

https://dspace.org/

Gene expression studies in Arabidopsis in response to South African Cassava Mosaic Virus infection utilizing microarrays

Pierce, Erica Joanna

2006-11-16T12:39:26Z
http://hdl.handle.net/10539/1813

Downloaded from a DSpace repository. https://dspace.org/

GENE EXPRESSION STUDIES IN *ARABIDOPSIS* IN RESPONSE TO *SOUTH AFRICAN CASSAVA MOSAIC VIRUS* INFECTION UTILIZING MICROARRAYS

Erica Joanna Pierce

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science

Johannesburg, 2005

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

_____ day of _____ 2005

ABSTRACT

Cassava Mosaic Disease is the most devastating disease affecting cassava (*Manihot esculenta* Crantz) crops worldwide. This disease is associated with eight species of geminiviruses, all belonging to the genus *Begomovirus* of the family *Geminiviridae*. In South Africa, in particular, CMD is caused by *South African cassava mosaic virus* (SACMV). Currently, there are no adequate methods for control of this disease as mechanisms within virus-host interactions are poorly understood. This brings about the need for development of virus-disease control strategies. This study was therefore conducted to identify the host's response to an invading virus. The model plant, *Arabidopsis* was chosen as it is a well-characterized plant system, with expression databases readily available as its entire genome has been sequenced. This study was conducted, firstly, to phenotypically determine if *Arabidopsis* was resistant or susceptible to SACMV infection, and secondly, to identify the host's response to pathogen infection on a molecular level through gene expression studies utilizing microarrays. Results from the symptomatology study revealed that *Arabidopsis* plants were fully symptomatic 28 days post-inoculation, displaying characteristic disease symptoms such as stunting, yellowing, and leaf deformation. This indicated that *Arabidopsis* was susceptible to SACMV infection. Microarray analyses revealed 86 differentially expressed genes, of which 48 showed up-regulation and 38 down-regulation. Relative quantification real-time PCR was performed on selected genes to confirm these results. Many up-regulated genes were shown to be primarily involved in a general stress response induced by the host, whereas those genes that were down-regulated seemed to be involved in more specific responses to viral invasion, probably a consequence of suppression of host genes by SACMV to enhance its own replication. The majority of genes identified fell under the predominant functional categories involved in metabolism, transcription, and transport. To our knowledge, this is the first study in which a DNA geminivirus has been used in a host-pathogen interaction utilizing microarrays.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Professor Christine Rey for her advice, encouragement, enthusiasm and support throughout the course of this study.

Special thank-you to Daniel Theron and Sally-Ann Walford for their microarray assistance.

I would also like to thank the Council for Scientific and Industrial Research, the National Research Foundation, and the University of the Witwatersrand Postgraduate Merit Award for the financial support.

Lastly, I would like to thank all my family and friends for their support and encouragement throughout my studies.

CONTENTS

DECLARATION……... 2
ABSTRACT……... 3
ACKNOWLEDGEMENTS……... 4
CONTENTS……... 5
LIST OF FIGURES……... 8
LIST OF TABLES……. 10
LIST OF ABBREVIATIONS…… 11

CHAPTER ONE – INTRODUCTION

1.1 Importance of Cassava…….. 13

1.2 Constraints to Cassava Production…….. 15

1.2.1 Pests and Diseases……. 15
1.2.2 Cassava Mosaic Disease (CMD) ……. 16
1.2.3Geminiviruses…… 17

1.3 Towards Improving Cassava-Based Systems……. 22

1.3.1 Host-Pathogen Interactions……. 22
1.3.2 *Arabidopsis* as a Model Plant System in Host-pathogen Interactions……... 28

1.4 Analysis "Tools" for Host-Pathogen Interaction Studies…….. 31

1.4.1 Analysis of Host-pathogen Interactions through Microarray Technology……. 31
1.4.2 *Agrobacterium*-Mediated Gene Transfer (Agroinoculation) of SACMV into *Arabidopsis*……. 36
1.4.3 Validation of Microarray Results through Real-Time RT-PCR…. 36

1.5 Objectives and Specific Aims……... 39

CHAPTER TWO – METHODS AND MATERIALS

2.1 Plant Growth…… 40

2.2 Virus Inoculations (Agroinoculation)…….. 43

2.3 Confirmation of Symptoms……. 44

2.3.1 Total Nucleic Acid Extraction (TNA)…… 44
2.3.2 Polymerase Chain Reaction (PCR)……... 44
2.3.3 Absolute Quantitative Real-Time PCR……..... 45

2.4 RNA Extractions……. 45

2.4.1 Purification and Quantification of RNA……. 48
2.4.2 PCR……. 48

2.5 Labeling, Hybridization, Scanning and Washing……... 49

2.5.1 Fluorescent Target Preparation……... 49
2.5.2 Hybridization…….. 50
2.5.3 Target Preparation…….. 50
2.5.4 Washing…….. 50
2.5.5 Scanning……. 51

2.6 Data Analysis……... 51

2.6.1 MIDAS-Normalization……... 53
2.6.2 Standard Deviation (SD) Regularization…… 53
2.6.3 Flip-dye Consistency Checking…….. 53
2.6.4 Virtual Trim…… 53
2.6.5 MEV – T-test, HCL Clustering and TAIR……..... 54

2.7 Validation of Microarray Results Using Real-Time RT-PCR…… 54

CHAPTER THREE – RESULTS

3.1 Symptom Development……... 57

3.2 Confrimation of SACMV Infection……. 61

3.2.1 Total Nucleic Acid Extraction (TNA)…… 61
3.2.2 PCR……. 61
3.2.3 Absolute Quantification Real-Time PCR…… 62

3.3 RNA Extractions……. 66

3.3.1 Concentration and Purity Determination…… 66
3.3.2 RNA Formaldehyde Gel Electrophoresis……... 67
3.3.3 PCR……... 67

3.4 Microarrays……... 70

3.4.1 Locally Weighted Scatterplot Smoothing (LOWESS) Normalization... 70
3.4.2 Standard Deviation (SD) Regularization…… 73
3.4.3 Flip-Dye Consistency Checking……. 77
3.4.4 MEV and TAIR…….. 79

3.5 Real-time RT-PCR ……. 82

CHAPTER FOUR – DISCUSSION..... 85

CHAPTER FIVE – REFERENCES..... 93

LIST OF FIGURES

Figure 1.1 (A) A field of healthy cassava plantings, and (B) starchy cassava roots..... 13

Figure 1.2 Cassava leaves showing the characteristic yellow mosaic patterns, leaf reduction and distortion, as a result of CMD..... 16

Figure 1.3 Bipartite genome organization of begomoviruses (particular species shown is SACMV) 18

Figure 1.4 Interference of geminivirus proteins with host cell pathways.... 19

Figure 2.1 Schematic representation of overall methodology used in this study..... 41

Figure 2.2 Schematic representation of methodology performed in section (A) showing: Plant Growth, Virus Inoculations, Symptom Development and Symptom Confirmation..... 42

Figure 2.3 Schematic representation of methodology performed in section (B) showing: RNA Extractions, Fluorescence-Labeling of cDNA, Hybridization, and Scanning..... 47

Figure 2.4 Schematic representation of methodology performed in section (C) showing data analysis using: GenePix version 5.1, MIDAS, MEV, TAIR, and Relative Quantification Real-Time RT-PCR……... 52

Figure 3.1 Showing (A) Healthy *N.benthamiana* (mock-inoculated control); (B) Infected *N.benthamiana*. Typical symptoms observed is the stunting of plants; (C) Infected *N.benthamiana* leaves showing stunting, leaf curling, crinkling, and chlorosis.. 58

Figure 3.2 BR1 showing (A) Typical symptom such as stunting of the entire plant; (B) Healthy *Arabidopsis*; (C) Yellowing and distortion of young SACMV-infected leaves; (D) Leaf deformation……..... 59

Figure 3.3 BR2 showing (A) and (C), Healthy *Arabidopsis*; (B) and (D) Typical symptom such as stunting of the entire plant; infected plants appear flaccid, diameter of rosettes are smaller than healthy controls and the plants are reduced in height; F) Severely reduced leaf size compared to (E) healthy leaf……... 60

Figure 3.4 BR1 showing a 1% agarose gel of (A) TNA extracted from SACMV-infected *Arabidopsis* leaf samples and (B) Amplification of the ~550bp CCP fragment from SACMV-infected *Arabidopsis* leaves……. 63

Figure 3.5 BR2 showing a 1% agarose gel of (A) TNA extracted from SACMV-infected *Arabidopsis* leaf samples and (B) Amplification of the ~550bp CCP fragment from SACMV-infected *Arabidopsis* leaves……. 64

Figure 3.6 (A) Standard curve obtained from plotting log of known DNA concentrations of plasmid SACMV DNA-A ; (B) 1% Agarose gel…… 65

Figure 3.7 BR1 (A) 1% MOPS/Formaldehyde gel electrophoresis of healthy *Arabidopsis* RNA and B) 1% agarose gel showing band amplification of *Arabidopsis* DNA using ubiquitin primers……. 68

Figure 3.8 BR2 (A) 1% MOPS/Formaldehyde gel electrophoresis of healthy *Arabidopsis* RNA and B) 1% agarose gel showing band amplification of *Arabidopsis* DNA using ubiquitin

primers……. 69

Figure 3.9 BR1 LOWESS pre-(blue) and post (red) normalization ratio-intensity (RI) plots……... 71

Figure 3.10 BR2 LOWESS pre-(blue) and post (red) normalization ratio-intensity (RI) plots……... 72

Figure 3.11 BR1 Box Plots displaying the intensity log-ratio distribution for each of the 32 blocks on an array before (pink) and after (green) scale normalization……. 74

Figure 3.12 BR2 Box Plots displaying the intensity log-ratio distribution for each of the 32 blocks on an array before (pink) and after (green) scale normalization……. 75

Figure 3.13 Standard deviation (SD) normalization RI plots before SD regularization (Black) and after regularization (Purple) …… 76

Figure 3.14 Flip-dye consistency checking for I(b)/I(a) ratios or (Cy5/Cy3) ratios. Black indicates data before replicate filtering and blue shows filtered data resulting in a better correlation between the flip-dye pair……. 78

Figure 3.15 Hierarchical Clustering of 86 differentially expressed *Arabidopsis* genes……... 81

Figure 3.16 Relative Quantification Real-Time RT-PCR…….. 84

LIST OF TABLES

Table 2.1 Primer and Probe Sequences used for Relative Quantification Real-Time RTPCR…….. 56

Table 3.1 RNA concentration and purity determination for each biological replicate…….. 66

Table 3.2 Probability (*P*) of exceeding the critical value ……... 79

Table 3.3 Differential up or down-regulated genes in SACMV-infected *Arabidopsis* based on mean $\log_2$ ratios above 0.5 or below 0.5…… 80

Table 3.4 A comparison made between ratios obtained from real-time RT-PCR and microarray analysis……..... 83

LIST OF ABBREVIATIONS

AA-dUTP	amino allyl d-UTP
ACMV	*African cassava mosaic virus*
avr	avirulence
BCTV	*Beet curly top virus*
BR1	Biological replicate 1
BR2	Biological replicate 2
CaMV	*Cauliflower mosaic virus*
CCD	charge-coupled device
CCP	core coat protein
cDNA	complentary DNA
cf	confidence factor
CMD	Cassava Mosaic Disease
CMGs	Cassava Mosaic Geminiviruses
CMV	*Cucumber mosaic virus*
CP	coat protein
CP	crossing point
CTAB	cetyltrimethyl ammonium bromide
DD	differential display
df	dispersion factor
dsDNA	double-stranded DNA
EACMV-UG	Ugandan variant
EST	expressed sequence tag
ET	ethylene
EtBr	ethidium bromide
HCL	hierarchical clustering
HC-Pro	helper component proteinases
HR	hypersensitive response
ISR	induced systemic resistance

JA	jasmonate
LOWESS	locally weighted linear regression
LRR	leucine rich repeat
MEV	MultiExperiment Viewer
MHC	Microarray high-speed centrifuge
MSV	*Maize streak virus*
NSP	nuclear shuttle protein
ORFs	open reading frames
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PTGS	post-transcriptional gene silencing
pRbs	retinoblastoma-like tumour suppressor protein
PVX	*Potato virus X*
R	resistance
RBR	retinoblastoma related
RCR	rolling circle replication
REn	replication enhancer
Rep	replication protein
R-I	ratio intensity
ROI	reactive oxygen intermediate
rpm	revolutions per minute
RT	reverse transcription
RT	room temperature
SA	salicyclic acid
SA	South Africa
SACMV	*South African cassava mosaic virus*
SAR	systemic acquired resistance
SAGE	serial analysis gene expression
SD	standard deviation
siRNAs	short interfering RNAs
ssDNA	single-stranded DNA
TAIR	The Arabidopsis Information Resource
T-DNA	transferred DNA
TMV	*Tobacco mosaic virus*
TNA	total nucleic acid
TrAP	transcriptional activator
TR	technical replicate
TYLCCNV	*Tomato yellow leaf curl China virus*

CHAPTER 1

INTRODUCTION

1.1 Importance of Cassava

Cassava, (*Manihot esculenta* Crantz, Euphorbiaceae), also commonly known as Manioc, Tapioca, Brazilian arrowroot, and Yuca, is a short-lived perennial, woody shrub that grows up to 5 metres tall, and produces enlarged tuberous roots (Alleman and Coertze, 1996). It is a major carbohydrate source, well-known for its starchy root, providing the primary calorie source for over 500 million people in the tropics, sub-tropical Africa, Asia and Latin America (El-Sharkway, 2004; Olsen, 2004). Cassava has therefore become the major source of carbohydrates in sub-Saharan Africa and the fourth most important tropical crop worldwide (Olsen, 2004) (Figure 1.1).



Figure 1.1 (A) A field of healthy cassava plantings, and (B) starchy cassava roots (IITA, 2001).

Annually, cassava is able to produce root yields of more than 170 million tons, as it has acreage of more than 16 million hectares worldwide. During the last 30 years, cassava production has increased 75% (Anderson *et al.*, 2004). As a monocrop cassava can yield as much as 90 tons of fresh roots per hectare, under favourable experimental conditions. It is, however, usually grown in poor soils and harsh climates

and in association with other crops, such as maize, beans, or cowpeas. The average yield in tons of fresh roots per hectare is much lower under these conditions: 9.6 tons worldwide; 7.7 tons in Africa; 12.7 tons in Latin America; and 12.9 tons in Asia (CIAT, 2001_a).

Cassava is generally grown by small-holding farmers as a subsistence crop. It is valued particularly for its drought tolerance and ability to grow in poor acid soils. It can be safely left in the ground for 7 months to 2 years after planting, providing there is effective security against famine. Furthermore, it is not season-bound and can be planted and harvested at any time of the year (Nestel, 1980; CIAT, 2001_a). After harvesting, fresh cassava roots are either eaten on the farm, processed for starch extractions, dried for flour production, roasted for human and/or animal feed, and marketed for consumption. These processed food products are commonly known as *farinha da mandioca* in Brazil, *gablek* in Indonesia, and *gari* or *foufou* in West Africa (El-Sharkway, 2004).

For human food consumption, the cassava root is prepared in many ways, i.e. boiling, baking, frying, as meal, flour as well as in beer. In West and Central Africa, fresh leaves are eaten as a vegetable. A wide range of sweet and savoury foods such as crackers, tapioca pearls, noodles, and cheese breads are made from the starch extracted from cassava roots. When utilized as animal feed, the fresh roots provide a first-rate source of carbohydrate and the leaves may be used as a protein supplement for cattle. Concentrates of dried cassava are also used for poultry, pigs, and cattle as well (Alleman and Coertze, 1996). Cassava's role varies in different parts of the world. It is grown mainly by women and used mostly for food in sub-Saharan Africa. As a result, it is an important source of employment and income, since most of the processing of this crop into food is done on a small scale in rural areas (CIAT, 2001_a). In Asia and Latin America, cassava starch is used in industry in the manufacture of many chemical products such as citric acid, sorbitol, mannitol, monosodium glutamate, high fructose syrup, glucose, and alcohol. It may also be used in paper-making, food processing, as a lubricant in oil wells, adhesives and textiles (Alleman and Coertze, 1996).

The uses of cassava described above have major applications in South Africa (SA). It is the secondary staple food to maize, and cassava produce is sold by small-holder farmers in provinces such as Mpumalanga, Limpopo and Kwazulu-Natal. In addition to this, cassava starch is used for paper, wood,

textile, pharmaceutical, chemical, corrugated cardboard and mining industries (J. Casey, personal communication; Mathews, 2000). About 90 000 tons of starch is produced every year for these purposes in SA (J. Casey, personal communication). Cassava starch could become the preferred source (if introduced successfully) of glucose production for raw materials as it produces a higher yield per unit area of land. The yield of cassava fresh tuber per hectare is 10-30 tons whereas maize grains are only 1-7 tons on dry land. Irrigated cassava tubers produce 30-70 tons per hectare whereas maize grains produce 5-10 tons per hectare (Mathews, 2000). In addition to this, the low protein content of cassava starch makes it comparatively slightly easier to hydrolyse. SA also utilizes cassava in the making of ethanol where 1 ton of fresh tuber supplies 180 litres of ethanol, this is less (70 litres) than maize but cassava's yield per hectare of available raw material is up to 7-10 tons more than maize. Cassava's food market is expanding at a rapid rate with increasing profitability and sustainability of cropping systems of the small-holder farmers due to its drought tolerance, especially in semi-arid regions of SA (Mathews, 2000).

1.2 Constraints to Cassava Production

1.2.1 Pests and Diseases

The main diseases affecting cassava are cassava bacterial blight, superelongation disease, frog skin disease and most importantly Cassava Mosaic Disease (CMD). *Xanthomonas campestris* pv. *manihotis* is one of the main biotic constraints in cassava cultivation world-wide causing cassava bacterial blight. Heavy infestations of bacterial blight can thus destroy the whole crop. Repeated or continual use of pesticides to prevent subsequent attacks of pests is firstly, environmentally unsound and secondly, economically prohibited as cassava is grown by subsistence farmers. Cassava is therefore susceptible to prolonged and repeated attacks from several insect pests due to its long growth period of 8-24 months. Nematodes are becoming an increasing problem worldwide in severity where crop rotation and fallow periods are either shortened or abandoned causing losses of up to 98% (Puonti-Kaerlas, 1998). The main insect pests of cassava in Africa are mealybugs (*Phenacoccus manihoti* Mat. Ferr.) and green spider mites (*Mononychellus tanajoa* Bondar), where green mites cause losses of up to 80% (Dahniya, 1994). Successful control of the cassava mealybug and green mite has been achieved through stable host-plant resistance, offering a long-term solution for control, as it may also be used in conjunction with other

control measures (Dahniya, 1994). The main attention, however, has been drawn to CMD caused by geminiviruses. It is the most important disease affecting cassava in Africa and no adequate control for this disease has been established (Hong *et al.,* 1993).

1.2.2 Cassava Mosaic Disease (CMD)

CMD was first described in Tanzania in 1894, where the causal agent was assumed to be a virus. In 1936, Storey demonstrated that the disease was transmissible and inferred that a virus was responsible. However, proof of this theory was not obtained until the 1970`s and 1980`s following isolation and visualization by electron microscopy of geminivirus particles. In addition, successful mechanical transmission studies from cassava to the experimental herbaceous host, *Nicotiana benthamiana* and back to a susceptible Brazilian cassava cultivar was also achieved (Bock and Woods, 1983). Fulfilling Koch's postulates, these isolates were therefore shown to be the cause of CMD (Bock *et al.*, 1978). Characteristic leaf mosaic patterns, resulting from cassava mosaic geminiviruses (CMGs), are determined at an early stage of development. The plants are stunted and the leaves are reduced in size, misshapen and twisted with yellow areas separated by areas of normal green colour. The leaf chlorosis may be paler than normal, pale yellow or nearly white. The chlorotic areas vary in size from the whole leaflet to small flecks or spots and are clearly demarcated. Leaflets show either a localized pattern which is often at the base of the leaf or a uniform mosaic pattern. Secondary effects associated with symptom severity are distortion, reduction in leaflet size and general growth retardation. Symptoms may vary even for the same plant variety and virus strain in the same locality due to differences in virus species and strain, sensitivity of the host, plant age, environmental factors as well as mixed infections (Legg and Thresh, 2000; Hillocks and Thresh, 2001) (Figure 1.2).



Figure 1.2 Cassava leaves showing the characteristic yellow mosaic patterns, leaf reduction and distortion, as a result of CMD (IITA, 2001).

1.2.3 Geminiviruses

Genome Organization of Geminiviruses

Geminiviruses cause several plant diseases of great economic importance. The family, *Geminiviridae* is separated into four genera, *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus*, based on their biological and molecular properties. These properties include: - genome organization, insect vector taxon and host range (Gafni and Epel, 2002; Harrison and Robinson, 2002). Mastreviruses infect monocotyledonous plants mainly (those belonging to the family, *Poaceae*) and are transmitted by leafhoppers in a persistent circulative manner. There are about a dozen species in this genus, an example of which is *Maize streak virus* (MSV). This virus causes maize streak which is one of the most economically important plant virus diseases found in Africa. The mastrevirus genome consists of a monopartite circular, single stranded DNA (ssDNA) molecule of 2.6kb, encoding four proteins: - two on the viral DNA strand (virion-sense) and two on the complementary strand (Harrison and Robinson, 2002). Curtoviruses infect dicotyledonous plants and are also transmitted by leafhoppers, namely, the beet leafhopper, *Eutettix tenellus. Beet curly top virus* (BCTV) is a type species belonging to this genus which causes disease in sugar beet and several other crops in North America. Like mastreviruses, curtovirus genomes consist of ssDNA. The genome is approximately 3kb in size, encoding three proteins in the viral strand and four proteins in the complementary strand. The genome organization of topocuviruses resembles that of BCTV but contains only two genes in the viral strand. The only species belonging to this genus is *Tomato pseudo-curly top virus* which is transmitted by the treehopper, *Micrutalis malleifera,* and occurs in southern United States (Harrison and Robinson, 2002).

Of primary importance in this study is the fourth geminivirus genus, *Begomovirus.* Begomoviruses infect dicotyledonous plants and are transmitted by the whitefly, *Bemisia tabaci* (*B. tabaci*), in a persistent circulative manner. *B. tabaci* is considered to be a species complex occurring worldwide in tropical, subtropical and warm temperate regions (Harrison and Robinson, 2002). Begomovirus genomes may be either monopartite (ssDNA A of about 2.6kb) or bipartite (ssDNA A and DNA B each about 2.6-2.8kb in size) (Gafni and Epel, 2002). The DNA A and DNA B nucleotide sequences in bipartite genomes differ from one another except for a short “common region” of 200-400 nucleotides that is very similar, even identical in the two DNAs. This region includes a stem-loop structure (loop containing the nonanucleotide

TAATATTAC) which is conserved in all geminivirus genomes. The origin of rolling circle DNA replication is the last A in the nonanucleotide (Harrison and Robinson, 2002) (Figure 1.3).

Bipartite-genome organization of begomoviruses encodes at least four proteins on the DNA A: the viral strand contains the coat protein (CP or AV1) and the pre-CP (AV2), which is found only in Old World begomoviruses (like SACMV). The complementary strand contains three proteins; AC1, AC2 and AC3 from overlapping open reading frames (ORFs). AC1 is required for initiation of DNA replication and is termed the replication-associated protein (Rep), AC2 (TrAP) activates transcription in both the DNA A and DNA B of the viral sense genes, and AC3 is the DNA replication enhancer (REn). DNA B encodes two proteins, namely BC1 and BV1 which are involved in intracellular, intercellular and systemic virus movement. BC1 is found on the complementary strand and mediates cell-to-cell movement of the virus. BV1 is the nuclear shuttle protein (NSP) which controls movement of viral DNA between the nucleus and cytoplasm (Gafni and Epel, 2002; Harrison and Robinson, 2002) (Figure 1.3).

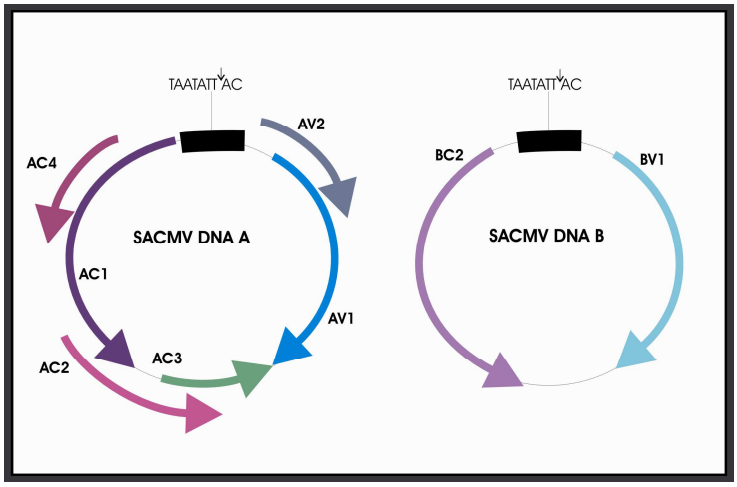


Figure 1.3 Bipartite genome organization of begomoviruses (particular species shown is SACMV). DNA A containing 6 ORFs and DNA B containing 2 ORFs (indicated in coloured arrows). Direction of transcription depicted by arrows (Diagram modified from Berrie *et al.*, 2001).

DNA Replication and Cell-Cycle Interactions

Geminivirus DNA replication occurs in the nucleus of the host, requiring two stages for replication. These stages include: i) ssDNA conversion to dsDNA, and ii) rolling-circle replication (RCR). Viral circular genomic ssDNA (positive strand) is converted into supercoiled covalently dsDNA intermediates through a priming event activating the negative strand origin of DNA replication. These dsDNA intermediates are

then amplified through a RCR mechanism (Gutierrez, 1999). The initiation site for RCR has been mapped to the intergenic region which contains an invariant nine nucleotide sequence (TAATATTAC) which is common among all geminiviruses (Figure 1.3). Viral proteins are expressed from the transcriptionally active dsDNA forms. Generally, genes encoded on the v-sense have movement and structural functions whereas those encoded on the c-sense function in DNA replication, regulation of transcription, and most probably interfere with cellular processes required for replication (Gutierrez *et al.*, 2004). Viruses require both host cellular factors and machinery for replication, systemic spread as well as for suppression of antiviral defense mechanisms (Petty *et al.*, 2000).

Geminiviruses have been implicated in many processes such as transcriptional regulation, DNA replication, control of the cell cycle, cell proliferation and differentiation, and macromolecular trafficking in whole plants (Gutierrez, 2002). In order to complete infection in a host, geminiviruses need to modify certain host-cell pathways. Such changes include:- modulation of plasmodesmata structure and function, host silencing-related defense mechanisms, interactions with proteins such as NAC-domain (*NAM, ATAF1/ATAF2*, and *CUC2)* containing proteins which are involved in growth and development regulation, host gene expression changes, and retinoblastoma-related (RBR) pathway interference (Gutierrez, 2002) (Figure 1.4).

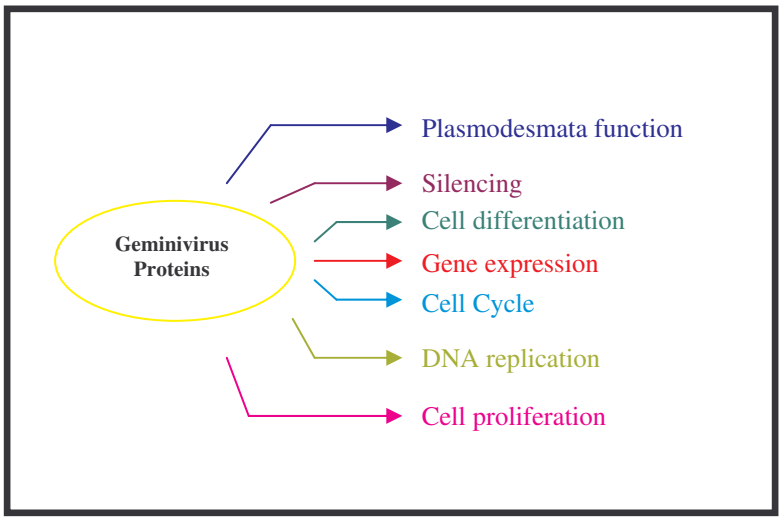


Figure 1.4 Interference of geminivirus proteins with host cell pathways (Gutierrez *et al.*, 2004).

Geminiviruses utilize host DNA replication factors such as DNA polymerase in combination with a virus-encoded replication protein (Rep or AC1), for its own replication. Viral and host factors controlling DNA replication, early and late gene expression are thus integrated through activities controlled by the Rep/AC1 protein. The Rep protein is multifunctional in that it is responsible not only for DNA replication

initiation, binding to the origin, and induction of host replication machinery, but also for regulating its own expression at the transcriptional level and in ATP hydrolysis (Selth *et al.*, 2004).

The Rep/AC1 protein functions by nicking the DNA, once bound to the stem structure at the replication origin and initiates rolling-circle DNA replication. One problem encountered by geminiviruses is that they infect terminally differentiated cells at the resting state (G_0) which lack factors required for DNA replication. In order to overcome this problem geminiviruses induce host proliferating cell nuclear antigen (PCNA) which is a DNA polymerase accessory factor, normally found in the S-phase. Rep/AC1 is therefore required to induce PCNA. The process involves binding of Rep/AC1 to the viral replication enhancer (AC3), which then binds to PCNA (Arguello-Astorga *et al.*, 2004). Rep/AC1 in turn, physically interacts with host encoded retinoblastoma-like tumour suppressor proteins (pRbs). In mammals, the pRB protein functions as the G_1 checkpoint regulator which prevents completion of G_1 and entry into the S-phase. Cyclin-dependent kinases, have phosphorylating activity which stops the cell-cycle inhibition activity of pRb, therefore allowing progression into S-phase. It is believed that geminivirus Rep proteins interact with and either inactivate or divert the pRb-like protein in infected cells, allowing S-phase-specific mRNA production, also providing a pool of factors and enzymes required for viral DNA replication (Carrington and Whitham, 1998; Gutierrez, 2000; Egelkrout *et al.*, 2002; Kong and Hanley-Bowdoin, 2002). Plant geminiviruses are therefore analogous to animal DNA tumour-inducing viruses (such as SV40) and adenoviruss. These viruses also encode proteins which affect cell cycling apparatus (Carrington and Whitham, 1998).

Post-transcriptional gene silencing (PTGS) is a natural defense mechanism plants have against viruses, involving a homology-dependent mRNA degradation process (Vanitharani *et al.*, 2004). Several plant viruses are known to counter antiviral RNA silencing with silencing suppressor proteins. These proteins interfere with initiation, maintenance, and systemic signalling in the RNA-silencing process. Potyvirus helper component proteinases (HC-Pro) have been shown to interfere with initiation and maintenance of silencing where short interfering RNAs (siRNAs) are produced. siRNAs form an active multicomplex RNA-induced silencing complex which is responsible for homologous RNA cleavage (Vanitharani *et al.*, 2004). *Cucumber mosaic virus* (CMV) has been shown to inhibit long-range PTGS-signalling activity, thereby preventing initiation of PTGS in newly formed tissues. The production of the mobile silencing signal has also been shown to be suppressed by p25 of *Potato virus X* (PVX). RNA silencing inhibition by p19 of tombusviruses was shown to occur through physical interaction of p19 with siRNAs. In

geminiviruses in particular, the transcriptional activator protein (TrAp) or Ac2 in both the monopartite *Tomato yellow leaf curl China virus* (TYLCCNV) and bipartite *African cassava mosaic virus* (ACMV), Kenyan strain (ACMV-[KE]) have been identified as suppressors of PTGS (Vanitharani *et al.*, 2004).

Recombination, Biodiversity and Taxonomy of Begomoviruses

It was suggested that the viruses infecting cassava had separate non-overlapping distributions (Hong *et al.*, 1993). However, findings on *South African cassava mosaic virus* and the Ugandan variant (EACMV-UG) revealed that a greater variability in cassava begomoviruses exists. Apart from transmission by whiteflies, extensive trafficking of cassava stakes (legally and illegally) has led to multiple introductions of highly diverse begomoviruses into and around South Africa from neighbouring countries. Movement of infected stakes therefore increases the possibilities for mixed infections, reassortments, and recombination between viruses since "new" viruses are introduced into previously uninfected or existing infected areas (Berry and Rey, 2001). EACMV-UG consists of most of the CP gene of ACMV inserted in an EACMV-like A component (Zhou *et al.,* 1997). This suggests the occurrence of natural inter- and intraspecies pseudorecombinations (reassortment of genome components) (Pita *et al.,* 2001). Interspecific recombination is likely to have occurred in EACMV-UG due to the ability of ACMV and EACMV to co-infect cassava (Harrison *et al.,* 1997). SACMV on the other hand has shown a close relatedness to EACMV in both its DNA A (85%) and DNA B (90%) components. The entire AC4 ORF contains a unique sequence derived from an unknown begomovirus (Berrie *et al.,* 1998). Recombination plays an important role in the evolution and divergence of begomoviruses, leading to biodiversity of species (Zhou *et al.*, 1998; Berrie *et al.*, 2001) and therefore an increase in incidence of CMD, resulting in a greater loss of cassava crops (Fondong *et al.,* 2000).

Currently, there are eight CMG species causing CMD, six African and two Indian. Sequence homologies between species of begomoviruses have been set at 89%, based on the full DNA A component for each species (Fauquet *et al.*, 2003). A species is considered to be distinct if its full-length nucleotide sequence identity is less than 89% (Fauquet *et al.*, 2003). The six African begomovirus species therefore include: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), and *South African cassava mosaic virus* (SACMV), while the two Indian begomoviruses are, *Indian cassava mosaic virus* (ICMV) *and Sri-Lankan cassava mosaic virus* (SLCMV) (Fauquet *et al.*, 2003).

According to Trench and Martin (1985), the prevalence of CMD in SA in the early 1980`s was generally low and controllable with the occurrence of ACMV. However, more recently, commercial growth and utilization of cassava has increased due to the starch-processing factory construction in the Limpopo Province. This and whitefly transmission as well as infected cassava stakes has brought about the prevalence of EACMV, the recombinant EACMV-UG variant, and the more recent SACMV in SA. Mixed infections and recombination has also caused an increase in symptom severity and greater incidence of cassava begomoviruses in SA (Berry and Rey, 2001).

1.3 Towards Improving Cassava-Based Systems

1.3.1 Host-Pathogen Interactions

Evolution of Plant-Pathogen Interactions

Close communication between a plant and a pathogen is encountered upon contact between the two organisms. Generally, plants are able to detect the presence of a pathogen and mount appropriate defense responses. Pathogens in turn, focus on colonization and utilization of host resources. However, due to the high co-evolution of plant and pathogen species, a particular pathogen species may circumvent plant defenses, or plants may adapt in such a way that successful pathogens may be blocked by adaptive responses. During the infection process, a shifting in the plant's metabolism results in a mixture of disease resistance responses and disease susceptibility responses (Wan *et al.*, 2002).

It was proposed that pathogens have evolved virulence on a particular host plant resulting in a compatible reaction. When a host is able to specifically recognize and resist infection of the pathogen, selection then favours evolution and spread of the host (i.e. modification of a host receptor to specifically recognize an avirulent gene product). However, the pathogen may respond by mutating its avirulence gene (becoming virulent) resulting in susceptibility, with the host requiring new resistance (*R*) gene specificities for defense. Gene-for-gene evolution between host and pathogen is therefore constantly evolving with a

diverse array of avirulence (*avr*) genes found in different pathogen races as well as in *R* genes found in different host species (Staskawicz *et al.*, 1995).

Plants are able to defend themselves against viruses in a mechanism known as post-transcriptional gene silencing (PTGS). In turn, viruses are able to suppress PTGS. This was observed in potato virus Y whereby a helper component proteinase (HC-Pro) blocks PTGS in tissues where silencing has already been established. On the other hand, viruses such as cucumber mosaic virus (Cmv2b) encoding a 2b protein is able to prevent PTGS initiation at growing tips of the plant. Both HC-Pro and 2b proteins are therefore important for virulence and systemic spread throughout the plant. These virus-suppression mechanisms therefore provide another form of a pathogen overcoming plant defense responses. This mechanism compares to other pathogens that may accumulate avirulence (*avr*) genes to escape recognition from plants in gene-for-gene resistance mechanisms (Li *et al.*, 1999).

In gene for gene resistance, *R* genes are able to detect specific pathogen races through recognition of *Avr* proteins (pathogen-encoded). Some pathogens are therefore able to eliminate these *Avr* proteins to avoid detection by the plant, unless required for pathogen fitness (Feys and Parker, 2000).

Resistance (Incompatibility)

Plants are continuously threatened by a vast number of potential pathogens. In order to counter the attack by pathogens, intricate plant defense mechanisms have evolved to recognize and respond to invading pathogens (Mysore and Ryu, 2004).

A broad spectrum of plant defense molecules are activated upon pathogen detection. This early response is controlled by plant disease resistance (*R*) genes. *R* genes encode proteins that either directly or indirectly recognize pathogen *Avr* proteins in a plant defense mechanism known as gene-for-gene disease resistance. A host plant that carries an *R* gene will give the pathogen that carries the corresponding *avr* gene an avirulent phenotype (Staskawicz *et al.*, 1995; Bent, 1996; Li *et al.*, 1999; Tang *et al.*, 1999; Feys and Parker, 2000; Dangl and Jones, 2001; Feys *et al.*, 2001). It has been hypothesized that *R* proteins “guard” plant proteins targeted by the pathogen *Avr* proteins. These *R* proteins therefore trigger the hypersensistive response (HR) and other defense related responses upon *R-avr* detection (Glazebrook, 2001).

Induction of the plants defense responses leading to the HR is initiated by elicitor molecules produced by the pathogen which are recognized by the plant. Upon recognition, a cascade of host genes are activated leading to induction of the HR (Staskawicz *et al.*, 1995). The HR is associated with rapid necrosis of plant cells at the site of invasion resulting in local containment of the pathogen, rendering the infection unsuccessful (Li *et al.*, 1999; Feys and Parker, 2000).

Yu *et al.* 1998, suggested that defense responses not involved in gene-for gene resistance are often activated at a lower level without initiation of a programmed HR (cell death). These defense responses are therefore less effective in preventing pathogen growth. It has been shown that although a strong association exists between HR cell death and gene-for-gene resistance, gene-for-gene interactions are still possible without HR cell death. This was observed in an *Arabidopsis* mutant, *dnd1* that did not develop an HR in response to the avirulent pathogen, *P. syringae*, while exhibiting gene-for-gene restriction to pathogen growth. It was therefore suggested that an HR may strengthen, or enhance stimulation in gene-for-gene interactions, therefore providing more of a complete restriction of pathogen growth (Yu *et al.*, 1998).

Early changes within the plant associated with localized resistance responses (HR) include:- reactive oxygen intermediates (ROI) resulting from an oxidative burst, alterations in cell wall structure, signalling molecule accumulation, nitric oxide (NO) production, endogenous salicyclic acid (SA) increase, and the transcriptional activation of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Feys *et al.*, 2001). Similarly, uninfected portions of the plant also establish a heightened resistance throughout the plant against subsequent challenge. This is known as systemic acquired resistance (SAR), acting effectively against a broad spectrum of pathogens. SAR is a long-lasting form of resistance which requires the phenolic signalling molecule, SA. Plants are also able to express a set of PR proteins during the onset of SAR. Alternatively, a different form of systemic resistance, induced systemic resistance (ISR), also exists. This form of resistance is independent of SA, requiring plant growth hormones such as jasmonic acid (JA) and ethylene (ET), which are effective against a broad spectrum of pathogens (Feys and Parker, 2000). A given pathogen can cause a physiological state of elevated defensiveness and potentiation of the defense activation machinery by SAR and ISR (related in function

but act independently), resulting in stronger and more rapid responses to subsequent infections (Wan *et al.*, 2002).

There are many examples of *avr* and *R* gene specificities found in fungi, bacteria and viruses. *Cladosporium fulvum* is an extracellular growing fungal pathogen that contains *Avr4* and *Avr9* genes encoding pre-proteins that become small secreted peptides which are able to elicit *R* gene-dependent defense responses. The type III secretory system encoded by bacterial *Hrp* gene cluster (required for HR induction and pathogenesis) is required for movement of *Avr* proteins in plant cells and is responsible for the recognition event of the bacterial *avr* gene products and corresponding plant *R* gene products occurring inside plant cells (Li *et al.*, 1999). Generally, for most cloned bacterial *avr* genes, the *Avr* protein itself is the elicitor which when injected into the plant encodes hydrophilic proteins lacking signal sequences, not inducing an HR. Viral *Avr-R* recognition occurs inside plant cells as viruses enter through existing wounds and replicate intracellularly, encoding genes for replication, movement and encapsidation. The coat protein, RNA replicase, or movement protein are all avirulence determinants. This has been observed in tobacco mosaic virus (TMV) in which the *Avr* protein for which a matching *R* gene, *N*, has been cloned. This *N* gene has been putatively identified as a cytoplasmic protein belonging to the nucleotide binding site, leucine rich repeat (NBS-LRR) family of resistance genes (Li *et al.*, 1999). *RCY1* has recently been cloned from *Arabidopsis* ecotype C24 which confers resistance to *cucumber mosaic virus* (CMV) strain Y (Takahashi *et al.*, 2002). This gene encodes a CC-NBS-LRR (CC, coiled coil domain) protein which is allelic to both *RPP8* and *HRT*. *RPP8* and *HRT* have been shown to be resistant against *Peronospora parasitica* and turnip crinkle virus (McDowell *et al.*, 1998; Cooley *et al.*, 2000).

Host and Nonhost Resistance

Two types of resistance have been proposed to exist. The first type of resistance is referred to as host resistance and is cultivar or accession specific. The second type of resistance, a less understood mechanism is referred to as nonhost resistance which provides resistance against pathogens for all members of a plant species. A nonhost pathogen therefore is a pathogen that cannot cause disease on a nonhost plant. The most common and durable form of plant resistance is nonhost resistance of an entire plant species to a specific parasite or pathogen (Scholthof, 2001). Host resistance therefore involves gene-for-gene resistance *(R-avr)* involving products of single *R* genes specifically acting directly or indirectly with elicitors produced by *avr* genes of pathogens. A less understood nonhost resistance is therefore still

unclear as to why a fully virulent pathogen on one plant species does not cause disease on others (Mysore and Ryu, 2004). Nonspecific resistance mechanisms (not mediated by *R* genes) may therefore involve pathogens capable of inducing a broad spectrum of defense responses to overcome infection. The plant environment, however, may not be compatible for the pathogen or may not have sufficient machinery to enable pathogen growth and spread. This is non-host resistance, restricting the pathogen to a limited host range (Scholthof, 2001).

Basal Resistance and Tolerance

Another phenomenon in resistant and susceptible responses is that susceptible hosts possess what is called "basal resistance", an innate defense response that limits but does not stop pathogen growth. Unlike the HR in gene-for-gene resistance occurring at the site of infection, this response results in cell death at the site of infection, which is preceded by a spreading of chlorosis as well as a secondary necrosis in surrounding, uninfected tissue. As with the resistance responses, susceptible responses also undergo changes in gene expression such as reactive oxygen (ROS) production and cell wall composition, the only difference in the susceptible response is that these changes are delayed. Effective resistance is therefore dependent on the speed in which induced defense reactions occurs in susceptible (compatible) and resistant (incompatible) interactions (O´Donnell *et al.*, 2003).

This is all dependent on how quickly the pathogen can replicate and cause disease or how fast the plant may respond with corresponding levels of defenses. Immediate recognition by a plant of an invasive pathogen (as in gene-for-gene resistance) results in a rapid defense mechanism (such as an HR). Susceptibility (disease) results when the pathogen recognition is not rapid enough to mount the appropriate defense responses required to block pathogen infection. After an HR (resistance) or a successful infection (susceptibility) has been initiated, SAR is induced by the plant to prevent infection from spreading further or to prevent a secondary infection from occurring, resulting from a broad spectrum of pathogens (Dong, 1998). It has been hypothesized that the host may therefore suppress disease in an incompatible response in a regulated manner or alternatively, the host may not immediately recognize the pathogen and induce effective resistance responses, resulting in disease. Disease symptoms are not always associated with pathogen growth, thus in the absence of symptoms, pathogen growth may still be observed in a process referred to as tolerance (O´Donnell *et al.*, 2003).

Studies on compatible interactions may explain why certain plants develop mild symptoms (tolerance), while others suffer severe symptoms sometimes leading to plant death. Most of the disease problems are caused by systemic infections which explain the need to study compatible interactions in plants and pathogens (Scholthof, 2001).

Susceptibility (Compatibility)

Virulent pathogens are able to infect particular plant species or cultivars, reinforcing their highly specialized ability to cause disease. Successful disease formation depends on the pathogen's ability to actively suppress or avoid plant defense responses therefore inducing susceptibility in a host that would normally be either resistant or tolerant to the pathogen (Abramovitch and Martin, 2004). A susceptible reaction is the result of the plant not being able to detect the pathogen, or if detected, the pathogen is able to cope with plant defense mechanisms. Alternatively, the response may either not be activated or activated too late leading to spread of the pathogen (Venisse *et al.*, 2002). Examples of pathogenicity factors include small molecule suppressors from phytopathogenic fungi, phytopathogenic bacteria containing type III effectors and toxins, as well as post-transcriptional gene silencing suppression by plant viruses (Abramovitch and Martin, 2004).

O'Donnell *et al.* 2003, suggested that a susceptible host plays a vital role in the interaction with a virulent pathogen due to its participation in basal resistance and disease symptom development. Two signaling intermediates, ethylene (ET) and salicyclic acid (SA) have been found to play a role in both susceptible and resistant responses of hosts to pathogens (O´Donnell *et al.*, 2003). Increased levels of ET and SA in the *Arabidopsis Xanthomonas campestris* pv. *campestris (Xcc)* compatible interaction proved that these two signals are essential in disease symptom production (O´Donnell *et al.*, 2003).

Virus infections in plants

Disease in plants is caused by a compatible interaction between a plant and a virus resulting from the expression of specific host and viral genes. Disease components in a host-virus interaction can therefore be studied individually or in combination by exploring genetic variation between the two genomes of both a plant and pathogen (Cecchini *et al.*, 1998). Susceptibility is the final outcome once a virus has

successfully completed genome replication, local cell-to-cell movement and vasculature dependent long-distance movement in the host plant (Carrington and Whitham, 1998). Disease results when the preformed plant defenses are inappropriate, the plant does not detect the pathogen, or activated defense responses are ineffective (Hammond-Kosack and Jones, 1996). Host plant cells therefore do not die but retain large quantities of virus, thus supporting a complete infection "cycle" and allowing progressive spread of the virus to adjacent tissue. The appearance of symptoms is the final result of virus infection in susceptible tissues (Maule *et al.*, 2002). Symptom development requires extensive changes in gene expression resulting in stunting, vein-clearing, mosaics and chlorosis (Geri *et al.*, 2004). Symptoms therefore represent structural and physiological changes at the cellular level resulting in whole plant physiology changes such as reduced growth and development (Maule *et al.*, 2002).

Once viruses have mechanically disrupted the cell wall and plasma membrane, they remain within the symplast until they move into the plasmodesmata where they continue to infect cells. The virus then continually moves to adjacent tissue, progressively spreading either to most or even all of the susceptible tissue, resulting in the appearance of symptoms. Symptoms such as reduced growth and development of the plant are visible due to the physiological and structural changes in the plant caused by the virus (Maule *et al.*, 2002).

1.3.2 *Arabidopsis* as a Model Plant System for Host-Pathogen Interactions

Arabidopsis as a Model Plant System

Arabidopsis thaliana is a member of the mustard family (Cruciferae or Brassicaceae), naturally distributed throughout Europe, Asia, and North America (Meinke *et al.,* 1998). *Arabidopsis* has many attributes as a model plant system. It has a rapid generation time where its entire life cycle is completed in 6 weeks, starting from seed germination, rosette fromation, main stem and flowering development to seed maturation. It is small in size, with a rosette diameter ranging from 2 to 10cm with mature plants reaching 15 to 20cm in height (Meinke *et al.,* 1998). Apart from its short generation time and small size, it also has a high efficiency of transformation, having the smallest genome among higher plant species (125Mb) (Mysore *et al.,* 2001).

The model plant system, *Arabidopsis* has been chosen in many studies because its' entire genome has been sequenced, making expressed sequence tags (ESTs), gene expression databases and cDNA microarrays readily available. Progress in plant development and physiological response discoveries has thus been attained which is not possible in less-characterized plant systems (Horvath *et al.,* 2003). For example, *Arabidopsis* cDNA microarrays have been used to compare gene expression patterns in non-model plant systems such as wild oat (*Avena fatua*), poplar (*Populus deltoidsies*), and leafy spurge (*Euphorbia esula*) to identify common signaling pathways and conserved genes that contribute to transcriptome functions in less-characterized plant systems (Horvath *et al.,* 2003).

Arabidopsis in Host-Pathogen Interactions

Arabidopsis-virus interactions

Arabidopsis has been used to identify general changes in plant gene expression by a variety of pathogens. This study investigated changes in gene expression upon induction of five different positive-stranded RNA viruses. These viruses included: turnip vein clearing tobamovirus (TVCV), oilseed rape tobamovirus (ORMV), potato virus X potexvirus (PVX), cucumber mosaic cucumovirus (CMV), and turnip mosaic potyvirus (TuMV). Findings from this study confirmed that plant RNA viruses elicit general plant stress responses either specifically or non-specifically, causing an increase in stress- and defense-associated genes. Promoter analysis of co-regulated genes revealed that viruses also may activate uncharacterized signalling pathways in susceptible hosts leading to the common changes in gene expression (Whitham *et al.,* 2003).

Gene expression profiles of *A. thaliana* ecotype Shahdara have been investigated in both inoculated and systemically *Tobacco mosaic virus* (TMV) infected leaf tissues, identifying a diverse array of functional proteins such as transcription factors, antioxidants, metabolic enzymes and transporters (Golem and Culver, 2003). *Arabidopsis* has also been used in transgenic studies whereby a viral pathogenic determinant was transgenically expressed in *Arabidopsis* in order to develop new strategies for mutant plant pathogenic loci screening (Geri *et al.,* 1999). Studies involving the geminivirus, *Beet curly top virus* (BCTV) were shown to differ in their ability to infect certain ecotypes of *Arabidopsis.* A series of reciprocal crosses between the resistant ecotypes Ms-O and Pr-O with the susceptible ecotype Col-O were carried out. F_1 plants from both crosses accumulated viral DNA and developed symptoms, thus indicating

that resistance was not due to a dominant gene, but to a single, recessive locus. The primary finding identified the first single resistance plant locus associated with geminiviruses (Lee *et al*., 1994).

Bacterial and fungal-Arabidopsis interactions

The transcriptome of *Arabidopsis* have been investigated after infection with the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, during an HR. This study was conducted in order to relate the changes observed at four time points, to monitor the change in genes from a housekeeping function to defense metabolism (Scheideler *et al.,* 2002). Disease resistance responses associated with regulatory pathways have been monitored in *Arabidopsis* after inoculation with an incompatible fungal pathogen, *Alternaria brassicicola.* Findings illustrated that considerable interactions among these pathways exists, especially between salicyclic acid (SA) and methyl jasmonate (MJ) (Schenk *et al.*, 2000). *Arabidopsis* as a model plant system has therefore contributed widely to the complicated network of biochemical and regulatory interactions that occur in host-pathogen interactions.

Disease resistance genes in Arabidopsis

Arabidopsis has been extensively used to study the signaling pathways and structural features of resistance (R) genes. Sequence comparisons have revealed that R genes show extensive conservation in structure, all containing leucine-rich repeats (LRRs), a protein domain associated with protein-protein interactions, and ligand binding. Mapping of the *Arabidopsis* genome has enabled R gene identification based on interactions with a variety of pathogens. These include: *Erysiphe cichoracearum* and *Erisyphe cruciferarum* (RPW loci), *Albugo candida* (RAC loci), *Peronospora parasitica* (RPP loci), caulimovirus (CAR1) and *Pseudomonas syringae* (RPS loci). An *Arabidopsis* CIC-YAC (Yeast Artificial Chromosome) library was used to identify the position of diseases resistance homologs on the *Arabidopsis* genome. Map positions have therefore correlated with the disease resistance loci *RPS5, RAC1, RPP5, CAR1, RPP7, PPW2, RPP1, RPP10, RPP14, RPP5, RPP4, RPS2, RPW6, HRT, RPS4, RPP8, RPP21, RPP22, RPP23, RPP24* and *TTR1.* To date, 21 resistance gene loci have been mapped, 12 of which belonging the *RPP* loci (Speulman *et al.*, 1998).

1.4 Analysis "Tools" for Host-Pathogen Interaction Studies

1.4.1 Analysis of Host-Pathogen Interactions through Microarray Technology

Gene Expression Techniques

Functional roles of gene products are determined through gene expression patterns that vary among diverse biological processes (Rishi, 2002). In order to understand how gene products function, it is important to know where and when a gene is expressed and how the expression level is affected. Genes do, however, have an additive function of working together, therefore expression levels of these large gene numbers can be monitored through gene expression studies. Microarrays therefore provide insight into how functional pathways and cellular components work together to regulate and carry out cellular processes (Lipshutz *et al.*, 1999).

Currently, differential expression between two sets of conditions are carried out utilizing techniques such as Northern blot analysis, serial analysis of gene expression (SAGE), differential display (DD) and dotblot analysis (van Hal *et al.*, 2000; Rishi, 2002). The current problem with these techniques is that large numbers of genes can not be analyzed in parallel. Problems which arise are: a limited number of mRNAs may only be studied simultaneously using Northern blot analysis. SAGE requires extensive DNA sequencing which is technically difficult and involves complex sample preparation and is very laborious. Simultaneous detection of multiple differences in gene expression is possible with DD, however, screening is not based on identity but in mRNA length. Likewise, this method is not quantitative, allowing a limited number of conditions for comparative purposes (van Hal *et al.*, 2000).

DNA microarray technology has the potential to overcome these limitations, as differences in mRNA abundance between two differing sets of conditions are determined simultaneously on a large scale. This provides valuable information on the regulation and expression patterns of thousands of genes at the molecular level (Rishi, 2002).

Types of Microarrays

Two forms of microarrays exist, namely complementary DNA (cDNA) microarrays and oligonucleotide microarrays. cDNA microarrays are usually generated from genomic DNA, cDNA libraries or subtracted library clones, selecting unique clones which are amplified by the polymerase chain reaction (PCR) using vector-specific primers (Rishi, 2002). Expressed sequence tags (ESTs) are predominantly used in microarray analysis which consists of partial sequences taken from a cDNA clone corresponding to an mRNA sequence, ideally 150bp or more (Adams *et al.*, 1991). More than 71% of GenBank entries as well as 40% of individual nucleotides in the database use ESTs as a source of sequence information (Hegde *et al.*, 2000).

Oligonucleotide microarrays consist of synthetic oligonucleotides, not more than 80 nucleotides in length. This small oligonucleotide length enables greater specificity among members of gene families (Wullschleger and Difazio, 2003). Affymetrix oligonucleotide microarrays consist of 25mer oligonucleotides, directly synthesized on the array surface in parallel using a process called photolithography. Internal controls are required for this array as non-specific hybridization patterns may arise from such a small oligonucleotide array (Rishi, 2002).

Manufacturing of Microarrays

There are two main methods for manufacturing DNA microarrays. Firstly, this consists of a photolithography based synthesis method of oligonucleotides on a solid surface. High density microarrays of more than 250 000 spots per cm^2 can be manufactured using this method, producing a large number of identified spots per array. The downfall to this array is that it has no flexibility in design and is expensive (van Hal *et al.*, 2000). The second method in the manufacturing of DNA microarrays is DNA micro-dispensing which involves aliquoting a small volume (minimum of 50pl) of DNA solution onto a solid surface. Micro-dispensing machines may either be active or passive dispensers. The DNA solution in passive dispensers is applied with multiple pins spotting the surface of the array. Either solenoid valves or piezoelectric delivery is used in active dispensing where no direct surface contact occurs. The surface for which DNA sequences are fixed, are either glass or membrane (van Hal *et al.*, 2000). Glass slides used are either poly-L-Lysine or aminosilane coated (Hegde *et al.*, 2000).

Preparation of Microarrays

Microarrays are constructed with DNA sequences immobilized on a glass slide. Sample preparation entails: cDNA synthesis of two different samples from extracted mRNAs, labeling of the two fluorescent dyes (usually Cyanine dyes) to the cDNA, and hybridization to the immobilized probes (known cDNA sequences) on the surface of the array where competitive binding of the cDNA target (unknown, labelled cDNA) to the probe takes place. The slides are then washed and scanned whereby fluorescence measurements are made, enabling transcript level ratio determination for each gene on the array (Rishi *et al.*, 2002; Wullschleger and Difazio, 2003). The abundance of target molecules and/or the binding efficiency between the probe and target molecules are determined through the strength of signal of each fluorescent dye which is quantitatively determined through a laser scanner or charge-coupled device (CCD) (Zhu, 2003). The abundance and/or binding abilities of hundreds to thousands of DNA probes to complementary DNA or RNA target molecules on a solid surface may therefore be monitored simultaneously through the use of DNA microarray technology (Zhu, 2003).

Application of Microarrays

cDNA microarrays have become the predominant method of choice in gene expression studies and have been used in a variety of biological processes (Zhu, 2003). These processes include: plant disease resistance and environmental stress responses, circadian rhythm maintenance, nitrate assimilation, photomorphogenesis signalling, as well as in fruit and seed development (Aharoni and Vorst, 2002; Donson *et al.*, 2002). A particular study conducted by Horvath *et al.*, 2003 cDNA microarrays were utilized for species such as wild oat (*Avena fatua*), poplar (*Populus deltoidsies*), and leafy spurge (*Euphorbia esula*) with limited genomic information. Comparisons of gene expression patterns enabled identification of common signaling pathways and conserved genes, which was not previously possible without cDNA microarrays (Horvath *et al.*, 2003).

Additionally, microarrays have been used in many applications involving qualitative differences between plants exposed to temperature, drought and aluminium toxicity, as well as in spatial and temporal changes in gene expression associated with fibre and root elongation, in cotton and maize. Studies on plants response to oxidative stress and metabolic changes induced by carbon dioxide concentrations have also been carried out through microarray analysis. Global studies of gene expression in plants have also been

initiated through the completion of the *Arabidopsis thaliana* genome sequencing project (Wullschleger and Difazio, 2003).

Because of the vast amount of data that is generated from gene expression studies, microarrays are used in a number of different processes. These include annotating genes to functional pathways, analyzing the biochemical processes leading to a disease state, and gene function identification as possible drug targets (Reynolds, 2002).

Advantages of gene expression microarrays include: (i) transcript abundance of thousands of genes may be measured at once; (ii) a tight connection between expression patterns of a gene and gene product exists. This gene product contributes to fitness due to the fact that genes are expressed in specific cells under specific conditions; (iii) information from transcript profiling may be obtained from promoters which control expression of a gene. Therefore, by altering the level of transcription of specific genes, information such as identity, environment and internal state of a cell may be obtained; (iv) gene expression studies may also reveal what a cell is composed of from the genes that are expressed in that cell, gaining insight into underlying biochemical and regulatory systems (Brown and Botstein, 1999). DNA microarrays therefore allow the simultaneous screening of large gene numbers. A higher sensitivity is also achieved due to the small size of the array, and a smaller amount of starting material is required for each array because of this parallel screening of large gene numbers compared to conventional techniques (van Hal *et al.*, 2000).

1.4.2 *Agrobacterium*-Mediated Gene Transfer (Agroinoculation) of SACMV into *Arabidopsis*

One of the problems encountered by begomoviruses, in particular SACMV, is that they cannot be transmitted mechanically by rubbing healthy plants with virus-infected leaf sap. This is because, in nature, SACMV is transmitted by the whitefly, *Bemisia tabaci*, which delivers the virus directly into the plant's vascular system. One approach for virus introduction into a plant host without the use of the insect vector is by agroinoculation. This is a form of mechanical inoculation in which *Agrobacterium tumefaciens* cells

are transformed with a full-length copy of the virus genome which is injected into the plant (Leiser *et al.,* 1992; Mayo *et al.,* 2000).

Agrobacterium tumefaciens is a soil-born pathogen well-known for its ability for *trans*-kingdom DNA transfer (Ward and Zambryski, 2001; Eckardt, 2004). Besides its natural ability to transform host plants with foreign DNA, it also causes crown gall disease on several important crop species such as grapevines, stone fruit, and nut trees (Eckardt, 2004).

Agrobacterium has a tumour-inducing (Ti) plasmid which contains a specific DNA fragment called transferred DNA (T-DNA), which is delimited by a left and right T-DNA border (25bp direct repeats) (Tzfira and Citovsky, 2002). It is within this T-DNA region that foreign DNA is inserted which is then transferred to the plant cell and integrated into the plant genome (de la Riva, 1998). The process involves *Agrobacterium* VirD1 and VirD2 proteins which are induced by host signals where both T-DNA borders are nicked, resulting in a single stranded transfer strand (T-strand). A channel is then formed by the VirD4 and VirB4 proteins where the T-strand and several Vir proteins are exported into the cytoplasm of the host cell. Once integrated, a T-DNA transport complex (T-complex) is formed consisting of one VirD2 molecule covalently attached to the 5´end of the T-strand (coated with many VirE2 molecules), which is then translocated into the host nucleus with the aid of VirD2 and VirE2 proteins. This entire process requires both host factors and bacterial Vir proteins for successful transformation of plant cells (Tzfira and Citovsky, 2002).

Two different strategies are utilized for foreign gene introduction into the T-DNA region. These involve an indirect method of cloning whereby the gene of interest in *cis* is cloned on the same plasmid as the *vir* genes (co-integrative vectors); or the gene of interest is cloned into the T-DNA region on a separate plasmid from the *vir* genes (*trans*-acting *vir* genes), also known as binary vectors (Gelvin, 2003).

Grimsley *et al.* 1996, were the first to describe a transformation system for dicotyledonous plants with cloned viral DNA from *Agrobacterium tumefaciens* to plants via the Ti plasmid using *Cauliflower mosaic virus* (CaMV). This study showed that viral DNA was infectious once excised from the bacterial vector, as

it was able to replicate and systemically infect the plant. Geminiviruses have been successfully introduced using agroinoculation methods into different host tissue types. These include: host leaf discs, mainly using model plant systems such as *Nicotiana* spp., germinating seeds, and whole plants, based on direct injection of transformed bacterial cultures into the plant vascular system (Pico *et al.*, 2001).

It has been documented in many studies that cloned geminivirus DNAs are infectious as tandemly repeated copies present on a Ti plasmid and delivered into plants via agroinoculation (Grimsley *et al.*, 1986; Hayes *et al.*, 1988; Stenger *et al.*, 1990). Geminiviral partial tandem repeats are constructed and inserted into the T-DNA region of a binary vector. This construct is then introduced into *A.tumefaciens* and released into the plant genome through mechanism described previously (Jacob *et al.*, 2003). The release of infectious unit-length circular replicative viral genomes into plants from the partial tandem repeats may be explained by two mechanisms. Either intramolecular homologous recombination within the tandem viral genome repeats which result from a single cross-over event (occurring at random locations) leads to the release of circular dsDNA (Stenger *et al.*, 1991), or consequently, when the viral origin of replication is duplicated, the viral replication-associated protein replicates the full-length viral genome from the T-DNA region containing the partial tandem repeat portion (Stenger *et al.*, 1991).

Agroinoculation has also been used to infect dicotyledonous plants with *African cassava mosaic virus* (ACMV) and *Tomato golden mosaic virus* (TGMV) as well as monocotyledonous plants with *Maize streak virus* (MSV), *Digitaria streak virus* and *Wheat dwarf virus*. Agroinoculation of *Beet curly top virus* (BCTV) has successfully been introduced into *N. benthamiana* and *D. stramonium* (Briddon *et al.*, 1989). Plants where agroinoculation with SACMV have been successful are from the species *Phaseolus vulgaris*, *Malva parviflora*, and cassava (*Manihot esculanta* Crantz) (Berrie *et al.*, 2001).

1.4.3 Validation of Microarray Results through Real-Time RT-PCR

Comparison of Real-Time RT-PCR to Conventional Quantitative Methods

Differences in gene expression may be validated with methods such as Northern blot hybridization and RNase protection assays. These methods are however, time- consuming, and require large amounts of RNA. Another method of choice is reverse transcription (RT) followed by the polymerase chain reaction (PCR). This method requires a smaller amount of RNA but quantification relies on endpoint analysis of

the PCR product. Real-time RT-PCR on the other hand also uses smaller quantities of RNA but product accumulation is determined during the log-linear (exponential) phase of the reaction (Rajeevan *et al.*, 2001). Data produced from the log-linear phase is thus more accurate than end-point PCR, and in addition, constant amplification efficiency is achieved allowing a more precise determination of starting material (Roche Applied Science Technical Note, www.roche-applied-science.com/lightcycler/). Reliable and rapid quantification results are thus achieved through its high sensitivity and ability to identify and quantify small changes in gene expression and rare transcripts (Pfaffl, 2001).

The Process Involved in Quantitative Real-time RT-PCR

RNA is reverse transcribed into single-stranded complementary DNA (cDNA) through the retroviral enzyme, reverse transcriptase [either Moloney murine leukemia virus (MMLV-RT) or avian myeoblastosis virus (AMV-RT)]. First strand cDNA synthesis is initiated by an oligonucleotide primer which anneals to the RNA, where the reverse transcriptase (having RNA dependent DNA polymerase activity) extends the cDNA toward the 5'end of the mRNA. Primers involved in this process may either be non-specific such as hexamer primers (which bind to all RNAs present) and deoxythymidine residues [oligo(dT)] (annealing to the polyadenylated 3' tail on the mRNA) or specific (gene-specific). Sequence-specific primers are used to increase specificity especially in cases where messages are rare. This eliminates the possibility of spurious transcripts when elevated RT reaction temperatures are used (Freeman, 1999).

Once the RNA is reverse transcribed into a single cDNA strand, PCR is carried out in a three step process involving denaturation, annealing and elongation. Two distinct phases occur during a PCR reaction. This includes the exponential phase and plateau phase. The exponential phase involves cDNA denaturation, primer binding and strand extension, occurring in early to middle cycles, lasting from ten to twenty cycles. The amount of starting material dictates the amount of cycles before a reaction enters the exponential phase. The plateau phase results when components of the reaction mixture becomes limiting. This may be due to the competition of cDNA for primers and DNA amplification product concentration increases to the extent that single-stranded products may re-anneal to each other rather than to the primers. Inhibitors may also accumulate in this phase, along with a depletion of nucleotides and primers, resulting in a less predictable quantification reaction (Freeman, 1999). The five major chemistries used in real-time RT PCR are: - DNA-binding fluorophores, Linear oligoprobes, 5' Nuclease oligoprobes, Hairpin

oligoprobes and self-fluorescing amplicon. These may be classified into amplicon sequence specific or non-specific methods of detection (Mackay *et al.*, 2002).

The fluorescent detection format used in this study was hybridization probes. The naming convention for "probes" and "targets" are switched for real-time PCR in comparison to microarrays. This probe method is for specific product identification that uses two oligonucleotide probes, labelled with different marker dyes (e.g. Fluorescein and LightCycler (LC) -Red 640). These donor and acceptor dyes hybridize to the target sequences on the amplified DNA fragment in a head-to-tail arrangement. This arrangement results with the dyes falling in close proximity to each other causing excitation of the donor dye (Fluorescein) by the external light source (LED source), passing on part of its excitation energy to the acceptor dye (LC-Red 640). This fluorophore then emits measurable light at a different wavelength at the end of each annealing step. The hybridization probe is then released during elongation, resulting in a double-stranded PCR product (Roche Molecular Biochemicals Technical Note, www.roche-applied-science.com/lightcycler/).

Data Analysis

Data may be analyzed in two ways in quantitative real-time RT-PCR, namely: - absolute quantification and relative quantification. The exact transcript copy number is determined from absolute quantification where the input copy number is obtained by relating the PCR signal to a standard curve. Relative quantification is determined through gene expression changes in a target sample relative to a reference sample. A reference sample is usually a housekeeping gene (for example, GAPDH, ß-actin, B2-microglobulin, and rRNA). The purpose of these housekeeping genes is to normalize the PCR samples to account for the experimental variation in the amount of RNA added to the RT reactions (Livak and Schmittgen, 2001). A housekeeping gene is an "unchanged" gene that is not differentially expressed (i.e. neither up-regulated nor down-regulated) throughout the plant genome.

1.5 Objectives and Specific Aims

Geminiviruses are emerging plant pathogens, severely affecting important food crops worldwide. In sub-Saharan Africa, continued movement of infected stakes, recombination, and whiteflies has led to the introduction of highly diverse begomovirus strains and species (Berry and Rey, 2001). In SA, cassava is grown by subsistence farmers and also by large-scale commercial farmers for starch production. It does however, suffer major crop losses due to Cassava Mosaic Disease (CMD) caused by SACMV (Berry and Rey, 2001). SACMV and plant virus diseases in general are very difficult to control as mechanisms of virus pathogenicity within a host are poorly understood (Golem and Culver, 2003). This brings about the need for development of effective virus disease control strategies. Currently, the approach in controlling SACMV in SA involves pathogen-derived resistance (PDR). This approach involves genetic engineering, where transgenes containing virus-derived sequences are expressed in crop plants. An alternative to this approach is to identify the plants response to an invading pathogen, elucidating the basic underlying mechanisms involved in both resistant and susceptible host responses to pathogens, so that host-derived genes can be exploited in developing resistant germplasm.

Cassava is not a well characterized plant system due to its large genome of 36 chromosomes. The model plant system, *Arabidopsis*, was therefore selected for gene expression studies as its entire genome has been successfully sequenced, making expression databases and expressed sequence tags (EST's) readily available (Horvath *et al.*, 2003).

The overall objective of this study was to identify differentially expressed *Arabidopsis* genes in response to SACMV infection using microarrays.

The specific aims of this study were:

(i) To determine if *Arabidopsis* was resistant or susceptible to SACMV infection on a phenotypic level.

(ii) To investigate host-pathogen interactions at the molecular level utilizing microarray technology, since it was previously unknown whether *Arabidopsis* was a non-host or a susceptible host for SACMV infection. An infectivity study needed to be conducted to identify differentially expressed *Arabidopsis* genes in response to SACMV infection.

CHAPTER 2

MATERIALS AND METHODS

Section A-Infectivity Study

An overview of the procedure used in this study can be seen in Figure 2.1, with the methodology performed for Section A shown in Figure 2.2.

2.1 Plant Growth

Arabidopsis thaliana (ecotype Columbia-0) and *Nicotiana benthamiana* (*N. benthamiana*) seeds were planted in seed trays containing thirty peat pellets (Jiffy Products International), covered with plastic wrap and placed at 4°C for 3 days to eliminate dormancy and ensure uniform germination. These plants were then transferred to growth chambers (Specht Scientific) operating at 22°C under a 10h photoperiod in a humid environment at an intensity of $100\mu Em^{-2}$ sec^{-1}. In order to acclimatize the plants, two-to-three cuttings were made in the plastic covering ten days after planting. This procedure was repeated daily for ten days in order to maintain humidity and avoid air flow around the plants. Once acclimatized, the plastic covering was removed and plants were fertilized and watered as required, until ready for virus inoculations.

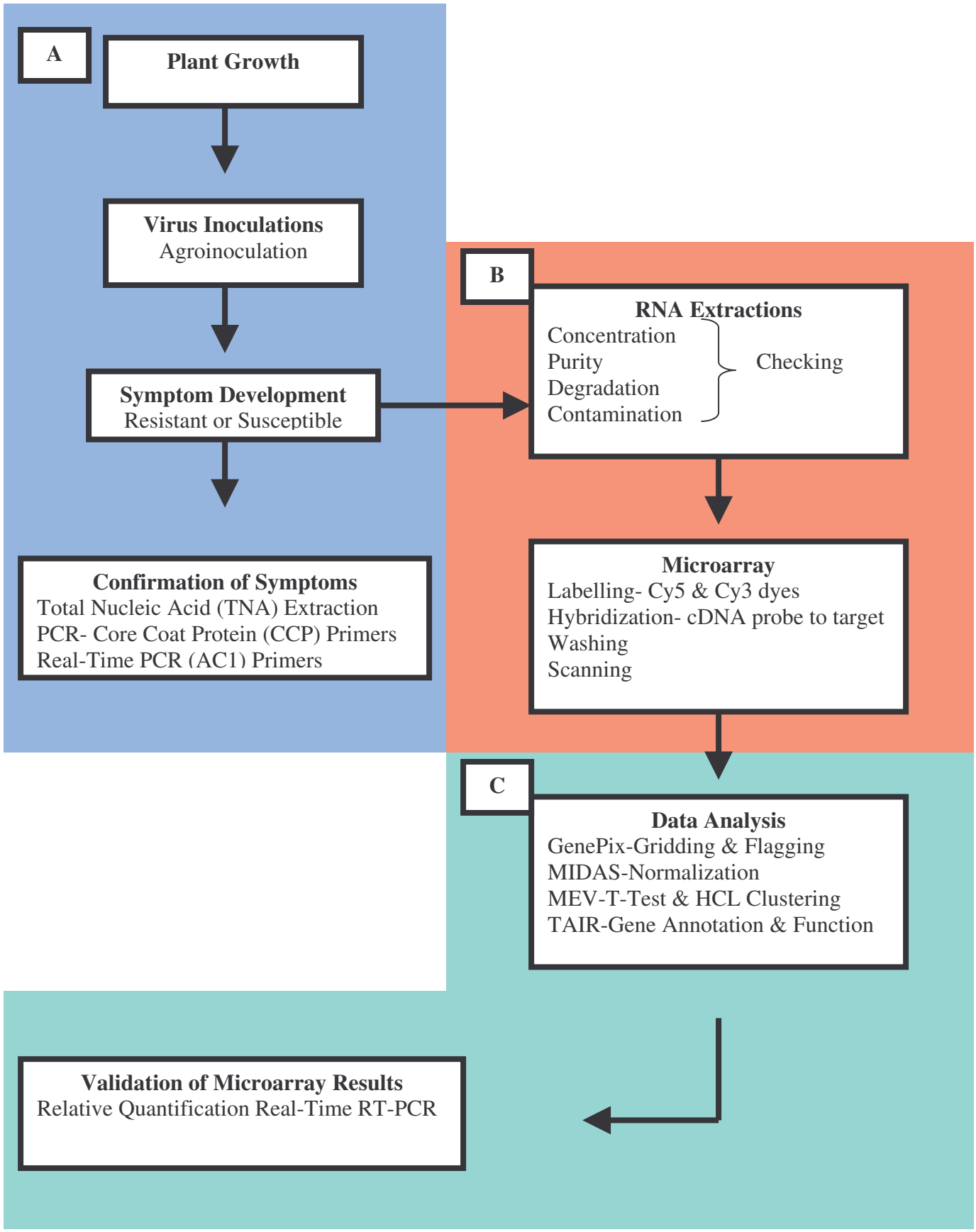


Figure 2.1 Schematic representation of overall methodology used in this study

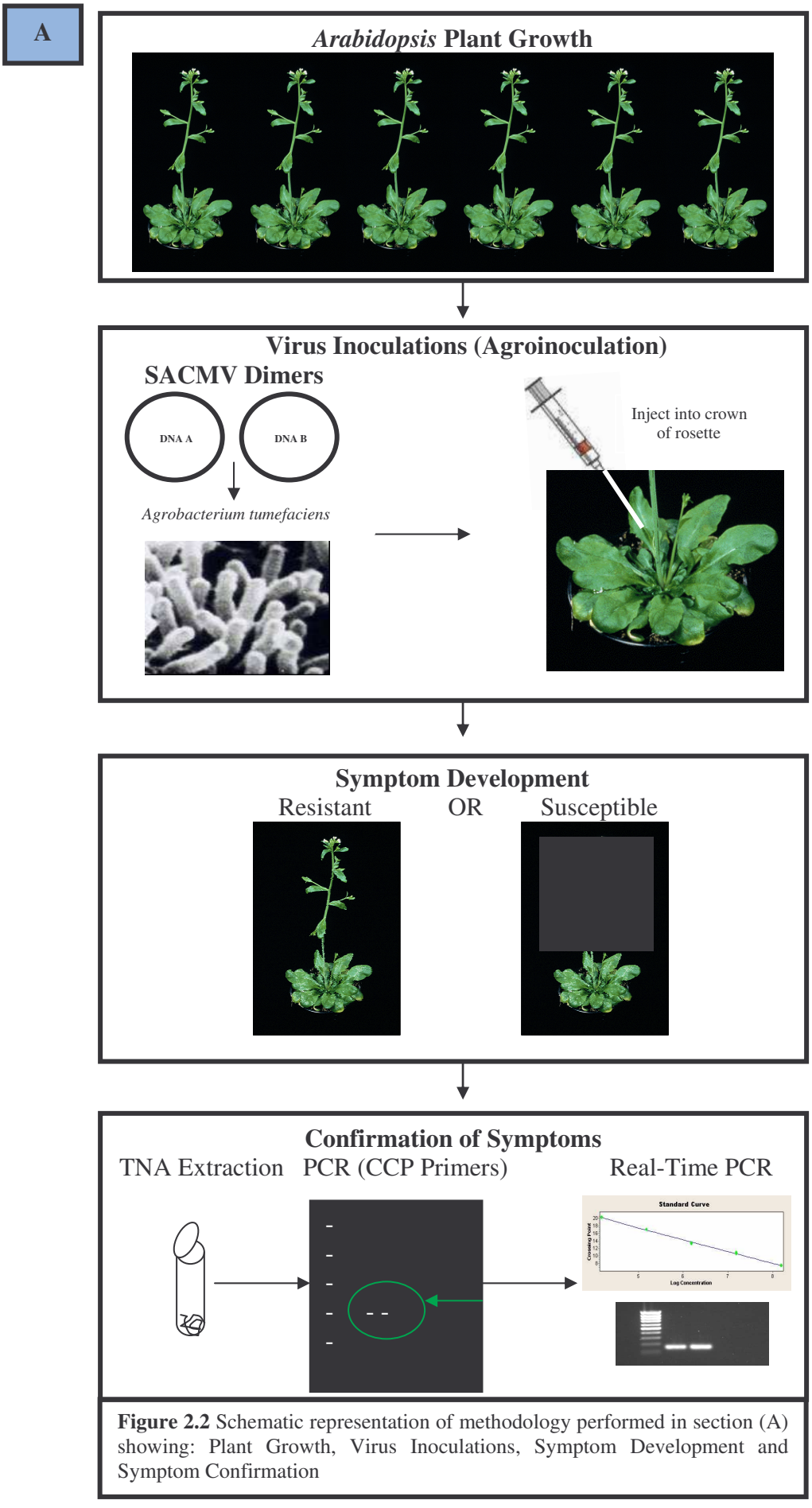


Figure 2.2 Schematic representation of methodology performed in section (A) showing: Plant Growth, Virus Inoculations, Symptom Development and Symptom Confirmation

2.2 Virus Inoculations (Agroinoculation)

Five-week-old *Arabidopsis* plants were co-inoculated with 23-24µl of full-length head-to-tail SACMV DNA A and DNA B dimers, mobilized into *Agrobacterium tumefaciens* strain C58C1 (pMP90) (constructed by L.Berrie) by the adapted agroinoculation method of Hayes *et al.* 1988, injected into the crown of the rosette. Two *Agrobacterium* cultures (one containing SACMV DNA A and the other SACMV DNA B) were incubated at 30ºC until an optical density (OD) of ± 0.4 was reached. One milliliter aliquots of each culture was pelleted at 8000 rpm, washed in sterile water, and spun at 8000 rpm. Each culture was then resuspended in 200µl Luria Bertani Broth and mixed together. Two biological replicates were conducted, in each biological replicate, sixty *Arabidopsis* and ten tobacco (*N. benthamiana)* plants were inoculated at the ten- to-twelve leaf stage using a Hamilton syringe. Control plants were mock-inoculated with filtered sterilized water (negative control). Tobacco plants (positive control) were inoculated with 10µl of virus suspension three times along the stem from bottom to top.

It was imperative that consistency between biological replicates was maintained, therefore virus inoculations and harvesting of leaves were done at the same time of day (differences in gene expression will be observed between replicates if consistency is not maintained).

2.3 Confirmation of Symptoms

2.3.1 Total Nucleic Acid Extraction (TNA)

TNA was extracted from symptomatic *Arabidopsis* plants according to the CTAB (cetyltrimethylammonium bromide) method of Doyle and Doyle (1987). Fifty milligram young leaf samples were ground in liquid nitrogen and TNA was extracted by the addition of 0.5ml pre-heated CTAB extraction buffer (2% CTAB, 20mM EDTA, 1.4M NaCl, 100mM Tris pH 8.0) and β-mercaptoethanol (to a final concentration of 0.1% v/v). The aqueous layer containing the TNA was extracted using chloroform:isoamyl (24:1) in a two step process and the nucleic acids precipitated with an equal volume of isopropanol. The pellet was then washed with 70% ice-cold ethanol, vacuum dried and resuspended in 12µl 1XTE buffer (10mM Tris pH 8.0, 1mM EDTA) containing 20µg/ml RNase A. TNA extractions were then examined and viewed for concentration determination by electrophoresis on a 1% agarose gel run at 80V.

2.3.2 Polymerase Chain Reaction (PCR)

PCR using degenerate core coat protein (CCP) primers was carried out in order to confirm SACMV infection in *Arabidopsis* plants. The CCP primers consisted of the following sequences: AC1048 (5′GGRTTDGARGCATGHGTACATG3′) and AV514 (5′GCCCWTGTAYAGRAAGCCMAG3′) (Wyatt and Brown, 1996). Between 40-90ng TNA was added to each reaction consisting of 2.5mM $MgCl_2$, 200µM of each dNTP, 0.001% gelatin, 0.5% Tween-20, 0.1 volumes *Taq* buffer and 2.5 units *Taq* DNA polymerase (Roche) of which 20pmoles of each primer was added, making up a final reaction volume of 50µl. Amplification was carried out utilizing the MyCycler™ Thermal Cycler (Bio-Rad) with cycling conditions programmed for 1 cycle at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, this was followed by a 10 min extension at 72°C.

2.3.3 Absolute Quantitative Real-Time PCR

A standard curve was constructed using 5 known concentrations (in duplicate) of plasmid SACMV DNA A. Copy numbers of the standards were calculated, (Xg/µl DNA / [plasmid length in basepairs x 660]) x Avogadros's number x 10^{-12}. Standard curves based on Crossing Points cycles (CP) for a 10-fold dilution series ranged from $1.54x10^{8}$ molecules/µl to $1.54x10^{4}$ molecules/µl. Plasmid DNA A and total nucleic acid (TNA) samples extracted from *Arabidopsis* leaf tissues at 13dpi and 35dpi were quantified on the LightCycler (Roche Applied Science) using Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen). The primer pair; (5′ GGC TAG TTC CCG GAT TAC AT 3′; 5′ GAC AAG GAC GGA GAC ACC 3′) was designed to bind to a 150bp region of the AC1 gene on SACMV DNA A. Quantitative PCR was performed using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche Applied Science). Each sample was prepared in LightCycler capillaries containing an optimal $MgCl_2$ concentration of 4mM, 0.5µM of each primer, and 2µl of LightCycler FastStart DNA Master SYBR Green I (10x conc), and 2µl sample DNA. Cycling conditions consisted of an activation mode of 95°C for 10 min. Then 32 amplification cycles run at 95°C for 5 sec, 60°C for 10 sec, and 72°C for 10 sec for a single acquisition (fluorescence detection at 520nm at the end of the elongation phase for each cycle). A melting curve was then performed by heating to 95°C, cooling to 65°C for 30 sec, and slowly heating to 95°C at 0.1°C/s with continuous measurement of fluorescence at 520nm. A final cooling step was carried out at 40°C for 10 sec.

Section B-Microarrays

A schematic overview of methodology performed for Section B may be seen in Figure 2.3.

2.4 RNA Extractions

Two independent biological replicates were carried out, each containing four technical replicates. For each biological replicate, total RNA was extracted from pooled (20 to 25 plants) SACMV-infected and healthy

Arabidopsis plants at 35 days post-inoculation (dpi), using Tri® Reagent (Sigma) according to a modified method originally described by Chomczynski and Sacchi (1987).

One gram of plant tissue was ground in liquid nitrogen with a mortar and pestle and transferred to a 15ml solution containing Tri® Reagent (Sigma). Samples were then incubated at 60°C for 5 min followed by centrifugation at 8750 rpm for 10 min at 4°C. The supernatant was then treated with 3 ml of chloroform, vortexed for 15 sec, left at room temperature (RT) for 2-3 min and centrifuged at 8100 rpm at 4°C for 15 min. The aqueous phase was carefully pipetted into a clean screw-cap centrifuge tube and precipitated by adding isopropanol and 0.8M Sodium Citrate/1.2M NaCl, half volume of aqueous phase of each. The tubes were then mixed by gentle inversion and incubated for 19 min at RT, followed by another centrifugation step at 8100 rpm for 10 min. The RNA pellet was washed with 75% ethanol, centrifuged at 8100 rpm at 4°C for 10 min and resuspended in 100µl of sterile water (Sabax water for injections, Adcock Ingram) containing 1µl (20U/µl) RNase Inhibitor (SUPERase.In™, Ambion).

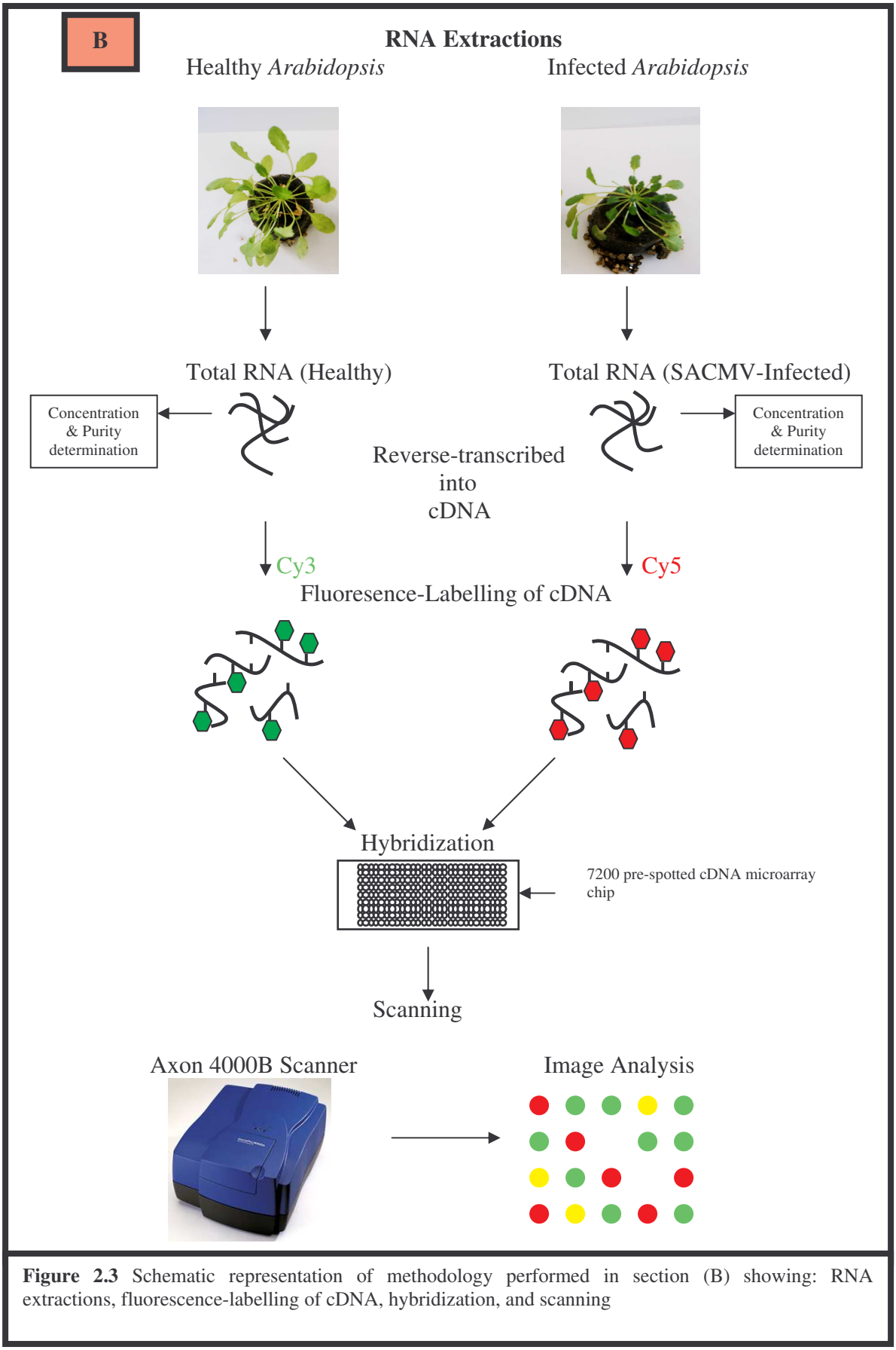


Figure 2.3 Schematic representation of methodology performed in section (B) showing: RNA extractions, fluorescence-labelling of cDNA, hybridization, and scanning

2.4.1 Purification and Quantification of RNA

In order to purify the RNA samples, the RNeasy Mini Protocol for RNA cleanup (Qiagen) was performed according to manufacturer's instructions (RNeasy ® Mini Handbook, Qiagen). For concentration determination, both healthy and SACMV-infected RNA samples were diluted 500X and measured spectrophotometrically (Ultraspec 3000, Pharmacia Biotech) using quartz cuvettes to a total volume of 1ml. Purity and concentration readings were taken at $A_{260}/A_{280.}$

Calculations for spec readings were determined as follows:

Concentration of RNA sample = spectrophotometric conversion X A_{260} X dilution factor
= x µg/ml
Total yield = concentration X volume of sample in milliliters
= x µg

2.4.2 PCR

In order to determine if contaminating DNA was present in the RNA samples, PCR was carried out using ubiquitin primers (Inqaba). The ubiqitin primer sequences were designed as follows: - UB Forward (5′ATTTCTCAAAATCTTAAAAACTT3′) and UB Reverse (5′TGATAGTTTTCCCAGTCAAC3′).
Either 60ng of *Arabidopsis* TNA (positive control), or 40ng-100ng of RNA samples to be tested were added to a reaction mixture containing 0.5µM of each primer, 1.5mM $MgCl_2$, 0.5% Tween 20, 0.001% gelatine, 200µM dNTPs and 2.5U *Taq* DNA Polymerase (Roche) to make up a final reaction of 50µl. Amplification was carried out utilizing the MyCycler™ Thermal Cycler (Bio-Rad) with cycling conditions of DNA denaturation and *Taq* DNA Polymerase activation for 2 min at 95°C, and then 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C and extension for 60 sec at 72°C. The amplification products were examined by electrophoresis on a 1% agarose gel stained with ethidium bromide (EtBr) to a final concentration of 10µg/µl in a 0.5M TBE (Tris-Borate EDTA) electrophoresis buffer containing 50µg of EtBr run at 80V.

2.5 Labeling, Hybridization, Scanning and Washing

2.5.1 Fluorescent Target Preparation

Pre-spotted *Arabidopsis* Mendelbio L35 chip sets containing 7200 (6912 samples with 288 repeats) cell-signalling and some defense-related genes were received from Capar (Cape array opportunities) (www.capar.uct.ac.za) and stored in a dessicator at RT until ready for use. Fluorescence-labelled cDNA targets were prepared from SACMV-infected and healthy *Arabidopsis* total RNA (50µg) by indirect incorporation of amino allyl-dUTP (AA-dUTP) labelled cyanine dyes (Cy5 and Cy3) using the CyScribe Post-Labeling Kit (Amersham Biosciences) according to the manufacturer's instructions.

Briefly, this consisted of a two step process whereby, firstly, AA-dUTP was incorporated into the RNA template during cDNA synthesis. Unincorporated AA-dUTP was removed and remaining AA-dUTP cDNA was then purified using the PCR purification kit protocol obtained from the QIAquick® Spin Handbook (Qiagen). Modifications to the protocol involved replacing the phosphate wash (PB) buffer (Qiagen supplied) with a freshly prepared phosphate wash buffer (5mM KPO_4, pH8.0, 80% Ethanol-EtOH). The cDNA was eluted with phosphate elution buffer (4mM KPO_4, pH 8.5) replacing phosphate elution buffer (EB) (Qiagen supplied). The reaction was incubated at 42°C for 1.5 hrs, then treated with 2µl (2.5M) NaOH and incubated at 37°C for 15 min. To each reaction, 10µl (2M) HEPES was added for neutralization to occur.

The next step involved chemical labeling of the Cy3 and Cy5 NHS-esters to the AA-dUTP modified cDNA. The reaction was then incubated in the dark (due to the light sensitivity of the dyes) for 60 min. After CyDye labeling, 15µl (4M) Hydroxylamine was added to each coupling reaction and incubated in the dark for 15 min to inactivate any unreacted CyDye NHS-ester molecules. Purification of synthesized targets and removal of unincorporated CyDye molecules was then carried out using the PCR purification kit protocol obtained from the QIAquick® Spin Handbook (Qiagen) according to the manufacturer's instructions. Modifications to the protocol were as follows: 35µl (100nM) NaOAC, pH5.2 was added to the reaction mix prior to step one. The step was repeated a total of four times. Once the required 30µl of

buffer EB (Qiagen supplied) was added to the centre of the column, an incubation step for 5 min at RT was carried out.

2.5.2 Hybridization

(Modified from UCT (www.capar.uct.ac.za) and UP (http://microarray.up.ac.za)
Printed slides were treated in a preheated solution containing 3.5X SSC (Sigma), 0.2% SDS (Sigma), and 1% BSA (Roche) for 20 min at 60°C, followed by a rinsing step in DEPC treated, RNase free water. The slides were then dried using the ArrayIt™ Brand Microarray High-speed Centrifuge (MHC) for 8 seconds and then placed in a locally manufactured four slide hybridization chamber (Hyb-Up) for target to probe hybridization.

2.5.3 Target Preparation

For each slide, 30µl of Cy3 and Cy5 dyes were combined (final reaction volume of 60µl) and dried down in a Speed Vac Concentrator (Savant) to 30µl. The target pair was then resuspended in a 35µl hybridization solution containing 50% deionized formamide (Sigma), 25% hybridization buffer (Amersham), and 25% sterile water (Sabax water for injections, Adcock Ingram). The target solution was then denatured at 92°C for 2 min and placed on ice immediately. Coverslips were sprayed with compressed air and lowered onto the array area. In order to create a humid environment, 10µl of sterile water was added to each well in the chamber (20µl total), sealed and covered in foil (light-tight), and incubated at 42°C for 16h in a water bath.

2.5.4 Washing

Hybridized slides were washed in a low stringency buffer containing 1.0X SSC (Sigma) and 0.2% SDS (Sigma) for 4min at 42°C, then in a high stringency buffer containing 0.1X SSC (Sigma), 0.2% SDS (Sigma) for 4 min at 42°C (repeated), and 0.1X SSC for 1min at RT (repeated twice). Slides were then rinsed in DEPC treated, RNase free water a few times and dried with the MHC (ArrayIt™).

2.5.5 Scanning

Axon Instruments GenePix 4000B array scanner using GenePix Pro version 5.1 was used to scan the hybridized arrays at the African Centre for Gene technologies (ACGT) Microarray Facility, University of Pretoria, South Africa (http://microarray.up.ac.za). Grids were constructed (service supplied by Microarray Facility) and manually adjusted (by user) to ensure optimal spot recognition. Measurements of fluorescence and local background fluorescence for each spot were calculated. Local background fluorescence was subtracted from the fluorescence intensity on each array. Spots with low signal intensity, covered in dust particles, saturated spots and spots with high background areas were flagged for exclusion from downstream analysis (Figure 2.4).

Section C-Data Analysis

A schematic overview of methodology performed for Section C may be seen in Figure 2.4.

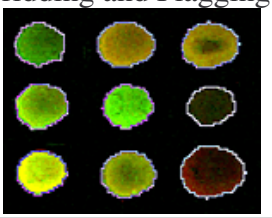
2.6 Data Analysis

In order for microarray files to be analyzed in TIGR MIDAS and TIGR MEV(MultiExperiment Viewer) [The Institute for Genomic Research (TIGR), Rockville, Md; http://www.tigr.org/software], data created in GenePix (.gpr) files was converted to a tab-delimited (.mev) file using TIGR Express Converter (version 1.4.1) (TIGR, http://www.tigr.org/software). This is a file transformation tool that reads microarray data from a variety of file formats, generating files suitable for MIDAS and MEV programs.

C

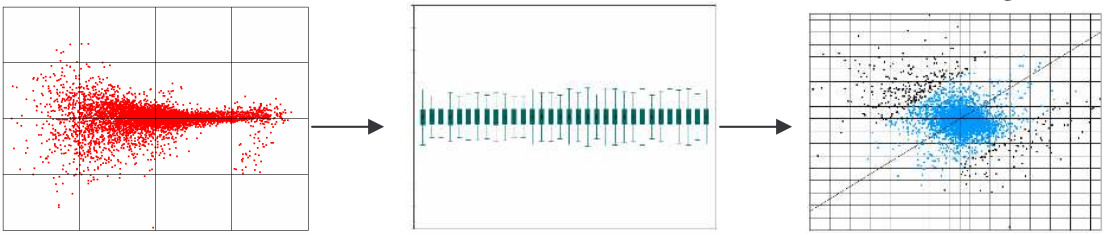
Data Analysis

GenePix Version 5.1
Gridding and Flagging of Spots



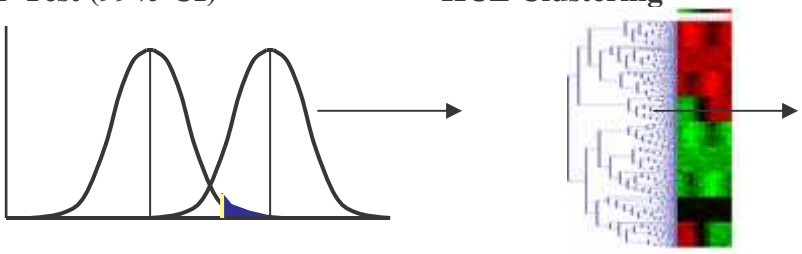
MIDAS

LOWESS Normalization | SD Regularization | Flip Dye Consistency Checking



MEV (MultiExperiment Viewer) + **TAIR**

T-Test (99% CI) **HCL Clustering**



Gene Annotation + Function

Validation of Microarray Results
Relative Quantification Real-Time RT-PCR

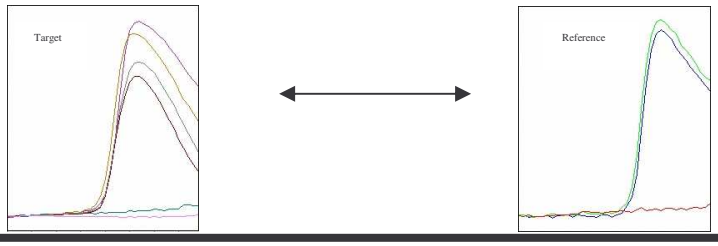


Figure 2.4 Schematic representation of methodology performed in section (C) showing data analysis using: GenePix version 5.1, MIDAS, MEV, TAIR, and Relative Quantification Real-Time RT-PCR

2.6.1 MIDAS-Normalization

Normalization procedures were carried out utilizing the TIGR MIDAS software. Four sets of dye-swap experiments (8 slides in total) were normalized simultaneously in MIDAS, correcting for experimental errors within and between repeated hybridizations. To correct for intraslide variation, a locally weighted linear regression (LOWESS) algorithm was applied with a smoothing parameter set at 0.33 (33%) on a block-by-block basis. The Cy5 channel intensity was adjusted by the calculated LOWESS factor.

2.6.2 Standard Deviation (SD) Regularization

SD regularization was then applied to the data set to scale channel intensities of each spot so that the spots within each block of a slide had the same spread (SD for $\log_2$Cy5/Cy3). Cy5 was the channel intensity selected for adjustment by the calculated SD regularization factor for all spots within each block of each slide.

2.6.3 Flip-dye Consistency Checking

In order to show consistencies between flip-dye replicates, the flip-dye consistency parameter was applied. The SD cut option was carried out which excluded spots within a SD below or above 2. These spots were marked as flip-dye inconsistent and intensities set to zero, excluding them from downstream analysis.

2.6.4 Virtual Trim

The virtual trim option was applied to include “bad” spots in the output files but set to zero to distinguish them from “good” spots thereby maintaining original spot numbers.

2.6.5 MEV-T-test, HCL Clustering and TAIR

Normalized and scaled data was then imported into TIGR MEV for differential gene expression analysis. A *t*-test was carried out selecting p-values based on the *t*-distribution, set with an overall alpha (no correction) critical p-value of 0.01 (99%). The mean value tested against was zero as $\log_2$ transformed data was used for gene expression analysis. Hierarchical Clustering (HCL) was applied to cluster genes based on average linkage clustering according to Euclidean distance parameters. Genes with $\log_2$ values above 0.5 or below -0.5 were selected for further analysis and annotation on The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org), which is a comprehensive database and web-based information retrieval, analysis and visualization program.

2.7 Validation of Microarray Results Using Real-Time RT- PCR

First strand cDNA was synthesized in a total volume of 20µl containing 1µg of total RNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche) according to the manufacturer's instructions. PCR amplifications were carried out using probes and primer sets listed in Table 2.1, (designed by Roche). A master mix
cocktail was prepared for each gene using the LightCycler FastStart DNA Master Hybridization Probe Kit (Roche) according to manufacturer's instructions. To this reaction, 0.2µM of each probe (LC Red and Fluorescein), 0.5µM of each primer (forward and reverse), and 100ng of cDNA was added to obtain a final reaction volume of 20µl. A negative control was run with each sample by replacing template cDNA with PCR-grade water.

Relative quantification real-time RT-PCR reactions were performed on the LightCycler 2.0 System (Roche Applied Science) with thermal cycling conditions consisting of an initial activation step of 95°C for 10 min, followed by a cycling step repeated 40 times consisting of 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec with a single fluorescence measurement. A melting curve analysis was then carried out at 95°C for 0 sec, 45°C for 30 sec, and 95°C for 0 sec at a heating rate of 0.1°C per second and a continuous fluorescence measurement. A final cooling step was then carried out at 40°C for 30 sec.

Crossing Points (CP) were then determined with the LightCycler software version 4.0 (Roche Applied Science). This is the point at which the fluorescence of the product rises above background fluorescence. Four genes were analyzed and normalized with a selected reference gene shown to be constitutively expressed from microarray analyses.

Calculations were determined as a function of the PCR reaction efficiency of 2 (this value corresponds to a perfect doubling of PCR production with each cycle). The following equation was thus applied to determine relative quantification of the target gene versus the reference gene (Pfaffl, 2001):

$$\text{Ratio} = \frac{(E_{target})^{\Delta CPtarget(control-sample)}}{(E_{ref})^{\Delta CPref(control-sample)}}$$

E_{target} = real-time PCR efficiency of a target gene transcript

E_{ref} = real-time PCR efficiency of a reference gene transcript

ΔCP_{target} = CP deviation of control minus the sample of the target gene transcript

ΔCP_{ref} = CP deviation of control minus the sample of the reference gene transcript

Table 2.1 Primer and Probe Sequences used for Relative Quantification Real-Time RT-PCR

Gene Name	Primer/Probe	Melting Temperature (Tm in°C)	No. of bases	Primer Sequence (5`-3`)
AGP6 Primer set	F	52.4	19	GCA CGT CAA TTT GTC GTT T
	R	58.4	22	GAA GAG GCC GAA GAT GAT TTA G
AGP6 Probe set	FL	61.8	21	GCT CCA ACT GCC ACA ACC AAG-Fluo
	LC Red	66.3	25	LC Red 640-TCC TTC AGC TCC AAC CAA GGC TCC-Pho
Carbamoyl Primer set	F	57.9	21	CCG GAA CCT TGT CAG AGA TTA
	R	55.2	17	GCT TCG AAC TCT GCA GC
Carbamoyl Probe set	FL	62.1	22	GAA GAA GTC CGT ACC AAG CGG A-Fluo
	LC Red	68.3	35	LC Red 640-TCT CTA GGA GTT GTT CCA TCT TAC AAG AGA GTG GA-Pho
Dynamin Primer set	F	52.8	17	AGG TCT TGA ACC TTC GC
	R	56.0	18	CTC TCA GGC TCC ACA GTA
Dynamin Probe set	FL	61.8	21	GGG AAC TAA AGC CGT GGA GCA-Fluo
	LC Red	67.0	33	LC Red 640-CAA TGC AA CAG TCA AAT CTT CTA GGA TTC CGC-Pho
Peroxidase Primer set	F	55.3	20	ACA TAC GAT TGG AGT AGC GA
	R	55.3	20	GAA ATA TTG TTG TCA CCG CC
Peroxidase Probe set	FL	64.8	26	TAA CCA ACC AGA CGA GAC GCT AGA GA-Fluo
	LC Red	68.3	35	LC Red 640-TCT TAC TAC TAT GGT CTC AGG TCA ATT TGT CCA CC-Pho
WD-40 Primer set	F	55.9	21	TTG ATT GGA ATC CTC ATG ACG
	R	58.4	22	GGA GAC CAC TGA ACA CAA AGA A
WD-40 Probe set	FL	62.7	24	CAA CAC TGT CCG GTT GTT TGA TCG-Fluo
	LC Red	64.9	27	LC Red 640-GGA AGC TTA CCG CTA ATG GAG TTG GTT-Pho

CHAPTER 3

RESULTS

3.1 Symptom Development

Agroinoculation was used as the method for inoculation since some geminiviruses such as SACMV are not mechanically transmissible and whitefly inoculation facilities were not available. It should be noted that *Agrobacterium* was used as a transformation vector only in this study, and may stimulate transient expression changes when inoculated into the plant but it is unlikely to cause symptom expression at 35 dpi. This present study assessed the reaction of *Arabidopsis* to disease caused by SACMV by monitoring the progression of SACMV replication from inoculation to symptom development by scoring symptoms phenotypically and using absolute quantitative real-time PCR. No symptoms were observed between 0-14 and 14-21 dpi. Symptoms of stunting began to emerge in 26 of the 30 infected plants, between 21-28dpi, but no typical leaf curl and yellowing was observed. SACMV-infected *N.benthamiana* (tobacco) plants, used as a positive control, displayed characteristic symptoms such as leaf curling, crinkling and chlorosis (Figure 3.1 C). Stunting of the entire plant was also observed (Figure 3.1 B) as seen compared to the healthy control (Figure 3.1 A). From 28-35 dpi, some of the infected *Arabidopsis* plants in both biological replicates began to display typical geminivirus symptoms such as severe leaf curl, deformation and reduction in size (Figure 3.2 D and Figure 3.3 F). A slightly yellow mosaic pattern was also observed in a minority of infected plants (4 of the 30 plants) (Figure 3.2 C). Infected plants appeared flaccid, diameter of rosettes were smaller than healthy controls (Figure 3.2 A and Figure 3.3A) and the plants were reduced in height (Figure 3.3 B, D). In addition, infected *Arabidopsis* plants in biological replicate 1 (BR1) showed little or no inflorescence formation (Figure 3.2 A) which was clearly visible in all healthy control plants (Figure 3.2 B).

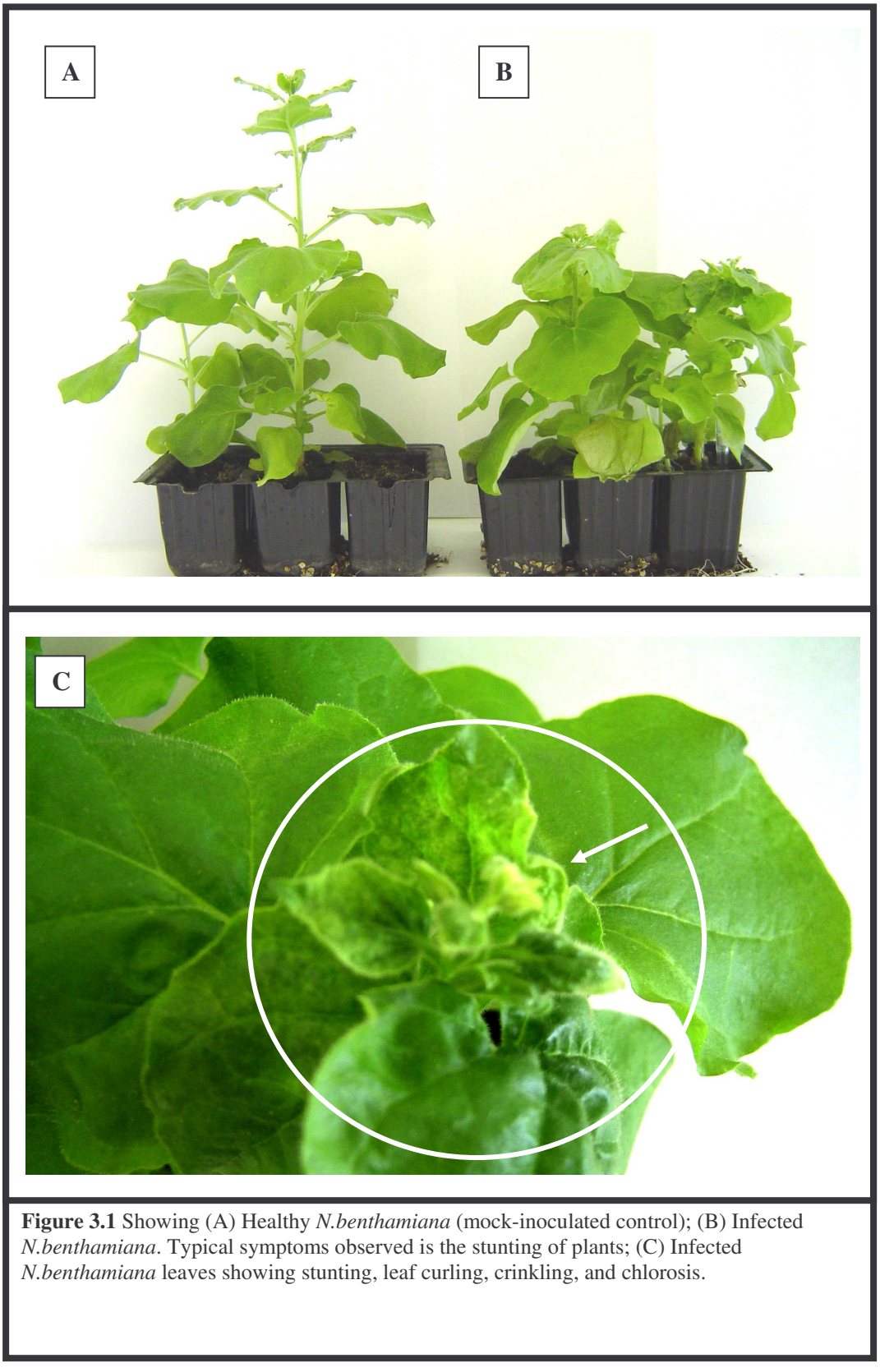


Figure 3.1 Showing (A) Healthy *N.benthamiana* (mock-inoculated control); (B) Infected *N.benthamiana*. Typical symptoms observed is the stunting of plants; (C) Infected *N.benthamiana* leaves showing stunting, leaf curling, crinkling, and chlorosis.

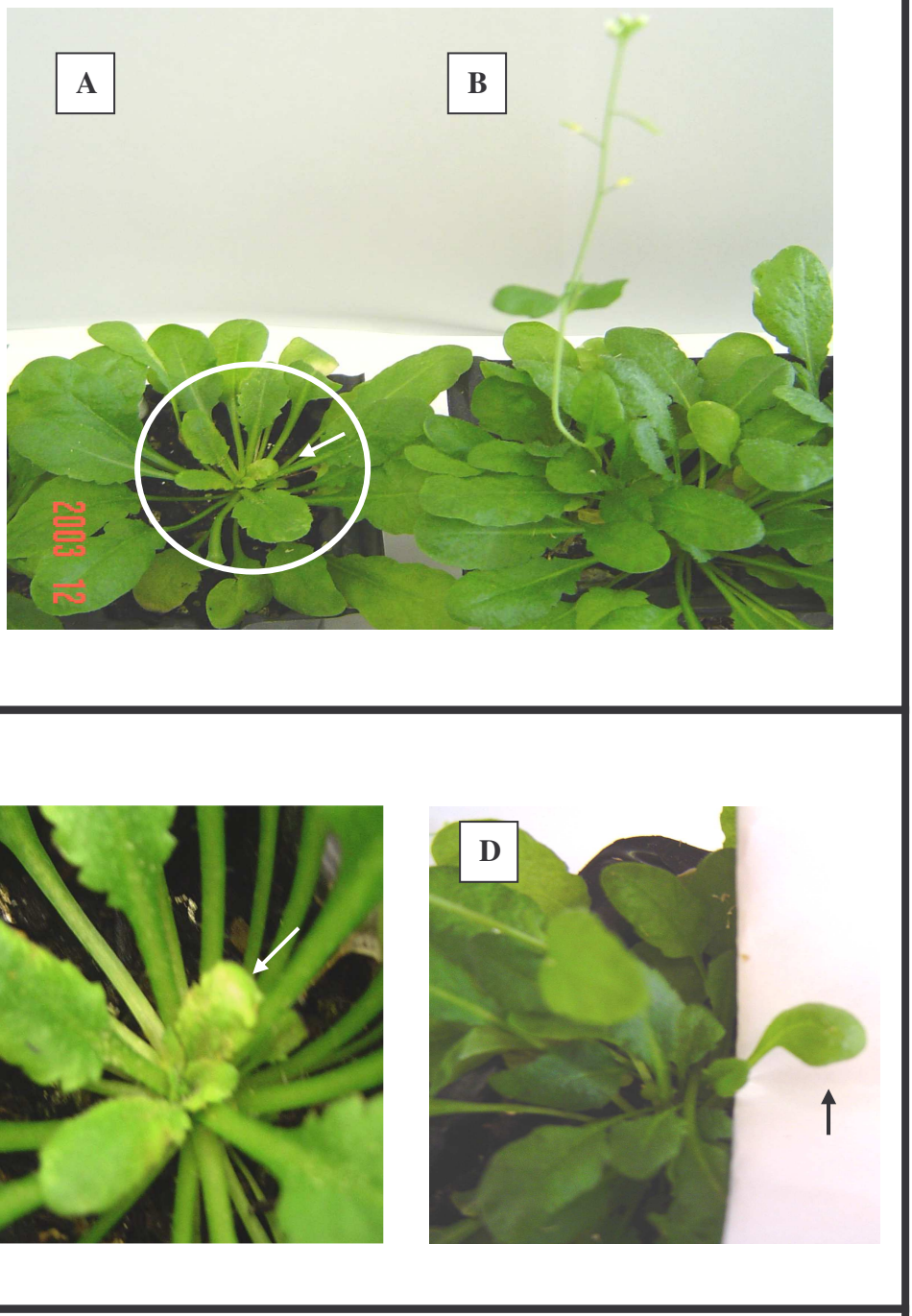


Figure 3.2 BR1 showing (A) Typical symptom such as stunting of the entire plant; (B) Healthy *Arabidopsis*; (C) Yellowing and distortion of young SACMV-infected leaves; (D) Leaf deformation.

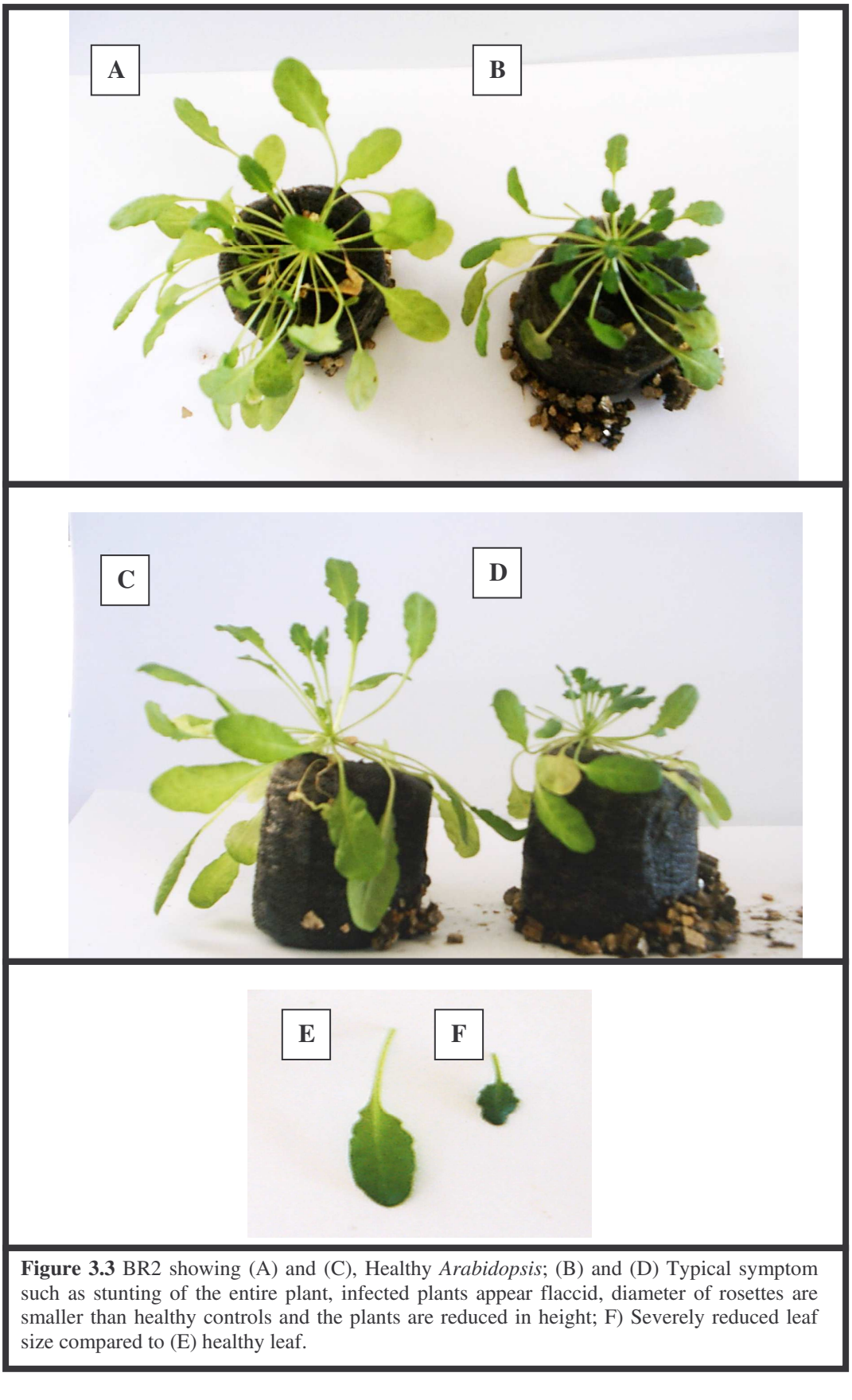


Figure 3.3 BR2 showing (A) and (C), Healthy *Arabidopsis*; (B) and (D) Typical symptom such as stunting of the entire plant, infected plants appear flaccid, diameter of rosettes are smaller than healthy controls and the plants are reduced in height; F) Severely reduced leaf size compared to (E) healthy leaf.

3.2 Confirmation of SACMV Infection

3.2.1 Total Nucleic Acid Extraction (TNA)

Concentrations were determined on a 1% agarose gel using MassRuler™ DNA Ladder (2ng-150ng) (Fermentas Life Sciences) which have been calibrated for DNA concentration determination. Concentration(s) for BR 1 (samples 3 and 4) were 15ng/µl and may be seen in Figure 3.4 A, lane 5 and 6. BR 2 (sample 4) was also 15 ng/µl and may be seen in Figure 3.5 A, lane 6.

3.2.2 PCR

The expected ~550bp core coat protein (CCP) primer region of SACMV was successfully amplified using CCP degenerate primers specific for begomoviruses. Because SACMV had not previously been tested in *Arabidopsis,* a concentration gradient was constructed to determine at what concentration SACMV would amplify at in BR 1. It was ascertained that using 34ng (sample 1) was not sufficient for PCR amplification (Figure 3.4 B, lane 5) whereas, 42.5ng (sample 2) and 51ng (sample 3) was optimal for ~550bp band amplification (Figure 3.4 B, lane 6 and 7). The positive control used was cassava TNA infected with *East African cassava mosaic virus* (EACMV) (45ng) (Figure 3.4 B, lane 3). The negative control showed no amplification as expected (Figure 3.4 B, lane 8). Optimal concentrations were determined for PCR band amplification in BR 1, therefore no concentration gradient was required for BR 2. The resulting ~550bp CCP region was successfully amplified

from concentrations of 42.5ng and 51ng, respectively (Figure 3.5 B, lanes 5 and 6). The positive control used was also cassava TNA infected with EACMV (45ng) (Figure 3.5 B, lane 3) and no band amplification visible in the negative control (Figure 3.5 B, lane 7).

3.23 Absolute Quantification Real-Time PCR

Absolute quantitative real-time PCR showed that an increase in virus titer correlated with fully susceptible *Arabidopsis* tissues. The virus titer levels of fully symptomatic plants at 35 dpi were approximately 3-fold higher (14522 virus copies/100ng of TNA) than at 13dpi (845 virus copies/100ng of TNA) where no symptoms were visible. The predicted PCR product length of 150 bp was confirmed by both the melting curve analysis (data not shown) and agarose gel electrophoresis (figure 3.6 B) as a single product was amplified. The standard curve was constructed by plotting the log of known plasmid SACMV DNA-A concentrations against crossing point values showing an error of 0.0255 and efficiency of 2.087 (Figure 3.6 A).

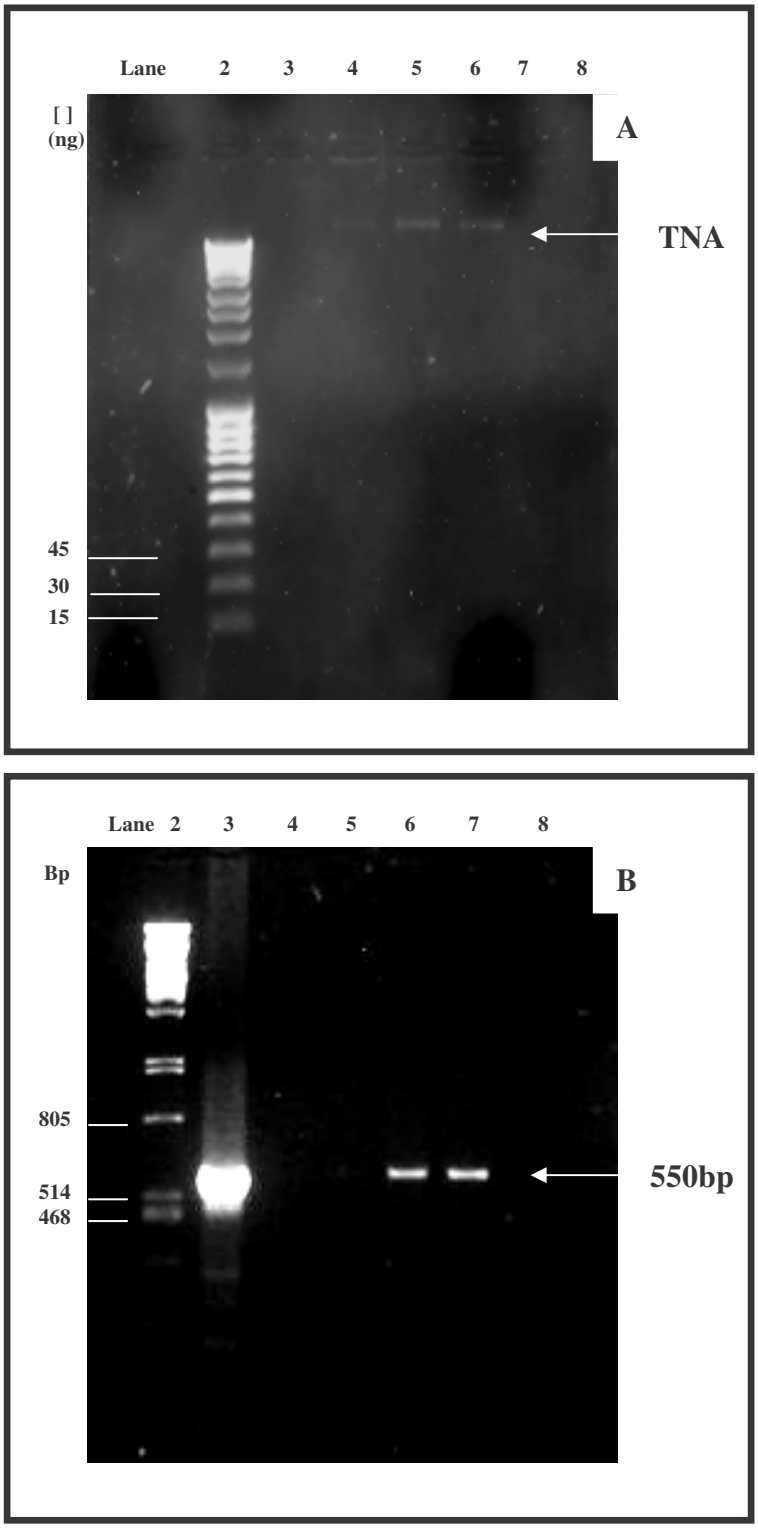


Figure 3.4 BR1 showing a 1% agarose gel of (A) TNA extracted from SACMV-infected *Arabidopsis* leaf samples, lane 5 and 6. Lane 2, 15µl MassRuler™ DNA Ladder (Fermentas Life Sciences) and (B) Amplification of the ~550bp CCP fragment from SACMV-infected *Arabidopsis* leaves. Lane 2, Pst1-λ DNA marker; Lane 3, EACMV positive control; Lane 4 and 5, Empty; Lane 6 and 7, Infected *Arabidopsis;* Lane 8, Negative control.

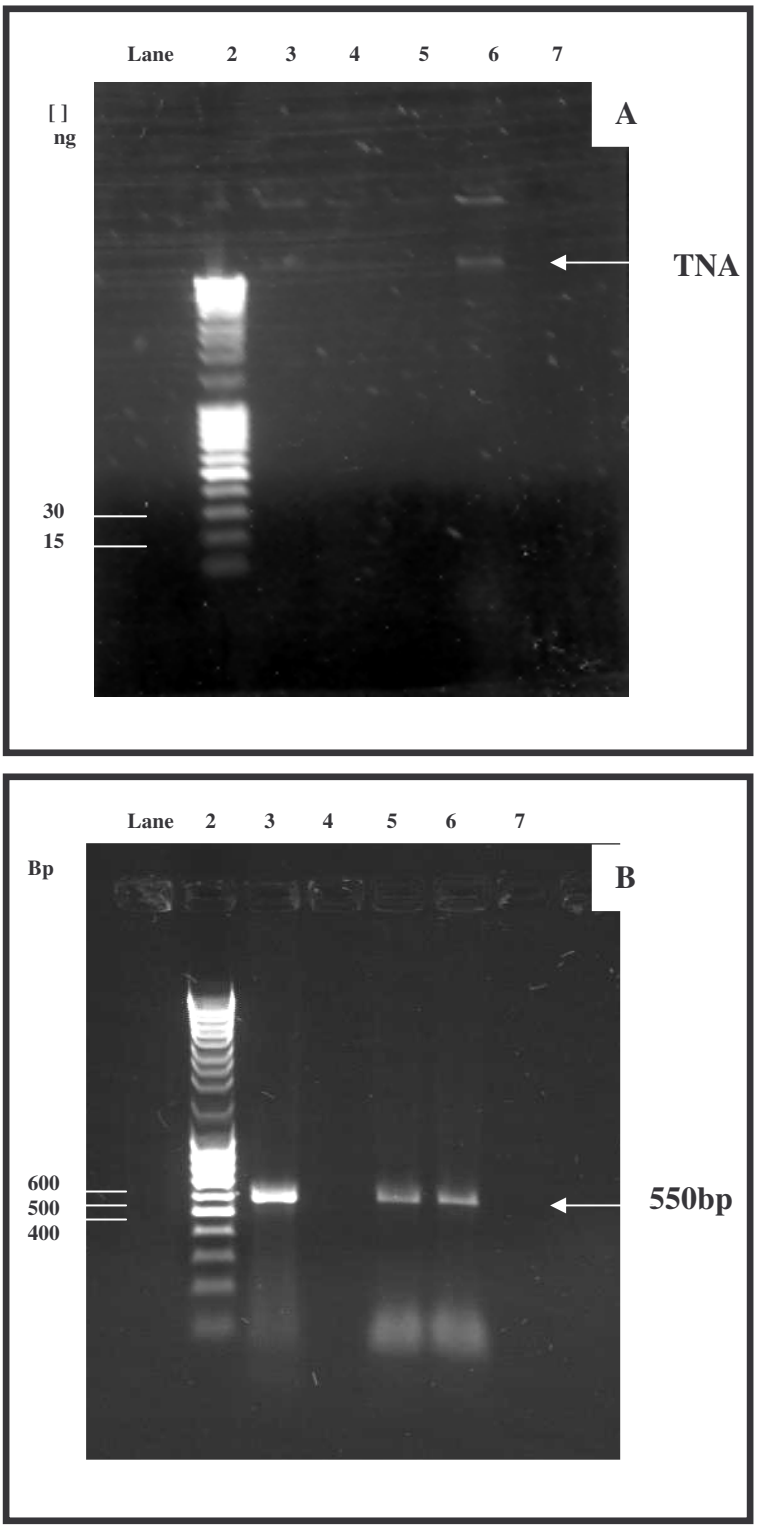
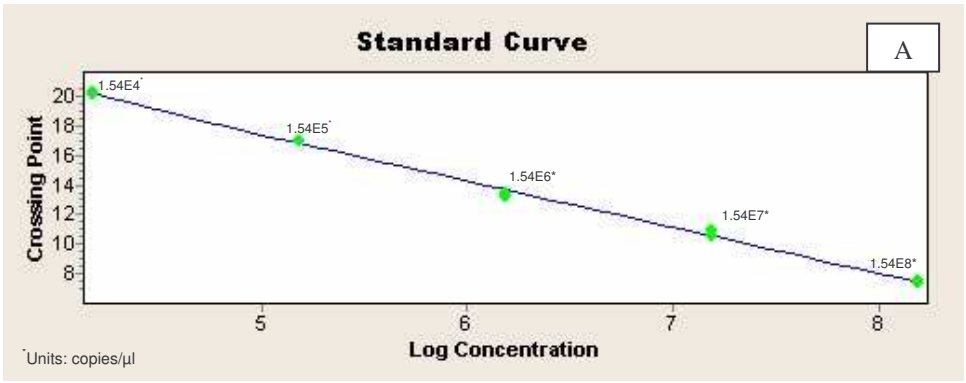


Figure 3.5 BR2 showing a 1% agarose gel of (A) TNA extracted from SACMV-infected *Arabidopsis* leaf samples, lane 6. Lane 2, 15µl MassRuler™ DNA Ladder (Fermentas Life Sciences) and (B) Amplification of the ~550bp CCP fragment from SACMV-infected *Arabidopsis* leaves. Lane 2, 15µl MassRuler™ DNA Ladder (Fermentas Life Sciences); Lane 3, EACMV positive control; Lane 4, Empty; Lane 5 and 6, Infected *Arabidopsis* DNA*;* Lane 7, Negative control.



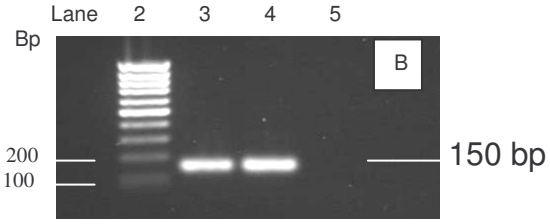


Figure 3.6 (A) Standard curve obtained from plotting log of known DNA concentrations of plasmid SACMV DNA-A (1.54E8-1.54E4 copies/µl) against Cp values obtained from real-time quantitative PCR; (B) 1% Agarose gel showing the 150 bp end-point PCR product at 13 dpi (lane3) and 35 dpi (lane4). Negative control (lane 5). O′GeneRuler100bp DNA Ladder (Fermentas Life Sciences) (lane 2).

3.3 RNA Extractions

3.3.1 Concentration and Purity Determination

For each biological replicate, 20-25 healthy and SACMV-infected *Arabidopsis* leaves were pooled separately, successfully isolating between 350µg to 430µg (3.50µg/µl – 4.26µg/µl) of total RNA respectively for microarray analyses (50µg of total RNA is required per labelling reaction for array hybridizations) (Table 3.1). The purity of total RNA was determined by A_{260}/A_{280} ratios, revealing purity ratios of between 1.9 and 2.3. A high degree of purity was therefore observed among the RNA samples for both healthy and SACMV-infected *Arabidopsis* total RNA in each biological replicate (Table 3.1).

Table 3.1: RNA concentration and purity determination for each biological replicate

Sample	Concentation (µg/µl)	Purity (1.9-2.3)
BR1		
Healthy RNA	4.26	2.06
Infected RNA	3.60	2.10
BR2		
Healthy RNA	3.56	1.94
Infected RNA	3.62	1.94

3.3.2 RNA Formaldehyde Gel Electrophoresis

A 1% formaldehyde agarose gel revealed intact, non-degraded total RNA in both biological replicates (Figure 3.7 A, lane 4 and 5, and Figure 3.8 A, lane 3 and 4), indicating that no degradation had occurred. Distinct 25S and 18S rRNA bands demonstrated high quality, intact total RNA.

3.3.3 PCR

Results obtained from PCR analysis revealed that no contaminating plant DNA was present in the RNA samples (Figure 3.6 B, lanes 4-11 and Figure 3.7 B, lanes 4-11). Only positive band amplification was observed, which was expected (Figure 3.6 B, lane 3 and Figure 3.7 B, lane 3). Furthermore, band amplification was also not observed in the negative controls (Figures 3.6 B, lane 12 and 3.7 B, lane 12).

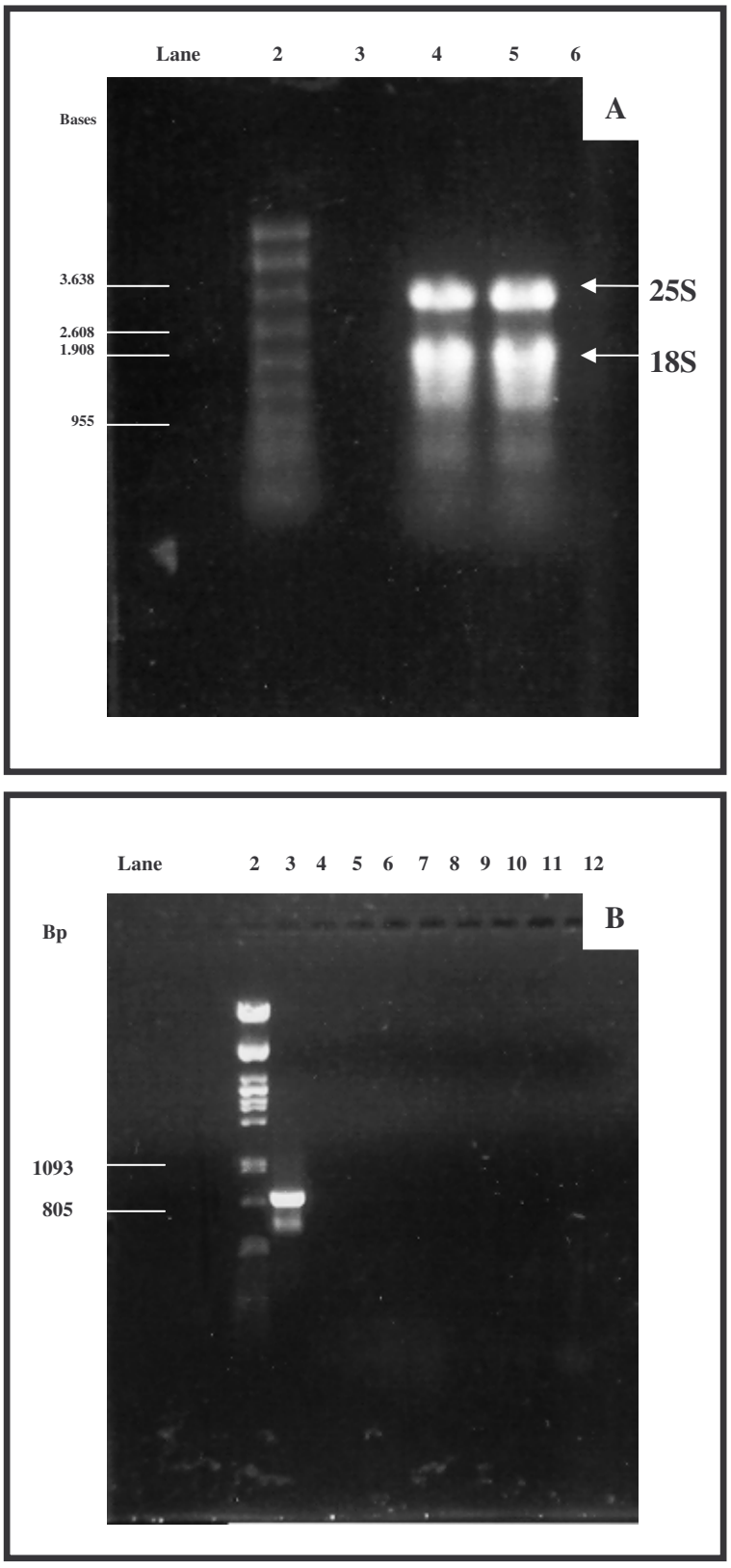


Figure 3.7 BR1 (A) 1% MOPS/Formaldehyde gel electrophoresis of healthy *Arabidopsis* RNA (8.52µg), lane 4. SACMV-infected *Arabidopsis* RNA (7.20µg), lane 5. Lane 2, 3µl RNA Marker (Promega); B) 1% agarose gel showing band amplification of *Arabidopsis* DNA using ubiquitin primers (positive control), lane 3. Lanes 4-7, healthy *Arabidopsis* total RNA. Lanes 8-11, SACMV-infected *Arabidopsis* total RNA. Lane 12, negative control. Lane 2, Pst1-λ DNA marker.

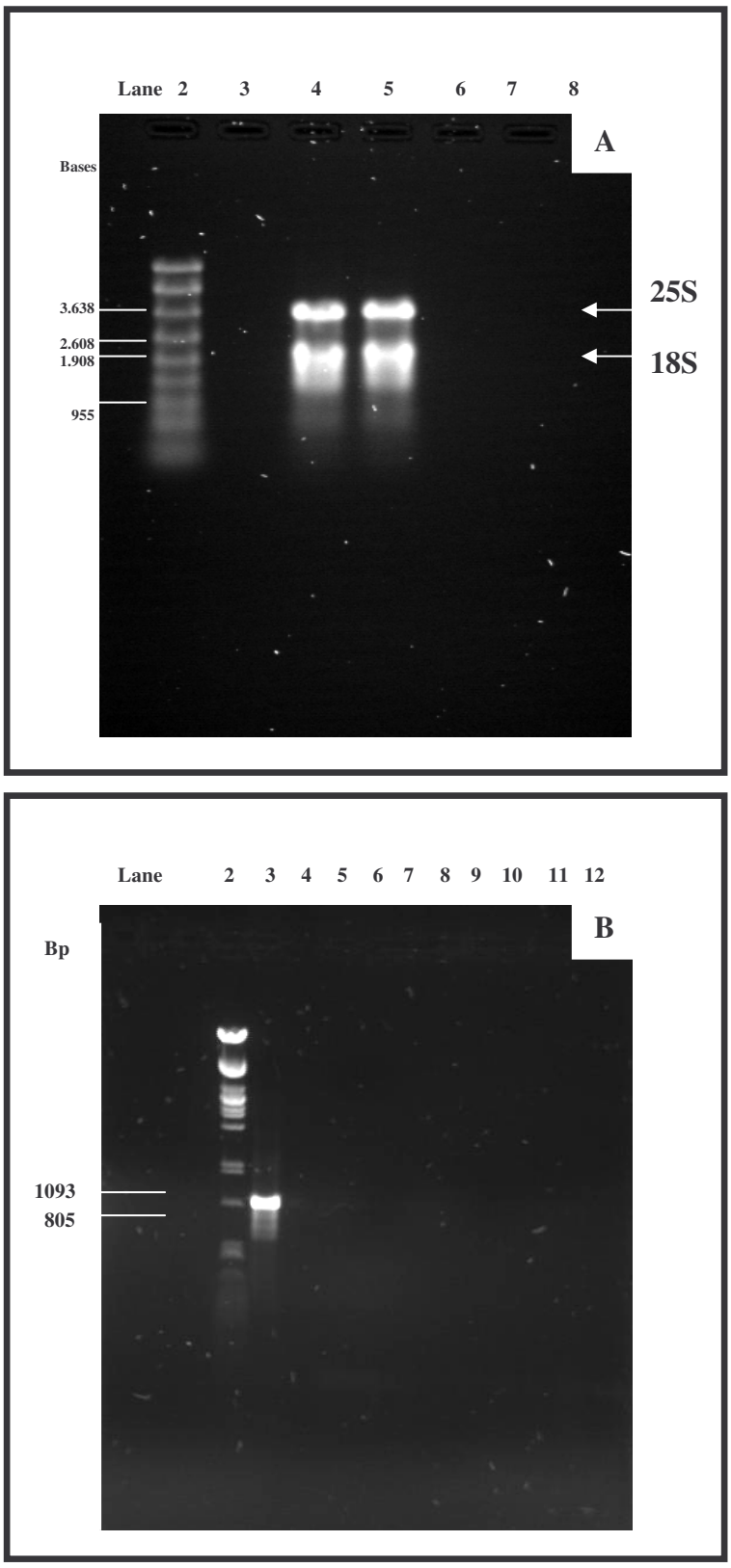


Figure 3.8 BR2 (A) 1% MOPS/Formaldehyde gel electrophoresis of healthy *Arabidopsis* RNA (3.56µg), lane 3; SACMV-infected *Arabidopsis* RNA (3.62µg), lane 4; Lane 1, 3µl RNA Marker (Promega); B) 1% agarose gel showing band amplification of *Arabidopsis* DNA using ubiquitin primers (positive control), lane 3. Lanes 4-7, healthy *Arabidopsis* total RNA. Lanes 8-11, SACMV-infected *Arabidopsis* total RNA. Lane 12, negative control. Lane 2, Pst1-λ DNA marker.

3.4 Microarrays

3.4.1 Locally Weighted Scatterplot Smoothing (LOWESS) Normalization

Ratio-Intensity (R-I) scatter plots were created from eight complete hybridizations of the *Arabidopsis* 7200 cDNA microarray chips, consisting of two biological replicates (independent RNA) named BR1 and BR2. Within each biological replicate, 2 technical replicates (pooled RNA) each with dye-swap experiments (totalling 4 technical replicates in each biological replicate) were constructed.

Fluorescence intensities were measured from both channels (i.e. Cy5 and Cy3), displaying $\log_2$ (Cy5/Cy3) ratios for each spot as a function of the $\log_{10}$ (Cy5*Cy3) product intensities (Figure 3.9 and 3.10). Raw data generated from GenePix is indicated by the pre-processed scatter plots for each biological replicate. From figures 2.9 and 2.10 (indicated in blue), it may be seen that these spots are deviating from zero, indicating a systematic dependence on intensity, and showing an even greater variation at lower intensities. LOWESS was therefore applied to remove intensity dependent dye-specific artifacts in the $\log_2$ (ratios). This algorithm subtracted the average ratio from the experimentally observed ratio, producing a balanced distribution of expression ratios around zero, independent of intensity (Figure 3.9 and 3.10, shown in post-normalization plots, red). Similar patterns of expression were also observed among LOWESS normalized signal intensity ratios among the technical replicates in BR1, distributed around a mean of zero (Figure 3.9, indicated in red). This was also observed among the technical replicates in BR2, also centered around a mean of zero after LOWESS normalization (Figure 3.10, shown in red). Differences among BR1 and BR2 indicate tightly packed profiles in BR1 (Figure 3.9) as opposed to BR2 (Figure 3.10) suggesting more variation in gene expression in

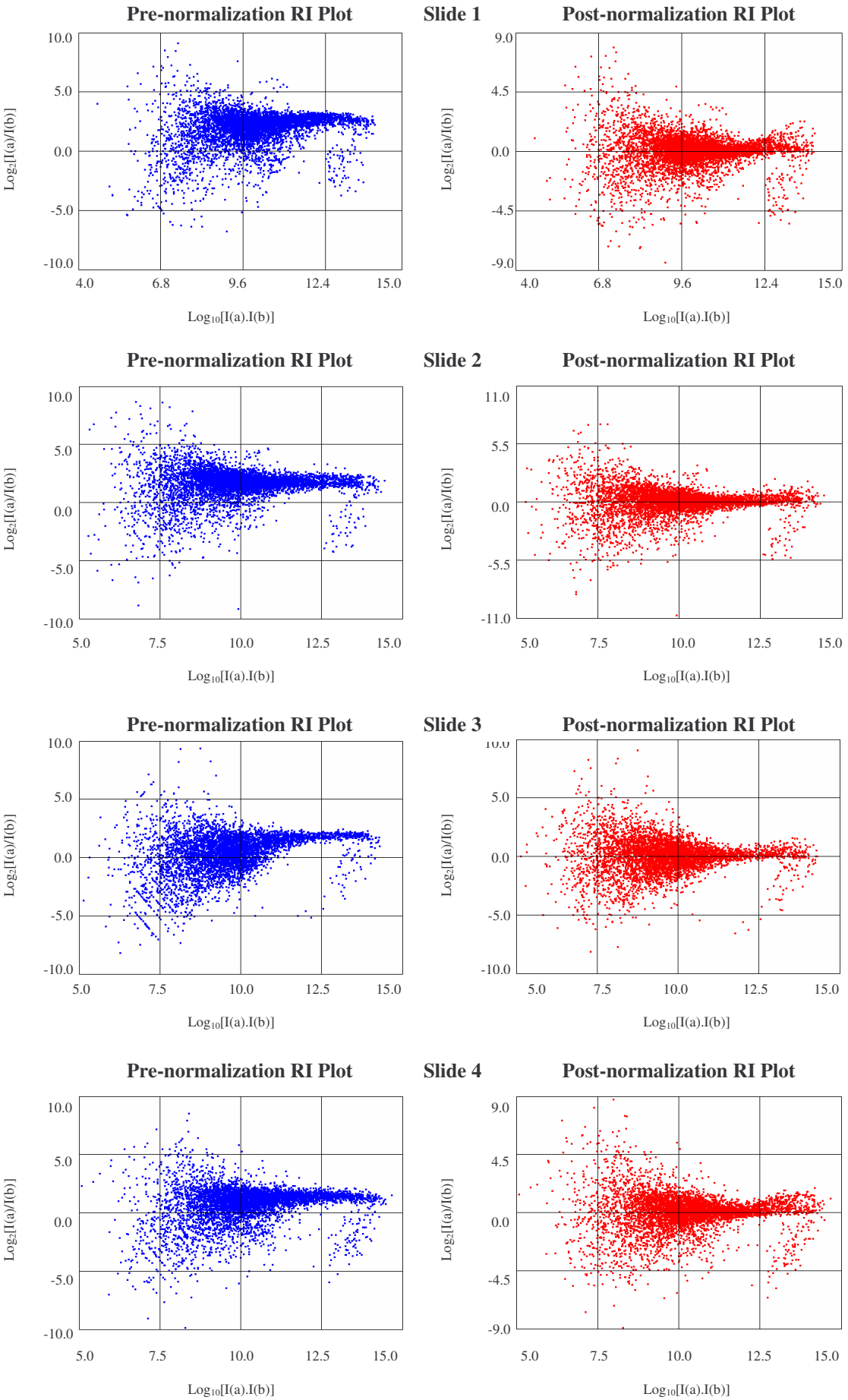


Figure 3.9 BR1 LOWESS pre- (blue) and post (red) normalization ratio-intensity (RI) plots.

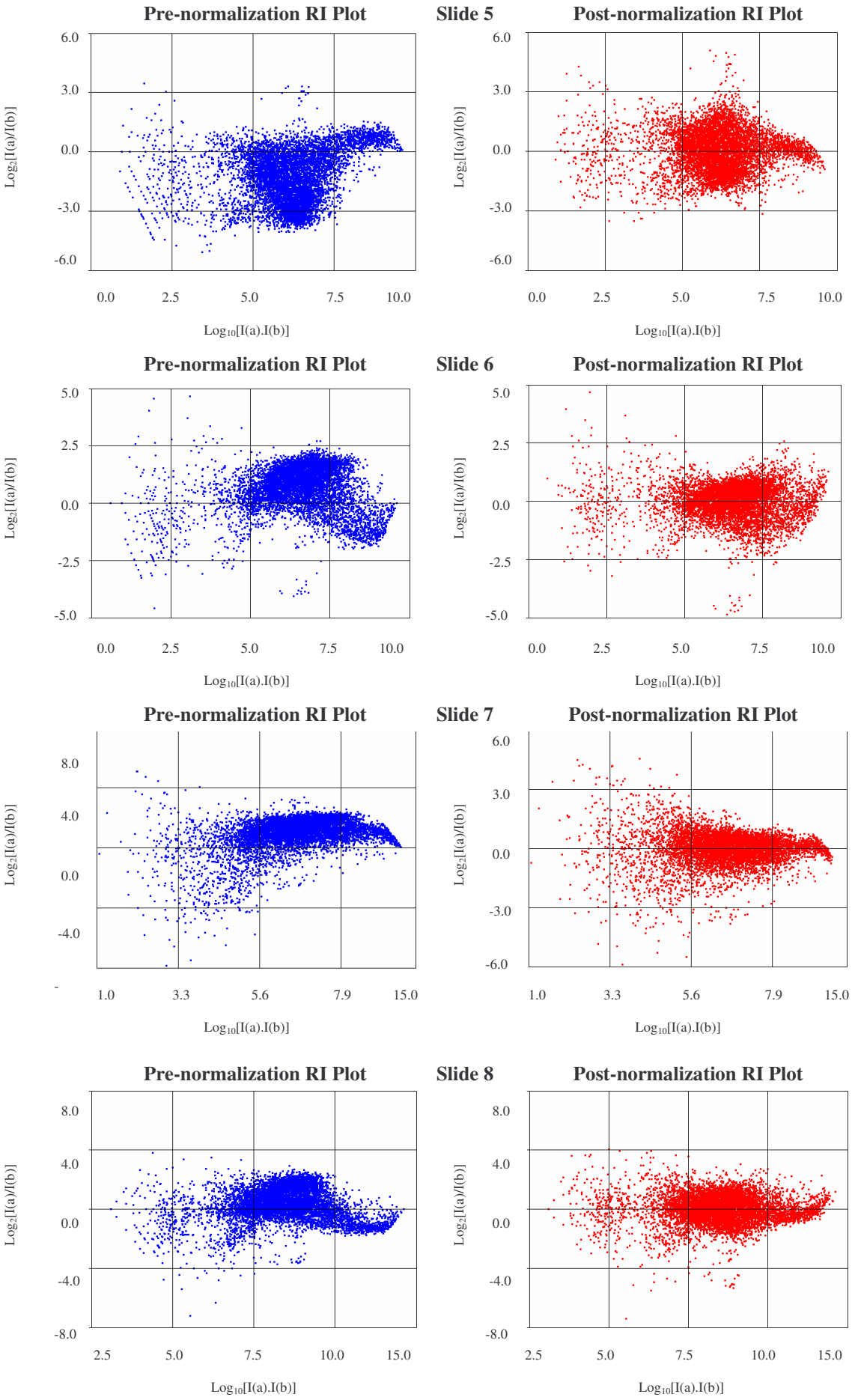


Figure 3.10 BR2 LOWESS pre- (blue) and post (red) normalization ratio-intensity (RI) plots.

BR2 after normalization. BR2 was more successful in terms of balancing fluorescence intensities, as less systematic errors were removed from BR2 (average input spots among 4 slides, 7200 and output spots 7038), as computed by the program, revealing a loss of 162 spots. BR1 contained more systematic errors as post-normalization output spots was 5652 from an input of 7200 spots (1548 spot loss) (Figure 3.9 and 3.10).

3.4.2 Standard Deviation (SD) Regularization

After LOWESS normalization, scale adjustment was required by SD regularized normalization as log ratios now centred around zero have slightly different spreads (Figure 3.11 and 3.12, shown in pink). Differences in spread among the 32 blocks in one array may result in misidentification in genes that are differentially expressed in infected tissue when compared to healthy controls. An average SD block log-ratio was therefore computed for all 32 blocks on each array (each block containing 225 spots). After SD regularization was applied, log ratios became evenly distributed on each array, resulting in each block having the same SD for $\log_2$ (Cy5/Cy3) distribution (Figure 3.11 and 3.12, indicated in green).

From Figure 3.13 it may be seen that scatter plot smoother LOWESS was constructed to identify intensity and spatial normalization problems, calculating SD before (Figure 3.13, black) and after LOWESS correction (Figure 3.13, purple). It was observed that after SD regularization, SD values were decreased (Figure 3.13, purple). Only a small number of outliers outside 3 SD from the mean was observed, suggesting that the distribution of $\log_2$ (ratio) values were not deviating badly from the mean of zero (Figure 3.13, purple).

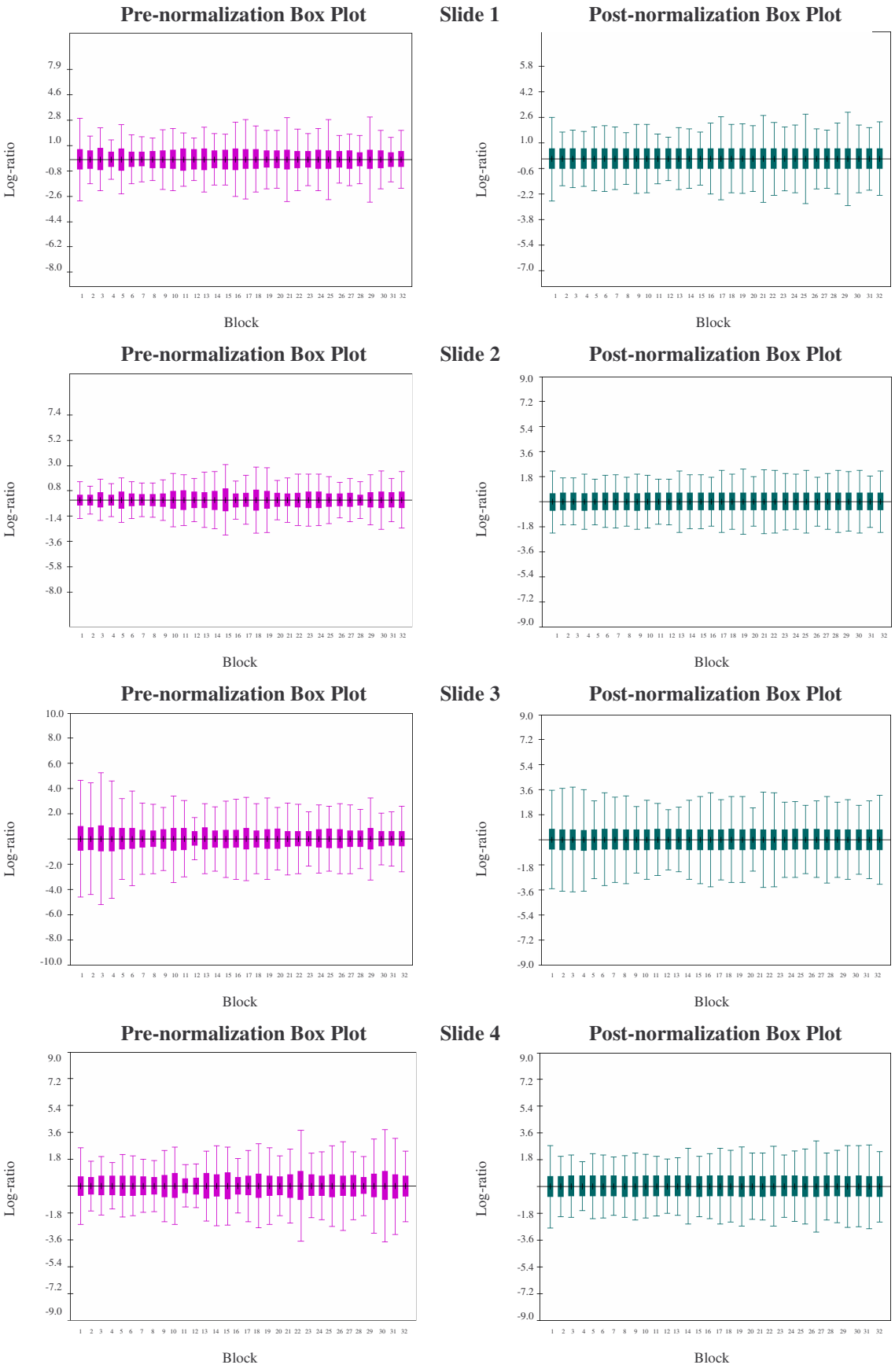


Figure 3.11 BR1 Box Plots displaying the intensity log-ratio distribution for each of the 32 blocks on an array before (pink) and after (green) scale normalization.

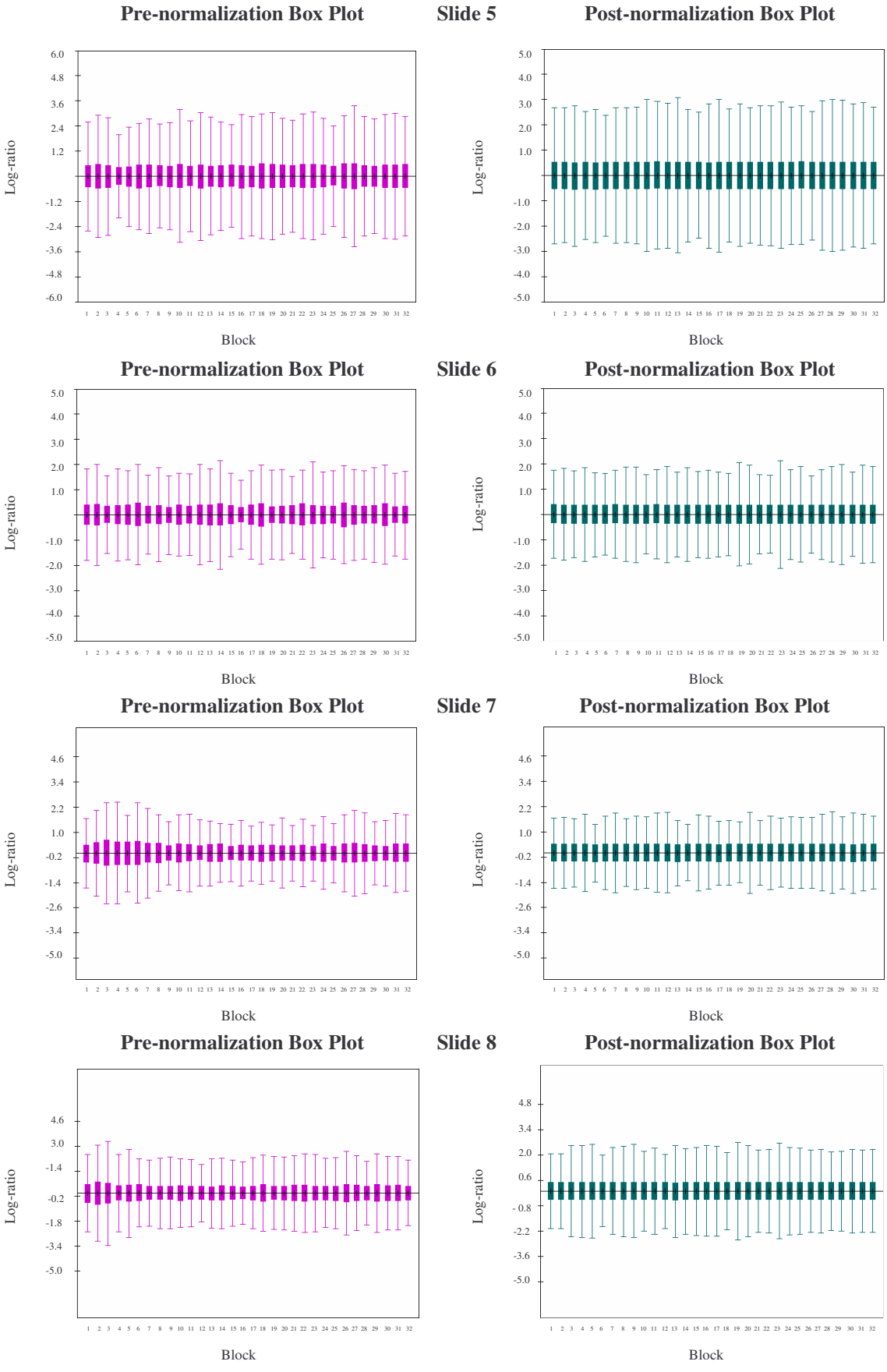


Figure 3.12 BR2 Box Plots displaying the intensity log-ratio distribution for each of the 32 blocks on an array before (pink) and after (green) scale normalization.

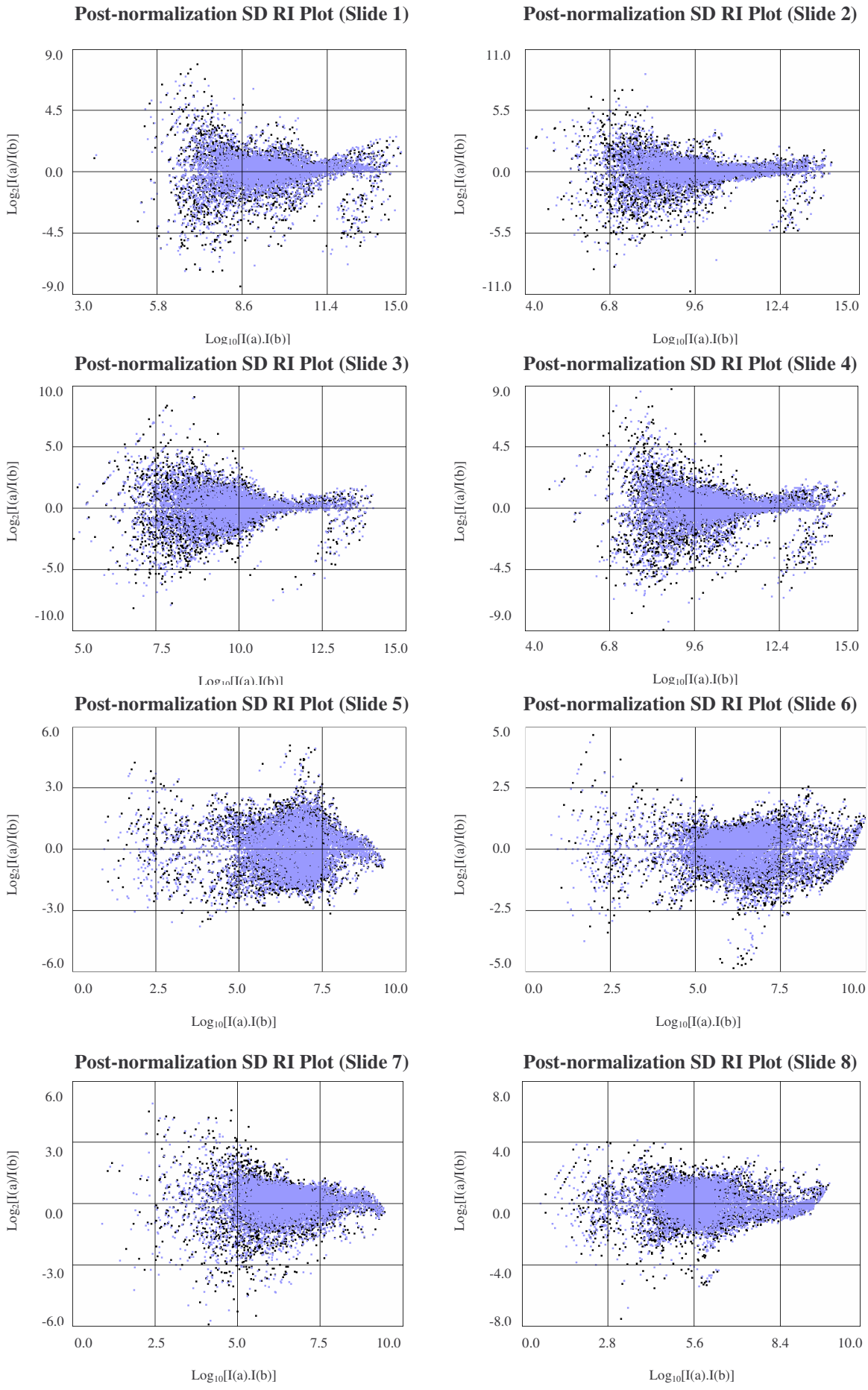


Figure 3.13 Standard deviation (SD) normalization RI plots before SD regularization (Black) and after regularization (Purple).

3.4.3 Flip-Dye Consistency Checking

Flip- dye consistency checking was performed on flip-dye replicates. Log_2 (Cy5/Cy3) values formed a "sphere" centred around zero (Figure 3.14). From Figure 3.14, it may be seen that data points greater than 2 SD from the mean were eliminated using outlier criterion (black, representing poor quality spots in one or both replicates), resulting in a much tighter dataset (light blue). Flip-dye replicate consistency checking therefore reduced variability between flip-dye replicates (Figure 3.14, seen in light blue). Before replicate filtering was applied, a number of outliers was observed (Figure 3.14, in black) which was filtered (Figure 3.14, light blue) resulting in a better correlation between flip-dye replicates. Flip-dye inconsistent values were then adjusted to zero and excluded. The overall consistency between the flip-dye pair was described by the confidence factor (cf) and dispersion factor (df). Cfs for the SD cut-off range of ±2 SD should be very close to 95%, if log_2 values follow a normal distribution. Results indicated that the cf for pair 1 was 0.94 (94%), pair 2 was 0.94 (94%), pair 3 was 0.95 (95%) and pair 4 was 0.96 (96%) indicating that the log_2 values for each flip-dye pair do follow a normal distribution as a value close to 95% was obtained for each flip-dye pair. Dfs are those percentages of spots having log_2 values not between -1 and 1. Df differs to cf in that the distribution of log_2 values is not taken into account. The percentage of spots having more than 2 fold ratio inconsistencies between replicate pairs was therefore calculated. Results obtained a dispersion factor of 0.39 (39%) for pair 1, 0.53 (53%) for pair 2, 0.20 (20%) for pair 3, and 0.47 (47%) for pair 4. Flip-dye replicate pair 2 and 4 resulted in a high df, indicating that close to or including half the spots had a 2-fold ratio inconsistency between replicate pairs. Remaining flip-dye consistent data was then merged as one dataset and used in downstream analysis. From Figure 3.14, it was observed that the output number of spots was 4948 for Slide pair 1, 4351 for Slide pair 2, 6564 for Slide pair 3, and 6689 for Slide pair 4.

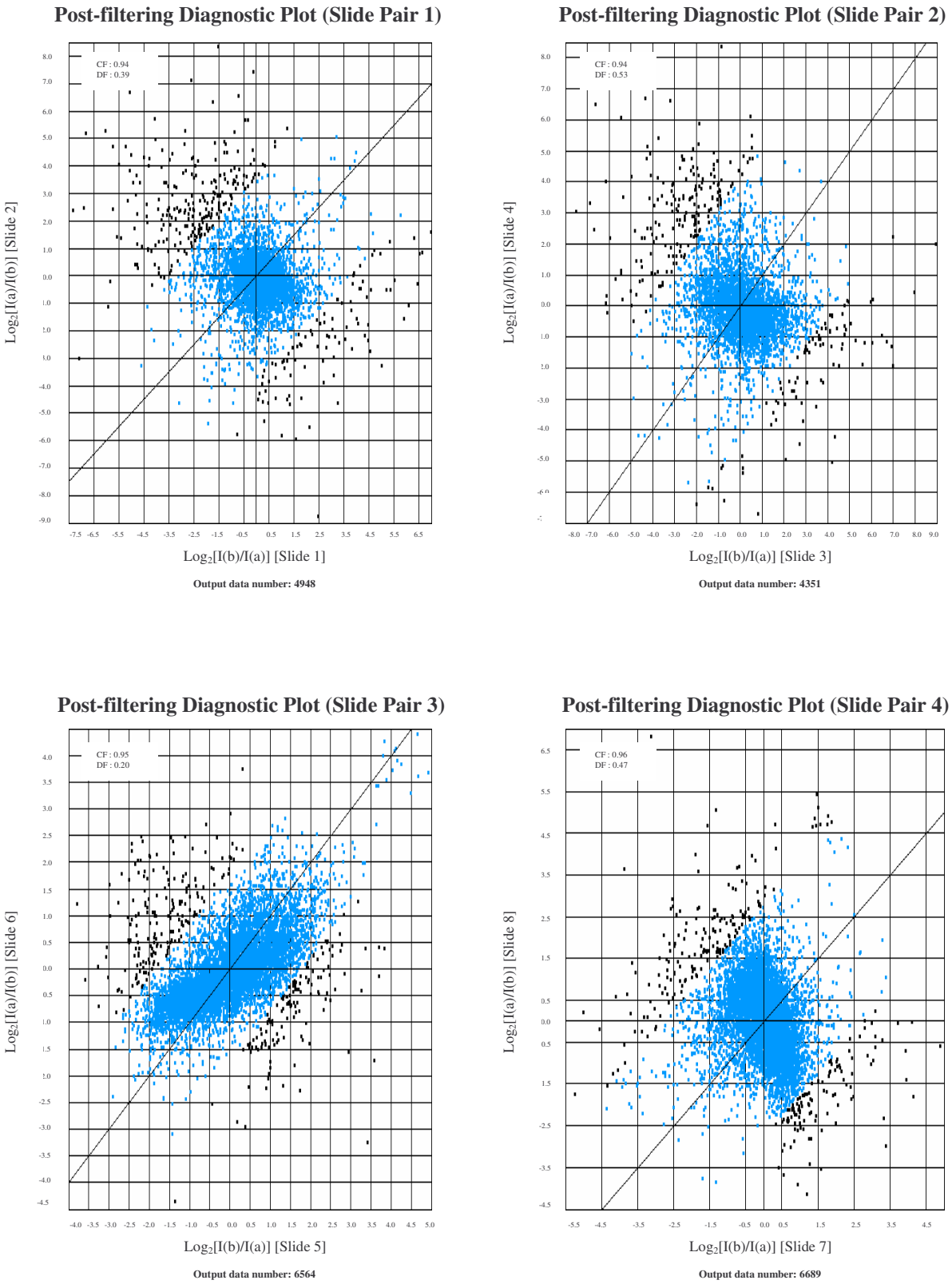


Figure 3.14 Flip-dye consistency checking for I (b)/I (a) ratios or (Cy5/Cy3) ratios. Black indicates data before replicate filtering and blue shows filtered data resulting in a better correlation between the flip-dye pair.

3.4.4 MEV and TAIR

Results from the one-sample *t*-test carried out at the 99% confidence interval (CI) in TIGR MEV (http://www.tigr.org/software), identified 86 differentially expressed genes. Genes were considered differentially expressed if ratios (Cy5/Cy3) deviated from a mean of 0. Hierarchical clustering (HCL) analysis revealed that from the 86 differentially expressed genes, 48 genes were shown to be up-regulated and 38 down-regulated (Figure 3.15). Gene ratios were considered significant if calculated *t* values exceeded the tabulated *t* values with respect to their relative degrees of freedom (df) at $P = 0.01$ (Table 3.2 and 3.3). Significant genes with a mean $\log_2$ value above 0.5 or below -0.5 were therefore selected for further analysis and are displayed in Table 3.3.

Table 3.2 Probability (*P*) of exceeding the critical value (selected values taken from Clarke, 1994).

df	*P* = 0.1	0.05	0.02	0.01	0.002	0.001
1	6.314	12.706	31.821	63.657	318.31	636.62
2	2.920	4.303	6.965	9.925	22.327	31.598
3	2.353	3.182	4.541	5.841	10.214	12.924
4	2.132	2.776	3.747	4.604	7.173	8.610

Table 3.3 Differential up or down-regulated genes in SACMV-infected *Arabidopsis* based on mean log_2 ratios above 0.5 or below 0.5.

Gene Acc No.	Description	*t*-Ratio	df	Log_2 value	Locus Identifier	Description/Putative involvement/Activity
AC011698	putative T-complex protein 1, theta subunit (TCP-1-Theta)	8.40	3	0.5	At3g03960.1	DNA recombination, DNA repair, branched chain family amino acid biosynthesis, glycerol metabolism, cellular protein metabolism
D88377	ATP SYNTHASE EPSILON CHAIN, MITOCHONDRIAL >dbj|BAA13602.1|	12.30	2	0.5	At1g51650.1	ATP synthesis coupled proton transport
D85339	hydroxypyruvate reductase [*Arabidopsis thaliana*]	12.61	3	0.5	At1g68010.1	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
	PROTEASOME ALPHA SUBUNIT (MULTICATALYTIC ENDOPEPTIDASE COMPLEX ALPHA	6.04	3	0.9	At5g35590	Endopeptidase activity, threonine endopeptidase activity
	ELONGATION FACTOR 1-ALPHA (EF-1-ALPHA) >pir||S06724 translation	618.84	1	1.0	At1g07920.1	Amino acid metabolism, protein biosynthesis, translational elongation, selenocysteine incorporation protein biosynthesis, translational elongation, translational termination, selenocysteine incorporation
AC006931	putative MAP kinase [*Arabidopsis thaliana*]	6.62	3	0.6	At2g42880.1, At5g10630.1	MAP kinase activity Catalysis of the phosphorylation of proteins. Mitogen-activated protein kinase; a family of protein kinases that relay signals from the plasma membrane to the nucleus.
X98804	peroxidase ATP18a [*Arabidopsis thaliana*]	6.85	3	0.6	At1g44970.1	Response to oxidative stress, often resulting from exposure to high levels of reactive oxygen species, e.g. superoxide anions, hydrogen peroxide (H2O2), and hydroxyl radicals.
AC002332	unknown protein [*Arabidopsis thaliana*]	6.54	3	0.7	Many	Several putative functions
AC008263	ESTs gb|AA042183 and gb|R86825 come from this gene	8.82	3	0.8	At1g74880.1	Encodes subunit NDH-O of NAD(P)H:plastoquinone dehydrogenase complex (Ndh complex) present in the thylakoid membrane of chloroplasts. This subunit is thought to be required for Ndh complex assembly
AC009177	putative sugar transporter [*Arabidopsis thaliana*]	9.25	3	0.8	At3g05150.1	Carbohydrate transport, inorganic anion transport, ion transport, organic anion transport, phosphate transport, transport, ATP synthesis coupled electron transport
	probable cytochrome b PA4430 [imported] - Pseudomonas aeruginosa	13.15	2	0.7		metabolism
AC006931	En/Spm-like transposon protein [*Arabidopsis thaliana*]	109.86	1	0.8	At2g42840	Encodes a putative extracellular proline-rich protein is exclusively expressed in the L1 layer of vegetative, inflorescence and floral meristems and the protoderm of organ primordia. structural constituent of cell wall
	hypothetical protein A_TM018A10.2 - *Arabidopsis thaliana*	10.52	2	-1.1	At4g00950	Regulation of transcription
AC005169	putative ribosomal protein L28 [*Arabidopsis thaliana*]	7.07	3	-1.0	At2g19730	Protein biosynthesis
AB018107	GDSL-motif lipase/hydrolase-like protein [*Arabidopsis*]	5.86	3	-0.7	At5g37690	Has catalytic activity. Located in endomembrane system.Members of the endomembrane system pass materials through each other or though the use of vesicles
AP001306	emb|CAB10291.1~gene_id:MKA23.7~similar to unknown protein	8.43	3	-0.8	At3g22160.1	VQ motif-containing protein, contains PF05678: VQ motif
U93215	Putative VP1/AB13 family regulatory protein *(A.thaliana)*	7.96	3	-0.8	At2g30470	Protein ubiquitination, regulation of transcription (DNA-dependent)
AC005727	putative membrane channel protein (*Arabidopsis thaliana*]	7.07	3	-0.8	At2g28900	Intracellular protein transport, protein transport
AC007369	Unknown protein *(A.thaliana)*	5.95	3	-0.5	Many	Several putative functions
L34546	CALMODULIN-RELATED PROTEIN 3, TOUCH-INDUCED >gb|AAC37419.1|	94.43	1	-0.6	At2g41100	Encodes a calmodulin-like protein, with six potential calcium binding domains. Calcium binding shown by Ca(2+)-specific shift in electrophoretic mobility. Expression induced by touch and darkness. Expression may also be developmentally controlled.
	hypothetical protein F13G24.110 - *Arabidopsis thaliana*	8.75	3	-0.5	At5g07910.1	Leucine-rich repeat family protein, contains leucine rich repeat (LRR) domains, Pfam:PF00560
	aspartate--tRNA ligase homolog F6E21.100 - *Arabidopsis thaliana*	9.29	3	-0.7	At4g31180.1	tRNA aminoacylation for protein translation, asparaginyl-tRNA aminoacylation, aspartyl-tRNA aminoacylation, lysyl-tRNA aminoacylation
	ketoconazole resistance protein - *Arabidopsis thaliana*	9.09	3	-0.6	At5g66040	Senescence-associated family protein, almost identical to ketoconazole resistant protein GI:928938 from (Arabidopsis thaliana) full-length cDNA: Ceres:101608
	probable DNA-binding protein T27E13.1 - *Arabidopsis thaliana*	8.01	3	-0.5	Many	Several putative functions
AC007197	dynamin-like protein [*Arabidopsis thaliana*]	6.16	3	-0.5	At2g14120	GTP binding, GTPase activity

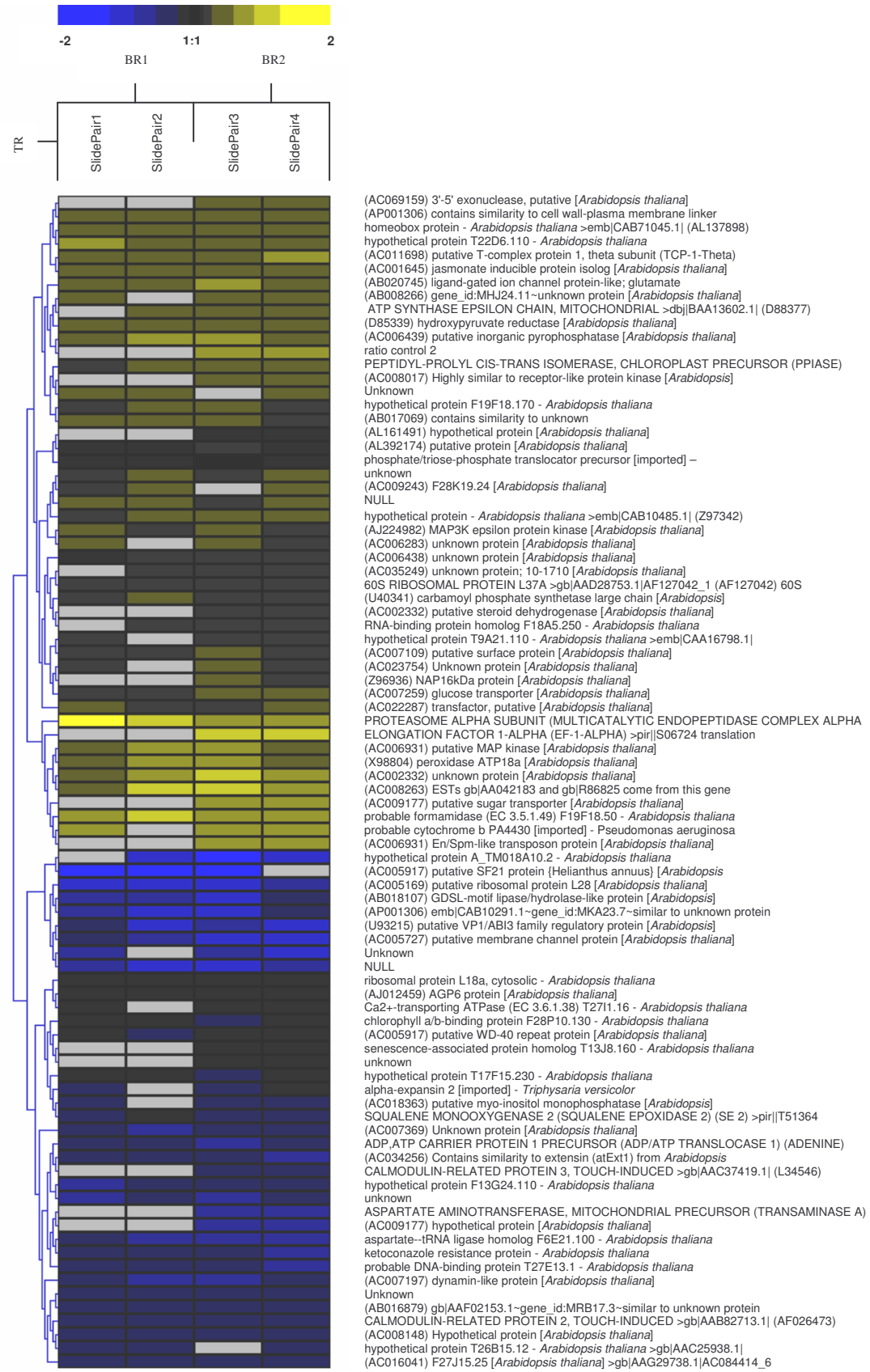


Figure 3.15 Hierarchical Clustering of 86 differentially expressed SACMV-infected *Arabidopsis* genes. Yellow blocks represent up-regulation and blue blocks down-regulation. Gray blocks indicate missing data and black blocks show no differential gene expression. Each column represents a different hybridization experiment and each row represents a specific gene. BR=Biological Replicate. TR=Technical Replicate.

3.5 Real-time RT-PCR

Five differentially expressed genes (including the reference, AGP6 protein [AJ012459]) from biological replicate 2 were randomly selected from the 86 differentially expressed genes (Figure 3.15) for relative quantification real-time RT-PCR. Expression ratios were determined from crossing points from real-time RT-PCR amplification plots of target versus reference genes (Figure 3.16 A). Microarray and real-time RT-PCR were in agreement in terms of up- and down-regulated genes (three out of four), except for the putative WD-40 repeat protein (AC005917) (Table 3.4). Therefore a 75% confirmation of these results was obtained. Specificity of real-time RT-PCR products was determined by gel electrophoresis. Results indicated that a single product was obtained with the desired amplicon length for each gene (Figure 3.16 B). Primer and probe sequences used for relative quantification real-time RT-PCR are depicted in Table 2.1.

Table 3.4 A comparison between ratios (from biological replicate 2) obtained from real-time RT-PCR and microarray analysis

Accession No.	**Gene Name**	**Amplicon length (bp)**	**Real-time RT-PCR Expression ratios**	**Relative Expression (Up-or down-regulated)**	**Microarray Expression ratios**	**Relative Expression (Up-or down-regulated)**
	Reference					
AJ012459	AGP6 protein	197	1.00*		0.92*	
	Target					
X98804	peroxidase ATP18a	150	1.10	Up	1.63	Up
U40341	carbamoyl phosphate synthetase large chain	181	1.43	Up	1.13	Up
AC005917	putative WD-40 repeat protein	160	1.45	Up	0.86	Down
AC007197	dynamin-like protein	183	0.78	Down	0.72	Down

* Real-time RT-PCR and microarray expression values relative to the reference gene (1.00/0.93). Values above the reference gene represent up-regulation and values below the reference gene, down-regulation.

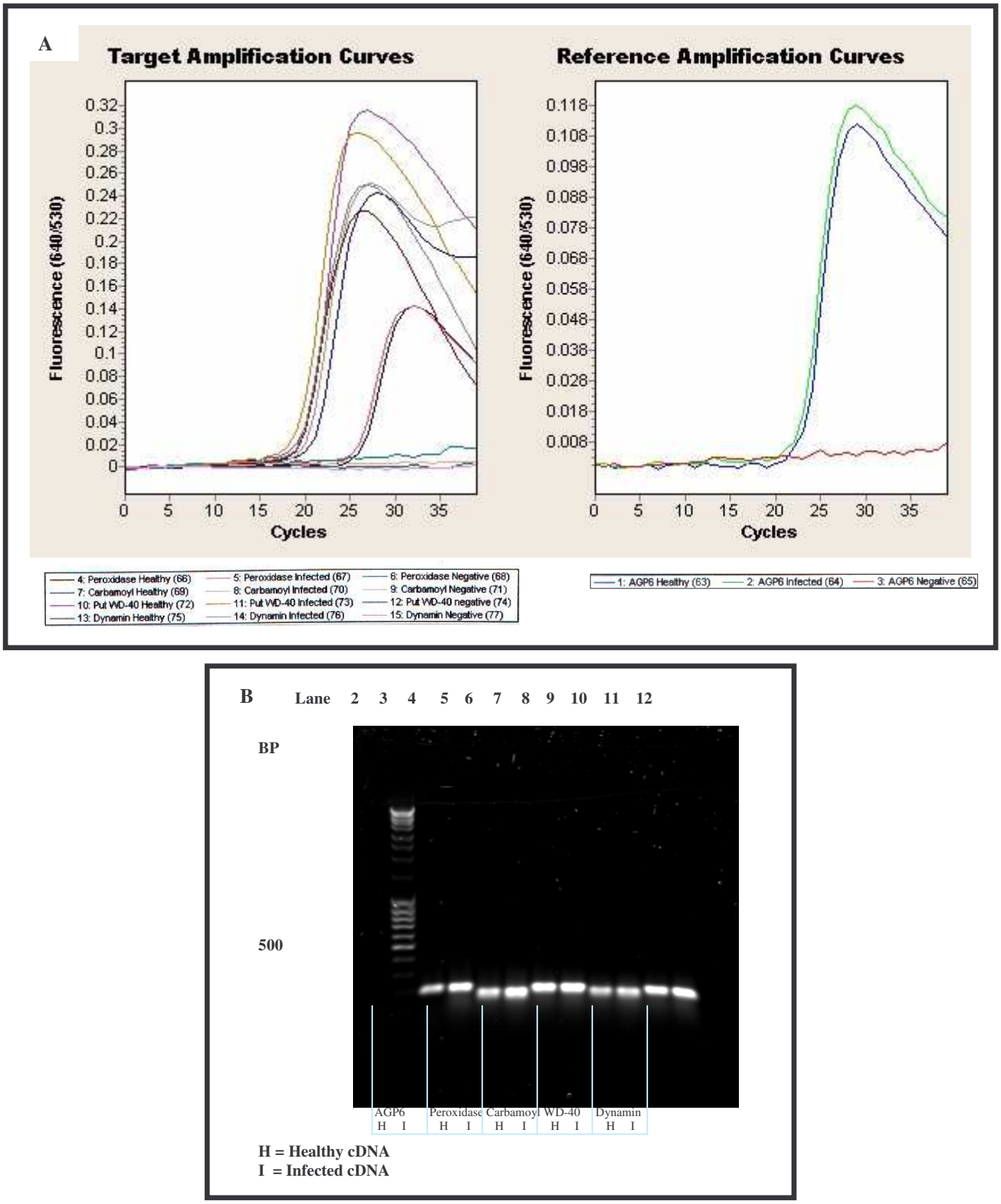


Figure 3.16 Relative quantification real-time RT-PCR showing: (A) an increase in fluorescence with PCR cycle number in amplification plots of both healthy and infected cDNA. The four target genes are represented on the left plot and the reference gene on the right plot; (B) Single products separated on a 1% agarose gel revealing expected fragment sizes. Selected base pair size of MassRuler™ DNA Ladder (Fermentas Life Sciences) is indicated on the left.

CHAPTER 4

DISCUSSION

This study was conducted to investigate the effects of SACMV infection in fully susceptible *Arabidopsis* at 35 dpi. Gene expression studies were conducted using cDNA microarrays to determine both direct and indirect effects of virus replication in fully susceptible *Arabidopsis* plants. An infectivity study was carried out to monitor disease symptoms as well as virus progression in infected plants.

Infectivity Study

Arabidopsis plants were observed to be fully symptomatic 35 dpi, although symptoms started appearing at approximately 28 dpi. Symptoms such as stunting of the entire plant, leaf reduction and deformation were observed in infected *Arabidopsis* from both biological replicates (Figure 3.2 A,C,D and Figure 3.3 B,D,F). The infectivity and host range of SACMV was previously assessed using *Agrobacterium*-mediated inoculation of *Phaseolus vulgaris, Malva parviflora,* and cassava (*Manihot esculenta* Crantz) with SACMV; symptoms of leaf curl, chlorosis and stunting were observed (Berrie *et al*., 2001). These findings correlate with symptoms observed in this study, indicating for the first time that *Arabidopsis* is a susceptible host for SACMV infection. A previous study conducted with the geminivirus, *Beet curly top virus* (BCTV), infecting *Arabidopsis* showed that different strains (Logan and California) of BCTV differ in their pathogenicity characteristics on susceptible hosts. *Arabidopsis* plants infected with BCTV-Logan were clearly symptomatic three- to- four weeks post-inoculation with symptoms such as leaf curling and deformation, development of stunted and deformed inflorescence structures, and the accumulation of anthocyanin pigments (Lee *et al.*, 1994). The occurrence of chlorotic symptoms can be viewed as the direct result of the plants attempt to rescue resources from infected tissues via basal resistance mechanisms. It has been suggested that if chlorosis is absent in infected tissues, a loss of basal resistance has occurred (O´Donnell *et al.*, 2003). In our study, chlorosis was only observed in four of the thirty infected *Arabidopsis* plants at 35 dpi. A high level of viral replication was noted in infected parts of the leaf tissues, as observed from quantitative real-time PCR, showing an approximated 3-fold increase in virus titer from 13 dpi to 35 dpi. This suggests that the plant is able to no longer rescue resources from surrounding tissues, as more metabolites are diverted toward SACMV replication. A study conducted by

Nagar *et al.* 2002, showed that a high level of the geminivirus, TGMV, replication correlated with symptom formation in *N. benthamiana*, indicating that a strong association between active viral DNA accumulation and symptom development exists. Similarly, in a gene expression study conducted by Golem and Culver 2003, of *Tobacco mosaic virus* (TMV) in *Arabidopsis* Shahdara at both 4 dpi and 14 dpi, a greater fold change in TMV-responsive genes was observed at the later time point, suggesting that higher levels of TMV were present at 14 dpi.

Controlling systematic variation in microarrays

Two biological and four technical replicates (within each biological replicate) were carried out to reduce inherent biological inconsistencies between plants so that variation in gene expression for statistics calculation was a true reflection of SACMV infection and not just natural plant variation. Biological replicates (independent biological sources) measure the natural biological variability and account for any random variation used in sample preparation. Furthermore, evaluation of RNA integrity (Figures 3.7A and 3.8A), purity (Table 3.1), and ensuring that no contaminating plant DNA was present in the RNA samples (Figures 3.7B and 3.8B) were critical to achieve optimal hybridization efficiency. Technical replicates (pooled RNA samples) account for the natural and systematic variability carried out during experimental procedures (Quackenbush, 2002). Stringent criteria were applied to all steps of data analysis. Normalization was applied to all intensity data sets as microarray experiments are normally subjected to a variety of random and systematic errors. These errors may arise from various sources such as dye labeling efficiencies, heat and light sensitivities, as well as scanner settings (Leung and Cavalieri, 2003). In particular, locally weighted linear regression (LOWESS) was the normalization method of choice for microarray assays as it removes intensity-dependent dye-specific effects in $\log_2$ ratios (Yang *et al.*, 2002). In this study, LOWESS corrected all deviations from the expected behaviour for each data point in the R-I plot by applying a local weighted linear regression by subtracting the average ratio from the experimentally observed ratio. A balanced distribution of expression ratios around zero was produced therefore reducing the dye-specific artifacts most commonly occurring at low-intensity data points.

Standard deviation (SD) regularization normalization was applied to scale the data points so that extreme log ratios became evenly distributed. This algorithm corrects for systematic variation in the array, including slight local differences in hybridization conditions across the array, slide surface variability, and

inconsistencies among the spotting pens applied (Yang *et al.,* 2002). Flip dye consistency checking filtered out genes whose mean and SD for each replica pair was greater or less than two from the mean (Yang *et al.,* 2002). Good quality spots in each replicate pair were combined and used for downstream analysis.

Validation of microarray results with real-time RT-PCR

In order to confirm microarray results, an independent experiment was required to validate the results obtained from microarray analyses. Real-time RT-PCR was chosen as an alternate quantitative method as it is an extremely sensitive method for detecting and quantifying gene expression levels. It is also particular suitable for quantifying low abundance mRNA and is also able to elucidate small changes in mRNA expression levels (Pfaffl *et al.*, 2002). This method was particularly suitable for this study as the five genes (including the reference gene) selected from microarray analyses had low expression levels (Table 3.4). Microarray and real-time RT-PCR were in excellent agreement in terms of up- and down-regulated genes (four out of five), except for the putative WD-40 gene. Therefore an 75% confirmation in these results was obtained. The discrepancy between the putative WD-40 gene in real-time RT-PCR (1.45) and microarray results (0.86) could be due to an error occurring in the microarray result. Allen *et al.* 2000, suggested that if redundant copies of the sequence on the microarray slide are present, then the fluorescent signals corresponding to a specific mRNA target could be titrated out. This could explain why the putative WD-40 gene was showing a down-regulated value in microarray results as opposed to up-regulated in real-time RT-PCR. It doesn't seem feasible that the error would lie in real-time RT-PCR as both the primers and probes were specifically designed for each gene. It does not however, rule out this possibility as there is never a 100% efficiency of RNA that is reverse transcribed into cDNA. According to Freeman *et al.* 1999, most of the variability existing in real-time RT-PCR occurs during the RT step, implying that the signal output may be variable in different reactions. However, due to the increase in transcripts in real-time RT-PCR as opposed to the decrease in transcripts in microarray data, it is likely that the error lies in the microarray result. If there was an efficiency problem in the real-time RT-PCR result, then the ratio would be lower than the microarray value.

Identification of differentially expressed SACMV-responsive *Arabidopsis* genes

A one-sample *t*- test was performed at the 99% confidence interval to identify differentially expressed genes by comparing the means of healthy versus infected *Arabidopsis* leaf tissues. Of the 86 differentially expressed genes, 48 were shown to be up-regulated and 38 down-regulated (Figure 3.15). Gene groups were hierarchically clustered by related regulation patterns and expression amplitudes. In this study, genes with $\log_2$ ratios of above 0.5 or below -0.5 were selected for functional analysis (Table 3.3). A general overview of selected genes such as the up-regulated ATP synthase epsilon chain (D88377), up-regulated putative MAP kinase (AC006913), down-regulated putative membrane channel protein (AC005727), and down-regulated probable DNA binding protein (T27E13.1) in Table 3.3 suggests that a variety of metabolic processes in the *Arabidopsis* genome have been affected. According to Escaler *et al*. 2000, this general induction and suppression of genes implies that viruses may induce common stress responses in order to produce cellular responses sited for replication. Viruses may also suppress host gene expression to avoid direct competition for host cellular factors to enable efficient viral gene expression and replication.

Analysis of up-regulated genes

The majority of up-regulated genes in SACMV-infected *Arabidopsis* apppeared to be involved in a general stress response of the host (Table 3.3). Genes such as the putative MAP kinase (AC006931) showed up-regulation with a $\log_2$ value of 0.6. MAP kinases (mitogen-activated protein kinases) have been implicated in both biotic and abiotic stress responses in plants. Abiotic stresses include environmental stresses such as dehydration, salinity, chilling, and wounding. Biotic stresses on the other hand, are caused by pathogen infection. Cumulatively, these factors affect the growth and metabolism of the plant (Yuasa *et al.*, 2001). MAP kinases have therefore been implicated as mediators in response to abiotic or biotic sources for reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, which are associated with a number of physiological disorders in plants (Yuasa *et al.*, 2001).

Likewise, in this study, another gene that was up-regulated, which is also associated with ROS, was peroxidase ATP18a (X98804) (Table 3.3). Peroxidase is associated with the oxidative burst that is usually

induced within minutes of contact between a host and pathogen. ROIs act together with a number of other signalling molecules which are important for activating defense in adjacent cells as well as in the whole plant (Wan *et al.*, 2002). In this study, a continuous up-regulation of peroxidase up to 35 dpi, may suggest an attempt by the host plant, *Arabidopsis,* to mount some form of delayed basal defense response, but this is insufficient or ineffective to prevent SACMV replication and disease development. Previous studies have suggested that changes in gene expression, ROS production as well as cell wall composition also occurs in susceptible responses, but these responses are delayed, reiterating that effective resistance is dependent on the speed with which these responses are activated (O´Donnell *et al.*, 2003).

A very interesting finding in this study was the up-regulated Elongation Factor 1-Alpha (EF-1-Alpha) gene. This gene was found to have the highest $\log_2$ value of 1.0. In previous studies, EF-1-Alpha, along with beta tubulin and importin, has been implicated in intra- and inter-cellular virus trafficking. Beta tubulin (the building blocks of microtubules) has been implicated in cell-to-cell spread and cytoplasm-to-nucleus movement of viruses (Fregene *et al.*, 2004). This is an important association since geminivirus DNA must move into the nucleus for replication to take place. EF-1-Alpha has been shown to deliver aminoacyl-tRNA to ribosomes and is therefore an essential part of the translation machinery (Fregene *et al.*, 2004). It was also previously shown that the 3'-terminal stem-loop of West Nile virus binds to EF-1-Alpha, incorporating it into the viral replication machinery (Cimarelli and Luban, 1999), and we can therefore speculate that the up-regulation of this gene by SACMV may be acting in a similar manner as in the case of West Nile virus. The faster SACMV is able to replicate, the more host cells become infected, leaving *Arabidopsis* with less time to mount appropriate defense responses. Effective resistance is dependent on the speed in which induced defense reactions occurs in susceptible (compatible) and resistant (incompatible) interactions (O´Donnell *et al.*, 2003). Alternatively, if EF-1-Alpha is not incorporated into the viral replication machinery it may still assist in viral translation, enabling viral replication in the host cell. EF-1-Alpha has recently been identified in cassava, the natural host for SACMV, using SAGE analysis, in both resistant (TME3) and susceptible (TMS30555) cassava cultivars (Fregene *et al.*, 2004). This finding may have important implications for future studies in SACMV-cassava interactions as this gene was found to be the most up-regulated gene in this study due to viral infection, and has also been implicated as an essential part of the host translation machinery (Fregene *et al.*, 2004).

Down-regulated gene analysis

GDSL-motif lipase/hydrolase-like protein (AB018107) was shown to be down-regulated with a $\log_2$ value of -0.7. It has been assigned to be putativley involved in protein ubiquitination, DNA repair, and zinc ion binding. Particular attention was drawn to this protein's involvement in protein ubiquitination. This process is responsible for controlling protein abundance in the plant. Degradation of proteins thus occurs via the ubiquitin 26S proteasome pathway, which is essential for entry into the S phase, sister chromatid separation, and exit from mitosis (Skowyra *et al*., 1997). The ubiqitin (Ub)/26S proteasome pathway is the main proteolytic pathway in eukaryotes. Ub serves to bind specific protein targets via an ATP-dependent reaction cascade, where the 26S proteasome recognizes this Ub-protein pair, releasing Ub for rescue in the cell. This cycle therefore removes abnormal proteins by performing a housekeeping function. We therefore hypothesize that SACMV may be exerting a suppressive response to prevent its own coat protein degradation. In addition we can speculate that by ubiquitin suppression, entry into the S phase can take place where the virus can actively replicate. Previous studies have shown that *Tomato golden mosaic virus*, along with inducing the expression and accumulation of proliferating cell nuclear antigen (PCNA; processivity factor for DNA polymerase delta), also interferes with host cell cycle regulation (Hanley-Bowdoin *et al.*, 2004). This occurs through interaction with the retinoblastoma-related protein (pRBR) which shifts mature cells to the S phase in the cell cycle, creating a more favourable cellular environment for viral DNA synthesis (Egelkrout *et al.*, 2002).

The protein F13G24.110 (Table 3.3) was found to be down-regulated by SACMV. This protein has been identified as showing similarity to the leucine-rich repeat (LRR) family protein (At5g07910.1). LRR proteins have been broadly identified in pathogen recognition with regards to intercellular/intracellular receptors and disease resistance (Bent *et al.*, 1994). The suppression of this LRR-related protein could suggest a putative strategy of SACMV to avoid induction of a defense response in *Arabidopsis.* Host repression during virus replication has also been observed in studies conducted with TMV in *Arabidopsis* ecotype Shahdara (Golem and Culver, 2003), as well as with other RNA viruses such as *Pea seed-borne mosaic virus* (PSbMV) (Wang and Maule, 1995; Aranda *et al.*, 1996) and *Cucumber mosaic virus* (CMV) (Havelda and Maule, 2000). In the TMV study, general gene repression was more evident in systemic tissues at 14 dpi, compared to 4 dpi, while an increase in the amount of differentially expressed genes was found at the 14 dpi time point. TMV responsive genes included those involved in transcription, metabolism, signal transduction and stress, indicating that a range of host functions becomes affected

(Golem and Culver, 2003). This correlates with our study in that SACMV responsive genes were involved primarily in metabolism, transcription, and transport. The recorded changes by SACMV in host gene expression in fully symptomatic systemically infected *Arabidopsis* leaves at 35 dpi correlate to highly active SACMV replication, reflected by a significant increase in virus titer.

Other genes of interest shown to be down-regulated included a putative membrane channel protein, and a touch-related Calmodulin protein 3 (>gb|AAC37419.1|). These proteins have been implicated in plant defense, in which an increase in ion fluxes has been observed across plasma membranes upon pathogen recognition, activating downstream defense responses (Wan *et al.*, 2002). In this study, Calmodulin down-regulation could be a result of a decrease in host mRNA transcripts due to induced degradation by SACMV. In studies conducted by Aranda *et al.* 1996, and Escaler *et al.* 2000b, *heat* shock protein 70 (HSP70) and polyubiquitin genes were shown to be upregulated whereas lipoxygenase and heat shock cognate proteins were down-regulated. It is therefore suggested from these studies that degradation of mRNAs may occur as part of a general host suppression strategy by plant viruses. It was also noted that the down-regulated Ketoconazole Resistance protein (associated with senescence protein family) in SACMV-infected *Arabidopsis* at 35 dpi was down-regulated. It is possible that SACMV might be exerting a suppressive effect on these resistance proteins through mRNA degradation strategies in order to ensure host support in replication. Host plant tissue senescence would not favour virus propagation. At 35 dpi, SACMV-infected *Arabidopsis* showed distinct stunting (and chlorosis in a few selected plants), but no evidence of leaf senescence was observed (Figure 3.3B). According to Maule *et al.*, 2002, in order to place virus gene expression at an advantage to host gene expression in a compatible host, a selective host-gene shut-off strategy must be employed to enable virus replication.

Summary

The interaction between fully susceptible *Arabidopsis* ecotype Col-0 and SACMV was examined by identifying plant genes that were differentially expressed through SACMV infection. An overall view of this study suggests that similarities and differences exist among virus-host interactions due to the tissue specificity of the virus, speed of infection as well as the timing of infection. This current study therefore provided insight into some of the genes that were up- or down-regulated as a result of a general host or a more viral specific host response due to SACMV infection in fully symptomatic *Arabidopsis* plants (at 35

dpi). Future studies will entail using time points to determine: if transient or long-lasting host gene expression will result, monitor possible increases or decreases in gene expression between the different time points, and to monitor gene expression patterns in a variety of host tissues. To our knowledge, this is the first microarray study involving a DNA geminivirus interaction in a compatible host. These findings therefore contribute to the limited knowledge of signalling mechanisms underlying plant-pathogen interactions leading to disease development or resistance**.** Gene identification and function in signalling pathways will eventually lead towards the engineering of tolerant plants. Tolerant plant varieties are able to produce high plant yields even when infected with pathogens.

Future gene expression studies in cassava, the natural host for SACMV using microarrays

Sequence conservation or similarities has been shown to exist among plant families. The next step involved in this area of research will entail utilizing SACMV-infected cassava cDNAs hybridized to *Arabidopsis* cDNA microarray chips for homology detection. Possible identification and function of genes may show similarities with genes already analyzed from this current study, providing underlying mechanisms that may contribute to breeding resistant or tolerant cassava lines.

To date, only one dominant resistance gene has been isolated from cassava. The next objective of this study will therefore entail suppressive subtractive hybridization (SSH) of a resistant and a susceptible cassava line. Enriched cDNA fragments will then be cloned into a vector to create a cDNA library containing only resistant or defense-related genes. These cDNAs will then be spotted onto an array providing a platform where resistant cassava lines may be hybridized to the array, allowing possible identification of resistance genes. Isolation of novel resistance genes will eventually lead to the underlying mechanisms controlling genes in resistance pathways that naturally occur in crops. Functions may therefore be assigned to novel resistance genes, which may eventually be used in cassava plant breeding systems or in genetically modified cassava for crop improvement.

CHAPTER 5

REFERENCES

Abramovitch, R.B. and Martin, G.B. (2004) Strategies used by bacterial pathogens to suppress plant defenses. *Current Opinion in Plant Biology* 7: 356-364.

Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., Moreno, R.F., Kerlavage, A.R., McCombie, W.R. and Venter, J.C. (1991) Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* 252: 1651-1656.

Aharoni, A. and Vorst, O. (2001) DNA microarrays for functional plant genomics. *Plant Molecular Biology* 48: 99-118.

Alleman, J. and Coertze, A.F. (1996) The Cultivation of Cassava. *Vegetable and Ornamental Plant Institute, Pretoria, 1-5.*

Allen, T.D., Dawe, A.L. and Nuss, D.L. (2002) Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypoviruses. *Eukaryotic Cell* 2(6): 1253-1265.

Anderson, J.V., Delseny, M., Fregene, M.A., Jorge, V., Mba, C., Lopez, C., Restrepo, S., Soto, M., Piegu, B., Verdier, V., Cooke, R., Tohme, J. and Horvath, D.P. (2004) An EST resource for cassava and other species of Euphorbiaceae. *Plant Molecular Biology* 56: 527-539.

Aranda, M.A., Escaler, M., Wang, D., and Maule, A.J. 1996. Induction of HSP70 and polyubiquitin expression associated with plant virus replication. *Proceedings of the National Academy of Sciences* 93:15289-15293.

Arguella-Astorga, G., Lopez-Ochoa, L., Kong, L., Orozco, B.M., Settlage, S.B. and Hanley-Bowdoin, L. (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma-related protein. *Journal of Virology* 4817-4826.

Bent, A.F., Kunkel, B.N.,Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J., Staskawicz, B.J. 1994. RPS2 of *Arabidopsis thaliana:* a leucine-rich repeat class of plant disease resistance genes. *Science* 265:1856-1860.

Bent, A.F. (1996) Plant Disease Resistance Genes: Function Meets Structure. *The Plant Cell* 8:1757-1771.

Berrie, L.C., Palmer, K.E., Rybicki, E.P. and Rey, M.E.C. (1998) Molecular characterization of a distinct South African cassava infecting geminivirus. *Archives of Virology* 143:2253-2260.

Berrie LC, Rybicki EP, and Rey MEC (2001) Complete nucleotide sequence and host range of *South African cassava mosaic virus:* Further evidence of recombination amongst begomoviruses. *Journal of General Virology* 82: 53-58.

Berry, S. and Rey, M.E.C. (2001) Molecular evidence for diverse populations of cassava-infecting begomoviruses in southern Africa. *Archives of Virology* 146:1795-1802.

Bock, K.R., Guthrie, E.J. and Meredith, G.C. (1978) Distribution, host range, properties and purification of cassava latent virus, a geminivirus. *Annals of Applied Biology* 90: 361-367.

Bock, K.R. and Woods, R.D. (1983) Etiology of African cassava mosaic disease. *Plant Disease* 67: 944-955.

Briddon, R.W., Watts, J., Markham, P.G. and Stanley, J. (1989) The coat protein of *Beet curly top virus* is essential for infectivity. *Virology* 172:628-633.

Brown, P.O. and Botstein, D. (1999) Exploring the new world of the genome with DNA microarrays. *Nature Genetics (supplement)* 21: 33-37.

Carrington, J.C. and Whitham, S.A. (1998) Viral invasion and host defense: strategies and counter-strategies. *Current Opinion in Plant Biology* 1: 336-341.

Cecchini, E., Al-Kaff, N.S., Bannister, A., Giannakou, M.E., McCallum, D.G., Maule, A.J., Milner, J.J. and Covey, S.M. (1998) Pathogenic interactions between variants of cauliflower mosaic virus and *Arabidopsis thaliana. Journal of Experimental Botany* 49(321): 731-737.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 162: 156-159.

$CIAT_a$ (Centro Internacional de Agricultura Tropical). (2001) Cassava: A Crop for Hard Times and Modern Times. www.ciat.cgiar.org/ciatinfocus/pdf/cassavafocus.pdf.

Cimarelli, A., and Luban, J. 1999. Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. *Journal of General Virology* (73)7:5388-5401.

Clarke, G.M. (1994) *Statistics and experimental design. An introduction for biologists and biochemists.* John Wiley and Sons Inc, New York.

Cooley, M.B., Pathirana, S.,Wu, H-J., Kachroo, P. and Klessig, D.F. (2000) Members of the *Arabidopsis HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. *The Plant Cell* 12: 663-676.

Dahniya, M.T. (1994) An Overview of Cassava in Africa. *African Crop Science Journal* 2(4): 337-343.

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

de la Riva, G., González-Cabera, J., Vázquez-Pardrón, R. and Ayra-Pardo, C. (1998) *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electronic Journal of Biotechnology* 1(3):118-133.

Dong, X. (1998) SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology* 1: 316-323.

Donson, J., Fang, Y., Espiritu-Santo, G., Xing, W., Salazar, A., Miyamoto, S., Armendarez, V. and Volkmuth, W. (2002) Comprehensive gene expression analysis by transcript profiling. *Plant Molecular Biology* 48: 75-79.

Doyle, J.J. and Doyle, J.L. (1987) A rapid isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19:11-15.

Eckardt, N.A. (2004) Host proteins guide *Agrobacterium*-mediated plant transformation. *The Plant Cell* 16: 2837-2839.

Egelkrout, E.M., Mariconti, L., Settlage, S.B., Cella, R., Robertson, D. and Hanley-Bowdoin, L. (2002) Two E2f elements regulate the proliferating cell nuclear antigen promoter differently during leaf development. *The Plant Cell* 14:3225-3236.

El-Sharkawy, M.A. (2004) Cassava biology and physiology. *Plant Molecular Biology* 56: 481-501.

Escaler, M., Aranda, M.A., Thomas, C.L. and Maule, A.J. (2000a) Pea embryonic tissues show common responses to the replication of a wide range of viruses. *Virology* 267: 318-325.

Escaler, M., Aranda, M.A., Roberts, I.M., Thomas, C.L., and Maule, A.J. (2000b) Acomparison between virus replication and abiotic stress (heat) as modifiers of host gene expression in pea. *Molecular Plant Pathology* 1(3):159-167.

Fauquet, C.M., Bisaro, D.M., Briddon, R.W., Brown, J.K., Harrison, B.D., Rybicki, E.P., Stenger, D.C. and Stanley, J. (2003) Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, an updated list of begomovirus species. *Archives of Virology* 148: 405-421.

Feys, B.J. and Parker, J.E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends in Genetics* 16(10): 449-455.

Feys, B.J., Moisan, L.J., Newman, M. and Parker, J.E. (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and Pad4. *The EMBO Journal* 20(19): 5400-5411.

Fondong, V.N., Pita, J.S., Rey, M.E.C., de Kochko, A., Beachy, R.N. and Fauquet, C.M. (2000) Evidence of synergism between *African cassava mosaic virus* and a new double recombinant geminivirus infecting cassava in Cameroon. *Journal of General Virology* 81: 287-297.

Freeman, W.M., Walker, S.J. and Vrana, K.E. (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 26: 112-125.

Fregene, M., Matsumura, H., Akano, A., Dixon, A. and Terauchi, R. (2004) Serial analysis of gene expression (SAGE) of host-plant resistance to the cassava mosaic disease (CMD). *Plant Molecular Biology* 56: 563-571

Gafni, Y. and Epel, B.L. (2002) The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. *Physiological and Molecular Plant Pathology* 60:231-241.

Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and Molecular Biology Reviews* 67(1): 16-37.

Geri, C., Cecchini, E., Giannakou, M.E., Covey, S.N. and Milner, J.J. (1999) Altered patterns of gene expression elicited by cauliflower mosaic virus (CaMV) infection and by a CaMV gene VI transgene. *Molecular Plant-Microbe Interactions* 12(5): 377-384.

Geri, C., Love, A.J., Cecchini, E., Barrett, S.J., Laird, J., Covey, S.M. and Milner, J.J. (2004) *Arabidopsis* mutants that suppress the phenotype induced by transgene-mediated expression of cauliflower mosaic virus (CaMV) gene VI are less susceptible to CaMV-infection and show reduced ethylene sensitivity. *Plant Molecular Biology* 56: 111-124.

Glazebrook, J. (2001) Genes controlling expression of defense responses in *Arabidopsis* – 2001 status. *Current Opinion in Plant Biology* 4: 301-308.

Golem, S. and Culver, J.N. (2003) *Tobacco mosaic virus* induced alterations in the gene expression profile of *Arabidopsis thaliana. Molecular Plant-Microbe Interactions* 16(8): 681-688.

Grimsley, N., Hohn, B., Hohn, T. and Walden, R. (1986) "Agroinfection," an alternative route for viral infection of plants by using the Ti plasmid. *Proceedings of the National Academy of Sciences* 83:3282-3286.

Gutierrez, C. (1999) Geminivirus DNA replication. *Cellular and Molecular Life Sciences* 56: 313-329.

Gutierrez, C. (2000) DNA replication and cell cycle in plants: learning from geminiviruses. *The EMBO Journal* 19(5): 792-799.

Gutierrez, C. (2002) Strategies for geminivirus DNA replication and cell cycle interference. *Physiological and Molecular Plant Pathology* 60: 219-230.

Gutierrez, C., Ramirez-Parra, E., Castellano, M.M., Sanz-Burgos, A.P., Luque, A. and Missich, R. (2004) Geminivirus DNA replication and cell cycle interactions. *Veterinary Microbiology* 98: 111-119.

Hammond-Kosack KE, and Jones JDG (1996) Resistance Gene-Dependent Plant Defense Responses. *The Plant Cell* 8:1773-1791.

Hanley-Bowdoin, L., Settlage, S.B., and Robertson, D. (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. *Molecular Plant Pathology* 5(2):149-156.

Harrison, B.D., Zhou, X., Otim-Nape, G.W., Liu, Y. and Robinson, D.J. (1997) Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. *Annals of Applied Biology* 131: 2437-2443.

Harrison, B.D. and Robinson, D.J. (2002) Green shoots of geminivirology. *Physiological and Molecular Plant Pathology* 60: 215-218.

Havelda, Z. and Maule, A.J. (2000) Complex spatial responses to Cucumber mosaic virus infection in susceptible *Cucurbita pepo* Cotyledons. *The Plant Cell* 12: 1975-1985.

Hayes, R.J., Coutts, H.A. and Buck, K.W. (1988) Agro-infection of *Nicotiana* spp. with cloned DNA of *Tomato golden mosaic virus. Journal of General Virology* 69:1487-1496.

Hillocks, R.J. and Thresh, J.M. (2001) Cassava Mosaic and Cassava Brown Streak Virus Diseases in Africa: A comparative guide to symptoms and aetiologies. *Roots* 7(1): 36-50.

Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Earl-Hughes, J., Snesrud, E., Lee, N. and Quackenbush, J. (2000) A concide guide to cDNA microarray analysis. *Biotechniques* 29(3): 548-562.

Hong, Y.G., Robinson, D.J. and Harrison, B.D. (1993) Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted geminiviruses in cassava. *Journal of General Virology* 74: 2437-2443.

Horvath, D.P., Schaffer, R., West, M. and Wisman, E. (2003) *Arabidopsis* microarrays identify conserved and differentially expressed genes involved in shoot growth and development from distantly related plant species. *The Plant Journal* 34: 125-134.

International Institute of Tropical Agriculture (IITA) (2001) Cassava Mosaic Disease pandemic mitigation in East Africa. Third Quarterly Technical Report. http://www.tropicalwhiteflyipmproject.cgiar.org/docs/ofdareport12.pdf.

Jacob, S.S., Vanitharani, A., Karthikeyan, S., Chincore, Y., Thillaichidambaram, P. and Veluthambi, K. (2003) *Mungbean yellow mosaic virus*-Vi agroinfection by codelivery of DNA A and DNA B from one *Agrobacterium* strain. *Plant Disease* 87(3): 247-251.

Kong, L. and Hanley-Bowdoin, L. (2002) A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. *The Plant Cell* 14: 1817-1832.

Lee, S., Stenger, D.C., Bisaro, D.M. and Davis, K. (1994) Identification of loci in *Arabidopsis* that confer resistance to geminivirus infection. *The Plant Journal* 6(4):525-535.

Legg, J.P., Thresh, J.M. (2000) Cassava mosaic virus disease in East Africa: a dynamic disease in a changing environment. *Virus Research* 71: 135-149.

Leiser, R.M., Ziegler-Graff, V., Reutenauer, A., Herbach, E., Lemaire, O., Guilley, H., Richards, K. and Jonard, G. (1992). Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. *Proceedings of the National Academy of Sciences* 89:9136-9140.

Leung, Y.F. and Cavalieri, D. (2003) Fundamentals of cDNA microarray data analysis. *Trends In Genetics* 19(11): 649-659.

Li, H, Lucy, A.P., Guo, H., Li, W., Ji, L., Wong, S. and Ding, S. (1999) Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *The EMBO Journal* 18(10): 2683-2691.

Lipshutz, R., Fodor, S.P.A., Gingeras, T.R. and Lockhart, D.J. (1999) High density synthetic oligonucleotide arrays. *Nature genetics (supplement)* 21:20-24.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25: 402-408.

Mathews, C. (2000) *Cassava production by small holder farmers in Mpumalanga:A case study*. Lowveld Research unit, Department of Agriculture Conservation and Environment, P/Bag x11318, Nelspruit-1200.

Mackay, I.M., Arden, K.E. and Nitsche, A. (2002) Real-time PCR in virology. *Nucleic Acids Research* 30(6): 1292-1305.

Maule, A., Leh, V. and Lederer, C. (2002) The dialogue between viruses and hosts in compatible interactions. *Current Opinion in Plant Biology* 5: 1-6.

Mayo, M., Ryabov, E., Fraser, G. and Taliansky, M. (2000) Mechanical transmission of *Potato leafroll virus. Journal of General Virology* 81: 2791-2795.

McDowell, J., Dhandaydham, M., Long, T., Aarts, M., Goff, S., Holub, E. and Dangl, J. (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of *Arabidopsis. The Plant Cell* 10: 1861-1874.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D. and Koornneef, M. (1998) *Arabidopsis thaliana*: a model plant for genome analysis. *Science* 282: 662-682.

Mysore, K.S., Tuori, R.P. and Martin, G.B. (2001) *Arabidopsis* genome sequence as a tool for functional genomics in tomato. *Genome Biology* 2(1):reviews1003.1-1003.4.

Mysore, K.S. and Ryu, C. (2004) Nonhost resistance: how much do we know? *Trends in Plant Science* 9(2): 97-104.

Nagar, S., Hanley-Bowdoin, L., and Robertson, D. (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. *The Plant Cell* 14:2995-3007.

Nestel, B. (1980) Optima 1: Cassava, A new outlook for an ancient crop. *United Nations Food and Agricultural Organisation, 53-68*

O´Donnell, P.J., Schmelz, E.A., Moussatche, P., Lund, S.T., Jones, J.B. and Klee, H.J. (2003) Susceptible to intolerance - a range of hormonal actions in a susceptible *Arabidopsis* pathogen response. *The Plant Journal* 33: 245-257.

Olsen, K.M. (2004) SNPs, SSRs and inferences on casssava's origin. *Plant Molecular Biology* 56: 517-526.

Petty, I.T.D., Carter, S.C., Morra, M.R., Jeffrey, J.L. and Olivey, H.E. (2000) Bipartite geminivirus host adaptation determined cooperatively by coding and noncoding sequences of the genome. *Virology* 277: 429-438.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29(9): 2002-2007.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* 30(9): 1-10.

Picó, B., Ferriol, m., Dĭez, M.J. and Viňals, F.N. (2001) Agroinoculation methods to screen wild *Lycopersicon* for resistance to *Tomato yellow leaf curl virus*. *Journal of Plant Pathology* 83(3): 215-220.

Pita, J.S., Fondong, V.N., Sangarĕ, A., Otim-Nape, G.W., Ogwal, S. and Fauquet, C.M. (2001) Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *Journal of General Virology* 82: 655-665.

Puonti-Kaerlas, J. (1998) Cassava Biotechnology. *Biotechnology and Genetic Engineering Reviews* 15: 329-364.

Quackenbush, J. (2002) Microarray data normalization and transformation. *Nature Genetics Supplement* 32: 496-501

Rajeevan, M.S., Vernon, S.V., Taysavang, N. and Unger, E.R. (2001) Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. *Journal of Molecular Diagnostics* 3(1): 26-31.

Reynolds, M.A. (2002) Microarray technology, GEM microarrays and drug discovery. *Journal of Industrial Microbiology and Biotechnology* 28:180-185.

Rishi, A.S., Nelson, N.D. and Goyal, A. (2002) DNA microarrays: gene expression profiling in plants. *Reviews in Plant Biochemistry and Biotechnology* 1: 81-100.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T, Somerville, S.C. and Manners, J.M. (2000) Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. *Proceedings of the National Academy of Sciences* 97(21): 11655-11660.

Scholthof, H.B. (2001) Molecular plant-microbe interactions that cut the mustard. *Plant Physiology* 127: 1476-1483.

Selth, L.A., Randles, J.W. and Rezaian, M.A. (2004) Host responses to transient expression of individual gebes encoded by *Tomato leaf curl virus*. .*Molecular Plant-Microbe Interactions* 17 (1):27-33.

Scheideler, M., Schlaich, N.L., Fellenberg, K., Beissbarth, T., Hauser, N.C., Vingron, M., Slusarenko, A.J. and Hoheisel J.D. (2002) Monitoring the switch from housekeeping to pathogen defense in *Arabidopsis thaliana* using cDNA microarrays. *The Journal of Biological Chemistry* 227(12):10555-10561.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S. and Harper, J.W. (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91: 209-219.

Speulman, E., Bouchez, D., Holub, E.B. and Beynon, J.L. (1998) Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana. The Plant Journal* 14(4):467-474.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G. and Jones, J.D.G. (1995) Molecular genetics of plant disease resistance. *Science* 268: 661-667.

Stenger, D.C., Carbonaro, D. and Duffus, J.E. (1990) Genomic characterization of phenotypic variants of *beet curly top virus. Journal of General Virology* 71: 2211-2215.

Stenger, D.C., Revington, G.N., Stevenson, M.C. and Bisaro, D.M. (1991) Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. *Proceedings of the National Academy of Sciences* 88: 8029-8033.

Storey, H.H. (1936) Virus diseases of East African plants. VI. A progress report on studies of the diseases of cassava. *East African Agricultural Journal* 2: 34-39.

Takahashi, H., Miller, J., Nozaki, Y., Sukamto, Takeda, M., Shah, J., Hase, S., Ikegami, M., Ehara, Y. and Dinesh Kumar, S.P. (2002) *Arabidopsis thaliana RPP8/HRT* family resistance gene, conferring resistance to *cucumber mosaic virus* requires salicyclic acid, ethylene and a novel signal transduction mechanism. *The Plant Journal* 32: 655-667.

Tang, X., Xie, M., Kim, Y.J., Zhou, J., Klessig, D.F. and Martin GB (1999) Overexpression of *Pto* Activates Defense Responses and Confers Broad resistance. *The Plant Cell* 11:15-29.

Trench, T.N. and Martin, M.M. (1985) An assessment of African Cassava Mosaic Disease in South Africa and Swaziland. *South African Journal of Plant Soil* 2:169-170.

Tzfira, T. and Citovsky, V. (2002) Partners-in-infection:host proteins involved in the transformation of plant cells by *Agrobacterium*. *Trends in Cell Biology* 12(3): 121-129.

van Hal, N.L.W., Vorst, O., van Houwelingen, A.M.M.L., Kok, E.T., Peijnenburg, A., Aharoni, A., van Tunen, A.J. and Keijer, J. (2000) The application of DNA microarrays in gene expression analysis. *Journal of Biotechnology* 78: 271-280.

Vanitharani, R., Chellappan, P., Pita, J.S. and Fauquet, C.M. (2004) Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *Journal of Virology* 9487-9498.

Venisse, J., Malnoy, M., Faize, M., Paulin, J. and Brisset, M. (2002) Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora. Molecular Plant-Microbe Interactions* 15(12): 1204-1212.

Wan, J., Dunning, F.M. and Bent, A.F. (2002) Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. *Functional and Integrative Genomics* 2: 259-273.

Wang, D., and Maule, A.J. (1995) Inhibition of host gene expression associated with plant virus replication. *Science* 267:229-231.

Ward, D.V. and Zambryski, P.C. (2001) The six functions of *Agrobacterium* VirE2. *Proceedings of the National Academy of Sciences* 98(2): 385-386.

Whitham, S.A., Quan, S., Chang, H., Cooper, B., Estes, B. and Zhu, T. (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. *The Plant Journal* 33:271-283.

Wullschleger, S.D and Difazio, S.P. (2003) Emerging use of gene expression microarrays in plant physiology. *Comparative and Functional Genomics* 4: 216-224.

Wyatt, S.D. and Brown, J.K. (1996) Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathology* 86:1288-1293.

Yang, I.V., Chen, E., Hasseman, J.P., Liang, W., Frank, B.C., Wang, S., Sharov, V., saeed, A.I., White, J., Li, J., Lee, N.H., Yeatman, T.J. and Quackenbush, J. (2002) Within the fold: assessing differential expression measures and reproducibility in microarray assays. *Genome Biology* 3(11): 1-13.

Yu, I., Parker, J. and Bent, A.F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proceedings of the National Academy of Sciences* 95: 7819-7824.

Yuasa, T., Ichimura, K., Mizoguchi, T. and Shinozaki, K. (2001) Oxidative stress activates ATMPK6, an *Arabidopsis* homologue of MAP kinase. *Plant Cell Physiology* 42(9): 1012-1016.

Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G.W., Robinso,n D.J. and Harrison, B.D. (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *Journal of General Virology* 78: 2101-2111.

Zhou, X., Robinson, D.J., Harrison, B.D. (1998) Types of variation in DNA-A among isolates of East African cassava mosaic virus from Kenya, Malawi and Tanzania. *Journal of General Virology* 79:2835-2840.

Zhu, T. (2003) Global analysis of gene expression using gene chip microarrays. *Current Opinion in Plant Biology* 6: 418-425.