatment while no significant regulation was recorded in the other treatments.

Moreover, the protein-dependent oligopeptide transport (*POT*) and homeobox-leucine zipper protein genes (*HAT22*) were only up-regulated when sprayed with 1.0 mM 2,4-D as showed by the brightness of the bands in Figure 2.9 compared with the other treatments and the control. The band intensities of the calmodulin-related touch (*TCH4*) and protein kinase (*PINOID*) genes
were found to be higher in both the surfactant only and the herbicide coupled with the surfactant treatments (Figure 2.9) in comparison with the control and the 1.0 mM 2,4-D treatments. In addition, there was a significant decrease in band intensity of the gene encoding the glutathione transferase (GST) (GSTU20) protein relative to the control, in response to both the 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 while in contrast the expression level of the other treatments did not change. The expression patterns of the chlorophyllide A oxygenase (CAO) gene, the 9-cis-epoxycarotenoid dioxygenase (NCED3) gene, the protein phosphatase 2C (PP2C) gene and the expansin (EXP1) gene showed a significant decrease in expression in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 compared to the control and the two other treatments. Duplicate PCR reactions performed with independent total RNA samples gave similar results. Overall the RT-PCR results were consistent with the microarray results.
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<th>1.0 mM 2,4-D + 0.2% surfactant Nul 1026</th>
<th>AGI ID</th>
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<td>D</td>
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<td>NC</td>
<td>I</td>
<td>I</td>
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<td>247925_at</td>
<td>P</td>
<td>I</td>
<td>NC</td>
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<td>I</td>
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<td>NC</td>
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<tr>
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<td>256299_at</td>
<td>P</td>
<td>NS</td>
<td>NC</td>
<td>D</td>
<td>At1g69530</td>
</tr>
<tr>
<td>Cngc2</td>
<td>246510_at</td>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>I</td>
<td>At5g15410</td>
</tr>
<tr>
<td>Gh3</td>
<td>262099_s_at</td>
<td>P</td>
<td>NC</td>
<td>I</td>
<td>I</td>
<td>At1g59500</td>
</tr>
<tr>
<td>Xtr6</td>
<td>254042_at</td>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>I</td>
<td>At4g25810</td>
</tr>
<tr>
<td>Iaa 19</td>
<td>258399_at</td>
<td>P</td>
<td>NC</td>
<td>I</td>
<td>I</td>
<td>At3g15540</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GAPDH_M_s_at</td>
<td>P</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

P=present, I= increase, D= decrease, NS= no change, NC= no change
Figure 2.9 Validation of microarray results using semi-quantitative RT-PCR.

Changes in gene expression levels from the microarray experiments compared to the control (water), 1 h after exposure to the different treatments are shown alongside. Regulation of a few selected genes (15 genes), as recorded during the microarray experiments, in response to the either of the 3 different treatments (1.0 mM 2,4-D, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026), 1 h post spraying were validated by semi-quantitative PCR using specific primers (Table 2.2). Total RNA was reverse-transcribed and PCR reactions were conducted for 25 cycles using equal quantity of cDNA template. The PCR reactions were repeated twice with independently extracted total RNA samples. GAPDH represented the housekeeping gene. Results obtained were consistent with microarray results. C- control; S- 0.2% (v/v) surfactant NUL1026; 1.0 - 1.0 mM 2,4-D; 1.0 + S -1.0 mM 2,4-D + 0.2 % (v/v) surfactant NUL1026.

2.4 Discussion

2.4.1 Phenotypic changes in response to the different spray solutions

Interestingly, the concentration of 1.0 mM 2,4-D, which had been previously shown to cause plant senescence (Raghavan et al., 2005, 2006) when root-applied did not induce plant death when the same concentration was foliar-applied, although growth was inhibited, leaf curling and petiole elongation were observed (Figure 2.5). These morphological changes may imply that although the plants had experienced an “auxin-overdose” when treated with the 1.0 mM 2,4-D, the amount of herbicide active ingredient that penetrated through the cuticular barrier of the Arabidopsis leaves, was not sufficient to kill the plants. In contrast, morphological changes in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant suggests that surfactant NUL1026 may have helped the active ingredient (2,4-D) to penetrate through the leaf cuticle and reach the target sites. The leaf epinasty and senescence observed imply increased levels of ethylene and
increased expression of senescence-inducing genes (Grossmann, 1998; Raghavan et al., 2005, 2006).

*Arabidopsis* seedlings appeared stressed after surfactant NUL1026 treatment and parts of the leaves exhibited senescence. This indicates that whilst the level of surfactant used (0.2% (v/v)) may improve herbicidal activity, surfactant NUL1026 alone may also potentially be phytotoxic to the plant. However, in this study, as shown in Figure 2.5, spray application of surfactant NUL1026 did not induce major growth inhibition and previous studies have shown that tissue damage caused by surfactants plays a key role in herbicide uptake and penetration (Feng et al., 1999). To further understand the molecular basis of these changes, gene expression levels were monitored in respond to the various spray solutions.

### 2.4.2 Microarray experiments and absolute analysis

A decade ago, techniques for the study of gene expression were limited both in breadth and efficiency, as these traditional methodologies enabled scientists to study only one or a few genes at a time (Schena et al., 1995; Kothapalli et al., 2002). However, newly developed procedures now allow investigators to relatively quickly monitor the expression patterns of tens of thousands of genes at a time. Oligonucleotide GeneChips (Affymetrix®) in particular provide a convenient and standard medium for the reliable screening for gene expression. The number of publications featuring gene expression data generated by microarray studies certainly highlights the powerful nature of this technique. However, in order to draw meaningful information from microarray studies and be able to compare array results, a number of preliminary issues need to be addressed. These issues include the creation of initial datasets for each microarray
experiment and the standardisation of array results across different treatments such that comparative studies can eventually be carried out.

The 12 microarray experiments using total RNA samples extracted from plants exposed to different treatments were successfully conducted. Most of the results obtained after conducting the absolute analysis (Tables 2.3 and 2.4) were according to the recommendations set by Affymetrix® (Affymetrix Technical Note, 2002a) and showed that the microarray results generated were reliable. Each oligonucleotide microarray read-out is often accompanied by a significant amount of assay noise (Wodicka et al., 1997; Hill et al., 2001) and to nullify this “noise”, expression results are normalised to an arbitrary value which is user-defined (Hill et al., 2001; Zien et al., 2001). Application of this type of normalisation or global scaling to a target intensity of 50 across the 12 arrays showed that there was insignificant variability between arrays in this study. Besides the scaling factor for all arrays was comparable and below the 3-fold value (Table 2.3 and 2.4). The overall intensity for a degraded RNA sample or a sample that has not been amplified or labelled properly will have a lower intensity compared to a “good” replicate sample and when the array is scaled to the same target intensity as the other arrays, the scaling factor of the “bad” sample will be much higher compared with the “good” arrays (Affymetrix Technical Note, 2002a).

Moreover, the signal values of both the Poly-A controls (lys, phe, thr and dap) and the Hybridisation controls (bioB, bioC, bioD and cre) reflected their respective relative concentrations that were used during the microarray procedures (Tables 2.3 and 2.4). These results indicated successful target labelling and hybridisation respectively. Replicate samples of the Hybridisation controls (Tables 2.3 and 2.4) also had approximately equal signal intensities.
In cases where the RNA sample was degraded or not amplified or labelled properly, when scaled, these signal intensity values of the hybridisation control probe sets would have been adjusted much higher than the normal replicate sample.

Furthermore, the ratio of the signals from the 3’ end to the 5’ end of the housekeeping genes, \(\text{GAPDH}\) and \(\text{ubiquitin}\) included on the GeneChip arrays were less than 3, indicating good quality of labelled target (Zhu and Wang, 2000) (Tables 2.3 and 2.4). The signal log ratios of these two housekeeping genes were also comparable across the other arrays (Tables 2.3 and 2.4). However, in the case of \(\text{actin}\), a big signal log ratio value was recorded across all 12 arrays (Tables 2.3 and 2.4). This data indicated a three prime bias for the gene encoding actin. This feature has been discussed by researchers at the 2nd GeneChip User’s meeting in Australia and it was concluded that the bias is probably due to probe design. According to Affymetrix\textsuperscript{®} (Affymetrix Technical Note, 2002a), there are times when the 3’ to 5’ ratio of one internal control gene might be normal while the 3’ to 5’ ratio of another control gene is high. This discrepancy in the 3’ to 5’ ratio is not indicative of the overall sample and assay quality but instead is likely to be due to specific transcript-related or an image artefact.

Further confirmation about the reliability of the microarray results obtained in this study was reflected by the scatter plots of the biological replicates (Figures 2.7 (a) - (e)). Signal values of both replicates from each treatment were plotted on 2-axis to generate the scatter plot and the concentration of the signals along a central line within the low fold change indicated the high reproducibility of the arrays (Figures 2.7 (a) - (e)). Other studies have also reported similar observations (Cheong et al., 2002; McClintick et al., 2003). Variation coefficient highlights the degree of variability and is used to assess the variability of probe sets between replicate arrays.
In this investigation, the signal intensities for the replicate arrays (Array 1 and Array 2) for each treatment at the two different time intervals were similar since in each of the treatments, a high percentage (> 90%) of genes exhibited a variation coefficient of $\leq 50\%$ (Figure 2.8).

Technical variability may arise as a result of sample preparation, labelling, hybridisation and other steps during the microarray experiment and this variation can significantly impact on array quality (Zakharkin et al., 2005). As such, in order to assess the extent of this technical variation, more than one target is usually prepared from a single RNA sample and these targets are individually hybridised on separate arrays. In this current study, technical replicates were not hybridised on separate arrays. Instead biological replicates were carried out since variations due to biological differences between samples have been shown to be more consequential (McClintick et al., 2003; Zakharkin et al., 2005). However, in this investigation, even while comparing arrays between the different treatments, the scaling factor and the signal log ratio of the housekeeping genes were comparable, thereby indicating low technical variability.

### 2.4.3 Validating microarray results by using semi-quantitative RT-PCR technique

A number of microarray experiments carried out with sufficient number of replicates (both biological and technical replicates) have highlighted the accuracy and reliability of microarray results (Tepperman et al., 2001; Cheong et al., 2002; Mussig et al., 2002a; Piper et al., 2002). However, in many cases, regular laboratories are unable to perform replicate microarray experiments due to its prohibitive cost and results drawn from just one replicate array experiment may not be reliable (Kothapalli et al., 2002; Kuo et al., 2002). As such more conventional methods such as northern blot analysis (Taniguchi et al., 2001; Pylatuik and Fobert, 2005), real-time PCR (Dallas et al., 2005) and semi-quantitative PCR (Nakabayashi et
al., 2005) have been introduced to verify the microarray results. These techniques are now being routinely used to validate the expression results despite the low sensitivity, unreliability and low reproducibility that have been reported for these traditional techniques (Peccoud and Jacob, 1996; Kuhn, 2001; Donson et al., 2002). There have been few reports whereby the results from northern blot analysis (Taniguchi et al., 2001), real-time PCR (Barrett and Kawasaki, 2003) and semi-quantitative PCR (Mutch et al., 2001) were not consistent with the microarray results.

Semi-quantitative RT-PCR is a technique being routinely employed in many laboratories to verify microarray results due to its low cost, technical simplicity and sensitivity (Bovet et al., 2003; Ji et al., 2003; Leonhardt et al., 2004; Villadsen and Smith, 2004; Nakabayashi et al., 2005). Verification of a subset of results by reverse transcription PCR can be thus used to establish an estimate of the variability of a given experimental system. Recently, Leonhardt et al. (2004) confirmed using RT-PCR the expression of 29 Arabidopsis genes, which showed differential expression to abscisic acid (ABA). In this present study, 15 genes were randomly chosen to verify microarray results using semi-quantitative RT-PCR. These genes showed differential expression across the treatments (1.0 mM 2,4-D, 0.2% (v/v) surfactant NUL1026, 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h post-spraying) relative to the control and overall the results obtained were consistent with the microarray results. However, the expression of SAUR and GH3, though being shown to be significantly up-regulated relative to the control, according to the microarray results, only showed a slight overexpression when validated by semi-quantitative RT-PCR (Figure 2.9). Despite repeated PCR, the same results were obtained for these two genes.
There have been reports of inconsistencies between microarray results from semi-quantitative PCR. Mutch et al. (2001) found only 73% correlation when they compared microarray data of the murine genome with expression results from semi-quantitative PCR. These discrepancies may be attributed to RNA quality, quantity, and efficacy of the reverse-transcription procedures and the reaction components which can all affect the final yield of the amplified products (Bustin, 2000, 2002). The secondary structure of mRNA has been shown to affect semi-quantitative PCR quantification and hence may be responsible for the unreliable results registered (Peccoud and Jacob, 1996; Bustin, 2000).

Furthermore, the loading and staining of the PCR products on agarose gene is not sensitive enough to detect small differences in transcript levels. Hence, semi-quantitative PCR can be used to detect qualitative difference (presence or absence) of a transcript, but it is not a reliable method to measure fold-change differences in transcript levels. Microarray results were only validated for those genes after 1 h of treatment applications since a high level of reproducibility was detected across the 12 arrays and besides all the 12 microarray experiments were conducted using the same equipment and under the same experimental conditions as much as realistically possible.

The following conclusions can thus be drawn from the absolute analysis:

- Global normalisation/scaling to the target intensity of 50 across the 12 arrays showed that there was insignificant variability between the arrays.
- Spike controls indicated successful target labelling and hybridisation efficacy across all arrays.
The ratio of the signals from the 3’ end to the 5’ end of the housekeeping genes indicated good quality of labelled target and the signal log ratios of these genes were also comparable across the other arrays.

Scatter plots highlighted the reproducibility of the duplicate arrays (Array 1 and Array 2) for each treatment.

Further confirmation of the biological reproducibility of the duplicate arrays was indicated by the high percentage (>90%) of probes, which showed a variation coefficient of less than 50%.

Technical reproducibility was indicated by similar scaling factors across all the arrays.

Semi-quantitative RT-PCR confirmed reliability of microarray results.

Since low technical and biological variability was recorded, this indicated that microarray results generated in this study were reliable. All 12 arrays were thus used for further analysis. Chapter 3 provides a more in-depth study of the preliminary results obtained in this Chapter in response to surfactant NUL1026 treatment. Comparison analysis to identify genes that were up-regulated and down-regulated in response to surfactant NUL1026 will be carried out and data mining will also be undertaken to look for early and late surfactant responsive genes. Chapter 3 will also focus on gene ontology and the function of regulated genes at the molecular and biological levels.
Chapter 3: Comparative and functional analysis of genes regulated by surfactant NUL1026

3.1 Introduction

Surfactants are used with post-emergence herbicides to help overcome the barriers that prevent movement of herbicide molecules from the leaf surface to the interior of the cell. A number of studies have highlighted the ability of surfactants to accelerate the penetration of herbicides into both isolated cuticles and in vivo (Foy, 1993; Schonherr and Baur, 1994). However, the effects of surfactants on foliar uptake of herbicides are complex and only partially understood. Based on a number of studies showing the phytotoxic and stimulatory effects of surfactants (MacDowall, 1963; Watson et al., 1980; Parr, 1982), it is evident that surfactants themselves have their own intrinsic biological functions. As yet, little information is available concerning the molecular mode of action of surfactants following foliar application. The overall goal of this chapter was to study the genome-wide response of Arabidopsis plants to an etheramine surfactant termed as NUL1026.

Absolute analysis data generated for each of the two time-points (1 h and 24 h) in Chapter 2, from plants sprayed with 0.2% (v/v) NUL1026, were compared with their corresponding control (also called baseline) data. Genes showing differential expression were selected, data mining performed, selected genes were annotated and inferences were drawn.

3.1.1 Comparison Analysis

Comparison analysis is performed to compare expression profiles and measure the changes in gene expression between experiments (e.g. treatment versus control). After selecting for arrays showing good sample quality and reproducibility, absolute analysis is carried out to build
individual databases for each experiment. A comparison analysis is then carried out using the Microarray Suite version 5.0 (MAS 5.0) software. This type of analysis compares the difference values (PM - MM) of each probe pair in the baseline array to its matching probe on the experimental array. Data generated includes a change call (increase, decrease or no change) in expression level of each probe set as compared to the control. Moreover, when the experimental and the baseline array are compared, a signal log ratio value, which indicates the magnitude and the direction of change of a transcript, is also generated for each probe set (Affymetrix Technical Note, 2002a).

3.1.2 Data mining

Oligonucleotide chips generate large data sets for all the 24,000 transcripts simultaneously. However, one of the biggest challenges is to manage and determine the most robust changes in gene expression that can be deduced from a comparison analysis. Not all the probes sets on the chip set will exhibit a fold change in response to a particular treatment or experimental condition. Hence data mining is used with the aim of identifying those genes that are significantly regulated in response to a particular experimental condition when compared to the baseline. This generates smaller and significantly more meaningful data lists for further analysis (Bassette Jr, 1999).

3.1.2.1 Mining for significantly regulated genes-metrics for analysis

Significantly regulated genes include genes that are either up regulated or down-regulated in response to a treatment when compared to the baseline. A standardised technique of filtering data that are informative involves the use of the following selection criteria (Affymetrix Technical Note, 2002a):
“Detection” is a measure of presence or absence of a particular transcript. When searching for significant increases in gene expression, it is important to look for transcripts with “Present” calls in the experimental sample. Alternatively, when looking for significant decreases, transcripts with “Present” calls in the baseline should be chosen. These guidelines help to eliminate “Absent” to “Absent” calls that are insignificant.

“Change” is described as a measure of increase or decrease for a particular transcript. Hence, those probes giving “No Change” call are eliminated for future analysis since they are uninformative.

The relative change in transcript abundance is denoted as Signal log ratio (SLR). Based on the research carried out by Wodicka et al. (1997), it has been suggested that a two fold change in expression levels should be regarded as a significant change (Wodicka et al., 1997; Girke et al., 2000). Therefore, transcripts with a fold change of >2 indicate significant increase and decrease for <2. This two-fold change corresponds to a SLR of 1 and –1 respectively. The above-mentioned cut-off values can, however, vary and largely depend on the interest of the researchers. As yet, there is no consensus among researchers concerning cut-off values though a two-fold change is generally applied in selecting for regulated genes.
3.1.2.2 Mining for regulated genes across different experimental conditions

Once the genes that are significantly regulated in response to a particular treatment (in comparison to a control) have been identified, it is common practice to investigate the behaviour of the same genes across other experimental conditions. For instance, the same gene might either respond differently or show the same expression patterns to varying concentrations of a hormone or show variable expression at different time frames. Hence data mining and query building can be used to gain more in-depth information if there is more than one experimental condition. Venn diagrams are usually used to illustrate results of such mining (Seki et al. 2002). Data from such analyses provide insights on the gene expression patterns across several experimental conditions.

3.1.3 Functional analysis

The next step in analysis after the generation of large datasets involves associating genes to their function(s). This process, called functional analysis or functional genomics (Finkelstein et al., 2002), is an integral part of any microarray experiment as it enables investigators to draw conclusions about the system under examination on the basis of the role(s) each regulated gene plays.

In order to derive meaning from the vast amount of information generated by microarray analysis, a number of public databases such as The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) (Rhee et al., 2003), NetAffx™ (www.affymetrix.com) and Munich Information of Protein Sequences (MIPS) (namely the MIPS Arabidopsis database (MATDB)) are available free of charge to the research community. These databases are invaluable to
investigators as they help to manage large data sets and guide researchers through the functional
analysis of Arabidopsis genes.

3.1.3.1 Gene ontology

Because of the increasing demand for data exchange and experiment comparison, the Gene
Ontology Consortium developed “Gene Ontology” (GO). This GO database aims to collect and
annotate, using structured, controlled and consistent vocabularies, information that describes the
functions of genes and their gene products (The Gene Ontology Consortium, 2001). The use of
these vocabularies enables efficient usage of the biological databases (TAIR, NetAffx) as they
share a common terminology and hence allow cross-database queries (The Gene Ontology
Consortium, 2001). The GO consortium has developed a system that describes gene products in
terms of cellular component, molecular function and biological process (Rhee et al., 2003).

The GO project started in 1998 and maintains the largest database of gene function from a wide
variety of model organisms including Drosophila melanogaster (fruit fly), Rattus norvegicus
(rat), Danio rerio (zebrafish) and Saccharomyces cerevisiae (yeast) (The Gene Ontology
Consortium, 2001). TAIR became a member of the consortium in June 2000 and has
contributed significantly to the development of controlled vocabularies for describing the
function of Arabidopsis genes.

3.1.3.2 Gene annotation

The TAIR database (http://www.arabidopsis.org) is also referred to as the multiple data type
database as it consists of diverse data types at a single site for a single organism, in this case, the
model plant Arabidopsis. This database is updated weekly and it is rapidly growing with
information supplied by numerous research groups working on different aspects of this plant across the world. The TAIR database is often used for annotating *Arabidopsis* genes to cellular component, molecular function and biological processes. The locus identifiers (e.g. At4g37610) of genes selected for annotation are uploaded onto the database and the output files describe the gene ontology at cellular, molecular and biological levels.

Netaffx™ is a database maintained by Affymetrix® (www.affymetrix.com). This database can also be used to annotate genes and pick lists of probe sets associated with specific function (e.g. senescence). Researchers are given the option to either start with a list of genes to find functions or to select a list of genes with known functions and then check the regulation of these genes in the sets of microarray analysis data. Both the Netaffx™ and TAIR can be used for data mining and they enable keyword searches (e.g. abiotic stress) or searches based on gene families (e.g. cytochrome P450). Once information on genes associated with the keywords is available, investigators can analyse the regulation of these genes across the microarray project.

### 3.1.4 Aims

The main goals of this work were to:

1. carry out comparison analysis and the changes in gene expression after following the application of 0.2% (v/v) surfactant NUL1026 at 1 h and 24 after treatment. A comparison of the experimental data with the control/baseline data would generate a log value for each probe set and this log value would indicate the extent and direction of change of a mRNA transcript following foliar application of the surfactant.

2. perform data mining to identify the sets of genes that showed at least a two-fold change after treatment with surfactant NUL1026. These lists of genes included transcripts that
were regulated early (1 h post-treatment), late (24 h post-treatment) and genes that showed significant and constant expression at both 1 h and 24 h.

(3) associate the genes of interest from the data mining (2) above to their respective molecular function and biological processes in order to understand the molecular effect induced by surfactant NUL1026 on *Arabidopsis*.

### 3.2 Materials and methods

#### 3.2.1 Comparison Analysis

The MAS 5.0 (Affymetrix Technical Note, 2002a) was used to conduct the comparison analysis. Experimental data (0.2% (v/v) NUL1026, 1 h post-spraying) were compared with the baseline data (water, 1 h post-spraying). Comparison analysis was repeated for the *Arabidopsis* plants treated with 0.2% (v/v) NUL1026, 24 h post-treatment, and comparison was made with the expression data of control plants analysed 24 h after being sprayed with water. Since the first (1 h) and the last (24 h) time points of treatments were 23 hours apart, parallel control plants sprayed with water at both time-points were included to eliminate those genes that are regulated by the circadian clock. For example, if a gene is regulated by the circadian clock only, this gene should be regulated in the same manner in the treated or control sample at the same time point. As a result, this gene will not be selected as “differentially expressed” when comparing its expression level in the parallel control and the treated sample (Harmer et al., 2000).

In this study, the biological replicates (Array 1 and Array 2) for each treatment were individually compared to the control and all the probes on the array were used for comparison analysis. The average signal intensities for each probe sets of the biological replicates for each
treatment were calculated (y axis) and plotted over control samples (x axis). These scatter plots were used to illustrate the high number of genes that are both induced and repressed greater than 2-fold in response to the different treatments.

In order to filter out true variations due to experimental conditions, microarray data are usually normalised (Zien et al., 2001) and the MAS 5.0 software provides the user with the option of scaling arrays before performing a comparison analysis. Both normalisation and scaling can correct for variations between two arrays (Affymetrix Technical Note, 2002a). When normalisation is applied, the intensity of the probe sets from the experimental array are normalised to the probe sets on the baseline array. During scaling, both the intensity of the probe sets of the experiment and the baseline are scaled to a user defined target (Zien et al., 2001). Usually, scaling is the preferred method when comparing two arrays. As mentioned in Chapter 2, scaling was applied to all the arrays while performing absolute analysis and each array was scaled to a target value (TGT) of 50. Microarray data from all the treatments and the controls were comparable as indicated by the scaling factors which were within the 3-fold range (Chapter 2, Tables 2.3 and 2.4).

Comparison analysis also uses the Wilcoxon’s signed-rank test to calculate the change p-value. This p-value indicates the likelihood of the significant change and the direction of change (Affymetrix Technical Note, 2002a). The p-value ranges in scale from 0.0 to 1.0 and values close to 0.0 indicate the probability for an increase in transcript expression level in the experiment array compared to the control array, while values close to 1.0 show the possibility for a decrease in expression level (Affymetrix Technical Note, 2002a). In this study, the default
value for the parameter “gamma” (Appendix VI) was used to assign a call and this gamma value can be varied to increase or decrease the stringency of the change call made.

As with signal, the signal log ratio (SLR) for each probe sets was computed using a one-step Tukey’s Biweight method by calculating the mean of the log ratios of the probe pair intensities across the experiment versus baseline arrays for each treatment (Affymetrix Technical Note, 2002a). The SLR provides an estimate of the fold change in expression when two arrays are compared and is more accurate than a single array analysis since this approach helps to eliminate any differences in an individual probe, as ratios are derived at the probe level. A log scale of 2 was used and hence a SLR of 1.0 indicates an increase in transcript level by two-fold and -1.0 indicates a decrease by two-fold (Affymetrix Technical Note, 2002a).

3.2.2 MAS 5.0

The MAS 5.0 software consists of three components: MAS 5.0, Micro database (MicroDB™) and Data Mining Tool (DMT) version 3.0 (DMT 3.0). In this study, the MAS 5.0 was used for image, absolute and comparative analysis while data mining was carried out using the DMT software onto which data had been loaded via MicroDB™.

3.2.2.1 Publishing data via MicroDB™

Results from comparative analysis were “published” by transferring the data onto the MicroDB™ interface (Affymetrix MicroDB™ User’s Guide Version 3.0). The MicroDB™ serves as the interface between MAS 5.0 and the DMT and electronically transfers data for each array (both absolute and comparative analysis data) to DMT for data mining.
3.2.2.2 DMT 3.0 and EXCEL for selection of genes

Microarray data give signal (RNA) levels and induction ratios for each gene and these values were averaged using the biological replicates. Scatter plots were drawn to get an overall view of the expression data.

Both the DMT 3.0 and EXCEL softwares were used to facilitate the selection of genes that were commonly up- and down-regulated in both biological replicates (Array 1 and Array 2) in response to the different treatments at the two time points (Affymetrix Data Mining Tool User’s Guide) when compared to the controls. Prior to the selection of genes that were regulated significantly in response to the different treatments compared to the controls, those genes that showed inconsistent detection call (Present or Absent or Marginally Present or No Change) between replicates (Array 1 and Array 2) of the same treatment were eliminated. The steps involved in the selection of transcripts found to be significantly responsive with reliable change to the treatments are outlined in Table 3.1.

Table 3.1 DMT 3.0 and EXCEL were used to select significantly regulated genes compared to the control.

<table>
<thead>
<tr>
<th>Selection of up-regulated genes</th>
<th>Selection of down-regulated genes</th>
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<tbody>
<tr>
<td>Detection call 'Present' in treatment</td>
<td>Detection call 'Present' in control</td>
</tr>
<tr>
<td>Change call 'Increase' in treatment</td>
<td>Change call 'Decrease' in treatment</td>
</tr>
<tr>
<td>Signal Log Ratio $\geq 0.95^*$</td>
<td>Signal Log Ratio $\leq -0.95^*$</td>
</tr>
<tr>
<td>Signal value $\geq 100$ in treatment</td>
<td>Signal value $\geq 100$ in control</td>
</tr>
<tr>
<td>Same detection and change call in replicates</td>
<td>Same detection and change call in replicates</td>
</tr>
<tr>
<td>Significant up-regulation</td>
<td>Significant down-regulation</td>
</tr>
</tbody>
</table>

*A Signal Log Ratio cut-off of ±0.95 was used to select for genes that showed a 2-fold change in transcript expression.*
To accurately identify the genes induced by foliar application of the surfactant, genes giving a consistent detection call ‘Present’ in both duplicates were chosen. This dataset was further mined for those genes giving a change call of ‘I’ (increase) and showing a fold change greater than or equal to 2 with average signal values greater than or equal to 100 in both duplicate arrays. Selection of genes showing a decrease in expression in response to NUL1026 at 1 h and 24 h post-spraying was carried out by selecting for those genes giving a ‘P’ (present) call in the control, followed by those genes that decreased by at least a two-fold difference in both treatment duplicates and with signal values greater than or equal to 100 in the control.

The DMT and EXCEL were also used to identify surfactant responsive genes at both 1 h and 24 h treatments. Hence a number of meaningful queries that were relevant to the aims of this chapter were investigated and DMT and EXCEL were used to locate the genes of interest. Venn diagrams were drawn to illustrate results generated by the data mining.

3.2.3 Functional classification and GO annotations using TAIR

Each gene represented on the GeneChip array is assigned an identification termed as Probe_Set ID or array element. DMT also provides the locus identifier for each probe set (for instance, the locus identifier of Probe_Set ID 261766_at is At1g15580).

To classify a gene list into its respective functional categories, the locus identifiers were first extracted from DMT and saved as a text-delimited file (.txt). This file was then uploaded onto the GO annotation search tools of the TAIR database (www.arabidopsis.org). The relevant gene list was thus annotated to its functional and biological processes and pie-charts were used to
represent the frequency of the GO term in the list. Functional classification of genes described in this thesis was conducted in January 2006.

In this study, several queries were built based on the aims of this chapter and subsequently gene lists consisting of locus identifiers for each of those queries were loaded onto the GO annotations search tool of the TAIR to assign GO terms to each of the significantly regulated genes. The major gene lists that were investigated included:

- common genes whose transcripts levels were significantly regulated at both investigated time points (1 h and 24 h post-spraying) in response to 0.2% (v/v) surfactant NUL1026.
- genes which, under the influence of surfactant NUL1026, were exclusively up- and down-regulated at each of the two time points.

3.3 Results

3.3.1 Selection of significantly responsive genes

The scatter plots show that many genes were either induced or repressed greater than 2-fold in response to 0.2% (v/v) surfactant NUL1026 at both 1 h and 24 h (Figures 3.1 (a) - (b)).
Figure 3.1 (a)

Figure 3.1 (b)
The signal intensities were averaged from the biological replicates for each of the two time points and the signal intensities for (a) 0.2% (v/v) surfactant NUL1026, 1 h and (b) 0.2% (v/v) surfactant NUL1026, 24 h against their respective controls were plotted. Guide lines are given showing signal log ratios of 2 (induced) and (-2) repressed.

One hundred and eighty nine genes showed reliable significant up-regulation, 1 h after surfactant spraying while 216 gene transcripts showed increased expression after 24 h (Table 3.2). In contrast, 15 genes showed decreased expression after 1 h while 85 genes were down-regulated after 24 h (Table 3.3). Interestingly, at both time points, more genes were significantly up-regulated than repressed in response to the surfactant applications (Figure 3.2).

Table 3.2 Selection of significantly up-regulated genes with reliable expression in replicates (Array 1 and Array 2) in response to surfactant NUL1026, 1 h and 24 h.

<table>
<thead>
<tr>
<th>Conditions for selection</th>
<th>0.2% NUL1026 (Array 1 and Array 2), 1 h</th>
<th>0.2% NUL1026 (Array 1 and Array 2), 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of genes on the array</td>
<td>22,810</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called present in both</td>
<td>14,755</td>
<td>15,002</td>
</tr>
<tr>
<td>Total no. of genes with an increase call in both</td>
<td>913</td>
<td>1,700</td>
</tr>
<tr>
<td>Total no. of genes with an increase greater than or equal to 2 fold change in both</td>
<td>441</td>
<td>628</td>
</tr>
<tr>
<td>Total no. of genes with an increase greater than or equal to 2 fold change in both and signal value greater than or equal to 100</td>
<td>189</td>
<td>216</td>
</tr>
</tbody>
</table>
Table 3.3 Selection of significantly down-regulated genes with reliable expression in replicates (Array 1 and Array 2) in response to surfactant NUL1026, 1 h and 24 h.

<table>
<thead>
<tr>
<th>Conditions for selection</th>
<th>0.2% NUL1026 (Array 1 and Array 2), 1 h</th>
<th>0.2% NUL1026 (Array 1 and Array 2), 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of genes on the array</td>
<td>22,810</td>
<td>22,810</td>
</tr>
<tr>
<td>Total no. of genes with a present call under Control conditions</td>
<td>15,761</td>
<td>14,395</td>
</tr>
<tr>
<td>Total no. of genes with a decrease call in both</td>
<td>934</td>
<td>1,196</td>
</tr>
<tr>
<td>Total no. of genes with a decrease greater than or equal to 2 fold change in both</td>
<td>81</td>
<td>262</td>
</tr>
<tr>
<td>Total no. of genes with a decrease, greater than or equal to 2 fold change in both and signal value greater than or equal to 100 (signal should be greater than or equal to 100 in CONTROL)</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>
Figure 3.2 Genes differentially regulated in response to surfactant NUL1026 after 1 h and 24 h.

Histogram showing number of genes that were significantly and reliably induced and repressed in response to 0.2% (v/v) NUL1026 1 h post-spraying and 0.2% (v/v) NUL1026, 24 h post-treatment when compared to the controls, 1 h and 24 h respectively.

3.3.2 Data mining

The DMT 3.0 and EXCEL softwares were used to segregate up- and down-regulated genes into sets and subsets so as to identify and reveal potential insights into those genes that were exclusively or commonly regulated in response to the surfactant treatments. These datasets were displayed in Venn diagrams (Figures 3.3), which provided important overviews of the distribution of changes into shared and specific responses to the surfactant at the two time points.
Twenty-two gene transcripts of the 189 early response genes had increased transcripts levels at both investigated time points (1 h and 24 h) with surfactant NUL1026 (Figure 3.3 (a)). These genes were classified as “robust response genes”, while the remaining genes exclusive to each of the time period were classified as “transient response genes”. In contrast none of the 15 early reduced transcripts showed differential expression after 24 h (Figure 3.3 (b)). The highest number of genes that were exclusively regulated in response to the surfactant was recorded after 24 h treatment.

![Venn diagram showing gene expression comparison](image)

Figure 3.3 (a)
Figure 3.3 (b)

Figure 3.3 Venn diagrams represent the sets of genes that were regulated in response to spray application of 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment compared with the controls.

(a) The diagram shows that 22 genes were commonly induced 1 h and 24 h post-spraying while 167 transcripts were found to be exclusively up-regulated 1 h post-treatment and 194 genes were only expressed after 24 h treatment; (b) Venn diagram showing the significant down-regulation of transcripts in response to the surfactant, 1 h and 24 h post-spraying. This diagram highlights the fact that the genes that showed decreased expression after 1 h treatment did not show the same pattern of expression after 24 h.

3.3.3 Functional classification

To understand the nature of the changes in gene expression taking place when Arabidopsis plants were sprayed with surfactant NUL1026, the regulated genes were assigned functions based on automatically derived functional categories using the TAIR database. Functional classification of genes to high-level terms in the GO hierarchy was conducted for molecular and biological processes. Molecular function refers to what a gene product does at the biochemical
level, without specifying where or when the event actually takes place (Ashburner et al., 2001). In contrast, the biological function to which a gene product contributes to is referred to as a biological process (Ashburner et al., 2001).

### 3.3.3.1 Molecular function of genes exclusively regulated by surfactant NUL1026, 1 h and 24 h post-spraying

The pie-charts (Figures 3.4-3.7) show that transcriptional reprogramming in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment included genes from major functional classes such as enzyme activity, transcription factor activity, binding activity, transporter activity and genes with unknown molecular function. Figure 3.4 shows a summary of the functional categorisation of transcripts, which were exclusively up-regulated (167 genes, Figure 3.3 (a)) in response to the surfactant application, 1 h post-spraying. Apart from a large class of genes with unknown functions (13%) and genes predicted to function in binding activities (13%), the remaining genes were involved in transcription factor activity (9.9%) and other enzymatic activity (9.5%). In contrast, after 24 h treatment with surfactant NUL1026, the over-represented functional classes were the genes involved in enzymatic activity (23.7%) while 12.3% fell in the binding activity class and 10.6% had no known molecular function (Figure 3.5). Interestingly, genes belonging to the nucleic acid binding group (Figure 3.4) were not expressed after 24 h (Figure 3.5). Similarly, 0.4% of genes were only assigned to the structural molecule activity category after 24 hour (Figure 3.5).
Figure 3.4 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

Figure 3.5 Functional classification for Gene Ontology (GO) for molecular function.
This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

Functional classification of genes that were exclusively down-regulated in response to surfactant treatment, 1 h and 24 h (Figures 3.6 and 3.7 respectively), indicated that the majority of the genes could not be assigned to a specific molecular function (22.7% for the 1 h treatment and 24.1% for the 24 h treatment). The functional groups after 1 h spray application of the surfactant showed that the greatest number belonged to the transcription factor category (22.7%) followed by 18.2% of genes involved in DNA or RNA binding (Figure 3.6). Microarray analysis indicated that after 24 h treatment, categories that were well-represented included binding activity (12.7%), hydrolase activity (11.4%), enzyme activity (11.4%), transporter activity (8.9%) and transcription factor activity (7.6%) (Figure 3.7).

A higher number of genes (85 genes, Figure 3.3 (b)) showed reduced RNA levels after 24 h surfactant treatment as opposed to after 1 h (15 genes, Figure 3.3 (b)). These 85 genes were more widely distributed compared to those genes after 1 h surfactant application and included categories such as nucleotide binding (3.8%), structural molecule activity (2.5%), other molecular function (2.5%) and receptor binding or activity (1.3%) (Figure 3.7). These categories were not recorded at the 1 h time point (Figure 3.6). Overall, it should be noted that genes classed as being members of the structural molecule activity were differentially regulated only after 24 h of surfactant NUL1026 application.
Figure 3.6 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

Figure 3.7 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.
3.3.3.2 Molecular function of genes responsive to surfactant NUL1026, at both 1 h and 24 h time-points

Comparison of the gene lists consisting of down-regulated genes 1 and 24 h after being sprayed with surfactant NUL1026 revealed that there was no common gene between the two sets of genes responding to NUL1026. In contrast, molecular categorisation of the 22 up-regulated genes (Figure 3.3 (a)) that were common between the two time points showed that the majority of these genes had no known molecular function (28.6%) (Figure 3.8). The remaining genes belonged to the functional classes of: other enzyme activity (17.9%), other binding activity (17.9%), transcription factor activity (10.7%) while the least represented group was the “other molecular function” (3.6%) (Figure 3.8).

Figure 3.8 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were commonly up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.
3.3.3.3 Biological processes of genes exclusively regulated by surfactant NUL1026, 1 h and 24 h post-spraying

The pie-charts (Figures 3.9 - 3.12) show that where gene function could be assigned, the majority of the transcripts that were regulated in response to 0.2% surfactant NUL1026, at both 1 h and 24 h spraying, encoded proteins that were associated with a number of broadly defined but key biological processes. These biological activities included physiological processes, metabolism, cellular processes, transcription, response to stress, signal transduction, cell organisation and biogenesis and response to abiotic or biotic stimulus. At both 1 h and 24 h time points, in response to surfactant NUL1026, there was considerable up-regulation of genes that were classed in the “response to abiotic or biotic” category (Figures 3.9 and 3.10). Compared with the 1 h treatment, more genes (13.3%) were categorised in the “response to abiotic or biotic” group after 24 h (Figure 3.10). Likewise, a higher percentage of genes (7.4%) were classified under the stress response group after 24 h spraying as compared with after the 1 h treatment (3.9%) (Figure 3.9). However, there were a higher proportion of genes encoding transcription factor proteins after 1 h treatment (5.3%) (Figure 3.9) whereas after 24 h, only 1.4% of the RNA transcripts were associated with transcription (Figure 3.10).
Figure 3.9 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

Figure 3.10 Functional classification for Gene Ontology (GO) for biological processes.
This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

Functional classification of genes exclusively down-regulated after 1 h showed that the largest percentage of genes encoded proteins involved in metabolism (17.9%) (Figure 3.11) whereas after 24 h, genes classified as involved in cellular processes were the most highly represented (17%) (Figure 3.12). Moreover, a comparison of Figures 3.11 and 3.12 revealed that there were a higher proportion of down-regulated genes that were associated with cell organisation and biogenesis after 24 h (4.4%) than after 1 h (2.6%). It was also noted that the 85 significantly down-regulated transcripts after 24 h were more widely distributed and 2.2% of these late response genes were classed into the protein metabolism category while 1.5% were associated with the electron transport or energy pathways group (Figure 3.12). Genes belonging to such categories did not show reduced expression after the 1 h time period (Figure 3.11).

Figure 3.11 Functional classification for Gene Ontology (GO) for biological processes.
This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

![Functional Categorization by Genes for: GO Biological Process](image)

**Figure 3.12** Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

### 3.3.3.4 Biological processes of genes responsive to surfactant NUL1026, at both 1 h and 24 h time points.

TAIR (January 2006) was unable to assign 15.9% of the total 22 commonly up-regulated genes at both time intervals to any known biological function. However, the remaining genes were classified as belonging to key biological processes such as response to stress and abiotic or biotic stimulus, transcription, protein metabolism and signal transduction (Figure 3.13).
This pie-chart shows the frequency of GO terms in the gene lists that were commonly up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-spraying when compared with the control. Classification was carried out in January 2006 using the TAIR database.

### 3.3.4 GO annotations

Each of the gene expression data lists consisting of locus identifiers was annotated using the TAIR database (January 2006). The locus identifiers were assigned to GO terms and were also linked to one or more GO terms. The GO terms were classified into function, process and component and were presented as Directed Acyclic Graph (DAG) or networks. Results showed that biological processes regulated by surfactant NUL1026 included GO terms such as response to wounding, response to auxin stimulus, transcription factor activity and toxin catabolism (Tables 3.4-3.8). Other frequently occurring GO terms of genes regulated in response to the surfactant their association with cellular components- the nucleus, the mitochondrion and the chloroplast (Tables 3.4-3.8). Tables 3.4 to 3.8 are annotations of a representative group of differentially expressed genes by surfactant NUL1026, 1 h and 24 h post-application. Please
refer to the Supplementary data for the complete annotation of the list of genes regulated by the various treatments.

Table 3.4 Annotation of a representative list of genes that were up-regulated exclusively in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>261037_at</td>
<td>At1g17420</td>
<td>lipoxygenase, putative</td>
<td>response to wounding iron ion binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>lipoygenase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>jasmonic acid biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>defense response</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>chloroplast</td>
</tr>
<tr>
<td>248799_at</td>
<td>At5g47230</td>
<td>ethylene responsive element binding</td>
<td>ethylene mediated signaling pathway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>transcription factor activity</td>
<td>nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ethylene mediated signaling pathway</td>
<td>regulation of transcription, nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ethylene mediated signaling pathway</td>
<td>regulation of transcription, DNA-dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ethylene mediated signaling pathway</td>
<td>DNA binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ethylene mediated signaling pathway</td>
<td>nucleus</td>
</tr>
<tr>
<td>267154_at</td>
<td>At2g30870</td>
<td>glutathione-s- transferase, putative</td>
<td>toxin catabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cytoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>glutathione transferase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>response to water deprivation</td>
</tr>
<tr>
<td>257644_at</td>
<td>At3g25780</td>
<td>allene oxide cyclase, putative, AOC2</td>
<td>jasmonic acid biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>chloroplast</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>allene-oxide cyclase activity</td>
</tr>
<tr>
<td>264758_at</td>
<td>At1g61340</td>
<td>F-box family protein</td>
<td>molecular function unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>biological process unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mitochondrion</td>
</tr>
</tbody>
</table>
Table 3.5 Annotation of a representative list of genes that were up-regulated exclusively in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>263807_at</td>
<td>At2g04400</td>
<td>indole-3-glycerol phosphate synthase <em>(IGPS)</em></td>
<td>indole-3-glycerol-phosphate synthase activity, tryptophan biosynthesis, chloroplast</td>
</tr>
<tr>
<td>253046_at</td>
<td>At4g37370</td>
<td>cytochrome P450, putative</td>
<td>electron transport, iron ion binding, heme binding, oxygen binding, endomembrane system, monooxygenase activity</td>
</tr>
<tr>
<td>263948_at</td>
<td>At2g35980</td>
<td>harpin-induced family protein <em>(YLS9)</em></td>
<td>response to pathogen, cellular component, unknown, molecular function, unknown</td>
</tr>
<tr>
<td>264960_at</td>
<td>At1g76930</td>
<td>proline-rich extensin-like family protein</td>
<td>endomembrane system, structural constituent of cell wall, cell surface (sensu Magnoliophyta), endomembrane system, cell surface (sensu Magnoliophyta), response to abscisic acid, stimulus, response to salicylic acid, stimulus, response to wounding, response to jasmonic acid, stimulus</td>
</tr>
<tr>
<td>264787_at</td>
<td>At2g17840</td>
<td>senescence/dehydration-associated protein-related <em>(ERD7)</em></td>
<td>response to cold, chloroplast</td>
</tr>
</tbody>
</table>
response to salt stress
response to water deprivation
response to high light intensity
molecular function unknown

Table 3.6 Annotation of a representative list of genes that were down-regulated exclusively to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>260727_at</td>
<td>At1g48100</td>
<td>glucoside hydrolase family 28 protein</td>
<td>polygalacturonase activity</td>
</tr>
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<td></td>
<td></td>
<td>mitochondrion</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>carbohydrate metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>polygalacturonase activity</td>
</tr>
<tr>
<td>259751_at</td>
<td>At1g71030</td>
<td>putative myb family transcription factor</td>
<td>regulation of transcription, DNA-dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>transcription factor activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DNA binding</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>mitochondrion</td>
</tr>
<tr>
<td>250569_at</td>
<td>At5g08130</td>
<td>basic helix-loop-helix (bHLH) family protein (BIM1)</td>
<td>protein binding</td>
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<td>nucleus</td>
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<td>DNA binding</td>
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<td>247452_at</td>
<td>At5g62430</td>
<td>Dof-type zinc finger domain-containing protein (CDF1)</td>
<td>regulation of transcription</td>
</tr>
</tbody>
</table>
regulation of timing of transition from vegetative to reproductive phase
DNA binding
nucleus
DNA binding
negative regulation of transcription
transcription factor activity
protein binding

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
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<tbody>
<tr>
<td>247037_at</td>
<td>At5g67070</td>
<td>rapid alkalinization factor (RALF) family protein</td>
<td>apoplast</td>
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<td>248074_at</td>
<td>At5g55730</td>
<td>fasciclin-like arabinogalactan-protein (FLAI)</td>
<td>cell-cell signaling</td>
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<td></td>
<td></td>
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<td>248912_at</td>
<td>At5g45670</td>
<td>GDSL-motif lipase/hydrolase family protein</td>
<td>endomembrane system</td>
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<td></td>
<td></td>
<td>carboxylic ester hydrolase activity</td>
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<td></td>
<td>hydrolase activity, acting on ester bonds</td>
</tr>
<tr>
<td></td>
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<td>lipid metabolism</td>
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Table 3.7 Annotation of a representative list of genes that were down-regulated exclusively to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared to the control.
transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related regulation of transcription regulation of transcription, DNA-dependent transcription factor activity nucleus

Table 3.8 Annotation of a representative list of genes that were commonly up-regulated to 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>254331_at</td>
<td>At4g22710</td>
<td>cytochrome P450 family protein</td>
<td>oxygen binding</td>
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<td>electron transport</td>
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<td></td>
<td></td>
<td></td>
<td>monooxygenase activity</td>
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<td></td>
<td></td>
<td>iron ion binding</td>
</tr>
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<td></td>
<td></td>
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<td>N-terminal protein</td>
</tr>
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<td>myristoylation</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>endomembrane system</td>
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<td></td>
<td></td>
<td>heme binding</td>
</tr>
<tr>
<td>254432_at</td>
<td>At4g20830</td>
<td>FAD-binding domain-containing protein,</td>
<td>electron carrier activity</td>
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<td></td>
<td>cell wall (sensu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Magnoliophyta)</td>
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3.4 Discussion

Detailed analyses of genes significantly regulated 1 h and 24 h after surfactant applications have provided an intriguing insight into the nature of the early and late events triggered by surfactant NUL1026. This is the first comprehensive study of the genome-wide response of *Arabidopsis* to an etheramine surfactant.

3.4.1 Early surfactant responsive genes

The most striking observation is the predominance of transcription factor genes, 1 h after surfactant treatment (Figure 3.9). Their rapid induction or repression indicates that they are
important members of an early-response transcriptional network under surfactant control and may subsequently prompt a second wave of gene expression that may modulate more specific developmental or physiological processes.

3.4.1.1 Early surfactant responsive genes involved in transcriptional regulation

Figure 3.4 shows that the early response genes exclusively up-regulated in response to the surfactant contain a high percentage of genes involved in transcription regulation, namely 9.9% of the regulated genes and this value is higher than the 5.5% level at which these genes are present in the whole Arabidopsis genome (Riechmann et al., 2000). In particular, transcription factors from eight different transcription factor families were rapidly up-regulated, including three members of the Myb-domain protein (MYB51- At1g18570, At3g23250 and MYB73-At4g37260, nine members of the ethylene response factor (ERF)/APETALA2 (AP2) transcription factor (At1g28370, At2g44840, At5g51190, At3g23230, At5g61600, ERF6-At4g17490, ERF2- At5g47220, ERF5- At5g47230 and At4g34410), a member of the RAV2/AP2 domain protein (RAV2- At1g68840), three members of the WRKY family (the name of the WRKY family is derived from the most prominent feature of these proteins, a highly conserved amino acid sequence WRKYGQK) (Eulgem et al., 2000) (WRKY40- At1g80840, WRKY46- At2g46400 and WRKY18- At4g31800), a member of the Dof proteins (ADOF1-At1g51700), two members of the CCCH-type protein (At3g55980 and At2g40140), a member of the C2H2 type protein (ZAT11- At2g37430), two members of the no apical meristem (NAM) domain (NAC) (At3g49530 and At5g24590) and two members of the CCR4-NOT transcription complex protein (At3g44260 and At5g22250).
Fewer genes (15) were rapidly down-regulated following surfactant treatment (Table 3.3) among which, five genes code for transcription factor proteins. These included two members from the Myb family proteins (At3g71030 and At5g17300), two Dof transcription factors (*CDF1*-At5g62430 (and At3g47500) and a gene coding for the basic helix-loop-helix (bHLH) protein (*BIMI*- At5g08130).

Regulation of genes coding for transcription factors is of particular interest as they influence and modulate the coordinated expression of numerous downstream target genes and many have been shown to control entire metabolic and developmental processes within the plant (Chen et al., 2002; Davletova et al., 2005). These specific transcription factors, in particular those belonging to the WRKY, ERF/AP2 and Myb-domain proteins have previously been implicated in the plant response to general stress, pathogens, wounding, senescence, hormones (auxin, ethylene and abscisic acid) and secondary metabolisms and are further discussed in the specific sections below (Kranz et al., 1998; Eulgem et al., 2000; Chen et al., 2002; Nishiuichi et al., 2002; Yang et al., 2005).

### 3.4.1.2 Early surfactant responsive genes involved in signal transduction

TAIR classification of genes regulated in response to the surfactant, 1 h post-spraying showed that 3.3% (Figure 3.9) of the regulated genes were involved in signal transduction. A general signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates (IP), calcium and reactive oxygen species (ROS)), which subsequently initiate a protein phosphorylation cascade. This cascade then either targets proteins directly associated with cellular protection or target transcription factors controlling specific sets of stress and defence regulated genes. The products of these regulatory genes can
in turn initiate a second wave of signalling that follows the same generic pathway, which may or may not involve different components of the signal transduction pathways (Xiong et al., 2002).

Calcium signaling is an important component of plant response to diverse stimuli such as touch, light, pathogens, hormones and temperature (Reddy, 2001). Proteins that bind the Ca$^{2+}$ are important sensors that detect changes in cytosolic Ca$^{2+}$ and trigger specific cellular responses to different extracellular signals via interaction with target proteins (Delk et al., 2005). Significant induction of genes coding for Ca$^{2+}$ sensors namely the calmodulin related touch gene 2 (*TCH2*-At5g37770), the calcium dependent protein kinase (CDPK) 32 (*CPK32*-At3g57530) and CPK4 (At4g09570), and a C2 domain-containing protein showing similarity to a CDPK (At4g34150), implies the involvement of calcium as a second messenger in early surfactant response. Further supporting this likely theory of surfactant induced calcium mediated signaling is the significant up-regulation of the calcium - transporting ATPase 1 (*ACA1*-At1g27770) gene that encodes a Ca$^{2+}$ pump which maintains the cytoplasmic calcium levels low - a prerequisite for second messenger function (Bonza et al., 2000; Geisler et al., 2000).

Members of the mitogen activated protein kinase (MAPK) family were also found to be early-surfactant responsive as shown by the increased in transcript abundance of the MAP kinase 3 (*MPK3*-At3g45640), MAPK kinase 9 (*MKK9*-At1g73500) and the MAP kinase 4 (*MPK4*) substrate 1 (*MKS1*-At3g18690) genes. As with the calcium-signaling pathway, the MAPK signaling pathways have also previously been shown to be activated by various stimuli such as wounding, drought, pathogen, reactive oxygen and hormones. For instance, *MPK3* is highly reactive to hydrogen peroxide and a loss-of-function genotype of MPK3 has rendered mutant plants more ozone sensitive (Miles et al., 2005). These protein kinases play a critical role in
transferring information from sensors to cellular responses (Kovtun et al., 2000; Zhang and Klessig, 2001; Scherer, 2002; Chang, 2003; Ouaked et al., 2003; Teige et al., 2004; Andreasson et al., 2005).

Hence, up-regulation of components of the calcium signaling pathways and the MAPK cascades infers that different signal transduction pathways are involved in mediating intracellular transmission and amplification of surfactant responses, to subsequently result in the appropriate biochemical and physiological cellular responses.

3.4.1.3 Early surfactant responsive genes involved in cell wall reconstruction

Within the cell organisation and biogenesis category of Arabidopsis genes (Figure 3.9), the transcript levels of genes coding for cell wall components (At1g59910), cell wall modifying enzyme, xyloglucan endotransglycosylase (XTH) 22 (XTH22- At5g57560) and a putative XTH18 (At4g30280), showed a 2-fold increase in response to surfactant NUL1026 stimulus. XTHs consists of a large multigene family of enzymes involved in the construction and restructuring of the cell wall framework (Bourquin et al., 2002; Vissenberg et al., 2005). Surfactants function by altering or disrupting cell membrane structure and an increase in expression of the XTH genes in this investigation may imply a role in repairing and consolidating the damaged cell wall structure.

3.4.1.4 Early surfactant responsive genes involved in disease resistance

Interestingly, this study recorded an increase in RNA levels of a number of disease resistance (R) genes upon surfactant treatment. These R genes encode proteins that share one or more similar motifs such as the leucine-rich repeat (LRR) regions, nucleotide-binding sites (NBS) and
kinase domains (Century et al., 1997; Kobe and Kaja, 2001). In this investigation, the up-regulated \( R \) genes registered belonged to the Toll and Interleukin-1 receptor (TIR) - NBS- LRR class (At1g56510 and At3g44630), the TIR-NBS class (At1g66090, At1g72900 and At1g72940) and the TIR class (At1g65390). An \( R \) gene (At2g34930) containing the LLR domain also showed transcript abundance. The \( R \) genes allow plants to resist infection from a broad spectrum of pathogens such as bacteria, fungi, viruses and nematodes by expressing specific corresponding avirulence genes (Century et al., 1997).

The induced expression of the putative \( R \) genes in this study indicates their involvement in surfactant response as well. Foliar application of surfactant NUL1026 could potentially have induced the same pathogen-like effects on the leaves, thereby triggering the induction of the \( R \) genes. However, some scientists have argued that many of the \( R \) genes are not involved in defence (Katagiri, 2004). Rather, these \( R \) genes showed differential gene expressions due to alterations in cellular state that arise from pathogen infections (Katagiri, 2004); in this case, probably due to cellular changes from surfactant action. Further biochemical and molecular analysis is required to clarify the physiological role of the \( R \) genes as a response to surfactant NUL1026.

**3.4.2 Late surfactant responsive genes**

After 24 h of surfactant treatment, the number of induced and repressed genes increased considerably, with 216 up-regulated genes exhibiting \( \geq 2 \) fold increase in transcript abundance (Table 3.2) and 85 down-regulated genes showing \( \geq 2 \) fold decrease (Table 3.3). Twenty-two of the 189 up-regulated early response genes still showed \( \geq 2 \) fold increase in RNA levels 24 h
after treatment (Figure 3.3). These “robust response genes” may prove to be significant informative reporters about the surfactant status.

Different functional classes of genes were up- and down-regulated after 24 h among the regulated genes (Figures 3.10 and 3.12). In general, over-represented functional groups among the up-regulated genes included those genes responsive to abiotic or biotic stimulus with a total of 13.3% of the up-regulated genes, compared with 3.4% of the total *Arabidopsis* genome, stress-related genes which represented 7.4%, compared with a genomic portion of 1.9%, genes involved in transport (4.8% versus 2.8%), genes involved in electron transport and energy pathways (4.4% versus 1.8%) and signalling genes (2.1% versus 1.4%) (Figure 3.10). Among the down-regulated genes, genes involved in transport (7.4% versus 2.8%), developmental processes (7.4% versus 1.9%), cell organisation and biogenesis (4.4% versus 2.1%) and genes responsive to abiotic or biotic stimulus were more abundant (6.7% versus 3.4%) (Figure 3.12)

Collectively, this indicates that 24 h after surfactant treatment, specific transcriptomic changes have taken place that reflect the reprogramming of a number of cellular, physiological and metabolic processes (Figures 3.10 and 3.12). Some of these changes are discussed in more detail in the forthcoming sections.

**3.4.2.1 Late surfactant responsive genes associated with transcription factors**

Data analysis showed that the expression level of most of the transcription factors that were significantly up-regulated after 1 h of surfactant NUL1026 treatment were below the threshold after 24 h. Two transcription factors were the exceptions (*WRKY33*- At2g38470 and *ERF1*-At4g17500) and their RNA levels stayed elevated after 24 h. The WRKY33 transcription factor has been shown to be *in vitro* substrate of the *Arabidopsis MPK4* which is required for pathogen
defence responses (Andreasson et al., 2005). The ERF1 transcription factor is involved in ethylene and jasmonic acid (JA) signaling and is discussed further in the specific sections below. In contrast, none of the transcription factors that were strongly down-regulated post 1 h treatment were identified after 24 h. Overall, this suggests that the transcription factors that were up- and down-regulated 1 h after foliar application of surfactant NUL1026 worked transiently and have probably activated a second cascade of transcription factors.

Among the transcription factor genes that were strongly activated after 24 h spray application of the surfactant were two NAC (At3g10500 and At5g63790), one transcription factor showing similarity to bHLH (At1g10585), one TAZ zinc finger (At5g67480), one WRKY (WRKY70-At3g56400) and one basic region/leucine zipper motif (bZIP) 60 (bZIP60- At1g42990) family member. The plant specific WRKY70 has been demonstrated to be a component of the salicylic acid (SA) and JA- mediated signal pathways and acts by integrating these two signals (Eulgem et al., 2000; Li et al., 2004) while the transcription factor bZIP60 has been previously shown to regulate the endoplasmic reticulum stress response (Iwata and Koizumi, 2005). The transcription factor genes that showed decreased expression after 24 h are known to play a range of different roles in plant metabolism, cell and tissue development and these included a member of the bHLH family (At2g18300) and two members of the homoebox leucine zipper proteins (protodermal factor 1 (PDF1)- At4g04890 and PDF2- At4g21750) (Sessions et al., 1999; Abe et al., 2001; Abe et al., 2003; Heim et al., 2003)

3.4.2.2 Late surfactant responsive genes involved in cell wall breakdown

Data mining revealed that 24 h after treatment with surfactant NUL1026, a larger number of genes known to be involved in cell wall processes were significantly down-regulated than up-
regulated. These genes included the expansin (EXP) genes (EXP1- At1g69530, EXP3-AT2g37640, EXP8- AT2g40610 and EXPB3- At4g28250), which were not found to be expressed after 1 h treatment with the surfactant. Expansin genes belong to a complex multigene family and encode cell wall loosening proteins required for stress relaxation and cell wall extension (Lee et al., 2001a; Wu et al., 2001; Li et al., 2002).

This family has been subdivided into three categories and includes the α - expansin, β - expansin and expansin-like genes. Expansin genes are differentially regulated by environmental and hormonal stimuli such as auxin and ethylene (Lee et al., 2001a; Wu et al., 2001; Li et al., 2002) and the reduced expression of some of the expansin genes members in this investigation may be reflecting the modification and disassembly of the cell walls induced by the surfactant. A member of the XTH family (EXGT-A1- At2g 06850) and a putative XTH (At2g36870) were also found to be down-regulated after 24 h and this further highlights the fact that the surfactant may be causing a breakdown of the cell wall framework.

Surfactant NUL1026 was also found to regulate the xyloglucan endotransglycosylase (XET)-related (XTR) gene 6 (XTR6- At4g25810) which encodes cell wall modifying enzymes that control the properties of cell walls during development and environmental stress (Xu et al., 1996). Concomitant with this, the cellulose synthase-like (Csl) gene (CSLE- At1g55850) that is hypothesised to encode for Golgi-localised β-glycan synthases which polymerise the backbones of non-cellulosic polysaccharides in cell walls (Liepman et al., 2005) was overexpressed in response to the surfactant. Strong expression was also observed for the extension 4 (EXT4-At1g76930) gene previously shown to accumulate in response to abiotic and biotic stresses (Elliott and Shirsat, 1998; Yoshiba et al., 2001). Overexpression of these genes may be to
reinforce the cell wall structure that may have altered as a result of the physical or chemical damage caused by the surfactant. Previous studies by Feng et al. (1999) have highlighted the fact that certain surfactants cause tissue damage which subsequently promotes herbicide penetration and the differential expression of genes involved in the cell wall structure in response to surfactant NUL1026 may imply the probable mode of action of this surfactant on the leaf surface.

3.4.3 Surfactant regulated genes associated with detoxification

The up-regulation of a number of genes primarily known to be associated with detoxification of foreign compounds in plants was recorded at both time periods in this study. Metabolism of xenobiotic compounds in plants often includes 3 main stages, the first Phase (Phase I), generally catalysed by the cytochrome P450 monooxygenase superfamily, a xenobiotic compound may be oxidised, reduced or hydrolysed to reveal or introduce a functional group, thereby rendering the parent compound more water soluble, and usually less toxic than the original xenobiotic (Downie et al., 2004). Results in the present study showed that surfactant NUL1026 was capable of inducing transcriptional up-regulation of several Phase I enzymes; five isoforms of the early surfactant responsive cytochrome P450 transcripts were regulated after 24 h including \textit{CYP71B6} (At2g24180), \textit{CYP72A8} (At3g14620), \textit{CYP72A15} (At3g14690), At3g28740 and \textit{CYP81D8} (At4g37370).

In Phase II of xenobiotic detoxification, the activated metabolite is deactivated by conjugation with endogenous hydrophilic compounds such as malonate, glucose or glutathione (GSH) to form more hydrophilic and even less toxic compounds compared with the original parent compound (Wagner et al., 2002; Downie et al., 2004). These Phase II reactions are usually
catalysed by glycosyl-, malonyl- or glutathione S-transferases (GST). Interestingly, the up-regulation of the gene coding for the enzyme, glutathione synthetase (GSH2- At5g27380), involved in the biosynthesis of GSH was recorded only after 24 hour and not at 1 h post-treatment. GSTs are encoded by large gene families (Marrs, 1996; Nutricati et al., 2006) and the plant specific classes, namely the tau (GSTU) and the phi (GSTF) are the two largest groups (Edwards et al., 2000). The mammalian and non-mammalian theta (GSTT) and the zeta (GSTZ) groups have the least number of members (Edwards et al., 2000). An hour after surfactant application, only one gene encoding the enzyme GSTF4 (At2g30870) showed an increase in transcript abundance.

However, interestingly, after 24 h, the most highly inducible GST genes were found in the tau class (GSTU24- At1g17170, GSTU7- At2g29420, GSTU4- At2g29460, GSTU1- At2g29490, GSTU8- At3g09270, GSTU25- At1g17180 and GSTU22- At1g78340). Two GSTs from the phi class, namely, GSTF6 (At1g02930) and GSTF3 (At2g02930) and one GST (GSTZ1- At2g02390) from the zeta class were also up-regulated. Another enzyme involved in catalysing the conjugation process in the Phase II reaction, the UDP-glucose-dependent glucosyltransferase UGT72B1 gene transcript (At4g 01070) also had increased RNA levels after 24 h and this implies that different enzymes may be involved in rendering the surfactant molecules less toxic.

The third phase of detoxification involves the conjugated xenobiotic compound being carried from the cytosol by transport proteins to be sequestered into the vacuole (Coleman et al., 1997; Downie et al., 2004). The proteins performing the transportation and sequestration belong to the ATP binding cassette (ABC) transporter family and the multidrug-resistance (MDR) associated proteins. Across each of the examined time points (1 h and 24 h) in this study, a member of the
MDR associated proteins were up-regulated (At1g61890, after 1 h and \textit{MRP}4- At2g47800 and \textit{MRP}14- At3g59140 after 24 h) in response to surfactant NUL1026. An ABC transporter gene transcript also showed increased expression after 24 hour, namely the gene for pleiotropic drug resistance 12 (\textit{PDR}12- At1g15520).

The data presented in this section is consistent with other reports that show similar patterns of expression of members of the cytochrome P450 family, GSTs and ABC and MDR transporter proteins, in response to a xenobiotic compound (Mingot et al., 1999; Downie et al., 2004; Baerson et al., 2005). The level of surfactant used in these experiments may likely be exerting a xenobiotic perturbation when sprayed \textit{Arabidopsis} plants. However, burgeoning research has also shown that individual GSTs and P450 are not only involved in xenobiotic detoxification but are also differentially regulated in response to plant development (cell growth, senescence), various pathogens, environmental changes, heavy metals and chemical treatments (Edwards et al., 2000; Wagner et al., 2002; Almeras et al., 2003; Smith et al., 2004; Lee et al., 2005).

Hence an alternative possibility to explain the up-regulation of these genes could be as a result of accumulation of toxic metabolites brought about by the disruption of metabolic pathways and/or production of reactive oxygen species (ROS) induced by surfactant NUL1026. It is highly probable that application of surfactant NUL1026 had induced a secondary oxidative stress, as the respiratory burst oxidase protein D (\textit{rbohD}) gene (At 5g47910), involved in the production of reactive oxygen intermediates (Torres et al., 2005) had increased expression throughout the two time periods. Further studies based on assessing the metabolism-based degradation of surfactant NUL1026 by looking at metabolites of this surfactant may give a clearer idea of role of these “detoxification” genes in this study.
3.4.4 Surfactant-regulated genes associated with signaling of hormones and metabolism

Plant hormones such as auxin, ethylene, JA, abscisic acid (ABA), cytokinin and salicylic acid modulate a vast array of plant responses affecting growth and development and defence against abiotic and biotic stresses (Tiryaki and Staswick, 2002). These plant signals interact with each other in a complementary and antagonistic manner to achieve their signaling roles. Over the years, many hormone response mutants have been isolated and characterisation of these mutants has provided further evidence of interaction among plant hormones at the level of signal transduction (Mussig et al., 2002b; Swarup et al., 2002; Goda et al., 2004; Jalali et al., 2006). For instance, cross-communication between defence signaling pathways endow the plant with an elaborate regulatory potential thereby leading to the activation of the most suitable defence mechanism against the pathogen (Christensen et al., 2000; Van Wees et al., 2000; Lorenzo et al., 2003; Jalali et al., 2006; Li et al., 2006).

In some cases, there might be cooperation between different signal transduction pathways leading to an enhanced resistance against the pathogens. In other cases, antagonism between pathways allows the defence response to be controlled in an orderly manner (Spoel et al., 2003). Furthermore, none of these signal pathways are completely specific to a particular environmental stimulus and the involvement of multiple signals in response to multiple environmental cues implies that plants use signal networks rather than independent linear pathways. This highlights the fact that interactions between the pathways are the norms rather than an exception.
3.4.4.1 Jasmonic acid

JA, a plant signalling molecule, plays a major role in a number of abiotic and biotic stresses but is best known for its role in wound responses (Creelman and Mullet, 1997; Creelman and Mulpuri, 2002; Wang et al., 2005; Liu and Wang, 2006). Linolenic acid (LA), the precursor of jasmonic acid, is stored conjugated to phospho- and galacto-lipids within cell membranes. During wounding or when subjected to biotic stress, such as insect or fungal attack (Farmer and Ryan, 1992), cell membrane structure is altered or disrupted, releasing the bound LA. LA represents the main substrate for lipoxygenase (LOX) enzymes that catalyse the conversion of LA to lipid hydroperoxides by a process termed as lipid peroxidation (LPO).

Surfactants are known to function either as enhancers of cuticle penetration by predisposing the plant cuticular membrane to solute transfer or by functioning as co-solvents thereby enhancing the movement of herbicidal active ingredients into the plant cells. Surfactant NUL1026 may have altered the plant cell membrane structure, which in turn may have activated membrane bound phospholipases to cleave and release the linolenic acid from its conjugated form. At both 1 h and 24 h treatments with surfactant NUL1026, transcripts encoding key enzymes involved in JA biosynthesis and signaling pathways were up-regulated. These included the enzymes LOX (LOX3- At1g17420 and a putative LOX- At1g72520, 1 h; and LOX2- At3g45140, 24 h), allele oxide synthase (AOS- At5g42650, 1 h), allele oxide cyclases (AOC2- At3g25780, 1 h and AOC1- At3g25760, 24 h) and 12-oxophytodienoic acid reductases (OPR2- At1g76690, 1 h and 24 h; OPR3- At2g06050 and a putative OPR similar to OPR1 and OPR2- At1g17990, 24 h).

In the investigation, isozymes of the different enzymes involved in JA biosynthesis were recorded at the two time points. For instance, the transcript coding for the LOX3 enzyme was
up-regulated after 1 h of surfactant application but its expression faltered after 24 h and the regulation of the LOX2 gene was registered only after 24 h. Accumulating evidence seems to suggest that expression of specific isoforms of these enzymes may play an important role in providing specific signaling molecules depending on the stress factors and the stage of progression of the stress (He et al., 2002; Agrawal et al., 2004). Regulation of these isozymes might also be plant tissue specific. Moreover, studies have also shown that the intermediates in the jasmonate pathways, such as the 12-oxo-phytodienoic acid (OPDA) and LOX, also play important regulatory roles in defence and wound responses along with JA (Stintzi and Browse, 2000; Kachroo et al., 2001; Danon et al., 2005). Hence the specific regulation of the isozymes at the two time intervals may be as a result of the stress response induced by surfactant NUL1026, independent of JA biosynthesis.

*Arabidopsis* has 3 *OPR* genes, *OPR1*, *OPR2* and *OPR3* and the transcription of *OPR1* and *OPR2* genes are said to be wound induced (Biesgen and Weiler, 1999) while *OPR3* protein products has been reported as the sole enzyme catalysing the reduction of OPDA in the JA pathway (Schaller et al., 2000). Interestingly, this study did not register a detectable transcript signal of the *OPR3* gene on the microarray, 1 h after treatment. However, the fact that the gene *CYP79B2*, a member of the cytochrome P450 from the CYP 79 family was up-regulated at that time point strongly indicates the presence of JA.

The *CYP79B2* gene encodes one of the key enzymes involved in the production of glycosilonates in the tryptophan (Trp) pathway. Glycosilonates are natural plant metabolites that play a role in plant defence against insects and fungi (Duan et al., 2005) and their biosynthesis is strongly promoted by JA (Mikkelsen et al., 2003). Along with the increase in
expression level of the *CYP79B2* gene at 1 h and 24 h, several other genes whose products are involved in the Trp biosynthesis pathways and subsequent production of glycosilates were also up-regulated. These included genes encoding the anthranilate synthase alpha subunit (*ASAI*- At5g05730) and a putative ASA beta subunit (At5g57890) (which catalyse the conversion of chorismate into anthralinate, the first committed step in the biosynthesis of Trp), anthranilate phosphoribosyltransferase (*Trp1*- At5g17990) (which catalyses the second step in the tryptophan pathway) and the tryptophan synthase alpha chain (*TSA1*- At3gg54640) (involved in the formation of indole destined for Trp biosynthesis) (Rutherford et al., 1998; Ouyang et al., 2000; Woodward and Bartel, 2005). Furthermore, as mentioned previously, this study also recorded an increase in RNA level in the gene encoding the MYB51 transcription factor and previous research has shown that this transcription factor activates Trp gene expression in *Arabidopsis* (Bender and Fink, 1998).

### 3.4.4.2 Auxin

Interestingly, surfactant treatments also seemed to regulate genes associated with the plant natural hormone, indole-3-acetic acid (IAA). This is consistent with previous research reporting auxin synergism at low surfactant concentrations (Stowe, 1960; Stowe and Obreiter, 1962). Tryptophan and its indolic precursors are the sources of not only glycosilates but also IAA. The enzyme CYP79B2 converts Trp to indole-3-acetaldoxime (IAOX), and this IAOX is the precursor of either IAA or glycosilates (Hull et al., 2000; Mikkelsen et al., 2000; Ljung et al., 2005). At both 1 h and 24 h post-treatment, surfactant applications resulted in the up-regulation of two different calcium binding proteins (CBPs), namely an early pinoid (PID) binding protein 1 (*PBP1*- At5g54490) and touch 3 (*TCH3*- At2g41100) genes. The mRNA expression of these genes has been shown to be activated by auxin respectively (Benjamins et al., 2003). These two
auxin responsive genes function by interacting with PID, a protein serine/threonine kinase, that functions as efflux carriers in polar auxin transport and is a key component in auxin signaling (Benjamins et al., 2003). PBP1 products act as a cofactor and promote PID activity while TCH3 proteins negatively regulate PID activity in order to maintain auxin homeostasis (Benjamins et al., 2003).

The overexpression of two auxin efflux carriers implicated in polar auxin transport (At1g76520 and At2g17500) and the repression of the auxin-herbicide resistant 1 gene (AUX1- At2g38120), which mediates influx of IAA into cells (Swarup et al., 2004) were also registered 24 h after surfactant spraying. Also showing significant down-regulation was the auxin response factor 12 (ARF12- At1g34310) which belongs to a large gene family in Arabidopsis (23 members), involved in regulating auxin-mediated transcriptional activation/repression (Ellis et al., 2005; Okushima et al., 2005b).

It is interesting that a gene encoding the IAA glucosyltransferase (UGT75B2- At1g05560) was highly induced 24 h after surfactant application. This enzyme catalyses the formation of IAA-sugar conjugates (Jackson et al., 2001) and this conjugation leads to a decrease in free IAA levels. Activation of the UGT75B2 gene may be as a secondary response to the increased production of endogenous active free IAA as a result of surfactant treatments. Another gene (At2g04160) that most likely showed enhanced regulation due to Trp-dependent auxin biosynthesis was a subtilisin-like protease which is almost identical to the subtilisin-like protease AIR3 (AIR for Auxin- Induced in Root cultures). The mRNA levels of this gene accumulate during auxin-induced lateral root formation and studies have shown that mRNA
levels are elevated 8 hours following auxin induction and mRNA levels subsequently stay elevated for at least another 16 hours (Neuteboom et al., 1999a).

### 3.4.4.3 Ethylene

Ethylene has a highly pleiotropic role in plant growth and development and participates in germination, senescence, fruit ripening, defence and abiotic response (Ecker, 1995; Chen et al., 2005). After 1 h, treatment with surfactant NUL1026 showed an enhanced early expression of the gene encoding a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACS6 (At4g11280), the rate-limiting enzyme in the ethylene biosynthesis pathway (Yang and Hoffman, 1984). The production of ethylene gas may have occurred during the process of LPO whereby ethylene is generated from linolenic acid (Galliard et al., 1968) and this ethylene in turn promotes its own formation by the 1-aminocyclopropane-1-carboxylic acid pathway (Spiteller, 2003). This phenomena has also been observed in actively dividing plant cells whereby a LPO product of linolenic acid, an ethyl radical is converted to ethylene and this gas activates further ethylene production by inducing the genes involved in the ethylene biosynthesis pathway (Peters et al., 2000).

Another plausible explanation for the production of ethylene in this study could also be explained by IAA molecules that may have been generated in a Trp-dependent manner have induced ethylene biosynthesis, as demonstrated by Zhong and Burns (2003). Moreover, these scientists (Zhong and Burns, 2003) have also shown that ethylene gas is also able to increase IAA levels by influencing the up-regulation of the TSA gene involved in the production of auxin precursors in the Trp pathway.
Following the induction of ACC synthase gene, 24 h post surfactant application, interestingly, only one ethylene-regulated gene was regulated and this was the EIN3- Binding F-Box Protein 1 (EBF1- At2g25490) gene transcript. EBF1 encodes an F-box protein which is involved in the ubiquitin/proteasome dependent proteolysis of the EIN3 protein (Guo and Ecker, 2003, 2004; Chen et al., 2005). EIN3 is a key transcription factor in the ethylene signal transduction pathway and it positively regulates gene expression and plant responses to ethylene (Guo and Ecker, 2003, 2004; Chen et al., 2005) by triggering other transcription factors like the ERFs. Down-regulation of the EBF1 gene after 24 h of surfactant treatment suggests that degradation of EIN3 proteins was being inhibited and this inhibition was likely due to ethylene gas (Guo and Ecker, 2003).

### 3.4.4.4 JA and ethylene cooperation

A number of recent studies have shown that the signaling molecules, ethylene and JA, work in concert to regulate plant defence gene expression and increase the level of one another in response to external cues (i.e. to pathogen attack, wounding and ozone exposure) (O'Donnell et al., 1996; Turner et al., 2002; Farmer et al., 2003; Sasaki et al., 2003). The transcription factor ERF1 has been shown to participate in the regulation of these interactions (Lorenzo et al., 2003). ERF1 is considered as the convergent point between ethylene and JA pathways and it integrates and possibly amplifies JA and ethylene signals in the activation of plant defences (Gu et al., 2002; Almeras et al., 2003; Brown et al., 2003; Gutterson and Reuber, 2004).

The increased expression of the gene encoding ERF1 protein was a common response at both 1 h and 24 h treatments with surfactant NUL1026. Several other ERFs have also been shown to be induced by ethylene and JA (Brown et al., 2003; Lorenzo and Solano, 2005), implying that
other ERFs might share redundant functions with *ERF1* and likewise, treatment with NUL1026 showed the regulation of *ERF5, ERF6* and *ERF2* genes. This cross-talking between the JA and ethylene pathways may explain the induction of the defence response gene, the pathogenesis related 4 (*PR4* - At3g04720) whose expression was elevated after 24 h treatment to surfactant NUL1026.

### 3.4.5 Surfactant regulated genes associated with senescence

The expression level of the gene encoding the senescence associated family protein (At2g23810), which also shows similarity to the senescence-associated protein 5, also increased following foliar application of surfactant NUL1026, one hour post-treatment. It has been suggested that both ethylene and JA promote leaf senescence by inducing the transcription of a number of senescence-associated genes (SAGs) (Buchanan-Wollaston and Ainsworth, 1997; Park et al., 1998; He et al., 2002; Buchanan-Wollaston et al., 2003). In addition, 24 h after NUL1026 treatment, the yellow-leaf- specific (*YLS*) 4 (*YLS4* - At5g11520), *YLS9* (At2g35980) and the early responsive dehydration 1 gene (*ERD1* (also denoted as *SAG15*) - At5g51070) genes were up-regulated.

The transcripts of the *YLS* and *ERD1* genes were found to accumulate to maximum levels in senescing leaves in *Arabidopsis* and the *YLS* genes have been postulated as potential molecular markers for leaf senescence (Weaver et al., 1999; Yoshida et al., 2001; Hanaoka et al., 2002; Tran et al., 2004). Transcript of the dark inducible 11 gene, (*DIN11* - At3g49620), that has been demonstrated to increase in sugar-starved cells after 24 h of dark treatment and natural senescence (Fujiki et al., 2000; Fujiki et al., 2001; Gepstein et al., 2003), also accrued in response to the surfactant. Regulation of these senescence associated genes is consistent with
the phenotypic changes recorded in Chapter 2, Figure 2.5 whereby leaf senescence was recorded in parts of the leaves after treatment with surfactant NUL1026.

In summary, the expression profiles described here present an extensive assessment of the transcriptional response in *Arabidopsis* as a result of foliar application of an etheramine surfactant NUL1026. The data presented in this study not only identifies and catalogues to our knowledge for the first time, a large set (approximately 500 *Arabidopsis* genes) of novel early and late surfactant regulated genes, but most importantly have started to provide insights into the initial structure of the early transcriptional networks that modulate the genome-wide response to a surfactant signal. Expression results obtained in this study provide a starting point for future studies helping to understand the mechanism of action of herbicide surfactants. This will in the long run, enable the formulation of new surfactants which will permit lower use rates and hence be less stressful to the environment.

Inferences drawn from comparison analysis and functional analysis can be summarised as follows:

- A large number of genes were up-regulated in response to surfactant NUL1026 at both time points.
- Transcriptional regulators are predominant among the early response genes thereby suggesting a scenario in which a stepwise activation of different surfactant response pathways is initiated by the up-regulation or downregulation of transcription factors.
- Both the calcium signaling pathways and the MAPK cascades might be involved in mediating early intracellular transmission and amplification of surfactant responses to bring about biochemical and physiological cellular responses.
• Surfactant NUL1026 at 1 h post-treatment induced the up-regulation of a number of disease resistance genes.

• A number of genes involved in cell wall formation, consolidation and structuring were differentially expressed at both time points in response to the surfactant. This may indicate that surfactant NUL1026 may function by causing tissue damage on the leaf surface to promote herbicide uptake.

• A large number of genes involved in the detoxification process showed increased expression when plants were sprayed with the surfactants.

• Another theme emerging from the data was crosstalk between established plant chemicals (JA, ethylene and auxin). This interaction seemed to be achieved through pathways converging on the same transcriptional regulators (e.g. ERF1) or signalling components, ultimately affecting the levels of signaling molecules.

• Senescence associated genes were found to be up-regulated after 24 hour of foliar treatment with surfactant NUL1026 and this is consistent with the phenotypic changes that were recorded after plants were sprayed with the surfactant (Chapter 2, Figure 2.5).

• Approximately 500 Arabidopsis genes were found to be surfactant regulated and these genes may prove to be the starting point in helping to understand the mechanism of action of etheramine surfactants.

Chapter 3 has shown the global changes in gene expression in response to surfactant NUL1026. Chapter 4 will now focus on investigating the molecular synergistic effect of combining surfactant NUL1026 to herbicide 2,4-D at the subcellular level.
Chapter 4: Synergistic effect of combining surfactant NUL1026 to herbicide 2,4-D at the subcellular level.

4.1 Introduction

Auxinic herbicides like 2,4-D provide growers with the means to chemically manage weeds and these herbicides have been extensively used over the past decades to selectively control broadleaf weeds in grass crops. The efficacy of most herbicides relies on the ability of the herbicide molecules to successfully penetrate the plant tissues and reach the target sites. Surfactants are almost always added either as built-in formulation components or as tank-mix additives to improve the performance of such herbicides. Surfactants help to overcome the physical barriers that prevent movement of the herbicides from the leaf surface to the interior of the cell.

In the presence of surfactants, the efficacy of the herbicidal formulation depends mainly on a variety of factors including: the physiochemical properties of the active ingredients, the structure and concentrations of surfactants, the environmental conditions at the time the herbicide-surfactant formulation was sprayed and the type of leaf surface of individual target plants (Liu, 2004b). Not all surfactants can improve the uptake of a particular herbicide and as yet, there is no practical formula or model that can quantitatively predict the effect of a specific surfactant on the efficacy of a particular herbicide on a specific target or target weed (Liu, 2004b).

A variety of approaches have been used to study the consequences of combining surfactant and herbicide. These methods include basic observation of biological responses such as stem curvature, leaf necrosis, growth inhibition following droplet application to directly following the
movement of radiolabelled molecules into and through intact plants and isolated cuticles (Stock et al., 1992; Bukovac and Petracek, 1993; Schonherr and Baur, 1994; Sharma and Singh, 2000).

However, nowadays, along with the spray and rank methodology of assessing the effectiveness of a surfactant in promoting herbicidal toxicity, agrochemical industries are looking to use more science and technology to study the synergistic effect of surfactant-herbicide interaction on target plants. Approaches for the development of improved agrochemical formulations are already gradually shifting from the testing of agrochemicals for efficacy on whole plants towards the analysis of the genome response using expression arrays (Rishi et al., 2004). Results from such genomic studies may thus form the basis for the formulation of novel herbicides which are more target-specific and allow for lower rates of herbicide usage.

4.1.1 Aims

The overall aim of this current study was therefore to investigate whether combining surfactant NUL1026 to herbicide 2,4-D may have a synergistic effect at the subcellular level.

The individual goals of this chapter were to:

- carry out comparison analysis using the MAS 5.0 software on the changes in gene expression after treatment with 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h were compared with the 1 h control treatment. This analysis was repeated with the 24 h time point expression data for the 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatment.

- perform data mining to identify the sets of genes that showed a two-fold change in expression in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.
associate the genes of interest from the data mining (2) above to their respective molecular function and biological processes in order to understand the molecular effects induced by the two types of herbicide formulations on *Arabidopsis*.

In this chapter, the early (1 h) and late (24 h) genome-wide response in *Arabidopsis* following foliar-application of a spray formulation consisting of 1.0 mM 2,4-D coupled with 0.2% (v/v) surfactant NUL1026 was studied using the Affymetrix® ATH1-121501 arrays. This is the first study that provides an in-depth analysis of the genome response to such a spray treatment and it is expected that the expression profiling obtained will be related to weed response in field conditions.

The early (1 h) global transcriptomic response to 1.0 mM 2,4-D was also analysed using the same genomic tools of microarrays. Microarray results from these two treatments (1.0 mM 2,4-D and 1.0 mM 2,4-D combined with 0.2% (v/v) surfactant NUL1026) were compared and further comparisons were made with microarray data arising from surfactant NUL1026 applications alone (Chapter 3) in order to segregate those genes that were specific to each treatment from those transcripts that are part of general plant stress responses.
4.2 Materials and methods

The methodologies employed in this chapter were essentially as in Chapter 3, Section 3.2, except for the following differences.

4.2.1 Comparison analysis (corresponding to Section 3.2.1)

Results obtained from absolute analysis of gene expression (Chapter 2, Section 2.2.9), 1 h post-treatment, of the Arabidopsis plants separately sprayed with: 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026 were compared with the 1 h array control (baseline) data. This comparison analysis was repeated with absolute analysis data obtained from plants sprayed with 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-treatment, using the 24 h array control data as baseline.

4.2.2 DMT 3.0 and EXCEL for selection of genes (corresponding to Section 3.2.2.2)

The Data Mining Tool (DMT 3.0) and EXCEL softwares were used to compare and contrast the expressions of those genes showing significant response to not only the treatments being investigated in this Chapter (1.0 mM 2,4-D, 1 h and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h and 24 h) but also with those genes differentially regulated after foliar applications of 0.2% surfactant NUL1026, 1 h and 24 h (Chapter 3). Lists of genes showing specific or common regulations to the various treatments were identified and their numbers displayed onto Venn diagrams.
4.2.3 Functional classification and GO annotations using TAIR (corresponding to Section 3.2.3)

Major gene lists identified in Section 4.2.2 consisting of locus identifiers were loaded onto the Gene Ontology (GO) annotations search tool of the TAIR database in order to assign GO terms to each of the significantly regulated genes. The main gene lists that were investigated included:

genes that were specifically up- (116 genes, Figure 4.3) and down-regulated (132 genes, Figure 4.4) in response to 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h post-spraying.

genes that were specifically expressed (170 up-regulated genes, Figure 4.3 and 184 down-regulated genes, Figure 4.4) in response to 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-treatment.

gene transcripts that were specifically responsive to 1.0 mM 2,4-D spray application (3 up-regulated genes, Figure 4.3 and 4 down-regulated genes, Figure 4.4).

regulated genes that were exclusively common (7 up-regulated genes, Figure 4.3 and 1 down-regulated gene, Figure 4.4) to the following treatments: 1.0 mM 2,4-D + 0.2% (v/v) NUL1026 (1 h and 24 h post-spraying) and 1.0 mM 2,4-D, 1h post-treatment.

gene transcripts that were commonly expressed when sprayed with either surfactant NUL1026, 1 h and 24 h post-spraying or 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h and 24 h post-spraying (14 transcripts (Figure 4.3) were found to be commonly up-regulated after application of either surfactant NUL1026, 1 h and 24 h or 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h and 24 h; 7 genes (Figure 4.4) were commonly repressed after foliar application of either the surfactant, 24 h post-treatment or 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h and 24 h post-spraying.
genes that were specifically up- (53 transcripts, 1 h and 44 transcripts, 24 h, Figure 4.3) and
down-regulated (2 transcripts, 1 h and 29 transcripts, 24 h, Figure 4.4) in response to surfactant
NUL1026.

4.3 Results

4.3.1 Selection of significantly responsive genes

Microarray data gives signal (RNA) levels and induction ratios which were averaged for each of
the treatments (1.0 mM 2,4-D, 1 h and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h and 24 h)
using the biological replicates and scatter plots were drawn to get an overall view of the
expression data (Figures 4.1 (a) - (c)). The scatter plots show that many genes are both induced
and repressed greater than 2-fold in response to the different treatments and what is noticeable
however, is that the scatter is much greater for the 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h
post-spraying (Figure 4.1 (c)) than for either of the other treatments. This indicates that the
RNA response to this particular treatment is much larger under this condition at that time period.

Selection of genes that showed significant up- and down-regulation in response to each of the
treatments was carried out as outlined in Chapter 3, Section 3. 3. 1 and results are shown in
Tables 4.1 and 4.2. Interestingly with the exception of foliar application of 1.0 mM 2,4-D, more
genes were significantly overexpressed than repressed in response to the herbicide and
surfactant applications (Figure 4.2).
Figure 4.1 (a)

Figure 4.1 (b)
Figure 4.1 (c)

Figure 4.1 Scatter plots of the ATH1 arrays data.

The signal intensities were averaged from the biological replicates for each treatment and the signal intensities for (a) 1.0 mM 2,4-D, 1 h; (b) 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h; and (c) 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h were plotted against their respective controls. Guides lines are given showing signal log ratios of 2 (induced) and (-2) repressed.
Table 4.1 Selection of significantly up-regulated genes with reliable expression in replicates (Array 1 and Array 2) of the various treatments.

<table>
<thead>
<tr>
<th>Conditions for selection</th>
<th>1.0 mM 2,4-D (Array 1 and Array 2), 1 h</th>
<th>1.0 mM 2,4-D + 0.2% NUL1026 (Array 1 and Array 2), 1 h</th>
<th>1.0 mM 2,4-D + 0.2% NUL1026 (Array 1 and Array 2), 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of genes on the array</td>
<td>22,810</td>
<td>22,810</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called present in both</td>
<td>14,086</td>
<td>14,580</td>
<td>14,091</td>
</tr>
<tr>
<td>Total no. of genes with an increase call in both</td>
<td>242</td>
<td>1,691</td>
<td>2,161</td>
</tr>
<tr>
<td>Total no. of genes with an increase greater than or equal to 2 fold change in both</td>
<td>82</td>
<td>813</td>
<td>885</td>
</tr>
<tr>
<td>Total no. of genes with an increase greater than or equal to 2 fold change in both and signal value greater than or equal to 100</td>
<td>22</td>
<td>315</td>
<td>357</td>
</tr>
</tbody>
</table>
Table 4.2 Selection of significantly down-regulated genes with reliable expression in replicates (Array 1 and Array 2) of the various treatments.

<table>
<thead>
<tr>
<th>Conditions for selection</th>
<th>1.0 mM 2,4-D (Array 1 and Array 2), 1 h</th>
<th>1.0 mM 2,4-D + 0.2% NUL1026 (Array 1 and Array 2), 1 h</th>
<th>1.0 mM 2,4-D + 0.2% NUL1026 (Array 1 and Array 2), 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of genes on the array</td>
<td>22,810</td>
<td>22,810</td>
<td>22,810</td>
</tr>
<tr>
<td>Total no. of genes with a present call under Control conditions</td>
<td>15,761</td>
<td>15,761</td>
<td>14,395</td>
</tr>
<tr>
<td>Total no. of genes with a decrease call in both</td>
<td>740</td>
<td>2,558</td>
<td>2,193</td>
</tr>
<tr>
<td>Total no. of genes with a decrease greater than or equal to 2 fold change in both</td>
<td>81</td>
<td>442</td>
<td>876</td>
</tr>
<tr>
<td>Total no. of genes with a decrease, greater than or equal to 2 fold change in both and signal value greater than or equal to 100 (signal should be greater than or equal to 100 in CONTROL)</td>
<td>23</td>
<td>173</td>
<td>244</td>
</tr>
</tbody>
</table>
Figure 4.2 Genes differentially regulated by the various treatments.

Histogram showing number of genes that were significantly and reliably induced and repressed in response to 1.0 mM 2,4-D; 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 1 h post-spraying and 1.0 mM 2,4-D + 0.2% (v/v) NUL1026, 24 h post-treatment when compared to the controls, 1 h and 24 h respectively.

4.3.2 Data mining

The Venn diagrams (Figures 4.3 and 4.4) provided important insights into the distribution of genetic changes into shared and specific responses to the various treatments. Data mining revealed that the highest number of exclusively up- and down-regulated genes were registered after 24 h of foliar application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 (Figures 4.3 and 4.4).
In contrast, only 45 gene transcripts were regulated in response 1.0 mM 2,4-D treatment and 38 of these transcripts were also regulated in response to treatments with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h and 0.2% (v/v) surfactant NUL1026, 1 h and 24 h (Figures 4.3 and 4.4).
0.2% (v/v) surfactant NUL1026, 1 h post-spraying

53

1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying

109

116

5

5

11

112

170

1.0 mM 2,4-D, 1 h post-spraying

3

9

2

7

1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying

44

2

6

14

5

33
Figure 4.3 Significantly up-regulated genes.

Venn diagram shows a general overview of the genes that showed common and exclusive increased gene expression in response to the various spray applications, 1.0 mM 2,4-D, 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 and 0.2% (v/v) surfactant NUL1026, at the two time points (1 h and 24 h). Both DMT 3.0 and EXCEL were used to segregate up-regulated genes into set and subsets so as to identify those genes that were exclusively or commonly regulated in response to the surfactant and herbicide treatments.
0.2% (v/v) surfactant NUL1026, 24 h post-spraying

1.0 mM 2,4-D, 1 h post-spraying

1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying

1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying

0.2% (v/v) surfactant NUL1026, 1 h post-spraying
Venn diagram shows a general overview of the genes that showed common and exclusive decrease in gene expression in response to the various spray applications, spray applications 1.0 mM 2,4-D, 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 and 0.2% (v/v) surfactant NUL1026 at the two time points (1 h and 24 h). Both DMT 3.0 and EXCEL were used to segregate down-regulated genes into set and subsets so as to identify those genes that were exclusively or commonly regulated in response to the surfactant and herbicide treatments.

4.3.3 Functional classification

The gene transcripts that were commonly or exclusively over-expressed and repressed in response to the treatments being studied in this chapter were assigned functions using the TAIR database as in Chapter 3. Functional classifications of the regulated genes were carried out for both molecular and biological processes.

4.3.3.1 Molecular function of genes exclusively regulated by 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-spraying

Functional classification of RNA transcripts that were exclusively regulated in response to the following treatments at each of the two time points: 1.0 mM 2,4-D + 0.2% surfactant NUL102, 1 h and 1.0 mM 2,4-D + 0.2% surfactant NUL102, 24 h (Figures 4.3 and 4.4) resulted in the distribution of these genes in the following major groups: enzyme activity, transcription factor activity, binding activity, transporter activity and genes with unknown molecular function (Figures 4.5 and 4.6).

A large proportion of genes that were recorded as being exclusively up-regulated after 1 h had unknown molecular function (15.8%) while the smallest group was the receptor binding or
activity category (1%) (Figure 4.5). After 24 h treatment, the 170 genes that were exclusive to this time point were classified into two additional groups: nucleic acid binding and structural molecule activity (Figure 4.6). Genes belonging to these two categories were not recorded after 1 h treatment (Figure 4.5).

Figure 4.5 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 116 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.
Figure 4.6 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 170 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

TAIR classification (January 2006) of genes down-regulated exclusively in response to 1.0 mM 2,4-D + 0.2% surfactant NUL102, 1 h (132 genes in total, Figure 4.4) and 24 h (184 genes, Figure 4.4) revealed that transcripts classified into the structural molecule activity and nucleotide binding only showed reduced expression after 24 h (Figures 4.7 and 4.8). The molecular class representing the highest number of down-regulated genes had unknown molecular function at both time points (1 h and 24 h) and the remaining genes were distributed among categories such as transcription factor activity, transporter activity and kinase activity (Figures 4.7 and 4.8). It was noted that at both time points, TAIR assigned the least number of
differentially (up- and down-regulated genes) expressed genes to the receptor binding or activity category (Figures 4.5 - 4.8).

![Functional Categorization by Genes for: GO Molecular Function](image)

**Figure 4.7 Functional classification for Gene Ontology (GO) for molecular function.**

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 132 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.
Figure 4.8 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 184 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

**4.3.3.2 Molecular function of genes exclusively regulated by 1.0 mM 2,4-D**

Molecular functional classification of the three genes (Figure 4.3) that were exclusively up-regulated in response to 1.0 mM 2,4-D showed that 50% of the genes belonged to the transcription factor activity class and the remaining gene could not be assigned to any molecular function (Figure 4.9). TAIR could only categorise one of the exclusively down-regulated gene into the unknown molecular function category.
This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 1.0 mM 2,4-D, 1 h post-spraying when compared with the control. This gene list included 3 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

4.3.3.3 Molecular function of genes exclusively regulated by 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-spraying

The genes transcripts that exhibited increased expression only in response to 0.2% (v/v) surfactant NUL1026 at both 1 h and 24 h (Figure 4.3) and were not responsive to any of the other treatments were mainly classified into the following categories: transferase activity, enzyme activity, transcription factor activity, transporter activity, other binding activity and hydrolase activity (Figures 4.10-4.11). The over-expressed genes that were assigned to the following functional categories: receptor binding or activity and nucleic acid binding after 1 h of surfactant application (Figure 4.10) did not show significant up-regulation after 24 h of surfactant treatment (Figure 4.11). Likewise, genes that were categorised as having structural molecule function were represented after 24 h treatment but not at 1 h (Figures 4.10 - 4.11).
Figure 4.10 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene list that were exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 53 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

Figure 4.11 Functional classification for Gene Ontology (GO) for molecular function.
This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 44 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

The 2 genes (Figure 4.4) that were repressed in response only to the surfactant treatment after 1 h could not be assigned to any known molecular function whereas after 24 h, genes belonging to the transporter activity class were highly repressed and the least represented category at 24 h was the transcription factor activity (Figure 4.12).
Figure 4.12 Functional classification for Gene Ontology (GO) for molecular function.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively down-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 29 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

4.3.3.4 Biological processes of genes exclusively regulated by 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-spraying

RNA transcripts that were exclusively regulated by 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 24 h post-spraying were both associated with major biological functions including cellular processes, metabolism, physiological processes, response to abiotic or biotic stimulus, response to stress and transcription, among others (Figures 4.13 - 4.16).

Among the 116 genes (Figure 4.3) that were exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h post-application, 9.9% of the genes were assigned to the response to abiotic or biotic stimulus category and 4.2% were classed as responsive to stress
(Figure 4.13). After 24 h of herbicide and surfactant application, there was a slight decrease in the percentage of genes associated with the abiotic or biotic stimulus (9.2%) category and an increase in the stress response (5.7%) class (Figure 4.14). Interestingly, the percentage of genes encoding transcription factors was approximately similar at both 1 h and 24 h intervals (Figures 4.13 and 4.14).

![Functional Categorization by Annotations for: GO Biological Process](image)

**Figure 4.13 Functional classification for Gene Ontology (GO) for biological processes.**

This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 116 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.
Figure 4.14 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 170 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

In addition, functional categorisation of exclusively down-regulated genes at both time periods revealed that a higher proportion of genes belonging to the cell organisation and biogenesis group had decreased expression after 24 h (3.0%) than after 1 hour of 1.0 mM 2,4-D + 0.2% surfactant NUL1026 application (0.6%) (Figures 4.15 and 4.16). In contrast, more genes encoding transcription factor proteins were down-regulated after 1 h of treatment (5.2%) while 2.0% of the total number of exclusively down-regulated genes after 24 h belonged to the transcription factor class (Figures 4.15 and 4.16).
Figure 4.15 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 132 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.
Figure 4.16 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 184 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

4.3.3.5 Biological processes of genes exclusively regulated by 1.0 mM 2,4-D

Of the 3 genes that were exclusively up-regulated in response to 1.0 mM 2,4-D, 1 h post-application, 2 of the 3 genes were classed as being members of the transcription factor category while the remaining one gene was assigned to the protein metabolism group (Figure 4.17). Only one of the exclusively down-regulated genes could be associated to a biological process and TAIR (January 2006) classed that gene as having an unknown biological function.

![Functional Categorization by Genes for: GO Biological Process](image)

Figure 4.17 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene lists that were exclusively up-regulated in response to 1.0 mM 2,4-D, 1 h post-spraying when compared with the control. This
gene list included 3 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

4.3.3.6 Biological processes of genes exclusively regulated by 0.2% surfactant NUL1026, 1 h and 24 h post-spraying

Biological classification of genes exclusively up-regulated to surfactant treatments showed that at 1 h post-treatment, the highest percentage of surfactant-responsive genes showing increased expression belonged to the physiological process class while after 24 h, gene transcripts belonging to the “response to abiotic and biotic stimulus” category was more highly represented (21.1%) (Figures 4.18 - 4.19). In addition, a higher percentage of genes coding for transcription factors were registered after 1 h compared to after 24 h (Figures 4.18 - 4.19). None of the 44 RNA transcripts (Figure 4.4) that were over-expressed after 24 h surfactant application were classed under the cell organisation and biogenesis category while TAIR classified 1.6% of the 53 genes (Figure 4.3), up-regulated after 1 h, into this class (Figures 4.18 - 4.19).

![Functional Categorization by Annotations for: GO Biological Process](image)

**Figure 4.18** Functional classification for Gene Ontology (GO) for biological processes.
This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared with the control. This gene list included 53 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

![Functional Categorization by Annotations for: GO Biological Process](image)

**Figure 4.19** Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively up-regulated in response to 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 44 genes transcripts (Figure 4.3) that showed increased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

The 2 genes that were repressed in response to surfactant application after 1 h were not found to belong to any known biological process classification whereas the 29 down-regulated genes (Figure 4.4) were classed into a wide range of biological processes, as shown in Figure 4.20.
Figure 4.20 Functional classification for Gene Ontology (GO) for biological processes.

This pie-chart shows the frequency of GO terms in the gene list that was exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared with the control. This gene list included 29 genes transcripts (Figure 4.4) that showed decreased expression to this treatment alone. Classification was carried out in January 2006 using the TAIR database.

4.3.4 GO annotations

The gene expression data lists (mentioned in Section 4.2.3) consisting of locus identifiers were annotated using the TAIR database (January 2006) to some broad GO terms according to their positions in Directed Acyclic Graph (DAG) of GO. The GO terms were classified into function, process and component. Results showed that biological processes regulated by herbicide 2,4-D and surfactant NUL1026 included GO terms such as response to wounding, response to auxin stimulus and homeostasis, transcription factor activity and toxin catabolism (Tables 4.3 - 4.7).

Other frequently occurring GO terms of genes regulated in response to the surfactant and the herbicide highlighted their association with the nucleus, the mitochondrion and the chloroplast
Tables 4.3 - 4.7. Tables 4.3 - 4.6 are annotations of a representative group of genes that were only expressed by one of the following spray formulations: surfactant NUL1026, 1 h and 24 h post-application and 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-spraying. Table 4.7 is a representative annotation of those genes that were up-regulated both in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-application. Please refer to the Supplementary data for the complete annotation of the lists of genes investigated in this chapter.

Table 4.3 Annotation of a representative list of genes that were exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>264365_s_at</td>
<td>At1g03220</td>
<td>extracellular dermal glycoprotein, putative / EDGP</td>
<td>biological process unknown</td>
</tr>
<tr>
<td>263656_at</td>
<td>At1g04240</td>
<td>auxin-responsive protein / indoleacetic acid-induced protein 3 (IAA3)</td>
<td>response to auxin stimulus</td>
</tr>
<tr>
<td>263236_at</td>
<td>At1g10470</td>
<td>two-component responsive regulator / response regulator 4 (ARR4)</td>
<td>red or far red light signaling pathway</td>
</tr>
</tbody>
</table>

Please refer to the Supplementary data for the complete annotation of the lists of genes investigated in this chapter.
activity
response to stress
protein binding
cytoplasm
nucleus
cytokinin mediated signaling

263598_at At2g01850 xyloglucan:xyloglucosyl hydrolase activity, acting on glycosyl bonds
xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase / endo-xyloglucan transferase (EXGT-A3)
vascular tissue development (sensu Tracheophyta)
endomembrane system

263457_at At2g22300 ethylene-responsive calmodulin-binding protein, putative
calmodulin binding
mitochondrion
calmodulin binding
transcription regulator activity
Table 4.4 Annotation of a representative list of genes that were exclusively up-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>264467_at</td>
<td>At1g10140</td>
<td>expressed protein</td>
<td>mitochondrion, biological process unknown, molecular function unknown</td>
</tr>
<tr>
<td>259517_at</td>
<td>At1g20630</td>
<td>catalase 1</td>
<td>catalase activity, perixome, mitochondrion</td>
</tr>
<tr>
<td>261266_at</td>
<td>At1g26770</td>
<td>expansin, putative (EXP10)</td>
<td>structural constituent of cell wall, cell wall modification during multidimensional cell growth (sensu Magnoliophyta), cell wall loosening (sensu Magnoliophyta), unidimensional cell growth, molecular function unknown</td>
</tr>
<tr>
<td>255788_at</td>
<td>At2g33310</td>
<td>auxin-responsive protein / indoleacetic acid-induced protein 13 (IAA13)</td>
<td>transcription factors activity, response to auxin stimulus, nucleus</td>
</tr>
<tr>
<td>259018_at</td>
<td>At3g07390</td>
<td>auxin-responsive protein / auxin-induced protein (AIR12)</td>
<td>lateral root morphogenesis, response to auxin stimulus, extracellular matrix organisation and biogenesis, extracellular matrix structural component, extracellular region, anchored to membrane</td>
</tr>
</tbody>
</table>
Table 4.5 Annotation of a representative list of genes that were exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>259417_at</td>
<td>At1g02340</td>
<td>long hypocotyl in far-red 1 (HFR1) / reduced phytochrome signaling (REP1)</td>
<td>protein binding, DNA binding, nucleus, signal transduction, protein binding, transcription factor activity, nucleus, protein polyubiquitination, blue light signaling pathway, red, far-red light phototransduction, response to light intensity</td>
</tr>
<tr>
<td>244839_at</td>
<td>At1g03630</td>
<td>encodes for a protein with protochlorophyllide oxidoreductase activity</td>
<td>chlorophyll biosynthesis, oxidoreductase activity, protochlorophyllide reductase activity, NADPH dehydrogenase activity, protochlorophyllide reductase activity, chloroplast, oxidoreductase activity</td>
</tr>
<tr>
<td>262784_at</td>
<td>At1g10760</td>
<td>starch excess protein (SEX1)</td>
<td>cold acclimation, starch catabolism, mitochondrion</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Description</td>
<td>Activity/Function</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>266695_at</td>
<td>alpha-glucan, water dikinase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At2g19810</td>
<td>zinc finger (CCCH-type) family protein</td>
<td>nucleic acid binding regulation of transcription transcription factor activity</td>
<td></td>
</tr>
<tr>
<td>266225_at</td>
<td>mitochondrial import inner membrane translocase subunit</td>
<td>protein transport</td>
<td></td>
</tr>
<tr>
<td>At2g28900</td>
<td>Tim17/Tim22/Tim23 family protein</td>
<td>mitochondrial inner membrane protein transporter activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>protein transport activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>protein translocase activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mitochondrial inner membrane presequence translocase complex</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6 Annotation of a representative list of genes that were exclusively down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h post-spraying when compared to the control.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>260902_at</td>
<td>At1g21440</td>
<td>mutase family protein, similar to carboxyvinyl-carboxyphosphonate phosphorylmutase</td>
<td>catalytic activity, metabolism, chloroplast, isocitrate lyase activity</td>
</tr>
<tr>
<td>265076_at</td>
<td>At1g55490</td>
<td>RuBisCO subunit binding-protein beta subunit, chloroplast / 60 kDa chaperonin beta subunit / CPN-60 beta</td>
<td>chloroplast, protein binding, chaperone cofactor dependent, protein folding, systemic acquired resistance, cell death, protein folding, ATP binding</td>
</tr>
<tr>
<td>265444_at</td>
<td>At2g37180</td>
<td>plasma membrane intrinsic protein 2C (PIP2C)</td>
<td>response to desiccation, membrane, water channel activity, transport</td>
</tr>
<tr>
<td>267562_at</td>
<td>At2g39670</td>
<td>radical SAM domain-containing protein</td>
<td>iron ion binding</td>
</tr>
</tbody>
</table>

205
chloroplast
catalytic activity
iron ion binding
biological process unknown
catalytic activity

258957_at  At3g01420  pathogen-responsive alpha-dioxygenase, putative

Table 4.7 Annotation of a representative list of genes that were commonly up-regulated in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% surfactant NUL1026, 1 h and 24 h post-application when compared to the controls.

<table>
<thead>
<tr>
<th>Array element</th>
<th>Locus Identifier</th>
<th>Brief description</th>
<th>GO term</th>
</tr>
</thead>
<tbody>
<tr>
<td>262099_s_at</td>
<td>At1g59500</td>
<td>encodes an IAA-amino synthase</td>
<td>response to auxin stimulus cellular component unknown auxin homeostasis</td>
</tr>
<tr>
<td>258399_at</td>
<td>At3g15540</td>
<td>indoleacetic acid-induced protein 19 (IAA19)</td>
<td>response to auxin stimulus phototropism gravitropism nucleus transcription factor activity</td>
</tr>
<tr>
<td>245076_at</td>
<td>At2g23170</td>
<td>encodes an IAA-amino synthase</td>
<td>response to auxin stimulus auxin homeostasis</td>
</tr>
<tr>
<td>248282_at</td>
<td>At5g52900</td>
<td>expressed protein</td>
<td>molecular process unknown cellular component unknown biological process unknown</td>
</tr>
<tr>
<td>261766_at</td>
<td>At1g15580</td>
<td>indoleacetic acid-induced protein 5 (IAA5)</td>
<td>response to auxin stimulus nucleus transcription factor activity</td>
</tr>
</tbody>
</table>
4.4 Discussion

Results presented in Chapter 3 indicated that 0.2% (v/v) surfactant NUL1026 when administered without the herbicide active ingredient, has considerable effect on the transcriptome, with considerable transcript effects occurring at both 1 h and 24 h post-treatments. Work reported in this chapter demonstrates that the combination of surfactant NUL1026 and 1.0 mM 2,4-D has a synergistic influence on the overall toxicity on the plant and identifies those genes, the response of which, are specific to each of the spray treatments investigated in this project.

Data analysis conducted in Chapter 3 identified around 484 gene transcripts that showed significant expression modulation in response to 0.2% (v/v) surfactant NUL1026, at the 1 h and 24 h post-treatment time periods (Figure 3.3, Chapter 3). Furthermore, data mining revealed that 128 of the surfactant responsive genes that were regulated in response to the surfactant, were not expressed significantly in response to the other treatments examined in this study (Figures 4.3 and 4.4). Further in-depth analysis performed showed that 356 RNA transcripts that were differentially regulated in response to the surfactant were also overexpressed after treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-spraying and 1.0 mM 2,4-D, 1 h (Figures 4.3 and 4.4). Functional classification of the 484 gene transcripts have already been carried out in Chapter 3 and important physiological and metabolic processes have been extensively described in the Discussion section (Section 3.4) of Chapter 3 and will be therefore briefly mentioned in this current Discussion.

4.4.1 Specific expression of surfactant responsive genes

Functional analysis assigned the 128 genes that were specific to surfactant NUL1026 at both time points examined to an array of biological processes. Of note was the increased expression
of genes coding for transcription factors. At 1 h post-surfactant application alone, surfactant specific genes were found to code for transcription factors and these included the ethylene response factor (ERF)/APETALA2 (AP2) transcription factors (At3g23230, ERF5- At5g47230 and At4g34410), a member of the Dof proteins (ADOFI- At1g51700), a member of the C2H2 type protein (ZAT1- At2g37430), two members of the no apical meristem (NAM) domain (NAC) (At3g49530 and At5g24590) and a member of the CCR4-NOT transcription complex protein (At5g22250).

In contrast, 24 h post-treatment, only one member from the NAC family proteins (At3g10500) was found to be specifically up-regulated due to the surfactant treatment alone. Transcription factors are of particular interest as they coordinate the expression of many downstream target genes and subsequently entire metabolic and developmental pathways. Identification of these specific surfactant responsive transcription factors may considerably help in elucidating the mode of action of etheramine surfactants as their regulation indicates a possible scenario in which different surfactant response pathways are instigated by the differential regulation of these transcription factors.

Remarkably, genes coding for key enzymes such as the lipoxygenases (LOX3- At1g17420, 1 h and LOX2- At3g45140, 24 h), allele oxide synthase (AOS- At5g42650, 1 h), allele oxide cyclases (AOC2- At3g25780, 1 h and AOC1- At3g25760, 24 h) and the 12-oxophytodienoic acid reductases 3 (OPR3- At2g06050, 24 h), required for jasmonic acid (JA) biosynthesis were found to be exclusively surfactant responsive. Moreover, the significant down-regulation of auxin-related genes such as the auxin-herbicide resistant 1 gene (AUXI- At2g38120) and the auxin response factor 12 (ARFI2- At1g34310) were also recorded as being specific to the
surfactant treatment. In addition, this analysis also showed that the increased RNA expression recorded for the defence response gene (the pathogenesis related 4 (*PR4*- At3g04720)) and the dark inducible 11 gene (*DIN11* - At3g49620) seemed to be equally specific to this surfactant treatment.

Also showing over-expression only in response to the surfactant were genes coding for cell wall constituents (At1g59910, extension 4 (*EXT4*- At1g76930)). The cell wall loosening proteins, expansin 1 (*EXP1*), encoding transcript (At1g69530) was however repressed. Specific regulation of these genes coding for cell wall constituents as a result of the surfactant treatment may be indicating a special function under this condition. Further studies using plants deficient in or over-expressing these cell wall components may help in elucidating their specific role under the influence of an etheramine surfactant.

Interestingly, none of the genes (the cytochromes encoding proteins) involved in Phase I detoxification processes of foreign compounds in plants were specifically regulated to the surfactant and this implies that these genes may be functioning in a more indiscriminate manner. In contrast, a number of genes coding for Phase II detoxification enzymes (e.g. glutathione synthetase (GSH) (*GSH2*- At5g27380) and glutathione S-transferase genes (GST) (*GSTF4*- At2g30870, *GSTU25*- At1g17180, *GSTU22*- At1g78340) showed specific expression after application of the surfactant. Likewise, some of Phase III genes (At1g61890 and At2g47800) involved in the transport and sequestration of conjugated toxic compounds in the vacuole were also up-regulated after surfactant treatment only.

However, recent studies have shown that individual GSTs are not only involved in xenobiotic detoxification but are also regulated in response to abiotic and biotic stresses and individual
GST differentially regulates stress responses (Edwards et al., 2000; Wagner et al., 2002; Almeras et al., 2003; Smith et al., 2004; Lee et al., 2005). The regulation of these specific surfactant responsive GSTs may be due to secondary stress imposed on the plants by the surfactant application. In addition, regulation of the two Phase III transport proteins genes may be as a result of the accumulation of secondary products generated by surfactant assimilation.

4.4.2 Overlapping expression of the surfactant-, herbicide- and herbicide + surfactant-responsive genes

Further in-depth analysis performed in this current study revealed that of the 484 genes that were up- or down-regulated in response to the surfactant application (Chapter 3), 356 RNA transcripts showed overlapping gene expressions with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment and 1.0 mM 2,4-D, 1 h (Figures 4.3 and 4.4). The greatest number of genes showing similar patterns of expression was recorded between 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 (162 up-regulated genes and 44 down-regulated genes), both 24 h post-spraying (Figures 4.3 and 4.4). In contrast, there were no commonly up-regulated genes between 1.0 mM 2,4-D and 0.2% (v/v) surfactant NUL1026, 1 h post-spraying (Figure 4.3) and 2 genes were found commonly down-regulated between these two treatments at that 1 h time point (Figure 4.4).

Functional classification of the 356 genes carried out as part of the 484 surfactant-regulated genes in Chapter 3, had revealed that the majority of these gene transcripts were involved in transcription factor activity, signaling, disease resistance, cell wall organisation and biogenesis, detoxification, auxin and ethylene regulation and senescence (Section 3.4, Chapter 3). This common response between the surfactant and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026
at the two time points may in part be attributed to the presence of the surfactant in the 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 formulation. As demonstrated in Chapter 3, surfactant NUL1026 is not an inert compound and it has its own intrinsic biological effect at the subcellular level. Therefore, the common regulation of the majority of the 356 genes may have been solely triggered by the surfactant, independent of the influence of the herbicide molecules.

However, the majority of these overlapping genes have also been shown to be regulated in response to a plethora of abiotic and biotic stresses (Bray, 2002; Cheong et al., 2002; Kreps et al., 2002; De Paepe et al., 2004; Ludwikow et al., 2004; Narusaka et al., 2004; Coupe et al., 2006). Hence their regulation in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 may also be considered as being specific to this spray formulation and regulation of these common genes may be as a result of the stress response brought about by the coordinated action of both the surfactant and the herbicide. Besides, as shown in the Venn diagrams (Figure 4.3 and 4.4), there were overlaps between the different treatments, irrespective of the time at which RNA levels were analysed. For instance, 38 RNA transcripts showed common and specific up-regulation in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 responsive genes, 1 h and 24 h post-application and 0.2% (v/v) surfactant NUL1026 responsive genes, 24 h post-application (Figure 4.3). Taken together, these overlapping genes expressed in response to the various treatments, highlight their general stress response functions in plants.

4.4.3 Specific expression of either the herbicide- or the herbicide + surfactant responsive genes

Results obtained from the analysis of mRNA expression in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, at both time points examined, clearly showed the synergistic effect of
combining surfactant NUL1026 with 1.0 mM 2,4-D. Along with a considerable number of overlapping gene expressions with the surfactant at 1 h and 24 h, 248 and 354 genes were found to be specifically up- and down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 at the 1 h and 24 h time points respectively.

The following sections will take a closer look at some of the regulated genes that showed significant exclusive expression in response to either 1.0 mM 2,4-D, 1 h or 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h treatments.

4.4.3.1 Auxin-inducible genes showing overlapping expression to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h

Data mining and analysis revealed that the set of 7 overlapping genes that were up-regulated (Figure 4.3) exclusively in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, at the 1 h and 24 h time points were predominantly auxin response genes. As mentioned previously, auxinic herbicides like 2,4-D share several characteristics with the natural plant hormone auxin, indole-3-acetic acid (IAA). These characteristics include the fact that both auxin and auxinic herbicides induce growth by cell elongation at low amount while at high amount they display a range of abnormalities which can eventually lead to plant senescence (Grossmann, 1998; Grossmann, 2000). However, at the genomic level, research conducted by Raghavan et al. (2006) has shown that 2,4-D induced the expression of auxin- response genes when 2,4-D was applied at both auxinic and herbicidal levels.

Consistent with the results of Raghavan et al. (2006) this current study also showed the increased expression of several auxin-responsive genes such as members of the indole-3-
acid (IAA) genes (IAA1-At4g14560, IAA5-At1g15580 and IAA19- At3g15540). Other auxin-inducible genes involved in maintaining auxin homeostasis (growth hormone (GH)3.4-At1g59500 and GH3.3- At2g23170) (Staswick et al., 2005) and auxin-mediated morphogenesis (homeobox leucine zipper protein 2 (HAT2)- At5g47370) (Sawa et al., 2002) were also upregulated. The RNA expression of a putative glutathione S-transferase (GSTU20) gene (At1g78370) which also shows sequence similarity to 2,4-D inducible gene, was repressed after exposure to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h. The GSTU20 gene also showed ≤ 2 fold decrease after 24 h of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatment.

4.4.3.2 Other auxin-responsive genes specific to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h post-treatment

The genes involved in the biosynthesis, transport and regulation of auxin (Section 3.4.4.2, Chapter 3) found to be surfactant responsive were also regulated by 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h post-treatment. In addition to these genes and the auxin-related transcripts mentioned in the above section 4.4.3.2, data mining revealed the expression of a number of auxin-response genes that were distinctly regulated at either 1 h or 24 h post-spray application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. Among the gene transcripts that were specifically found to be regulated early upon exposure to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, IAA 3 (At1g04240) and a gene showing sequence similarity to the small up-regulated RNA (SAUR- At 1g72430) were up-regulated. Studies have shown that auxin rapidly and transiently triggers accumulation of the IAA/AUX, SAUR and GH3 transcripts and this response does not require de novo protein biosynthesis (Abel et al., 1994; Abel and Theologis, 1996).
Proper auxin response requires the degradation of auxin-induced proteins (such as the IAA/AUX proteins) and this is carried out via the 26S proteosome complex (Berleth et al., 2004; Schwechheimer and Schwager, 2004). An hour post-application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, two RNA transcripts which code for components of the 26S complex showed increased expression (At4g23570 and At5g43190). Concomitant with this, an hour post-treatment revealed the decrease in expression of the auxin response factor 2 gene (ARF2-At5g6200). ARF2 acts as a transcription repressor of auxin signalling and binds to AUX/IAA proteins which thus ensures transient auxin response (Okushima et al., 2005a). The down-regulation of this ARF2 gene may be explained by the fact that exposure to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 after 1 h may not have caused the AUX/IAA proteins level to plummet to an extent that would require the negative feedback mechanism instigated by AUX/IAA -ARF2 interactions.

Twenty-four hours post-treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, resulted in the additional decreased expression of the SAUR gene (At4g38840) and an increase in expression of the GH3.5 transcript (At4g27260) and other members of the AUX/IAA gene family including the IAA13 (At2g33310), IAA2 (At3g23030) and IAA17 (At3g23050). The IAA17 gene functions as a transcription repressor of auxin signalling (Liscum and Reed, 2002; Cluis et al., 2004) and its exclusive up-regulation at this time point seems to indicate that auxin-inducible gene expressions might be repressed. This treatment also showed the up-regulation of the flavodoxin-like quinine reductase 1 gene (FGQ1- At5g54500) which has been shown to be an auxin-response gene (Laskowski et al., 2002). However, in contrast to our study, previous research has shown that this gene is rapidly up-regulated and reaches a maximum accumulation 30 minutes after IAA treatment (Laskowski et al., 2002).
Also showing overexpression at this specific time point was the p-glycoprotein 4 (\textit{PGP4} - At\,2g4700) gene which is involved in the polar auxin transport (Noh et al., 2001). Interestingly, the down-regulation of two mRNA transcripts (the nucleotide diphosphate kinase 1A (\textit{NDPK1A})-At5g63310 and the chalcone synthase (\textit{CHS})-At5g13930) involved in regulating auxin transport were noted (Choi et al., 2005; Saslowsky et al., 2005). The induction and repression of transcripts involved in auxin transport might be to fine-tune the influx and efflux of the 2,4-D molecules.

The \textit{AIR3} gene (AIR for \underline{A}uxin- \underline{I}nduced in \underline{R}oot cultures) was found to be commonly up-regulated after application of both the surfactant (Section 3.4.4.2, Chapter 3) and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. In contrast, 24 h post-treatment, \textit{AIR12} (At3g07390), which has also been shown to be auxin-responsive (Neuteboom et al., 1999b), was specifically induced in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. Interestingly, the gene (At4g12550) encoding the \textit{AIR1} protein was down-regulated at 24 h, although studies by Neuteboom et al. (1999) have indicated that \textit{AIR1} and \textit{AIR12} are coordinately regulated and showed the same pattern of expression in response to both natural and synthetic auxins. However, these scientists (Neuteboom et al., 1999b) have suggested that \textit{AIR1} proteins has a structural role in a similar manner to extensins and other cell wall proteins. Therefore, down-regulation of \textit{AIR1} may be as a result of the breakdown of the cell wall due to the phytotoxic effects of the herbicide.
4.4.3.3 Specific regulation of ethylene and abscisic acid response genes in response to either 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h post-treatment

The proposed model for the mode of action of auxinic herbicides suggests that synthetic auxins such as 2,4-D raise endogenous ethylene levels by promoting the up-regulation of the 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (ACS), which encode key enzymes in ethylene biosynthesis. This ethylene subsequently triggers abscisic acid (ABA) biosynthesis (Hansen and Grossmann, 2000; Raghavan et al., 2005, 2006).

The ACS enzyme is encoded by a multigene family and members of this family show differential gene expression during development and in response to a variety of environmental stimuli (Liang et al., 1992). The ACS6 gene was commonly up-regulated by both the surfactant (Section 3.4.4.3, Chapter 3) and by 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-applications. The expression of ACS6 not only implies its involvement in ethylene biosynthesis but also points towards a more general stress response function of this gene. In contrast, transcriptomic analysis indicated the overexpression of the ACS11 (At4g08040) gene exclusively in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-exposure and the specific up-regulation of the ACS8 (At4g37770) gene at 24 h after the same treatment. Interestingly, Raghavan et al. (2006) have recently reported that they did not detect any significant expression of the ACS encoding genes at 1.0 mM 2,4-D treatment while the ACS6 gene was identified as responsive after treatment with lower 2,4-D concentrations.

The ethylene perception and signal transduction pathways involve sequential sets of events. None of the genes involved in ethylene perception (Guo and Ecker, 2004; Chen et al., 2005) were detected across the different treatments although the exclusive down-regulation of the
EIN3- Binding F-Box Protein 2 (EBF2- At1g53170) was registered after 1 h treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. This suggests that the ethylene-dependent signalling pathway may have been disrupted by the herbicidal level of 2,4-D used. The ERF transcription factors have been shown to be regulated in either an ethylene-dependent or ethylene-independent manner by abiotic and biotic stresses (Fujimoto et al., 2000). Hence, the specific and overlapping regulation of the various ERF genes may be occurring in an ethylene-independent manner and may be due to secondary stress response induced by surfactant NUL1026 and 2,4-D. ERFs regulated stress response by interacting with the cis-acting element GCC, present in defence-related genes (Fujimoto et al., 2000).

Interestingly, as opposed to other auxinic herbicides studies (Hansen and Grossmann, 2000; Grossmann and Hansen, 2001; Raghavan et al., 2005, 2006), a number of genes encoding abscisic (ABA)-induced proteins showed reduced levels of gene transcription in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. The known ABA-responsive genes that were down-regulated after 1 h are involved in ABA-mediated signal transduction and stress response (Lee et al., 2001b; Chen et al., 2002; Seki et al., 2002) and include the protein phosphatase 2C (PP2C- At3g11410) and the ABA insensitive 1 (ABAI1- At4g26080), the HVA22 (At4g24960) and the homeobox-leucine zipper protein 12 (ATHB-12- At3g61890) genes. Moreover, the expression of the gene (At4g19170) encoding the 9-cis-epoxycarotenoid dioxygenase (NCED3) enzyme involved in ABA biosynthesis was also reduced after 1 h treatment. In addition, the exclusive down-regulation of the ABA and salt stress- induced gene (At3g55610), which encode the key proline biosynthesis enzyme, delta-1-pyrroline-5-carboxylate synthase (P5CS), was recorded after 24 h. The down-regulation of these ABA-inducible genes was more pronounced after 1 h treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 than after 24 h.
4.4.3.4 Specific regulation of JA biosynthesis genes to either 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h post-treatment

Consistent with the findings of Liu and Wang (2006), none of the genes involved in JA biosynthesis was specifically regulated after exposure to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatments at either of the two times examined. The up-regulation of JA recorded in cleavers may have been due to plant stress response, as the authors have pointed out (Grossmann et al., 2004). The general regulation of a putative LOX (At1g72520) and a OPR2 (At1g76990) (Section 3.4.4.1, Chapter 3) across the surfactant and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatments are thus likely to be due to general plant stress response.

JA has been shown to accumulate in several types of stress conditions (Creelman and Mullet, 1997; Creelman and Mulpuri, 2002; Wang et al., 2005) such as wounding and pathogen infection and this hormone also plays a role in senescence. A number of experiments have documented the inhibitory effect of auxin on jasmonic acid biosynthesis (DeWald et al., 1994; Gutjahr et al., 2005) though Grossmann et al. (2004) have reported an increase (4.5 fold) in JA concentration 25 h after the application of 100 µmol/L IAA in cleavers. However, recently, microarray expression experiment which studied the effects of auxins on JA biosynthesis in Arabidopsis have confirmed the down-regulation of genes involved in JA biosynthesis (Liu and Wang, 2006)

4.4.3.5 Specific regulation of genes related to senescence to either 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h post-treatment

Senescence is often called the final stage of plant development and can be artificially induced by herbicides in target plants. Senescence involves massive changes both in cellular metabolism
and degeneration of cellular structures and the breakdown of cellular structures in senescing leaves begins with the chloroplast (Buchanan-Wollaston and Ainsworth, 1997; Buchanan-Wollaston et al., 2003). In agreement with this, application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 at 1 h was found to exclusively repress the expression of a number of genes involved in chlorophyll biosynthesis and photosynthesis. A few examples included genes encoding protochlorophyllide oxidoreductase (PORC- At1g03630), chlorophyll B synthase (CH1- At1g44446) magnesium chelatase 12 (CHL12- At5g45930) and lycopene epsilon cyclase (LUT2- At5g57030) (Beale, 2005; Kim et al., 2005). The down-regulation of genes involved in chlorophyll biosynthesis seemed to be early event since the differential regulation of none of these biosynthetic genes were detected after 24 hour. Microarray data analysis also revealed the decreased expression of some of the mRNAs that encode components of the photosynthesis complex.

Another consequence of senescence is the modification and disassembly of cell walls structure (Buchanan-Wollaston and Ainsworth, 1997). The spray application of the herbicide combined with the surfactant further resulted in the differential regulation of expansins (EXLA1- At3g45970, 1 h and EXPA10- At1g26770, 24 h) and xyloglucan endotransglycosylase encoding genes (EXGT-A3- At2g01850, 1 h and XTR8- At3g44990, 24 h).

Senescence also leads to the induction of a set of genes collectively termed as senescence-associated genes (SAGs) which are activated at various stages of senescence (Buchanan-Wollaston, 1997) and are induced in response to drought, salt, cold, heat, wounding, ethylene, ABA and auxin stress responses (Park et al., 1998; Arteca and Arteca, 1999; Miller et al., 1999; Gepstein et al., 2003; Schenk et al., 2005). Data mining revealed the up-regulation of the
SAG21 gene (At4g02380) after 1 h and this induced expression was maintained after 24 h whereas the expression level of the dark inducible 1 (Sen1- At4g35770) and the SAG20 (At3g10980) were specifically regulated after 24 h treatment. The Sen1 gene is now regularly used as a marker to characterise responses associated with senescence in Arabidopsis and this gene also contains an ABA responsive element and TCH motif common to stress-inducible genes (Oh et al., 1996; Quirino et al., 2000; He et al., 2002; Schenk et al., 2005). The up-regulation of these senescence responsive genes is consistent with the morphological changes observed after 24 h and 48 h treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 (Chapter 2, Figure 2.5).

4.4.3.6 Specific expression of 1.0 mM 2,4-D responsive genes

Microarray analysis revealed that the total number of genes regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 hour post-treatment was approximately 10 times more than the number of genes regulated after exposure to 1.0 mM 2,4-D only, 1 h post-spraying (Figures 4.3 and 4.4). A small number of genes (45 genes) were regulated by 1.0 mM 2,4-D treatment. This may likely to be due to a small amount of active ingredient penetrating through the cuticular barrier of the leaves. Comparison of the herbicide regulated genes with those genes regulated in response to the various other treatments under investigation in this study revealed that 3 up-regulated genes and 4 down-regulated genes were specific to the 1.0 mM 2,4-D treatment.

Importantly, the increase in expression of two transcription factors, the AP2 domain-containing transcription factor showing similarity to the AP2 domain containing protein RAP2.4 (At2g20880) and HAT22 (At4g37790) were recorded in response to 1.0 mM 2,4-D. Members of
the AP2 transcription factors are involved in hormone-dependent gene expression (Kizis et al., 2001) and HAT22 transcription factor proteins have been implicated in cytokinin response (Brenner et al., 2005). Several lines of evidence have shown that auxin and cytokinin control plant development by regulating each other’s abundance (Eklof et al., 2000; Swarup et al., 2002) and expression of the HAT22 gene may be as a result of cross-talk between auxinic level of 2,4-D and the plant endogenous cytokinin. The study conducted by Brenner et al. (2005) has shown the up-regulation of known early-auxin regulated genes in response to cytokinin.

Three of the 4 genes that showed reduced expression have yet to be assigned locus identifier numbers while TAIR classified At3g07350 as having unknown biological and molecular functions (Figures 4.13 and 4.23). Furthermore, 1.0 mM 2,4-D also induced the expression of a SAUR transcript (At4g38840). This early regulation of the SAUR gene was also shared by the 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatment, 1 h post-application. The decreased regulation of the proline-rich protein 2 (PRP2- At2g21140) in response to both 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h was also noted and this reduced expression implies that these two treatments may have affected the cell wall structure (Fowler et al., 1999). The increased expression of the auxin-response genes and the down-regulation of PRP2 after 1.0 mM 2,4-D application may explain the phenotypic changes (leaf curling and petiole elongation;Chapter 2, Figure 2.5) recorded. These symptoms are considered typical for auxin-type herbicides (Sterling and Hall, 1997; Grossmann, 2000).
Inferences drawn in this chapter can be summarised as follows:

- A large number of surfactant-responsive genes were also regulated in response to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026. These overlapping genes may have a more general function in plants.
- Surfactant NUL1026 exclusively regulates genes involved in JA biosynthesis.
- Surfactant NUL1026 has a synergistic effect on herbicide 2,4-D and has contributed to herbicide activity.
- Approximately 10 times more genes were regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 compared with 1.0 mM 2,4-D for the same time-frame.
- The extensive leaf curling and petiole elongation recorded in response to 1.0 mM 2,4-D are typical symptoms for auxinic herbicides and are probably due to the expression of auxin-response genes and PRP2 whose product is involved in cell wall structure.
- Overlapping genes regulated by 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h, 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 24 h and 1.0 mM 2,4-D were known auxin response genes.
- A large number of genes were exclusively regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h.
- Surfactant NUL1026 may have promoted uptake of 2,4-D molecules as shown by the specific regulation of additional auxin-inducible genes on exposure to either 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h.
- Genes specific to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h or 24 h were involved in ethylene biosynthesis, cell wall modification and chloroplast degradation.
ABA-biosynthesis and -response genes were down-regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment.

Treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 at both time points triggered senescence-response genes such as \textit{SAG 21} and \textit{Sen1}.

Having identified the genes that were found to be specifically responsive to herbicide 2,4-D, the next step is to monitor the expression of these genes in herbicide resistant and susceptible biotypes of an actual problematic weed called wild radish. This may help identify the gene(s) responsible for conveying herbicide resistance. However, wild radish is an obligate outbreeder and as such it is important to verify that the genetic framework of the resistant and susceptible populations are similar as this will ascertain that any difference in gene expression recorded may most likely be due to the resistance mechanism. Hence the next chapter looks at the genetic framework of the resistant and susceptible biotypes of wild radish using the technique of RAPD-PCR.
Chapter 5: Genetic variation between populations of Western Australian wild radish

5.1 Introduction

5.1.1 Wild radish – a weed of Australian dryland cropping

Wild radish (Raphanus raphanistrum L) is a prolific seed producing weed that is fast becoming problematic in wheat and lupin cropping areas in Australia (Walsh et al., 2004). Widespread throughout the world, it is thought to originate from the Mediterranean region and is considered a weed in cropping areas in the British Isles, USA, Europe, Canada and Chile (Piggin et al., 1978). It is the most troublesome broad leaf weed in Western Australia wheatbelt (Cheam and Code, 1998). Wild radish is an erect, outbreeding annual and the height of plants growing in crops commonly reaches 1 m in height (Cheam and Code, 1998). This weed is a highly competitive plant, especially in cereal and other winter crops resulting in up to 50% yield loss at wild radish density of 200 plants m\(^{-2}\) (Code and Reeves, 1981). As yet, there is limited knowledge concerning the genome sequence of this weed.

The effective practices for the control of wild radish are similar to those used for the control of most other weeds. Selective auxinic herbicides still remain the primary method of control. Code and Reeves (1981) reported that the ideal herbicide for controlling wild radish in wheat cultivation early in the growing season was bromoxynil/4-chloro-2-methylphenoxyacetic acid (MCPA) while 2,4-D and MCPA are effective in preventing seed production. However, Michael Walsh and his group (2004) have recently reported that farmers are experiencing herbicide resistance problems with 2,4-D in populations of wild radish (Walsh et al., 2004). To
date, the molecular basis of 2,4-D herbicide resistance in wild radish is largely unknown though there is a lot known about resistance to other herbicides.

Herbicide resistance may be described as “the evolved capacity of some plant biotypes (weeds) within a population to survive a herbicide treatment that would under normal circumstances be able to effectively control that weed population” (Heap, 1997). The development of herbicide resistance in weeds is said to be due to evolution and genetic variation for resistance must already be present in susceptible weed populations for resistance to be selected for and to subsequently evolve in plant populations (Jasieniuk et al., 1996). The major source of genetic variation is most likely to be gene mutations that confer resistance to a specific herbicide class (Jasieniuk et al., 1996). These mutations are not induced as a response to the application of herbicides but rather occur spontaneously (Jasieniuk et al., 1996). Repeated applications of herbicides do however impose a more intense selection pressure for the emergence of resistance in the susceptible population (Jasieniuk et al., 1996; Owen and Zelaya, 2005). Weed species with a high level of genetic diversity (e.g. weeds that are outbreeders) are considered to show significant potential for developing herbicide resistance (Holt and Hochberg, 1997).

5.1.2 Methods of assessing genetic diversity - a few commonly used techniques

Genetic diversity in organisms has traditionally been assessed by examining morphological and physiological traits. One such classic example is the experiment conducted by Gregor Mendel and his study of pea. This method of analysis laid the foundation for our understanding of genetics today. However, this technique has major drawbacks. Visible polymorphisms are highly influenced by the environment and thus limit our studies to specific taxon (Carvalho,
that express the phenotype under examination. These classical breeding experiments are also costly and time-consuming (Darmency, 1994).

The development of allozyme electrophoresis in the late 1960’s led to the emergence of the first set of molecular markers (Carvalho, 1998) and these markers examine protein polymorphisms to assess genetic differences. Their low cost and ease of use have made allozymes the method of choice for a wide range of studies (Jones et al., 2006; Moyle, 2006). However, despite their widespread use, allozymes have a number of disadvantages, including a lack of sufficient polymorphisms in some species, a need for fresh or frozen tissue samples, and an inability to detect a significant proportion of genetic variation (Ridgway, 2005).

Since the development of electrophoresis, a number of genetic analysis techniques have been introduced and a plethora of different molecular markers are available for genetic studies, each with a different method of assessing genetic differences. One such example is the restriction fragment length polymorphisms (RFLPs). This technique has been widely used in assessing genetic diversity in crop plants (El Karkouri et al., 2006; Ormeno-Orrillo et al., 2006). RFLPs are presumable neutral, highly polymorphic and co-dominant. However, RFLPs assays require computer-based systems which make this technique impractical due to laboratory restrictions. Furthermore the degree of polymorphism detected through RFLP among individuals within a species is so limited that it can be impossible to determine the origin of a plant species (Powell et al., 1996).

Amplified fragment length polymorphism (AFLP) analysis is a relatively novel technique that has been successfully applied to fingerprint a number of plant species (Datwyler and Weiblen, 2006; Wu et al., 2006). This approach is based on the selective polymerase chain reaction
(PCR) amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). Since fragments are PCR amplified, only a small amount of DNA is needed and DNAs of any origin or complexity can be used (Vos et al., 1995). Fingerprints are created without any prior knowledge of the genome sequence via the use of a limited set of generic primers (Vos et al., 1995). The number of fragments detected in a single reaction can then be “tuned” by selection of specific primer sets (Vos et al., 1995). This technique is considered more robust than any other genotyping methods since the annealing temperatures used during PCR amplification is stringent (Savelkoul et al., 1999). However, AFLP analysis also requires expensive computer software which is not accessible to most laboratories. Therefore practical limitations and costs related to AFLP analysis may restrict their use for routine identifications.

The development of another PCR-based technique known as random amplified polymorphic DNA (RAPD) has simplified the detection of DNA polymorphisms in a variety of species (Meikle et al., 1998; de Gruijter et al., 2004; Kim and Sappington, 2004; Rout, 2006) and has since proven to be a quick, easy and cost effective way of performing genetic analysis. This approach also requires a small amount of DNA and needs no prior knowledge of target DNA sequence, making RAPDs an ideal tool for the analysis of a wide range of plants whose genomic sequence information has yet to be completely unravelled. As with other molecular techniques, RAPDs also have certain disadvantages. If two amplified loci are of similar molecular weight, then they may be indistinguishable by electrophoresis of RAPD products. RAPDs are also dominant markers and hence heterozygous individuals; those with two different alleles at a locus cannot be distinguished from homozygous dominant individuals having two of the same allele at the locus. Allele frequencies estimated from RAPD data may, however, be still used under the
assumption that each locus has only two alleles, one present and one absent (Lynch and Milligan, 1994).

5.1.3 Aims

Genomic expression analysis has provided significant insight into the mode of action of 2,4-D. It may also provide a starting point in developing an understanding of the genes involved in the development of herbicide resistance in wild radish. However, the level of a specific gene transcript does not necessarily mean a corresponding alteration in protein level nor biological activity of final gene product. With this in mind, however, transcript analysis using *Arabidopsis* microarrays from herbicide resistant wild radish populations compared to transcriptomes from susceptible populations may shed some light on those genes involved in the development of resistance. Potentially, transcripts from resistant populations may be differentially regulated and it may be that mutation(s) in the genes found to be responsive to 2,4-D is likely to be causing 2,4-D resistance in wild radish.

However, before analysing the expression profiles of herbicide resistant and susceptible wild radish populations, it is important to investigate the genetic framework of these two wild radish biotypes. Wild radish is an obligate outbreeder and is therefore genetically very diverse. In order to ascertain that any differential gene expression recorded between resistant and susceptible wild radish populations is not because of their inherent genetic diversity, the genetic variability of the wild radish populations have to be assessed.

Previous genetic diversity studies by Huh and Ohnishi (2001) on East Asian wild radish (*Raphanus sativus var. hortensis f. raphanistroides*: the other wild radish species found in Japan and Korea), using allozymes, revealed a high level of genetic diversity within the wild radish
populations. However, the natural populations of the European and North American wild radish species were found to maintain significantly more variation when compared with the East Asian wild radish locus by locus. Further genetic studies on the East Asian wild radish species using AFLP also showed that the majority of genetic variation was observed within populations (Huh and Ohnishi, 2002). Huh and Ohnishi (2001, 2002) also found that the level of variation in the East Asian wild radish species was high compared with the other insect-pollinated outcrossing species.

The aim of this study was to gain an understanding of the genetic variability within and between two multiple herbicide resistant and one susceptible wild radish populations from the Northern wheatbelt of Western Australia (WA) using RAPD polymorphisms. This study may also identify RAPD markers that may be correlated to herbicide resistance.

5.2 Materials and methods

5.2.1 History and Plant Materials

Seeds of two wild radish populations (WARR 5 and WARR 6) showing multiple herbicide resistance and a susceptible biotype (WARR 7) were provided by Nufarm Australia Limited and Dr. Michael Walsh. WARR 5 and WARR 6 originated from fields on a farm situated in the Northern wheatbelt region of Western Australia (Table 5.1). These fields have been cultivated with either lupin (Lupinus angustifolius L.) or wheat (Triticum aestivum L.) for more than 17 years and have been exposed to annual herbicide selection pressure. WARR 5 and WARR 6 populations had received 36 and 34 applications of selective herbicides respectively by the end of the 1999 growing season (refer to Walsh et al. (2004) for information on the number and types of herbicides used to control these wild radish populations). Seeds of both WARR 5 and
WARR 6 populations were randomly collected at the end of 1999 from those wild radish plants that survived herbicidal treatments that year. WARR 7 seeds were collected from a nature reserve (located less than 20 km from the above mentioned farm) where no herbicide had previously been applied.

Table 5.1 Features of wild radish plants used for this study.

<table>
<thead>
<tr>
<th>Biotypes</th>
<th>Number of individuals</th>
<th>Year sampled</th>
<th>Crop at site in sampling year</th>
</tr>
</thead>
<tbody>
<tr>
<td>WARR 7</td>
<td>15</td>
<td>1999</td>
<td>N/A (bushland)</td>
</tr>
<tr>
<td>WARR 6</td>
<td>15</td>
<td>1999</td>
<td>wheat</td>
</tr>
<tr>
<td>WARR 5</td>
<td>15</td>
<td>1999</td>
<td>lupin</td>
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</tbody>
</table>

5.2.2 DNA extraction

Genomic DNA was isolated from WARR 7, WARR 6, WARR 5 leaf tissues using a modified CTAB (cetylmethyl ammonium bromide) method. Young leaf tissues were excised and frozen in 2 mL of liquid nitrogen. The frozen tissues were ground into a fine powder using sterile mortar and pestle followed by the immediate addition of 1 mL CTAB extraction buffer, transferred to a sterile 1.5 mL Eppendorf tube and incubated in a 55°C water bath for 20 minutes.

The solutions were centrifuged at high speed (13,000 rpm) for 10 minutes and 500 µL of supernatant from each tube was transferred to a clean tube. Five hundred µL of phenol: chloroform: isoamyl alcohol (25:24:1 by volume) was added to this tube, mixed by gentle inversion and then centrifuged for 5 minutes at high speed (13,000 rpm). Five hundred µL of the supernatant was removed and the process was repeated twice until a clear supernatant was obtained. The clear supernatant was transferred to a tube containing a double volume of chilled
absolute ethanol and a 1/10th volume of 7.5 M ammonium acetate. The tube was mixed by inversion and left for an hour at -20\(^\circ\)C for DNA precipitation. The tube was centrifuged at high speed (13,000 rpm) for 10 minutes to collect the DNA pellet. The pellet was washed in 200 µL of 70% ethanol before being thoroughly air-dried. The DNA pellet was resuspended in 100 µL of sterile TE buffer (1 mM EDTA, 10 mM Tris-HCL, pH 8.0) followed by incubation at 37\(^\circ\)C for 15 minutes with 0.5 µL of RNase-A to remove RNA. Extracted DNA was quantified by comparing band intensities with known standard of GeneRuler\textsuperscript{TM} 1 Kb DNA ladder on a 1% (w/v) agarose electrophoresis gel. Working solutions of DNA (1 ng µL\(^{-1}\)) were made using sterile, distilled water. Samples were stored at -20\(^\circ\)C.

5.2.3 RAPD reaction

Thirteen decamer primers of arbitrary sequence (Operon Technologies Inc.) were tested for PCR amplification (Table 2). DNA amplification was carried out in a total volume of 25 µL containing 10 ng µL\(^{-1}\) template DNA, 0.5 Units Taq DNA polymerase, 5mM MgCl\(_2\), 5 µL 10 x PCR buffer (500mM KCl, 100mM Tris-HCL pH 9.0, 1% Triton X-100), 1mM dNTP, 10 µM primer and sterile water. RAPD- PCR was performed via the Thermohybid PCR machine: 1 cycle of 3 minutes at 94\(^\circ\)C, 35 cycles of 15 seconds at 94\(^\circ\)C, 1 minute at 40\(^\circ\)C and 72\(^\circ\)C for 1 minute, and 1 cycle of final extension at 72\(^\circ\)C for 5 minutes. Negative controls without template DNA were included to ensure that there was no contamination or non-specific amplification.

5.2.4 Electrophoresis

The amplification products were separated on 1.5% (w/v) pre-stained (50 ng/mL ethidium bromide) agarose gel, in 1 x TBE buffer, at 90 V for 90 minutes. Gels were then viewed under a
UV transilluminator and amplification profiles were captured using the Biorad Gel Doc® system.

**5.2.5 Analysis of RAPD fragments**

**5.2.5.1 POPGENE**

Each PCR reaction was performed on two separate occasions to show that bands produced were reproducible. Reproducible bands were scored and were scored as present (1) or absent (0) for each sample and they were used to construct a data matrix. In cases where assignment of the presence or absence of bands was ambiguous, data was reported as “missing” in the assembly of the data matrix (17 ambiguous bands in total). The computer analysis package POPGENE version 1.31 (Yeh et al. 1999) was used to partition genetic variance into within and between populations and it was assumed that the marker alleles were in a Hardy-Weinberg equilibrium within each population. This may be a reasonable assumption as wild radish is a self-incompatible out-crossing species and there are usually numerous individuals within the dispersal range of its pollen. The POPGENE software was used to calculate genetic distances and similarities between populations. Two genetic distance (dissimilarity) matrices were generated based on calculations according to Nei (1972) by firstly grouping WARR 5, WARR 6 and WARR 7 as three separate populations and secondly by considering each individual of WARR 5, WARR 6 and WARR 7 as separate populations (45 populations in total).

**5.2.5.2 Generating dendrograms**

The resulting distance matrices were then used to generate dendrograms using the Unweighted Paired Group Method with Arithmetic Averages (UPGMA) and the computer package MEGA
version 2.1 (Kumar et al., 2001) and TREE-EXPLORER program version 2.1 (Kumar et al., 2001), thus providing a visual representation of the dissimilarity matrix.

5.2.6 Analysis of molecular variance (AMOVA)

The degree of variation between and within the 3 wild radish populations was examined by performing an analysis of molecular variance (AMOVA) using WINAMOVA 1.55 (Excoffier et al. 1992). Such hierarchical analyses are possible as AMOVA is based on a matrix of squared Euclidean distances between “chromosomes” which is equivalent to individual multilocus RAPD phenotypes and hence does not need complete genotypic information (Lougheed et al., 2000). The significance of the AMOVA variance components and corresponding $\phi_{ST}$ were tested using 1000 permutations (Excoffier et al., 1992). $\phi_{ST}$ summarises the degree of differentiation between population divisions and is analogous to Wright’s inbreeding coefficient, $F_{ST}$. Pairwise comparisons between the wild radish populations were also determined by pairwise $\phi_{ST}$ using WINAMOVA. Statistical significance was again determined using a permutation test.

5.2.7 Principal component analysis

Relationships within and between populations were also investigated using principal component analysis (PCA) to show the clusters of relatedness between the wild radish samples. This was performed using SPSS version 12.0 (SPSS, Inc., Chicago IL) and a 3-D graph was generated using the software Statistica version 5.1 (Statsoft, Tulsa, OK).
5.3 Results

Preliminary experiments with the thirteen RAPD primers revealed that a high level of polymorphism existed within and between the wild radish populations. Subsequently nine primers that gave an average of 11 clear and scorable bands per locus within a range of 4 to 17 bands per primer were chosen for further analysis (Table 5.2). A total of 118 polymorphic and non-polymorphic loci were examined. Bands examined ranged in size from 250 to 3000 base pairs (bp) and of the 118 bands, 97 were polymorphic, giving a percentage of polymorphic loci of approximately 82% in the three populations. The percentage polymorphic loci ranged from 67% in both the WARR7 population and WARR 6 population and up to 70% in WARR 5 population.

Table 5.2 List of RAPD primers, their respective nucleotide sequence and the number of polymorphic loci revealed by amplification of each WARR 7, WARR 6 and WARR 5 wild radish individuals tested.

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<th>Primer</th>
<th>Primer sequence 5' - 3'</th>
<th>Total number of bands</th>
<th>Total number of polymorphic bands</th>
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Diversity measures were estimated by Nei’s index (Nei, 1973) and ranged from $H = 0$ to $H = 0.5$ (Table 5.3). When averaged over all the markers, the WARR 5 population was found to be more diverse than both the WARR 7 and WARR 6 populations (Table 5.3).

Table 5.3 Genetic diversity within the three wild radish populations for 97 RAPD markers estimated by Nei’s ((Nei, 1973)) diversity measure.

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The average diversity within populations ($H_s$) was 0.24 and total diversity ($H_t$) was 0.27 (Table 5.4). The mean level of genetic differentiation ($G_{st}$) between populations over all loci was 0.1. Each of the markers contributed differently from the observed degree of population differentiation ranging from a low differentiation of almost 0% (0.002%) to a high of 42% (Table 5.4).

Table 5.4 Subdivision of genetic diversity into within and between wild radish populations for 97 RAPD markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>$H_t$</th>
<th>$H_s$</th>
<th>$G_{st}$</th>
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<td>0.02</td>
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<td>0.37</td>
<td>0.01</td>
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<td>0.10</td>
</tr>
</tbody>
</table>

Ht: genetic distance over all groups; Hs: genetic distance within populations; Gst: proportion of genetic diversity between populations.
The genetic distance between the three wild radish biotypes, derived from Nei’s (Nei, 1972) gene diversity index, varied from 0.01 between WARR 5 and WARR 6 to 0.05 between WARR 7 and WARR 5 (Table 5.5).

Table 5.5 Genetic distance between the three populations as estimated by Nei (1972).

<table>
<thead>
<tr>
<th>Population</th>
<th>WARR7</th>
<th>WARR6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WARR7</td>
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</tr>
<tr>
<td>WARR6</td>
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<td>-</td>
</tr>
<tr>
<td>WARR5</td>
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<td>0.01</td>
</tr>
</tbody>
</table>

5.3.1 Dendrograms

The dendrogram generated on the basis of Nei’s (1972) genetic distances and the UPGMA method showed two main clusters: WARR 5 and WARR 6 on one side and WARR 7 on the other side (Figure 5.1). Populations WARR 6 and WARR 5 showed 0.7% dissimilarity while WARR 7 was genetically different to the two resistant populations by 2.3%. The dendrogram generated from the 45 wild radish individuals (Figure 5.2) showed that all the samples had approximately 25% dissimilarity and that at the 20% level, the dendrogram branched into 2 groups. The smaller group contained more than half of the WARR 7 individuals and few of the WARR 6 population, while the bigger cluster consisted of all the individuals belonging to WARR 5, most of the individuals from WARR 6 and seven individuals from WARR 7.
Figure 5.1 Dendrogram indicating the relationships between the 3 wild radish populations.

The dendrogram was generated with MEGA and TREE-EXPLORER using the UPGMA method based on genetic matrices calculated according to Nei (1972).
Figure 5.2 Dendrogram showing the 45 individual from the 3 populations of wild radish based on percent dissimilarity.

The dendrogram was generated with MEGA and TREE-EXPLORER using the UPGMA method based on genetic matrices calculated according to Nei (1972).

5.3.2 AMOVA

The partitioning of molecular variance using AMOVA showed that most (87%) of the total genetic variance may be attributed to within population differentiation (Table 5.6). Mean pairwise $\varphi_{ST}$ for the RAPD dataset was 0.3 and $\varphi_{ST}$ comparison indicated significant genetic differentiation ($P<0.001$) (Table 5.7).

Table 5.6 Results from analysis of molecular variance

<table>
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<th>Level</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Variance</th>
<th>Total (%)</th>
<th>P</th>
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</thead>
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<td>0.48</td>
<td>0.02</td>
<td>13.04</td>
<td>$&lt;0.001$</td>
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<tr>
<td>Within</td>
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<td>0.15</td>
<td>86.96</td>
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</tbody>
</table>

Probability values are based on a null distribution from 1000 bootstrap replications

Table 5.7 Pairwise $\Phi_{ST}$ between populations of wild radish

<table>
<thead>
<tr>
<th>Population</th>
<th>WARR7</th>
<th>WARR6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WARR7</td>
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<td></td>
</tr>
<tr>
<td>WARR6</td>
<td>0.16</td>
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</tr>
<tr>
<td>WARR5</td>
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<td>0.05</td>
</tr>
</tbody>
</table>

5.3.3 Principal component analysis

This 3-D graph generated from the PCA analysis shows that most of the individuals belonging to either the resistant or susceptible biotype overlapped and the individuals could not be assigned to specific clusters (Figure 5.3). PCA analysis also revealed that three components
were required to account for a meaningful percentage of variance (16%, 7% and 6% respectively, with a cumulative total of 29%).

Figure 5.3 Scatter plot of the 45 wild radish individuals constructed from PCA data.

Numbers 5, 6 and 7 on plot represent individuals belonging to populations WARR 5, WARR 6 and WARR 7 respectively. The percentage of variance accounted for by each component is indicated in parentheses.
5.4 Discussion

5.4.1 Analysis of genetic diversity

As shown in Table 5.2, a high percentage of polymorphic loci were recorded within and between the wild radish populations. This observation is consistent with the reports that wild radish is a self-incompatible outcrossing species. Huh and Onishi (2002) studied the level of allozyme variation in East Asian wild radish and found that the isozyme variation was much higher compared with other insect-pollinated outcrossing annual species. The combination of an insect pollinated outcrossing breeding system, large population sizes, gene flow and prolific seed production (usually more than 300 seeds/individual) are all thought to contribute to the high level of genetic diversity observed in populations of wild radish (Huh and Ohnishi, 2001). The same mechanisms may be involved in the maintenance of the high levels of RAPD variation between biotypes of wild radish observed in this study.

In addition, WARR 5 population was found to show the highest diversity indices compared to both the WARR 7 and WARR 6 populations. This lower level of measured diversity displayed by WARR 7 and WARR 6 was mainly due to the presence of monomorphic loci such as OPBO4-4, OPC19-5, OPC19-8, OPX17-4, OPX17-6, OPNO4-6, OPBO8-13, OPBO8-14, OPVO6-8, OPAO11-6, OPAO11-13, OPAO11-19, OPAO11-20, OPUO3-4 and OPBO4-1, OPBO4-5, OPBO6-10 to OPBO6-14, OPBO6-16, OPAO11-19, OPAO11-16, OPUO3-1 respectively (Table 5.3). In contrast, WARR 5 population was the only population that displayed polymorphism at the OPBO6-8, OPBO6-9, OPC19-7 and OPX17-2 loci and was non-polymorphic at the OPC19-2 locus. Moreover, analysis of the genetic diversity within and between the wild radish populations (Table 5.4) revealed that only a low
proportion ($G_{st}$ value was 0.1 which equal to 10%) of overall diversity is observed between the 3 wild radish populations compared with diversity between individuals within each population (90%).

### 5.4.2 Dendrograms

The genetic distance values (Table 5.5) and subsequent dendrograms generated (Figures 5.1 and 5.2) correlated to the geographical locations where the wild radish seeds were collected. The WARR 5 and WARR 6 seeds were obtained from fields located on the same farm and as a result, had the smallest genetic distance (0.01). However, WARR 7, collected from a nature reserve (< 20 km) from the crop fields was characterised by the highest genetic distance (0.05). The close genetic distance observed for the weeds collected from adjacent fields may in part be due to the use of the same machinery on both fields, which is known to occur and which will aid in seed transfer and dispersal between the fields, consequently lowering the genetic distance between the populations (Michael Walsh, personal communication). Similar genotypes might have originally established the two wild radish populations. Pollen dispersal by insects which have small migratory distance may also explain the genetic distance observed between WARR 6 and WARR 5.

### 5.4.3 AMOVA

AMOVA showed that most (87%) of the total genetic variance may be attributed to within population differentiation (Table 5.6), as one would expect from an outbreeding plant given their breeding system (Huh and Ohnishi, 2001, 2002). These results are in agreement with the $G_{st}$ results, where only 10% of the total diversity was attributable to population differences, with 90% of the total diversity due to the variations between individuals within populations. Besides,
the genetic differences between the susceptible population and either of the two resistant populations (>0.15) were greater than the differences between WARR 6 and WARR 5 populations (0.05), the two resistant populations (Table 5.7). This significant difference (P < 0.001) between the resistant and susceptible wild radish populations could be attributed to the small migratory distances of most insects that pollinate wild radish plants, thereby limiting gene flow between the resistant and susceptible populations.

5.4.4 PCA analysis

Another perspective on the genotypic relatedness of wild radish populations is shown in Figure 5.3. Consistent with Figure 5.2, the PCA analysis showed that individuals within the three wild radish populations did not segregate into distinguishable clusters. This result is also consistent with the AMOVA results, which indicated that the genetic diversity was higher between individuals within a population than between populations. This occurrence of a larger amount of variation between individuals of each population was further highlighted by the fact that PCA analysis showed that three components were required to account for a meaningful percentage of variance (16.0%, 7.0% and 6.0% respectively, with a cumulative total of 29.0%) (Figure 5.3).

5.4.5 Populations are genetically similar

In most cases, according to Jasieniuk et al. (1996), herbicide resistance in weeds is conferred by a single gene and Moodie et al. (1997) further suggested that it is highly unlikely that herbicide application would select an herbicide resistant genotype of such significance that it would be detected by RAPD analysis. Moreover selection pressure on the two resistant wild radish populations confronted with these mixed herbicides and cultural practices for 17 years would be unlikely to result in the development of one particular form of herbicide resistance. Instead
Moodie et al. (1997) suggested that it is more likely that the variation detected between populations may be as a result of herbicide treatments giving rise to variations in phenotypes which may not be completely due to herbicide resistance. Hence the genetic diversity shown by WARR 6 and WARR 5 may simply be indicating the ability of the plants to survive and complete their life cycle and this variation may have little to do with herbicide pressure.

The genetically varied wild radish populations may be showing natural resistance, escaped from herbicide treatments or germinated from wild radish seeds whose dormancy broke at a time when herbicide pressure was not intensive (Moodie et al., 1997). However, it is noteworthy that WARR 5 and fourteen out of the fifteen WARR 6 individuals lack one allele generated by primer OPC19-8, which is present in the susceptible WARR 7 population. This particular marker may be correlated with herbicide sensitivity and hence be converted to sequence amplification region (SCAR) to be used as a marker for herbicide resistance testing on a segregating F1 population. However, it is also possible that this allele may simply be specific for WARR 7 population and hence absent in WARR 5 and WARR 6.

The three wild radish populations exhibited a high level of molecular polymorphisms characteristic of an outbreeding species. The genetic diversity between individuals within each population, by far, exceeded the diversity between populations. Hence the underlying genetic structure of the three wild radish populations appears to be the same despite the two populations exhibiting multiple herbicide resistance. This may imply that only a small number of loci might be controlling herbicide resistance in WARR 6 and WARR 5. At this stage, the slight genetic variation (as shown by the dendrogram, Figure 5.1) among the susceptible WARR 7 and the multiple herbicide resistant WARR 6 and WARR 5 wild radish populations can only be
attributed to geographical isolation and hence limited gene flow between the resistant and susceptible populations.

The molecular basis of resistance in wild radish is largely unknown and public databases contain little, if any information about the genome sequence of wild radish. In contrast, over the years, scientists have unravelled an enormous amount of information on the model plant *Arabidopsis* and comparative genomics studies using *Arabidopsis* microarrays have shown that such information are adaptable to plant species with uncharacterised genomes (Horvath and Olson, 1998; Girke et al., 2000; Horvath and Anderson, 2002; Horvath et al., 2003b; Horvath et al., 2003a). For instance, Horvath et al. (2003b) have successfully hybridised labelled cDNA from mature leaves and emerging tillers of wild oat onto *Arabidopsis* complementary DNA (cDNA) microarrays. More than 23% of the >11,000 cDNAs on the array, known to be involved the signal transduction pathways regulating growth and development, hybridised to the wild oat probe and these results shows that this well-characterised species can provide valuable leads into the physiological processes of less well characterised and agronomically important crops plants and weeds.

However, it was important to analyse the inherent gene diversity of this outbreeding weed before using the available *Arabidopsis* resources to investigate the differential gene expression between herbicide resistant and susceptible populations of wild radish in order to identify those genes which may play key roles in conferring resistance. This current study has shown that underlying genetic framework of the three wild radish populations appears to be similar. Hence any probable difference(s) in gene expression between resistant and susceptible populations of wild
radish may be correlated with herbicide resistance and may not be as a result of their intrinsic genetic difference.

Inferences drawn from this Chapter are as follows:

- A high degree of molecular polymorphisms exists between the 3 wild radish populations. This is characteristic of species that are outbreeders.

- Genetic diversity was higher within individuals between populations than between the WARR 5, WARR 6 and WARR 7 populations.

- Only one marker, OPC19-8, was found to be unique to the WARR 7 population and absent in WARR 5 and in most individuals of the WARR 6 populations. This marker may potentially be correlated to herbicide resistance.

- The two resistant wild radish populations were found to be closely related (0.7% dissimilar) to each other while the susceptible population was genetically dissimilar to WARR 5 and WARR 6 by 2.3%. This higher level of dissimilarity between the susceptible and resistant populations may be explained by limited gene flow between the populations since the susceptible population is geographically located further away from the resistant populations.

- The underlying genetic structure of the three wild radish populations appears to be similar despite that WARR 6 and WARR 5 have been exposed to mixed herbicide usage for over 17 years.
Chapter 6: General discussion

Traditional approaches of assessing surfactant performance in enhancing herbicidal activity have been limited to observing phenotypic changes in plants exposed to droplet application of active ingredients and the surfactant to directly studying the movement of radiolabelled molecules across intact plants or detached leaves (Stock et al., 1992; Foy, 1993; Stock and Holloway, 1993; Sharma and Singh, 2000) and isolated cuticles (Bukovac and Petracek, 1993; Schonherr and Baur, 1994). A number of studies have shown that surfactants are able to penetrate the leaf tissues (Holloway and Stock, 1989; Stock and Holloway, 1993) and based on data on the phytotoxicity (Spurrier and Jackobs, 1955; Stowe, 1960; Jansen et al., 1961; Schopmeyer, 1961; Temple and Hilton, 1963; Parr and Norman, 1964; Buchanan, 1965; Vieitez et al., 1965; Parr, 1982) and stimulatory effects of surfactants (Czeczuga et al., 1960; Stowe, 1960; Westwood and Batjer, 1960; Jansen et al., 1961; Stowe and Obreiter, 1962; MacDowall, 1963), it is clear that surfactants have their own intrinsic biological effects. Currently there is limited knowledge about the molecular mode of action of surfactants and the synergistic effect on the mechanisms of action of herbicides. A better understanding of the molecular mode of action of adjuvants/surfactants via the use of genomic tools such as microarray technology may contribute to the development of spray formulations that are target-specific and effective at low rates.

6.1 Phenotypic response to the different spray solutions

*Arabidopsis* seedlings grown in Petri dishes were sprayed with various spray solutions and the morphological changes induced were recorded at 1 h, 24 h and 48 h. Phenotypic changes in response to 1.0 mM 2,4-D did not cause plant death though plant growth was inhibited and leaf epinasty and petiole elongation were recorded (Chapter 2, Figure 2.5). The leaf curling and
petiole elongation recorded may be due to an “auxin-overdose” which promoted cell elongation. The most marked changes in the phenotype which included severe leaf curling, overall growth inhibition and subsequent plant death, were observed in response to the application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 (Chapter 2, Figure 2.5). This observation was consistent with the gene expression results as the genes associated with both ethylene biosynthesis and with senescence were up-regulated after treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.

Previous microscopic studies undertaken by Feng et al. (1999) have shown that tissue damage caused by surfactants is necessary for efficient herbicide penetration. The surfactant NUL1026 may function in the same manner. The senescence observed in parts of the leaves that were sprayed with surfactant NUL1026 may be due to tissue injury caused by the surfactant though plant growth and development were not hindered (Chapter 2, Figure 2.5). These observations correlate with the microarray results obtained from plants that had been sprayed with surfactant NUL1026 whereby a number of genes involved in maintaining cell membrane structure were differentially regulated and senescence-responsive genes were up-regulated.

6.2 Genomics to study mode of action of surfactants and auxinic herbicides

The use of the full genome arrays of *Arabidopsis* has led to the identification of a vast number of genes that potentially play important roles in the mode of action of surfactant NUL1026 and herbicide 2,4-D. This microarray technology has also highlighted the synergistic effect of surfactant NUL1026 when applied with herbicide 2,4-D on *Arabidopsis*. The higher number of genes involved in stress and senescence responses, that were expressed in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 compared with 1.0 mM 2,4-D alone and 0.2%
surfactant NUL1026 is a clear indication of the molecular synergy of the combination of surfactant NUL1026 and 2,4-D.

It is important to emphasise that changes in mRNA levels do not necessarily correlate to changes in protein product or enzyme activity levels. Despite the limitations of not being able to observe or elucidate the changes in proteins or the entire proteome, a great deal of valid and useful information can be gained from the examination of changes in the transcriptome and the inference of gene/transcript function from publicly available databases. The Gene Ontology (GO) databases were used to associate genes to function and the transcriptomic effects induced by the surfactant and herbicide are discussed based on the association of these responsive genes with their functions at cellular, molecular and biological levels. The robustness of this study lies not only in exploring the gene expression in the entire genome but also in aspects of analysis and application of functional genomics to the study.

6.3 Early and late response genes - kinetics of gene activation and pathway building

In this study, the global gene expression at two time points in response to 0.2% (v/v) surfactant NUL1026 and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 were investigated. Those genes that were responsive 1 h after treatment were considered as early response genes whereas those with significant changes in mRNA levels 24 h after treatment were considered late response genes.

Interestingly, when comparing the early and late response genes, the vast majority of these genes were either early or late response genes (Figures 3.3, 4.3 and 4.4), i.e., only a small number were present at both time points. For instance, only 22 genes still showed elevated mRNA levels after
24 h treatment with surfactant NUL1026, indicating that the elevated expression of most of the early genes that were induced was transient and not sustained through to 24 h post-treatment.

The time frame of gene induction of a particular gene by a signal may reflect the position of the gene product in a molecular response pathway. Hence early response genes often encode proteins involved in signalling or regulatory processes such as protein kinases and transcription factors while late response genes are effector genes coding for enzymes in metabolism. Several studies have shown that the protein products of early response genes regulate the expression of late response genes. For instance, it is known that in Arabidopsis, in response to pathogen and salicylic acid, both the WRKY and AP2/EREBP-type transcription factors are early response genes and their target genes which encode enzymes such as glutathione S-transferase are late response (Du and Chen, 2000). Based on the information gathered from the transcriptional profiling of early and late response genes, hypothetical models for gene regulation pathways can be built and further genetic and biochemical studies may subsequently be used to test the model pathways (Tepperman et al., 2001; Cheong et al., 2002; Brenner et al., 2005).

6.4 Molecular mode of action of surfactant NUL1026

The transcription profiling enabled the identification of 484 early and late surfactant responsive genes. These genes were found to be associated to a plethora of physiological and metabolic processes including transcription factor activity, signal transduction, cell wall expansion and organisation, disease response genes, detoxification, jasmonic acid (JA)-, ethylene- and auxin biosynthesis and senescence. A comparison of these 484 genes with those found to be responsive to 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h,
revealed that around 128 were specifically regulated in response to the surfactant when applied alone.

Of note was the up-regulation of JA biosynthesis inducible genes only in response to surfactant NUL1026 when applied alone. Surfactants are known to alter the cell membrane structure which in turn leads to the release of linolenic acid, the main substrate of the lipoxygenase enzyme and this enzyme is actively involved in JA biosynthesis. Treatment with surfactant NUL1026 also enhanced the expression of the gene encoding a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACS6 (At4g11280), the rate-limiting enzyme in the ethylene biosynthesis pathway (Yang and Hoffman, 1984) and the ethylene response factor 1 (ERF1- At4g17500) transcription factor gene. Both these ethylene response genes also showed induced expression in response 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.

The ERF1 transcription factor has been considered as the convergent point between ethylene and JA pathways and represents a plausible downstream component of both these signalling pathways (Lorenzo et al., 2003). Results of the present study have, therefore, led to the proposal of a model (Figure 6.1), to account for the molecular mode of action of surfactant NUL1026. This model briefly outlines the possible interaction between JA and ethylene in an ERF1-dependent manner to subsequently induce the expression of both defence (pathogenesis related 4 (PR4) - At3g04720) and senescence (e.g. dark inducible 11 gene (DIN11) - At3g49620) response genes.
Surfactant NUL1026

Interaction at cell membrane

Linolenic acid

Lipoxygenases (LOX)

Allele oxide synthase (AOS)

Allele oxide cyclase (AOC)

1-aminocyclopropane-1-carboxylate synthase 6 (ACS6)

Ethylene

12-oxophytodienoic acid reductase 3 (OPR3)

Jasmonic Acid

Ethylene response factor (ERF1)

Cross-talk

pathogenesis related 4 (PR4)
dark inducible 11 (DIN11)

Output response
This model proposes that surfactant NUL1026 caused an alteration in the plant cell membrane and this resulted in the release of bound linolenic acid (LA). Release of LA subsequently induced a cascade of enzymatic reactions involving the LOX, AOS, AOC and OPR3 enzymes to synthesise jasmonic acid (JA). Ethylene which might also have been generated by linolenic acid interacts with JA in an ERF1-dependent manner to induce the expression of defence and senescence response genes such as PR4 and DIN11 respectively. Dotted arrow indicates the possible positive feedback mechanism by ethylene, which may have induced the expression of ACS6 for further ethylene production. LOX- lipoxygenases, AOS- allele oxide synthase, AOC - allele oxide cyclase, OPR3- 12-oxophytodienoic acid reductase 3, ERF1- ethylene response factor 1, ACS6- 1-aminocyclopropane-1-carboxylate (ACC) synthase 6, PR4- pathogenesis related 4, DIN11 - dark inducible 11.

6.5 Transcriptomic effect induced by 1.0 mM 2,4-D

Microarray results revealed that 45 genes showed significant expression after treatment with 1.0 mM 2,4-D alone. This is in sharp contrast to the large number of genes being regulated in response to the other spray formulations being investigated in this study. The altered expression of this fewer numbers of transcripts may be due to the small amount of herbicide molecules able to pass through the cuticular barrier of the leaves. Another plausible explanation could be that this result may be a reflection of the short amount of time, at which transcriptomic analysis was carried out, by which time significant transcription had not taken place. Gene Ontology annotation of these 45 genes highlighted the auxinic nature of herbicide 2,4-D as shown by the induction of a series of known early auxin genes such as the small auxin up-regulated gene (SAUR) and members of the AUX/IAA genes.
6.6 Transcriptomic effect induced 1.0 mM 2,4-D + 0.2% surfactant NUL1026

Spray treatment of the 1.0 mM 2,4-D combined with 0.2% surfactant NUL1026 resulted in the up- and down- regulation of a vast number of genes, of which 118 and 199 genes were found to be specifically regulated in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 at 1 h and 24 h time points respectively. The addition of surfactant NUL1026 clearly had a synergistic influence on the mode of action of herbicide 2,4-D since approximately 10 times more genes showed altered expression in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 compared with 1.0 mM 2,4-D at the same time point after application.

A significant proportion of mRNAs that were responsive to the 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 treatment were also commonly induced by the surfactant treatment only. The expression of these overlapping genes may be solely due to the presence of the surfactant in the 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 formulation or may be as result of the combined effect of the surfactant and the herbicide. Further studies using mutant lines defective in surfactant or/and herbicide action may reveal which of the stress factor (surfactant alone or herbicide and surfactant combination) induced the expression of those overlapping genes. The mRNA transcripts that showed specific response after foliar application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 were those associated with auxin, ethylene and abscisic acid biosynthesis, senescence response genes and cell wall degradation. Photosynthesis was also profoundly affected as shown by the down-regulation of genes involved in chlorophyll biosynthesis and the process of photosynthesis.
6.6.1 Auxinic nature

Interestingly, treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, at both time points resulted in the induced expression of vast number of auxin response genes. Six of these genes, shown to be regulated as part of an early response to auxin (Grossmann, 2000; Hagen and Guilfoyle, 2002) were regulated in common with 1.0 mM 2,4-D. Additional auxin-responsive genes were specifically regulated 1 h post-treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 and these were related to auxin-signalling. Several of the exclusively up-regulated genes after 24 h treatment were also found to be induced as a result of auxin action and the overexpression of the IAA17 gene seems to indicate an overall repression of auxin-signalling at that time point.

These results are consistent with previous microarray studies conducted by Raghavan et al. (2005, 2006) which demonstrated that 2,4-D, at a toxic level, was still auxinic in nature and induced the expression of members of the auxin-response gene family. Hence, comparing the expression profiles of 1.0 mM 2,4-D and 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, the increase in the number of genes coding for auxin-response genes after treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026 may be indicating that surfactant NUL1026 had successfully promoted the uptake of additional 2,4-D molecules. This in turn resulted in the additional auxinic response by the plant cells.

6.6.2 Foliar - uptake versus root - uptake

Based on biochemical studies on cleavers by Hansen and Grossmann (2000), it was proposed that auxinic herbicides increase ethylene levels which in turn trigger abscisic acid (ABA) biosynthesis and this consequently results in growth inhibition and eventually senescence.
Recently, Raghavan et al. (2005, 2006) studied the transcriptomic changes in response to root-uptake of 1.0 mM 2,4-D in *Arabidopsis* for 1 h using microarray technology and have confirmed the regulation of genes involved in the production of ethylene and ABA. However, this current study which also looked at the molecular changes brought about by foliar-application of 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h post-treatment, revealed conflicting results when compared with those of Hansen and Grossmann (2000) and Raghavan et al. (2005, 2006). Of importance, this study registered the significant down-regulation of ABA-biosynthesis and ABA-responsive genes in response to 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026.

A number of plausible explanations may be attributed to this difference in gene expressions recorded in this study. Foremost, the proposed mode of action by these two groups of scientists (Hansen and Grossmann, 2000; Raghavan et al., 2005, 2006) have been based on the root-uptake of herbicide 2,4-D. However, this current study has investigated the gene expression profiles after foliar application of 2,4-D. Auxinic herbicides are said to have several modes of action, a situation that aggravates the identification of genes conferring auxinic herbicide resistance to plants. Thus it is likely that the mode of action of 2,4-D is organ specific and in leaves, growth inhibition and subsequent plant senescence by 2,4-D is brought about in an ABA-independent pathway. Studies have shown that response to drought and cold may occur both in an ABA-dependent (Kreps et al., 2002) and independent manner (Yamaguchi-Shinozaki and Shinozaki, 1994). Furthermore, several microarray experiments have highlighted the fact that roots and leaves have different transcriptome responses to stress (Kreps et al., 2002; Wang et al., 2003). Further studies should look into the difference in gene expression patterns between root and foliar-uptake of 2,4-D and identify genes which are unique to each organ.
Secondly, it is also possible that although all the experimental treatments (Raghavan et al. 2005 and this current study) were completed on the same genotype of *Arabidopsis* (Columbia) using 1.0 mM 2,4-D, the observed gene expression was finely tuned to the specific experimental conditions which therefore resulted in a set of genes induced by specific experimental conditions rather than a set of genes induced by 2,4-D. Besides, Bray (2002) have suggested that gene expression patterns are influenced by the severity, extent and rate of application of the stress. Hansen and Grossmann (2000) observed an increase in ABA levels after 5 hours of root application of 0.5 mM IAA while Raghavan et al. (2005) showed induction of ABA biosynthesis genes with 1.0 mM 2,4-D after 1 h of root-treatment. It would be interesting to conduct a time series experiment to see if there is induction of ABA response genes between the 1 h and 24 h time frame in response to foliar-application of 2,4-D.

6.7 Overlapping genes and hormone cross-talk

Among the 484 genes that showed altered mRNA levels in response to surfactant NUL1026, 356 transcripts were found to have similar expression levels after treatment with 1.0 mM 2,4-D + 0.2% (v/v) surfactant NUL1026, 1 h and 24 h post-treatment and 1.0 mM 2,4-D, 1 h. Functional categorisation of these 356 genes revealed that the majority were involved in transcription factor activity, disease resistance, cell wall organisation and biogenesis, detoxification, and senescence. Likewise, genes that were exclusively regulated in response to either the surfactant or the herbicide or the surfactant + herbicide combination were also associated to similar physiological and metabolic processes. In addition, the overlapping and specifically regulated genes were also shown to be involved in the biosynthesis and signal transduction pathways of auxin, ethylene, JA and abscisic acid. Expression profiles obtained in this study were found to show similarities with previous studies which looked at plant responses
upon abiotic, biotic and hormonal exposure (Bray, 2002; Cheong et al., 2002; Xiong et al., 2002; Bray, 2004; Goda et al., 2004; Brenner et al., 2005).

The commonality in expression profiles induced by different stimuli may be explained by the fact that plants do not have specific receptors for specific stimulus as such and do not tailor their response to the stimulus at hand; otherwise a fast response to a number of stimuli would be impossible and this may explain the common pathways or routes employed by plants in response to cold, drought, salt, pathogens, wounding, ethylene, auxin and abscisic acid (Bray, 2002; Cheong et al., 2002; Xiong et al., 2002; Bray, 2004; Goda et al., 2004; Brenner et al., 2005).

In addition, after signal perception, the secondary signals (e.g. reactive oxygen species (ROS)) generated may differ in specificity to the primary stimuli and this secondary signal may be shared by different stress pathways (Xiong et al., 2002). These pathways may converge and interact with one another in an antagonistic or synergistic manner using shared components such as transcription factors or signalling components in a complex intertwined network to bring about the appropriate response. For instance, both ethylene and JA converge via the transcription factor ERF1 to induce the expression of defence-related genes (Lorenzo et al., 2003). Communication among different signaling pathways is thought to render plants capable of defending themselves against a variety of different stresses (Glazebrook, 2001).

In order to discriminate and identify genes which are unique to each of the formulations investigated in this project a comparison could be made between the expression profiles reported in this study with those of published microarray experiments in which Arabidopsis plants have been subjected to a variety of abiotic and biotic stress treatments. Preliminary microarray analyses have shown that as opposed to passive root uptake of water, spraying Arabidopsis
plants with water alone in itself triggered a wide array of stress responses at the molecular level (data not shown). However, in this study, the general stress response triggered by water and spray application was subtracted from the genomic response observed as a result of the different spray treatments. Hence data reported in this study may already be providing specific cues to identifying surfactant and herbicide responsive genes rather than including genes responding to the stress of spray treatment as well.

6.8 Towards the development of improved spray formulations and other future prospects

This study has identified and catalogued for the first time, a list of plant genes that were found to be surfactant responsive. This list provides the starting point to dissect and understand the mode of action of an etheramine surfactant. Understanding how surfactants function at the transcriptomic level may help in the development of surfactants that have chemistries which complement those of the herbicides, hopefully leading to the development of spray formulations, that have better binding capacity, are more effective, are safer to the environment and pose less risk to the people working with such compounds on a regular basis.

Not all surfactants increase the uptake of any herbicide. Gaskin and Holloway (1992) have shown that glyphosate uptake into wheat and bean was improved by linear alcohol surfactant rather than by nonylphenol surfactants. Moreover, Liu (2004b) has recently demonstrated that \textit{C}_{13}\textit{C}_{15} surfactants are more efficient as opposed to octylphenol surfactants for both glyphosate and 2,4-D. Surfactant NUL1026 had a synergistic effect on 2,4-D as seen by the significant proportion of stress regulated genes induced when the surfactant was combined with 2,4-D.

Several of the surfactant and herbicide responsive genes identified in this study are potential targets for engineering plants with herbicide tolerance. Among these candidate gene lists, the
ABC transporter genes and glutathione S-transferases involved in detoxification are of special interest. Mutant plants overexpressing these detoxification genes may be able to show resistance to these spray formulations.

This study has thus highlighted that microarray technology may provide a novel way for manufacturers and scientists to test the efficacy of new surfactant formulations and estimate the performance of spray formulations. Moreover, it would be interesting to carry out similar biochemical tests as those undertaken by Hansen and Grossmann (2000) in order to verify whether foliar-application of 2,4-D follows an ABA-dependent or independent mode of action. The regulation of gene expression in response to foliar-application of different types of herbicides may also be investigated. This would help identify specific genes/proteins that hold the key to the mode of action of 2,4-D. Further work involving mutant lines defective in these 2,4-D specifically regulated genes will help determine the roles of these genes in bringing about the herbicidal effects and could prove to be important in the development of novel herbicides.

Furthermore, the lists of genes that showed differential regulation to 1.0 mM 2,4-D + 0.2% surfactant NUL1026 have provided the starting point to understanding and identifying genes involved in the mechanism of herbicide resistance in weeds. This will subsequently contribute to the design of more effective herbicide formulations and other weed control strategies. Arabidopsis microarrays may be used to monitor the expression profiles of these candidate genes in herbicide resistant and susceptible weeds, in particular in weeds belonging to the Brassicaceae family. Assuming that herbicide resistance is conferred by gene mutations, as it has been largely speculated, the gene expression patterns in response to 2,4-D are expected to be different for the susceptible and resistant biotypes.
Wild radish, a member of the Brassicaceae family, is a major weed in Australia and 2,4-D resistance is beginning to emerge in wild radish populations. The genome sequence of wild radish is largely uncharacterised and the molecular mechanisms of 2,4-D resistance is unknown. Hence *Arabidopsis* microarrays may be used to elucidate the mechanisms of 2,4-D resistance.

However, wild radish is an outbreeder and therefore it is important to first investigate the intrinsic genetic structure of the herbicide resistant and susceptible biotypes so as to be more confident that the differences in gene expression profiling that may be seen between the two biotypes are as a result of their resistance mechanisms. This study has revealed that there is a high degree of molecular polymorphism between the 3 wild radish populations (WARR 5, WARR 6 AND WARR 7), characteristics of outbreeders. Moreover, the genetic diversity within individuals between populations was higher than between the WARR 5, WARR 6 AND WARR 7 populations. Overall, despite that WARR 6 and WARR 5 have been exposed to mixed herbicide usage for over 17 years, the underlying genetic framework between the resistant and susceptible populations of wild radish is similar though not identical. Therefore, any likely difference(s) in gene expression between resistant and susceptible populations of wild radish may be associated with herbicide resistance rather than their intrinsic genetic difference.

**6.9 Concluding remarks**

To my knowledge this is the first comprehensive expression profiling of an etheramine surfactant over time. In addition, this is the first report to investigate the relationship between the action of a surfactant and an auxinic herbicide using a comprehensive profiling approach. The time course experiment revealed the overlap and divergence between the actions of these two components that play an active role in the spray formulation portfolio. Expression profiling results obtained in this current study also showed, not surprisingly, that the mode of action of
surfactant NUL1026 does not involve simple linear pathways but instead consists of an intricate and complex web of intersecting branches. Furthermore, the mode of action of foliar-applied 2,4-D might also be different to that of root-applied and likewise, this network is not linear. The microarray results in this study provide a useful starting point for more in-depth analysis. This study has created candidate gene lists to help prioritise the arduous task of using reverse genetics to assign functionality to genes and these lists will subsequently advance our understanding of the mode of action of surfactants and auxinic herbicides. Further investigation using biochemical and cell biology experiments will, therefore, be needed before applying the findings from this study to practical agriculture, that is to the development of novel herbicides with better efficacy.
Database references

www.arabidopsis.org

The *Arabidopsis* Information Resource (TAIR), funded by the National Science Foundation (NSF), is a collaboration of the Carnegie Institute of Washington Department of Plant Biology, Stanford, California and the National Center for Genome Resources (NCGR). This database is dedicated to scientific research in the model plant *Arabidopsis* and consists of various tools and databases for microarray experiments. The Gene Ontology (GO) annotations for the *Arabidopsis* genes in this study were retrieved from the TAIR database.

www.affymetrix.com

This is the website for the company Affymetrix® that manufactures oligonucleotide arrays for a number of organisms, including the whole genome array of *Arabidopsis* (ATH1-121501). The NetAffx™ database, which contains updated information related to the probes on the array, is also maintained by Affymetrix® and is accessible from this web-site. Affymetrix® developed the Microarray Suite (MAS 5.0) software and technical details pertaining to the software, which includes the MicroDataBase (MicroDB) and the Data Mining Tool is available on this web page.
Chapter 7 Bibliography


Appendix I

Murashige and Skoog medium (half strength) – 1 litre

Murashige and Skoog basal salts (Sigma M5524) - 2.15 g

Vitamin stock 1000x - 1 mL (The vitamin stock (1000x) stock was prepared by dissolving Murashige and Skoog vitamin powder (Sigma M7150) 10.3 g in 100 ml autoclaved MilliQ water and aliquots of 1 mL each were stored in microcentrifuge tubes in freezer))

Sucrose - 15g
MilliQ water - final volume brought to 1000 mL
pH adjusted to 5.7
Phytage - 5 g
Autoclaved

30 mL of the autoclaved medium was poured into deep Petri dishes (35mm x 10mm)
After the medium set the plates were stored at 4°C

Appendix II

1x TBE buffer (1 litre)

Tris hydroxymethyl aminomethane - 10.8 g
Boric acid - 0.98 g
EDTA disodium salt - 0.55 g

Appendix III

All buffers were prepared according to instructions in the course Affymetrix® GeneChip Expression Analysis manual.
Buffers:

5x RNA Fragmentation buffer (200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) 20 mL

1 M Tris acetate pH 8.1 (Trizma Base, pH adjusted with glacial acetic acid) 4mL

MgOAc 0.64 g
KOAc 0.98 g
DEPC treated water upto 20 mL
Filter sterilised through a 0.2 µm filter
Aliquots were stored at room temperature

1× MES Hybridisation buffer

a) 12× MES Stock (1.22 M MES, 0.89 M [Na+] 1000 mL

MES frees acid monohydrate 70.4 g
MES Sodium Salt 193.3 g
Distilled water 800 mL
pH 6.5 to 6.7
Volume finally made up to 1000 mL
Filter sterilised through 0.2 µm filter
Stored in dark at 4°C

b) 2× MES Hybridisation Buffer 50 mL

12× MES Stock 8.3 mL
5M NaCl 17.7 mL
0.5 M EDTA 4.0 mL
10% Tween 20 0.1 mM
Distilled water 19.9 mL
Stored in dark at 4°C
1× MES hybridisation buffer was prepared by mixing equal volumes of 2× MES hybridisation buffer and distilled water. Stored in dark at 4°C.

**Appendix IV**

**Non-stringent wash buffer 1000 mL**

20× SSPE

(3 M NaCl, 0.2 M NaH2PO4, 0.02 M EDTA) BioWhitaker #16-010

10% Tween 20 1 mL

Distilled water 698 mL

Filter sterilised through a 0.2 μm filter

**Stringent wash buffer 1000 mL**

12× MES Stock buffer (Refer preparation of 1× MES Hybridisation buffer) 83.3 mL

5 M NaCl 5.2 mL

10% Tween 20 1.0 mL

Distilled water 910.5 mL

Filter sterilised through a 0.2 μm filter, stored in dark at 4°C

**Appendix V**

**2× Stain buffer 250 mL**

12× MES Stock buffer (Refer preparation of 1× MES Hybridisation buffer) 41.7 mL

5 M NaCl 92.5 mL

10% Tween 20 2.5 mL

Distilled water 112.8 mL

Filter sterilised through a 0.2 μm filter, stored in dark at 4°C

1× stain buffer was prepared by mixing equal volumes of 2× stain buffer and distilled water. Stored in dark at 4°C.
Appendix VI

Default parameters for microarray data analysis using MAS 5.0

<table>
<thead>
<tr>
<th>Parameter</th>
<th># Probe pairs / Probe Set (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha1</td>
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<tr>
<td>Alpha2</td>
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<tr>
<td>Tau</td>
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<tr>
<td>Gamma1L</td>
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</tr>
<tr>
<td>Gamma1 h</td>
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</tr>
<tr>
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