THE BYSTANDER EFFECT: ANIMAL AND PLANT MODELS

FRANZ JOSEPH ZEMP

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ABSTRACT

Bystander effects are traditionally known as a phenomenon whereby unexposed cells exhibit the molecular symptoms of stress exposure when adjacent or nearby cells are traversed by ionizing radiation. However, the realm of bystander effects can be expanded to include any systemic changes to cellular homeostasis in response to a number of biotic or abiotic stresses, in any molecular system. This thesis encompasses three independent experiments looking at bystander and bystander-like responses in both plant and animal models. In plants, an investigation into the regulation of small RNAs has given us some insights into the regulation of the plant hormone auxin in both stress-treated and systemic (bystander) leaves. Another plant model shows that a bystander-like plant-plant signal can be induced upon ionizing radiation to increase the genome instability of neighbouring unexposed (bystander) plants. In animals, it is shown that the microRNAome is largely affected in the bystander cells in a three-dimensional human tissue model. *In silico* and bioinfomatic analysis of this data provide us with clues as to the nature of bystander signalling in this human '*in vivo*' model.

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(+) – positive sense

(-) – negative sense

½BS – half-bystander

24I – infected leaves 24 hours after infection

24S – non-infected (systemic) leaves 24 hours after infection

6dS – non-infected (systemic) leaves 6 days after infection

A – adenosine

ADAR – Adenosine Deaminase that acts on RNA

AFB – AUXIN SIGNALLING FBOX

AGO1 – ARGONAUT1

ARE – auxin response elements

ARF – AUXIN RESPONSE FACTOR

AUF – arbitrary units of fluorescence

AVR – avirulent protein

BS – bystander

BSMV – *Barley stripe mosaic hordeivirus*

BYV – *Beet yellows closterovirus*

CLL – chronic lymphocytic leukemia

CMV – *Cucumber mosaic cucumovirus*

CV – *Peanut clump pecluvirus*

DCL – DICER-LIKE

dpg – days post germination

dpi – days post-irradiation

ds – double-stranded

dsRNA – double-stranded RNA

HEN1 – HUA ENHANCER1

HFR – homologous recombination frequency

hpi – hours post-irradiation

HST – HASTY

HYL1 – HYPONASTIC LEAVES1

I – inosine

ISR – induced-systemic immunity

IR – ionizing radiation

IT- irradiation treatment

JA – jasmonic acid

LRR – leucine-rich repeats

MeSA – methyl salicylate

MRE – miRNA recognition element

miRNA – microRNA

miRNP – miRNA/AGO ribonucleoprotein

NPR1 – NON-EXPRESSOR OF PATHOGENESISRELATED1 (NPR1)

ORMV – *Oilseed rape tobamovirus*

P-body – processing-bodies

PI – proteinase inhibitors

PLV – *Pothos latent aureusvirus*

PPP – Promoter prediction programs

PVX – *Potato potexvirus X*

PTGS – post-transcriptional gene silencing

R – resistance protein

RB – rose Bengal

RDR6 – RNA-DEPENDENT RNA POLYMEASE6

RdRP – RNA-dependent RNA polymerase

RISC – RNA-induced silencing complex

RNAi – RNA interference

ROS – reactive oxygen species

SA – salicylic acid

SAR – systemic acquired resistance

SCL – scarecrow-like

SDE – SILENCING DEFECTIVE

SE – SERRATE

SGS3 – SUPPRESSER OF GENE SILENCING3

ss – single stranded

siRNA – small-interfering RNA

SMD – SILENCING MOVEMENT DEFICIENT

SRS – systemic recombination signal

SWS – systemic wound signalling

ta-siRNA – trans-acting small-interfering RNAs

TBSV – *Tomato bushy stunt tombusvirus*

TEV – *Tobacco etch potyvirus*

TCV – *Turnip crinkle carmovirus*

TGS – Transcriptional gene silencing

TIR1 – TRANSPORT INHIBATOR1

TMV – *Tobacco mosaic tobamoirus*

TNF – tumour necrosis factor

TRAIL – tumour necrosis factor-related apoptosis-inducing ligand

UV – ultraviolet light

VOC – volatile organic compounds

VSR – viral suppressors of silencing

1.0 INTRODUCTION

'Bystander effects' is a term originally used in cancer therapeutics, and refers to the attempt at affecting a single type of cell within a heterogeneous population resulting in several types of cells being affected by the treatment (Freeman, *et al.*, 1993; Morgan 2003a). The term bystander effect has now been applied to a number of different phenomena whereby unexposed 'units' exhibit the molecular symptoms of stress exposure when adjacent or nearby 'units' are a subject to a stress. Indeed, a unit seems a peculiar subject; however, the unit here can be referring to neighboring cells, systemic cells, or even entire organisms.

Modern bystander effects are non-targeted effects of ionizing radiation exposure, and refer to naïve cells that were either in direct contact with irradiated cells or received an irradiation 'distress' signal from irradiated cells (Zhou, *et al*., 2000; Morgan, *et al*, 2002; Morgan 2003a; Morgan 2003b; Mothersill and Seymour, 2003; Mothersill and Seymour, 2004; Mothersill and Seymour, 2006; Morgan and Sowa, 2007). In these instances, bystander effects can include a wide variety of genetic alterations such as gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations, and amplifications (Zhou, *et al*., 2000; Huo, *et al*., 2001; Lorimore, *et al*., 2001; Zhou, *et al*., 2002a; Zhou, *et al*., 2002b; Suzuki, *et al*., 2003; Klokov, *et al*., 2004; Lorimore, *et al*., 2005; Smilenov, *et al*., 2006; Hamada, *et al*., 2007), with further influences such as changes in gene expression, cellular proliferation, senescence, and cell death (Sawant, *et al*., 2001; Sawant, *et al*., 2002; Liu, *et al*., 2006; Lyng, *et al*., 2006b; Sedelnikova, *et al.*, 2007).

Other bystander-like effects may be induced by plasma- or blood-borne signals produced upon irradiation that direct chromosomal breakage and abnormalities in unirradiated tissues. These factors have previously been referred to in the literature as clastogenic factors (Mothersill and Seymour, 2001; Mothersill and Seymour, 2004; Morgan and Sowa, 2007). Similar to clastogenic factors are abscopal effects, in which radiation-induced changes occur outside the targeted area of irradiation, often in different organs (Mothersill and Seymour, 2001; Morgan, 2003a, Morgan and Sowa, 2007). A classic example is the bilaterally symmetric radiation-response, where only the left or right organ is exposed, but similar responses occur in both organs (Mothersill and Seymour, 2001; Morgan, 2003a; Koturbash, *et al.*, 2006).

Bystander-like effects have also been shown in different organisms. This can include the transgenerational transmission of radiation effects to offspring, especially in terms of 'bequeathing' genomic instability to cellular (Mothersill and Seymour, 2001; Morgan, 2003b; Morgan and Sowa, 2007) or organismal (Barber and Dubrova, 2006) progeny. Radiation-induced bystander phenomena have been shown to occur between cohabiting animals as well (Mothersill, *et al.*, 2006; Mothersill, *et al.*, 2007).

The field of radiation-induced bystander effects in plants, in the sense of cell-cell media transfer experiments, has yet to be explored. However, abscopal bystander-like effects are well known to occur in plants under a variety of biotic or abiotic stresses. The local application of biotic or abiotic stresses to plant tissue can lead to systemic changes in

pathogen resistance (Grant and Lamb, 2006), methylation pattern (Boyko, *et al.,* 2007), recombination rate (Kovalchuk, *et al.,* 2004), hormone levels (Schilmiller and Howe, 2006) and gene expression (Truman, *et al.,* 2006).

Bystander-like effects between separate plants have also been reported. Plants have long been known to communicate to one another via diffusible signals. The best known example being non-wounded (bystander) plants residing next to wounded plants emitting the same defensive signals and activating the same defensive machinery as the wounded plants (Baldwin, *et al.,* 2006).

As such, bystander and bystander-like effects are known to be involved in a number of different processes in different organisms. The commonality lies in non-effected 'units' receiving signals from effected 'units' to produce a response that modifies homeostasis in the non-effected 'units.'

Small regulatory RNA species play major roles in the gene regulation of most eukaryotic organisms (Mallory and Vaucheret, 2006; Vaucheret, 2006). They regulate such processes as cell patterning, differentiation, cell cycle progression, genome stability, and apoptosis (Jones-Rhoades, *et al.,* 2006*;* Mallory and Vaucheret, 2006; Liu, *et al.,* 2008), many of which are deregulated in bystander and bystander-like effects. Thus, the role these miRNAs may play in bystander tissues in plants and animals warrants investigation.

This thesis encompasses three independent experiments looking at bystander and bystander-like responses in both plant and animal models. The first study explores the relationship between regulation of miRNAs and the regulation of the plant hormone auxin in both stress-exposed and systemic (bystander) leaves. In the second study, I show that a bystander-like plant-plant signal can be induced upon ionizing radiation to increase the genome instability of neighboring unexposed (bystander) plants.

Finally, in the third study, I show that the microRNAome is profoundly affected in the bystander cells of a three-dimensional human tissue model. Bioinfomatic analysis of this data provide us with clues as to the nature of bystander signalling in this human '*in vivo*' model.

2.0 LITERATURE REVIEW

2.1 BYSTANDER AND BYSTANDER-LIKE EFFECTS

2.1.1 What are Bystander-effects?

Bystander effect, also known as non-targeted effects of radiation, is phenomenon whereby unexposed cells exhibit the molecular symptoms of stress exposure when adjacent or nearby cells are traversed by ionizing radiation. In mammalian cells, the primary effects may include genome rearrangements, chromosome aberrations, strand breaks, and increases in mutation, apoptotic, and transformation rate (Mothersill and Seymour, 2001; Mothersill and Seymour, 2004; Morgan, 2003a; Morgan, 2003b; Lyng *et al.*, 2006b; Morgan and Sowa, 2007). However, bystander cells are not always affected in a detrimental manner, as positive influences have been shown to occur, such as radio-adaptive response (Iyer and Lehnert, 2002; Lyng *et al.*, 2006b; Morgan and Sowa, 2007).

2.1.2 Ionizing Radiation and Targeted Effects

Ionizing radiation (IR), simply put, is high-energy waves (γ- or X-rays) or particles (α- or β-particles) that have the ability to ionize the atoms or molecules it comes into contact with. Although particular cells and tissues can be more or less susceptible to different types of radiation, common to all cell types is the genotoxic nature of radiation. Among mutagens, radiation has the unique ability to penetrate cells and randomly deposit its energy within the cell. DNA is affected when hit directly by IR, as the energy from the radiation can easily cleave the DNA phosphodiester backbone, resulting in single- or double-strand breaks (Little, 2000; Little. 2006). Further, IR traversing a cell deposits energy through atomic-molecular interactions and ionisations along what is known as

particle tracks (Feinendegen, *et al*., 2007). With these ionisations comes the radiolysis of water, thus producing reactive oxygen species (ROS). A single electron track from a 100-kV x-ray in an average cell (1 ng), the equivalent to an approximate 1 mGy dose, instantly generates up to 150 ROS molecules (Pollycove and Feinendegen, 2003). The consequence of this burst of ROS can include ROS-dependent signalling, activation of cellular oxidative stress response, or the induction of DNA damage (Wiseman and Halliwell, 1996; Feinendegen, *et al*., 2007), the results of which depend on the intercellular concentration (size) and location of the ROS burst. DNA damage resulting from ROS created from IR is considered indirect damage, whereas a collision between the particle track and a DNA molecule results in direct DNA damage. Thus, ionizing radiation is particularly cytotoxic, as well as a known carcinogen (Little, 2000). Ironically, the cytotoxicity of this radiation makes it one of the most successful modalities for cancer treatment.

2.1.2 Bystander Effects in Animals

Non-targeted effects of radiation were first documented in 1954, where it was found that low-doses of high-LET radiation to the spleen created a plasma-derived factor that directed chromosomal aberrations in the bone marrow and lymphocytes of the irradiated humans (Parsons, *et al.*, 1954). Further, this factor caused elevated tumour incidence in plasma-injected rats (Souto, 1962; Mothersill and Seymour, 2001). Plasma- or blood-borne signals produced upon irradiation that direct chromosomal breakage and abnormalities in unirradiated tissues, particularly lymphocytes, have subsequently been

termed clastogenic factors (Mothersill and Seymour, 2001; Mothersill and Seymour, 2004; Morgan and Sowa, 2007).

Similar to clastogenic factors are abscopal effects, in which radiation-induced changes occur outside the targeted area of irradiation, often in different organs (Mothersill and Seymour, 2001; Morgan, 2003a, Morgan and Sowa, 2007). A classic example is the bilaterally symmetric radiation-response, where only the left or right organ is exposed, but similar responses occur in both organs (Mothersill and Seymour, 2001; Morgan, 2003a; Koturbash, *et al.*, 2006). Interestingly, these clastogenic and/or abscopal effects have been linked to some cancer remissions in non-targeted tumours (Rees and Ross, 1983; Ohba, *et al.*, 1998). These results definitively point to some manner of soluble signal produced at the site of irradiation that travels to distal cells and tissues.

Much of the work studying bystander phenomena is done *in vitro*, using cell lines in culture. Examples of experiments showing *in vitro* bystander effects include media transfer experiments (where cells in culture are irradiated and their media is transferred to a non-exposed culture) and single- or few-cell irradiation of a colony (whereby one or a few cells are specifically irradiated using a precise beam of radiation), where effects are then observed in the non-irradiated cells. A number of *in vitro* experiments performed throughout that last decade point to a signal that is soluble and/or gap-junction-mediated (Mothersill and Seymour, 2001; Morgan, 2003b, Morgan and Sowa, 2007).

Whether there is a soluble or gap-junction-mediated cell-cell signal, or a systemic change in non-targeted tissues, these mechanisms fall under the umbrella of bystander effects. A further phenomenom that can be included is the transgenerational transmission of radiation effects to offspring, especially in terms of 'bequeathing' genomic instability to cellular (Mothersill and Seymour, 2001; Morgan, 2003b; Morgan and Sowa, 2007) or organismal (Barber and Dubrova, 2006) progeny. Indeed, bystander phenomena have been shown to occur between cohabiting organisms as well (Mothersill, *et al.*, 2006; Mothersill, *et al.*, 2007).

2.1.2.3 Postulated Mechanisms

The mammalian bystander work this literature review aims to cover is the traditional, cell-cell mediated bystander effects. As such, I will discuss mechanisms focusing on these types of experiments.

The exact mechanisms behind cell-cell mediated bystander effects remain largely enigmatic; however, some great insights have been gleaned to-date. Two possible types of signals are proposed to function in these cell-cell models, a gap-junction mediated signal, and a cell-cell contact independent signal.

A number of experiments have found that particular cell lines need the presence of gap junctions to facilitate the generation of bystander effects (Azzam *et al.*, 1998, 2001, 2003a; Shao *et al.*, 2003; Zhou, *et al.*, 2000, 2001; Hu *et al.*, 2006). Gap junctions are low-resistance channels that link adjacent cells, allowing for the passage of small

molecules involved in metabolism, growth control, and embryogenesis (Azzam, *et al.*, 2003b). In these studies, bystander effects such as increased sister chromatid exchanges and mutation rates only occurred in cells with functional gap junctions, while gap-junction deficient or inhibited cells failed to produce similar results (Azzam, *et al.,* 1998, 2001; Zhou *et al.,* 2000). Further work found that Connexin43 might be the gap junction protein mediating cell-cell bystander signals. In addition to a number of studies in which knocked-out Connexin43 showed an elimination or down-regulation of the effect (Azzam, *et al.,* 2001; Zhou, *et al.,* 2001), Connexin43 is significantly up-regulated and phospholyated in response to radiation and other oxidizing agents, further suggesting its role in stress response (Azzam, *et al.,* 2003a). In contrast, bystander responses have been found in the absence of cell-cell contact, suggesting a medium secreted, soluble factor may be responsible for the cellular communication of damage (Mothersill and Seymour, 1998; Mothersill, *et al.,* 2000; Vines, *et al.,* 2007).

One commonality that seems to be important to bystander effects is free radicals, particularly ROS (Azzam, *et al.,* 2003b, Morgan and Sowa, 2007, Vines, *et al.,* 2007). This was shown in early studies using radical scavengers to diminish the bystander effect in medium transfer experiments (Lehnert and Goodwin, 1997; Wu et al., 1999; Azzam et al., 2002; Azzam *et al.,* 2000b), and was shown more recently using knockout cell lines (Mothersill, *et al.,* 2000; Vines, *et al.,* 2007). While free radicals are indeed produced upon radiation, the role of oxidative metabolism is getting much attention in bystander studies. Specifically, NAD(P)H and NAD(P)H-oxidases have been found to be very important (Azzam, *et al.,* 2003a, b; Vines, *et al.,* 2007). NAD(P)H-oxidases are

membrane bound enzymes that oxidize NAD(P)H to reduce molecular oxygen to superoxide anions, and increase internal superoxide anion concentrations in both irradiated and bystander cells (Azzam, *et al.,* 2003a, b). Further, the decrease of NAD(P)H production through the inhibition of the pentose phosphate pathway resulted in cells unable to produce or receive the bystander signal (Vines, *et al.,* 2007). Hence, the roles of NAD(P)H and NAD(P)H-oxidases in the bystander response warrants further investigation.

Another cellular response to soluble bystander signals is an immediate and short-lived Ca^{2+} influx, as well as activation of the MAP kinase signalling pathway. Both these signalling stimuli can modulate changes in cell proliferation and the induction of apoptosis (Lyng, *et al.,* 2000; Lyng, *et al.,* 2006a). In these studies, a bystander signal triggered immediate calcium influx and MAPK activation, which led to increases in intercellular ROS and the induction of apoptosis (Lyng, *et al.,* 2006a). Again, however, the initial signal molecule remains enigmatic.

2.1.3 Bystander Effects in Plants

2.1.3.1 Radiation-Induced Bystander Effects in Plants

Radiation-induced bystander effects in plants, in the sense of cell-cell media transfer experiments, have yet to be explored. It would be interesting to examine this phenomenon in plant protoplasts, given the appropriate end-point is chosen. However, a few experiments to date suggest that abscopal and plant-plant bystander-like phenomena exist in plants.

The first published work referring to radiation-induced abscopal effects in plants occurred in 2007 (Yang, *et al.,* 2007). This laboratory demonstrated that direct and specific radiation to the shoot apical mersitem of embryonic Arabidopsis with high-LET α-particles resulted in post-embryonic developmental defects in root formation.

Further, unpublished data from our laboratory shows that the genomic instability generated by UV-C or X-Rays in Arabidopsis plants is transmitted through a volatile signal to neighbouring, un-irradiated plants (Zemp and Kovlachuk, unpublished data). These experiments are presented in Chapter 4.0.

2.1.3.2 Bystander-Like Effects in Plants

The systemic transmission of stress signals is a well-known phenomenon in plant molecular biology. The local application of biotic or abiotic stresses to plant tissue can lead to global changes in pathogen resistance (Grant and Lamb, 2006), methylation pattern (Boyko, *et al.,* 2007), recombination rate (Kovalchuk, *et al.,* 2004), hormone levels (Schilmiller and Howe, 2006) and gene expression (Truman, *et al.,* 2006). These may be considered bystander-like phenomena, as unexposed tissues are receiving signals from exposed tissues and attenuating cellular homeostasis.

2.1.3.2.1 Systemic Acquired Resistance

Systemic acquired resistance (SAR), also known as systemic immunity, is among the most well studied systemic signals in plants. It is roughly defined as the systemic

induction of a broad-based and long-lasting enhanced disease tolerance following pathogen recognition (Durant and Dong, 2004; Grant and Lamb, 2006)

The generation of SAR is believed to start with the innate inducible defence system in plants. In this highly specific system, a single host-encoded Resistance (R) protein is responsible for the detection of a single pathogen derived Avirulent (AVR) factor. Pathogen recognition leads to the signalling of immune responses such as localized cell death, lignin formation, production of antimicrobial compounds and the expression of pathogenesis-related genes, as well as the generation of the SAR signal (Nimchuk, *et al.*, 2003; Bent and Mackey, 2007).

The generation of SAR is reliant on the SA-dependent activation of NON-EXPRESSOR OF PATHOGENESISRELATED1 (NPR1) protein, which then translocates to the nucleus to stimulate the action of TGA and other transcription factors (Mou, *et al.*, 2003; Grant and Lamb, 2006). The activation of these transcription factors are, in part, responsible for the transcriptional reprogramming observed in infection-detected cells. However, an SA-dependent, NPR1-independent SAR response has also been observed (Desveaux, *et al.*, 2004).

A number of key experiments have placed the plant hormone jasmonic acid (JA) in the forefront of being involved in signalling (Durant and Dong, 2004; Grant and Lamb, 2006). JA is a lipid-derived phytohormone involved in various cellular responses such as growth inhibition, senescence, and leaf abscission (Halim, *et al.*, 2006). However, it was

recently shown that the volatile methyl salicylate (MeSA) is the SAR signal in the model system of tobacco (Park *et al.,* 2007). Thus, it is likely that the combined action of a number of phytohormones is required for the efficient response.

A similar mechanism to SAR is that of induced-systemic immunity (ISR). In this response, the symbiotic root bacterium *Pseudomonas fluorescens* induces increased tolerance to future bacterial infections in the remainder of the plant (Pieterse, *et al.*, 1998; Grant and Lamb, 2006). ISR is SA-independent mechanism, but does require NPR1 action. Further, systemic signalling has been found to involve the JA and ethylene phytohormones (Pieterse, *et al.*, 1998; Ton, *et al.*, 2002).

2.1.3.2.2 Systemic Wound Signalling

Systemic wound signalling (SWS) is a plant mechanism that is triggered in response to herbivory action, and functions to locally and systemically induce such defences as the excretion of toxic chemicals or, more interestingly, releasing volatile substances to attract parasitoids and predators of the herbivore (Kessler and Baldwin, 2002; Schilmiller and Howe, 2005).

The signal generated by SWS has been most widely studied in *Lycopersicon esculentum* (tomato) where local wound infection triggers global expression of defensive proteinase inhibitors (PI; Schilmiller and Howe, 2005; Wasternack, *et al.*, 2006). Forward genetics has produced a number of mutants impaired in the generation of global PI expression, and most of these mutants are involved in JA biosynthesis, JA perception, or systemin

function (Schilmiller and Howe, 2005). Systemin is a small, 18-amino acid peptide that has recently been found to bind a leucine-rich repeat receptor kinase SR160 (Scheer, *et al.,* 2002). A highly simplified model of the current SWS mechanism starts with the cleavage of Prosystemin to produce Systemin at the site of wounding. Systemin then binds SR160 in surrounding cells, which activate JA biosynthesis and accumulation. Increased JA concentrations trigger the expression of local wound responsive genes, such as PI, and JA then acts as the mobile signal to distal tissues to promote further JA synthesis and, thus, wound-inducible gene expression (Schilmiller and Howe, 2005; Wasternack *et al.,* 2006). Interestingly, a number of compounds, such as abscisic acid (Herde et al., 1996), ethylene (O'Donnell, *et al.,* 1996), ROS (Orozco-Ca´rdenas, *et al.,* 2001), UV-light (Conconi, *et al.,* 1996), and fatty acid conjugates (Turlings, *et al.,* 1995) have been found that activate the JA pathway, leading to JA-induced expression patterns (Schilmiller and Howe, 2005; Wasternack *et al.,* 2006).

2.1.3.2.3 Systemic Recombination Signal

The systemic recombination signal (SRS) was first discovered by Kovalchuk *et al.* (2003) and referred to a systemic and graft-transmissible increase in recombination rate in virus treated leaves. By using a combination of recombination reporter methods, a truncated reporter gene (see chapter 4.2.1) and an endogenous heterozygous chlorophyll-modulating gene, they showed that local infection resulted in the generation of a signal that traveled ahead of the virus to non-infected tissue. Further, viral-free, signal-receiving leaves grafted on to naïve plants propagated the signal to non-infected tissue. Further analysis of SRS-receiving tissue found globally hypermethylated genomes, with what

appeared to be sequence-specific hypomethyation to the leucine-rich repeat (LRR) regions of the N-gene-like R-genes (Boyko, *et al.,* 2007). These LRR regions were also found to be more unstable than control loci as measured by restriction fragment length polymorphisms. The actual signal responsible for the generation of the SRS has remained elusive, and further work involving the role of small RNAs (see Chapter 2.2.2) is underway in an effort to shed light onto the mechanism.

Alternate experiments utilizing the abiotic stresses UV-C and rose Bengal (a photoactivated dye that produces superoxide and other free radicles in plant tissues) found similar increases in recombination rate in systemic tissue (Filkowsi, *et al.,* 2004). Interestingly, the generation of this SRS was SA independent, but sensitive to radical scavenging compounds. It remains to be seen if the abiotic-generated SRS and the viral-induced SRS are similar mechanisms.

2.1.3.2.4 Plant-Plant Signalling

Plants have long been known to communicate to one another via diffusible signals, or volatile organic compounds (VOCs). This phenomenom was first discovered several decades ago when it was observed that non-herbivore attacked (bystander) plants residing next to herbivore-attacked plants were emitting the same defensive signals and activating the same defensive machinery as the attacked plants (Baldwin and Schultz, 1983; Baldwin, *et al.,* 2006). The reason for this signalling, or eavesdropping as some would put it, has a number of purposes including priming defences before the arrival of the herbivore, readying pathogen defences to disease, or attracting predators or parasitoids of

the organisms causing stress (Jiménez-Martínez, *et al.*, 2004; Baldwin, *et al.*, 2006). Interestingly, some of the phytohormones implicated in these plant-plant signals are similar to the hormones involved in both the SAR and SWS. Again, this plant-plant communication of damage or impending attack could be considered bystander-like phenomenon.

2.2 Small RNAs: Biogenesis and Function

2.2.1 Introduction

Since their discovery, small regulatory RNA species have been revealed to play major roles in the gene regulation of most eukaryotic organisms (Mallory and Vaucheret, 2006; Vaucheret, 2006). They have been shown to have essential functions in developmental processes from embryonic development through sexual maturity, regulating such processes as cell patterning, differentiation, cell cycle progression, genome stability, and apoptosis (Jones-Rhoades, *et al.*, 2006*;* Mallory and Vaucheret, 2006; Liu, *et al.*, 2008). Indeed, deregulation of these key players have been implicated in a number of human diseases including fragile X syndrome (Plante, *et al.*, 2006), DiGeorge syndrome (Landthaler, *et al.*, 2004), and, most notably, cancer (Cho, 2007; Croce, 2008). Further, in plants, loss of small RNA production can lead to enhanced disease susceptibility (Li and Ding, 2006; Ding and Voinnet, 2008).

Small regulatory RNAs are a group of single-stranded non-coding RNAs usually ranging in size from 20-24 nucleotides. They are derived from complementary or semi-complementary double-stranded RNA molecules and have functions in post-transcriptional and transcriptional gene silencing. Post-transcriptional gene silencing

(PTGS) refers to the down-regulation of gene expression after transcription and mRNA maturation. In animals and plants, this can refer to the sequence-specific binding to mRNA molecules in the cytoplasm resulting in the inhibition of translation. More commonly in plants, and rarely in animals, this sequence-specific binding can result in the degradation of mRNA transcripts. Transcriptional gene silencing is thought to be exclusive to plants, and refers to the methylation of DNA sequences and chemical modifications to histones surrounding the promoter of genes. These chemical modifications to the DNA and histones surrounding these genes result in down-regulation or complete cessation of transcriptional activity.

The biological activity of small RNAs has been harnessed with the advent of RNA interference (RNAi) as a tool used in molecular biology to knock-down genes using PTGS. Transient or stable expression of synthesized small RNA species can efficiently target particular genes of interest, allowing the study of phenotypes in the absence of the target transcript. As can be imagined, RNAi has already become a hot topic as a therapeutic agent, with sequences designed to control the expression of genes deregulated in disease (Martin and Caplen, 2007). Indeed, this technology has moved forward, as RNAi for the treatment of ocular disease reached phase I clinical trials in 2004 (Whelan, 2005), with phase III trials started in early in 2007.

Small RNA function and mechanism is somewhat conserved between the plant and animal kingdom; however, substantial changes in their biogenesis and function do exist, and they wil be discussed in separate sections.

2.2.2 Plants

2.2.2.1 Biogenesis

PTGS was first discovered in 1990, when the attempt to transgenically up-regulate pigment production in petunias resulted in the loss of both endogene and transgene activity in some of the transformants (Napoli, *et al.*, 1990). Further investigation showed that the loss of enzyme activity was the result of decreased concentrations of mRNA without decreased levels of transcription (Agrawal, *et al.*, 2003). Thus, it appeared some kind of mRNA degradation process was occurring between transcription and translation, and this phenomenon was entitled co-suppression. The phenomenon of co-suppression was subsequently found to be regulated by small regulatory RNAs.

There are two major classes of small RNAs in plants, microRNAs (miRNAs) and small-interfering (siRNAs), which differ more in their biogenesis than function (Bartel, 2004; Mallory and Vaucheret, 2006). Both classes are derived from double-stranded RNA (dsRNA) cleaved by an RNase III-type endonuclease (Dicer-like family; DCL), and associated into a complex consisting of a member of the Argonaut family. The differences in biogenesis derive from the origin and type of dsRNA and members of the Dicer and Argonaut family used in the processing. As miRNAs are the focus of this thesis, only they will be discussed.

miRNAs are derived from endogenous, single-stranded RNA gene products, termed pri-miRNAs, that fold to produce imperfect hairpin-like loops (Jones-Rhoades, *et al.*, 2006*;* Mallory and Vaucheret, 2006; Vaucheret, 2006). The areas of the genome coding for

these miRNA precursor transcripts are generally not associated with protein coding areas, suggesting most are under their own transcriptional control (Reinheart, *et al.,* 2002; Rhoades, *et al.,* 2006). Many of these pri-miRNAs contain typical TATA box motifs and undergo 3' polyadenylaton and 5' capping, suggesting they are transcribed by RNA polymerase II (Xie, *et al.,* 2005a; Rhoades, *et al.,* 2006).

The dsRNA arm of the hairpin loop formed by the pri-miRNA is recognized by DICER-LIKE1 (DCL1) in the nucleus where, with the dsRNA binding protein HYPONASTIC LEAVES1 (HYL1), cuts the hairpin twice to excise the mature miRNA/miRNA* duplex from the stem of the hairpin with a two-nucleotide 3' overhang on each strand (Ma, *et al.,* 2004; Vazquez, 2006). DCL1 and HYL1 are localized to a distinct region of the nucleus termed the nuclear dicing body where the excision of the mature miRNA occurs (Fang and Spector, 2007). It has also been postulated that the zinc-finger containing protein SERRATE (SE) is involved in the primary processing of miRNAs, however they are not specifically localized to nuclear dicing bodies nor has their function been characterized (Fang and Spector, 2007; Yang, *et al.,* 2006).

Given the fidelity in sequence of the mature miRNAs, there is believed to be a mechanism by which DCL1/HYL1 preferentially cuts from the stem; however, the mechanism behind this specificity remains enigmatic (Reinheart, *et al.,* 2002; Jones-Rhoades, *et al.,* 2006). It has been speculated that the secondary structure of the stem and not the primary sequence that grants specificity (Parizotto, *et al.,* 2004). Mature plant miRNAs can vary in size from ~20-24 nucleotides in length, but are most often 21-

nuclotides in length. The variance in size is thought to be dependent on the structure of the stem (Parizotto, *et al.,* 2004; Vazquez., 2006).

Once excised from the stem-loop structure, the mature miRNA/miRNA* duplex is methylated at the 2'-OH of the 3' ends of the duplex by the dsRNA methyltransferase HUA ENHANCER1 (HEN1), a process thought to impede polyuridylation and degradation (Vazquez, *et al.,* 2006; Mallory and Vaucheret, 2006; Jones-Rhoades, *et al.,* 2006; Vaucheret, 2006). Export of either the mature duplex or a single-stranded RNA to the cytoplasm is thought to occur through the exportin5 homologue HASTY (HST; Vazquez, 2006; Mallory and Vaucheret, 2006; Jones-Rhoades, *et al.,* 2006; Vaucheret, 2006)

When exported to the cytoplasm where they function, the miRNAs are loaded into a RNA-induced silencing complex (RISC), which facilitates the recognition and degradation of target mRNAs. The complex's central proteins are members of the Argonaut family, of which Arabidopsis has 10 members (Jones-Rhoades, *et al.,* 2006; Vaucheret, 2006). ARGONAUT1 (AGO1) is known to be the important member for miRNA biogenesis in Arabidopsis (Vazquez, 2006; Mallory and Vaucheret, 2006; Jones-Rhoades, *et al.,* 2006; Vaucheret, 2006).

Argonaut proteins contain an RNA-binding PAZ domain and an RNase H-like PIWI domain (Song, *et al.,* 2003; Song, *et al.,* 2004; Jones-Rhoades, *et al.,* 2006). It is currently thought that AGO1 recognizes the miRNA/miRNA* duplex via the two-nucleotide 3'

overhang, which nestles the 5' end of the miRNA into the PAZ-domain's binding pocket (Ma, *et al.*, 2004; Vasquez, 2006). As the miRNA is duplexed at this time, and the mature sequence will act as a guide to cleave target transcripts, the Argonaut protein must differentiate which mature sequence will be incorporated into the RISC. The asymmetry rule dictates which strand will be incorporated, and it states that whichever strand is most stably paired at its 5' end will incorporate into the final RISC (Schwarz, *et al.*, 2003; Vasquez, 2006). The miRNA* strand is then released and degraded.

Once in the RISC, the mature miRNA acts as a sequence-specific guide to reduce target transcripts through the perfect or near-perfect pairing between the miRNA and the mRNA transcript. This complementarity triggers the RNase activity of the PIWI-domain of AGO1 to generate a single cut of the target mRNA's phosphodiester backbone (Baumberger and Baulcombe, 2005; Jones-Rhoades, *et al.*, 2006). After cleavage of the target, the fragments are released and the RISC can target remaining complementary mRNAs.

MiRNA-mediated translational repression in plants is still debated in the field. Early evidence showed that members of the *APETALA* family of transcription factors may be regulated in this way (Chen, 2004), but subsequent studies may have disputed the finding (Schwab, *et al.*, 2005). Only future research will solve this debate.

MiRNAs also play an important role in the biogenesis of another type of small, regulatory RNA, the trans-acting small-interfering RNAs (ta-siRNA). These small RNAs

are very similar to miRNAs insofar as they are transcribed from specific loci by RNA polymerase II and function to direct the sequence-specific degradation of target mRNAs. However, this is the limit of their similarity, as their biogenesis is truly unique.

Ta-siRNAs derive from eight loci belonging to four families in Arabidopsis, *TAS1–4* (Howell, *et al.,* 2007; Xie and Qi, 2008). Following their transcription and mRNA maturation, these non-coding transcripts are targeted by certain miRNAs for cleavage in the miRNA pathway described above. miR-173 targets the *TAS1* and *TAS2* families, while miR-390 is responsible for the cleavage of *TAS3* (Vaucheret, 2005). The recently discovered miR-828 targets the *TAS4* loci (Howell, *et al.,* 2007). This TAS transcript cleavage, through a mechanism yet to be deduced, triggers the activity of RNA-DEPENDENT RNA POLYMEASE6 (RDR6), and through the coordinated action of RDR6 and SUPPRESSER OF GENE SILENCING3 (SGS3), the cleaved *TAS* transcripts are converted to dsRNA (Vaucheret, 2005; Vasquez, 2006; Xie and Qi, 2008). These dsRNAs are then cleaved by DICER-LIKE4 (DCL4) in 21-nucleotide succession from the dsRNA *TAS* ends to produce a phased array of mature siRNAs (Gasciolli, *et al.,* 2005; Xie, *et al.,* 2005b). These siRNAs are methylated by HEN1 and then loaded into RISC complexes with an Argonaut family member to direct mRNA cleavage as described above. Interestingly, the Argonaut family member recruited may be related to the family of *TAS* cleaved. For example, AGO7 has been found to be important in the biogenesis of tas3-siRNAs (Adenot, *et al.,* 2005), while other members have been shown to utilize AGO1 (Jones-Rhoades, *et al.,* 2006).

A number of hypothesis for the function of ta-siRNAs have been proposed. The most popular of which is that these ta-siRNAs can co-regulatate multiple non-homologous targets through the expression of a single miRNA (Vaucheret, 2005).

2.2.2.2 Functions

2.2.2.2.1 Development

Plant miRNAs have been found to have numerous roles in development in *Arabidopsis thaliana*. In fact, members of the small RNA biogenesis pathways were first identified through mutants with severe developmental defects (Mallory and Vaucheret, 2006; Jones-Rhoades, *et al.*, 2006). An extreme case is seen in the *dcl1-/-* plants, which result in embryo lethality.

miRNAs are important for development as their temporal and spatial regulation allows for the specific regulation of genes involved in cell fate and patterning. For example, miR-166/165 target the class III HD-ZIP members *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA*, allowing for the establishment of abaxial/adaxial leaf polarity (Xu, *et al.*, 2007); miR-172 targets *APETELA2* with roles in regulating stem cell fate in the shoot apical meristem as well establishing floral organ identity (Zhao, *et al.*, 2007); miR-160, -167 and -390 are involved in targeting different members of the *AUXIN RESPONSE FACTOR (ARF)* transcription factors, with roles in male and female reproduction (Wu, *et al.*, 2006), root cap formation (Wang, *et al.*, 2005), seed germination (Liu, *et al.*, 2007), and leaf polarity (Xu, *et al.*, 2007); abscisic acid induces miR-159 to control Myb and other transcription factors during seed germination (Reyes and Chua, 2007).

2.2.2.2.2 Anti-viral Resistance

dsRNA within a plant cell triggers the action of DCLs and associated molecules to cleave the dsRNA substrate. Interestingly, many plant viruses have dsRNA genomes, or have dsRNA intermediates during replication. Do viral derived dsRNAs serve as a substrate for small RNA biogenesis?

In short, the answer is yes. Plants and viruses have developed intricate networks of defence and counter-defence upon viral infection. Those that pertain to small RNAs are small RNA-mediated anti-viral immunity and viral suppressing of RNA silencing.

2.2.2.2.1.1 The Defence

Viral genomes can be composed of either DNA or RNA, and either single stranded (ss) or double-stranded (ds). Further, ssRNA viruses can be classified as either positive sense (+) or negative sense (-) Zaitlin and Palukaitis, 2000). Most plant viruses possess (+)ssRNA genomes (Hillman, 1998), although some well studied plant viruses contain ssDNA and dsDNA genomes.

The production of virus-derived small RNAs (viRNAs) from ssRNA viruses occurs in two ways. First, (+)ssRNA viruses contain their own RNA-dependent RNA polymerase (RdRP) in order to achieve error-prone genome replication. The dsRNA intermediates produced upon replication are most often recognized by DCL4 and cut into 21-nucleotide viRNAs; however, *dcl4*-/- plants have no increase in disease susceptibility, and it has been found that 22-nucleotide viRNAs are produced by DCL2 (Ding and Voinnet, 2008).

dcl4-dcl2 double knockouts have shown increased viral susceptibility (Bouche, *et al.*, 2006). Interestingly, DCL2 has not been found to have any other function in plants, implicating the role of DCL2 as a 'back-up' to DCL4, and displaying the importance of small RNA-mediated anti-viral immunity throughout plant evolution. (+)ssRNA and (-)ssRNA genomes can also produce dsRNA secondary structures that can be acted upon by DCL1 to generate viRNAs (Blevins *et al.*, 2006; Ding and Voinnet, 2008).

DNA viruses have alternative sources of dsRNA. In the case of *Cauliflower mosaic virus*, a dsDNA virus, a hairpin-like structure at the start of its 35S-driven polycistron triggers initial cleavage by DCL1 (Moissaiard and Voinnet, 2006; Blevins *et al.*, 2006), with subsequent cleavages by DCL4 and DCL3 generating viRNAs (Moissaiard and Voinnet, 2006). In the case of the circular ssDNA geminiviruses, which replicate to produce both sense and antisense transcripts, dsRNA is produced through sense-antisense pairing of these transcripts (Vanitharaini, *et al.*, 2005).

Initially, it was thought that the cleavage of viral genomes via DCL action could suffice for viral resistance. However, viRNAs were also found to be incorporated into RISC complexes to guide the additional cleavage of viral genomes, possibly providing a more complete elimination of the invading virus (Deleris, *et al.*, 2006). viRNA RISC complexes have mostly been shown to involve AGO1 (Baumberger and Baulcombe, 2005), but it is thought that there is much redundancy and specialization amongst the ten Arabidopsis Argonaut family members (Ding and Voinnet, 2008).

The production of initial or primary viRNAs, the viRNAs produced directly from the viral genomes, is no longer believed to suffice in providing anti-viral immunity to the rapid replication of plant viruses (Xie and Guo, 2006; Ding and Voinnet, 2008). Thus, two mechanisms involving RdRP to amplify the viral silencing mechanism have evolved. Primary viRNAs that associate with sufficiently homologous ssRNA can recruit plant-derived RdRPs to convert the entire strand to dsRNA, providing lengthy substrates for DCL4 cleavage (Donoyer, *et al.*, 2005; Voinnet, 2005; Ding and Voinnet, 2008). In this manner, areas of viral genomes that may have been free from small-RNA mediated anti-viral silencing activates due to a lack of secondary structure can now be targeted by this pathway.

The mechanism behind secondary viRNA production from primary viRNA involves RDR6, the RNA-helicase SILENCING DEFECTIVE3 (SDE3), and a putative mRNA export factor SILENCING DEFECTIVE5 (SDE5; Voinnet, 2005; Ding and Voinnet, 2008). However, a second method, independent of primary viRNAs, does exist to produce viRNAs. Aberrant transcripts, such as cap- or poly-A deficient transcripts commonly produced by viral transcription, can trigger the action of RDR6, SDE3, SGS3, and AGO1 to create *de novo* dsRNA (Xie and Guo, 2006; Broderson and Voinnet, 2006; Ding and Voinnet, 2008). This dsRNA is then used as a template by DCL4 to produce secondary viRNAs.

2.2.2.2.1.2 Non-infected Tissue Immunization

The production of primary and secondary viRNAs also function to 'immunize' non-infected cells by transporting viRNAs throughout the plant ahead of the replicating virus (Voinnet, 2005; Li and Ding, 2006; Ding and Voinnet, 2008). In short-range cell-cell signalling, 10-15 cells away from infection, primary or secondary viRNA can be transported through the plasmodesmata to neighbouring cells to target incoming viral genomes (Himber, *et al.,* 2003; Donoyer, *et al.,* 2005). This process is independent of the actions of RDR6 and SDE3, but does require the three *SILENCING MOVEMENT DEFICIENT (SMD1-3)* genes, which, to date, have uncertain functions (Himber, *et al.*, 2003; Donoyer, *et al.,* 2005; Ding and Voinnet, 2008). This type of short-range transport consists of 21 nucleotide and 24 nucleotide viRNAs, suggesting action via DCL4 and DCL3 (Li and Ding, 2006), respectively, and is often sufficient to export anti-viral viRNAs into the plant vasculature (Himber, *et al.,* 2003; Voinnet; Xie and Guo, 2006).

viRNAs that reach the plant vasculature are typically 24 nucleotide species, and these are systemically transported throughout the plant via the phloem (Li and Ding, 2006). However, sufficient systemic silencing does require the activity of RDR6, which is logical considering viRNA amplification is likely necessary for sufficient copies to be distributed throughout the plant. This systemic transport of anti-viral viRNAs is thought to function in inhibiting the potential spread of the infection and to immunize against future inoculation.

However, long-range cell-cell movement, greater than 15 cells away, has been found to require the action of RDR6 and SDE3, and consists exclusively of 21 nucleotide species (Donoyer, *et al.*, 2005). Thus, these observations suggest that signal amplification in distal cells through RDR6 activity is necessary for continued transport and signalling (Himber, *et al.*, 2003; Donoyer, *et al.*, 2005; Ding and Voinnet, 2008). It can be presumed, however, that this long-range cell-cell transport, as well as systemic phloem-dependent transport, would require a complementary host transcript for the action of RDR6 to produce secondary anti-viral viRNAs. As expected, it was found that viral immunity was greatly increased with the constitutive host expression of virus-derived transgenes (Gua and Garcia, 1997; Xie and Gua, 2006). This would provide copious amounts of anti-viral viRNAs for all cells given these plants's ability to efficiently produce secondary viRNAs. It is now thought that long-distance viRNAs are sent to systemic plant parts in order to 'prime' the cells for incoming viral infection (Ding and Voinnet, 2008). This line of reasoning is supported by the action of some viral suppressors of silencing that are subsequently discussed.

2.2.2.2.1.3 The Counter-Defence

The effectiveness of small RNA-mediated anti-viral immunity has forced plants viruses to evole elaborate and sophisticated strategies to elude these mechanisms. The most well-known and well-described counter-defence is the evolution of viral suppressors of RNA silencing (VSRs), which work by disrupting the mechanisms by which plants produce and/or export anit-viral viRNAs. VSRs are so important to the viral counter-defence strategy that they are present in some manner in every thoroughly described plant virus,

and occasionally with multiple types within a single virus (Li and Ding, 2006; Ding and Voinnet, 2008).

There are greater than 35 families of VSRs (Ding and Voinnet, 2008). These are defined through function and genomic position, as primary amino acid sequence similarity is rare even within viral families (Li and Ding, 2006). However, the exact number, type, and function of many VSRs are unknown.

DsRNA-binding proteins are among the most common VSRs, such as p19 from *Tomato bushy stunt tombusvirus* (TBSV), p14 from *Pothos latent aureusvirus* (PLV), p21 from *Beet yellows closterovirus* (BYV), P15 from *Peanut clump pecluvirus* (CV), γB from *Barley stripe mosaic hordeivirus* (BSMV), and HC-Pro from *Tobacco etch potyvirus* (TEV), CP from *Turnip crinkle carmovirus* (TCV), and 2b from *Cucumber mosaic cucumovirus* (CMV; Li and Ding, 2006; Mérai, *et al.*, 2006). Most of these dsRNA binding VSRs specifically target 21-nucleotide dsRNA, the predominantly produced viRNA, sequestering that size of sRNA from function (Li and Ding, 2006; Mérai, *et al.*, 2006). Further, some of the 21-nucleotide specific binders, BYV p21, TBSV p19, TEV HC-Pro, have shown to interfere with the HEN1 dependent methylation of the 3' ends of the duplex, suggesting that in addition to sequestering these molecules, VSR-directed destabilization might also be inhibiting the anti-viral silencing mechanism (Yu, *et al.*, 2006). Recent work has found that both the *Tobacco mosaic tobamoirus* (TMV) and *Oilseed rape tobamovirus* (ORMV) replicase proteins also act to interfere with the HEN1

mediated methylation of sRNAs, suggesting they too may be size-specific dsRNA binding VSRs (Vogler, *et al.,* 2007).

The size-unspecific binders, such as PLV p14, BYL p21, TCV CP, and CMV 2b function to sequester small RNAs, as well as to prevent the formation of viRNAs by binding longer dsRNA substrates such as viral hairpins. (Chao, *et al.,* 2005; Li and Ding, 2006; Mérai, *et al.,* 2006). More studies may lead to additional or altered functions, as have been found with the CMV 2b, which additionally targets AGO1-loaded RISC complexes, preventing their function (Zhang, *et al.,* 2006).

Related to these dsRNA binding proteins are ssRNA binding proteins, such as the AC4 protein found in many Gemini viruses. These VSRs are hypothesized to function in inhibiting mature RISC complex target cleavage (Bisaro, 2006; Li and Ding, 2006).

Another function of some VSRs includes the inhibition of systemic silencing. These VSRs function to impede the cell-cell and/or systemic signalling pathways plants utilize to export small RNAs. The first demonstrated of this class was found in *Potato potexvirus X* (PVX) infection of *Nicotiana benthamiana* which was found to only undergo systemic transgene silencing in the absence of the p25 protein (Voinnet *et al.*, 2000). Investigation into the mechanism behind p25 systemic silencing suppression has brought some mixed results. Initially, it was found that p25 from PVX in *N. benthamiana* strongly inhibited the production of 24-nucleotide RNAs while 21-nucletide species displayed only minor attenuation (Hamilton, *et al.,* 2002). This model fit well with the

observation that only 24-nucleotide siRNAs are involved in the phloem-dependent silencing signal. Indeed, a similar model has been proposed for the systemic silencer 2b from CMV, whereby the sequestering of the 24-nucleotide duplex prevents the phloem-dependent systemic spread of the silencing signal (Li and Ding, 2006). However, recent work has shown that PVX p25, which doubles as a cell-cell movement protein, also suppresses silencing in the infected cell, a process that is necessary, but not sufficient, for cell-cell infection (Bayne, *et al.*, 2005). However, this discrepancy in functions could easily be explained if p25 effects the processing or function of both 21-nucleotide and 24-nucleotide viRNA species, as do some of the size-unspecific dsRNA binding protein described above.

But what is the function of suppressing the silencing signal in distal tissues? In the case of the CMV 2b protein, it plays dual roles in systemic silencing suppression as well as phloem-dependent viral transport (Brigneti, *et al.*, 1998; Guo and Ding, 2002). The fact that CMV codes for both functions suggests that the 24-nucleotide RNAs are indeed 'priming' systemic cells for an effective anti-viral response (Bayne, *et al.*, 2005). This would seem to be an advantageous mechanism for many plant viruses, as most viruses use phloem-dependent systemic transport for infection. Unfortunately, it is assumed that the number of suppressor of systemic signalling VSRs are currently underestimated as the grafting experiments that must be performed to demonstrate this function are mechanistically challenging (Li and Ding, 2006).

2.2.2.2.1.4 The Symptoms – Off-target effects?

Given the mechanistic action of VSRs, and the importance of small RNAs to normal plant gene expression, it is not surprising that viral infection leads to an array of deleterious symptoms. Testaments to this are the infection-derived developmental abnormalities that resemble miRNA-biogenesis impaired or deficient mutants (Zhang, *et al.,* 2006; Ding and Voinnet, 2008). However, recent evidence has shown that attenuation of particular miRNA pathways can increase biotic resistance, as in the case of miR-398a and *Pseudomonas syringae* infection of *Arabidopsis thaliana* (Navarro, *et al.,* 2006). In this sense, one might consider that the alteration of host small RNA pathways may be a deliberate counter-defence. Further, the incorporation of these viRNAs into RISC complexes is responsible for another phenomenon termed viral-induced gene silencing (VIGS). When viRNAs contain sufficient homology to endogenous plant transcripts these transcripts are cleaved, causing viral symptoms that can mimic knockdown mutations of the gene (Ruiz, *et al.,* 1998; Ding and Voinnet, 2008). For example, it has been found that viRNAs derived from the 35S leader of CMV carry near-perfect homology to several host mRNA genes that are targeted during infection (Moissard and Voinnet, 2006). Further, hundreds of other host transcripts were calculated to have sufficient homology to be silenced. The question now remains as to whether these targets represent a legitimate viral or plant strategy, combating infection or resistance. Likely, it is a case of both, depending on viral and host factors.

2.2.2.2.3 Abiotic Stress Resistance

It has long been known that whole concerts of genes change in response to abiotic stresses such as temperature (Kreps, *et al.,* 2002; Oono, *et al.,* 2006), water (Kreps, *et al.*, 2002; Bray, 2004), and light extremes (Kimura, *et al.,* 2003), soil salinity (Kreps, *et al.*, 2002), heavy metals (Sahr, *et al.,* 2005; Herbette, *et al*, 2006; Chiang, *et al.*, 2006), radiation (Molinier, *et al.,* 2005), and nutrient deprivation (Mission, *et al.,* 2005). Traditionally, changes in gene expression in response to these factors were thought to take place at a transcriptional level; however, an ever-increasing role of post-transcriptional events is beginning to emerge.

Although many miRNAs have been found to be regulated in response to abiotic stresses (Jones-Rhoades, *et al.,* 2004; Sunkar and Zhu; 2004; Sunkar *et al.,* 2007), only a handful of small RNAs have been ascribed pathways in which they modulate resistance or acclimation. For example, miR-399(a-f) induction upon phosphate starvation targets a ubiquitin-conjugating enzyme (PHO2) releasing the regulation of a number of phosphate homeostasis mediating genes (Fujii, *et al.,* 2005; Bari, *et al.,* 2006; Sunkar, *et al.,* 2007); miR-395 is attenuated during sulphur starvation to target ATP sulfurylases (APS1, APS3 and APS4) and a low-affinity sulfate transporter (AST68) potentially modulating the rates of sulfate translocation and assimilation (Takahashi, *et al.,* 1997; Sunkar, *et al.,* 2007); The SRO5-P5CDH nat-siRNA, which are siRNAs produced from the overlapping *SRO5* and *P5CDH* transcripts, contribute to a regulatory loop that controls ROS production and proline homeostasis, leading to salt tolerance (Borsani, *et al.,* 2005; Phillips, *et al.,* 2007). Less well understood is the role of miR-417 effecting seed germination under salt stress (Jung and Kang, 2007), and the role of severe up-regulation

of miR-393 and down-regulation of miR-389a under cold, drought, and salinity stress (Sunkar and Zhu, 2004). Further, microarrays have revealed a number of stress regulated miRNAs in response to UV treatment (Zhou, *et al.*, 2007), and cold, drought, and salt stress (Liu, *et al.*, 2008). Further, with the advances in technology that may alleviate some of the technical and monetary problems associated with small RNA microarrays, we can expect this field to expand rapidly.

Common to drought, cold, salinity, high light, and heavy metals is an increase in reactive oxygen species (ROS) in the plant (Sunkar, *et al.*, 2007). Further, ROS is a photosynthetic by-product, making ROS scavenging within plant cells a very important mechanism. CU-ZN SUPEROXIDE DISMUTASE1 and 2 (CSD1 and 2) are up-regulated in response to oxidative stress (Kliebenstein, *et al.*, 1998), and are important players in maintaining intercellular ROS homeostasis (Sunkar, *et al.*, 2006; Lu and Huang, 2006; Sunkar, *et al.*, 2007). Interestingly, despite their up-regulation in response to abiotic stress, the transcriptional level of these genes remains static (Sunkar, *et al.*, 2006). It has subsequently been shown that miR-398 targets both *CSD1* and *CSD2*, and is responsible for the up-regulation observed during oxidative stress (Sunkar, *et al.*, 2006; Sunkar, *et al.*, 2007).

2.2.3 Animals

2.2.3.1 Biogenesis, Mechanisms of Suppression, and Regulation

There are currently more than 540 mature human miRNAs listed in the official miRNA database (miRBase) representing >1.0% of all the genes in the human genome, and

potentially targeting up to one-third of the human coding genes (Fujita and Iba, 2008; Griffiths-Jones, *et al.,* 2008). These mature miRNAs are produced from dsRNA hairpin formations, pri-miRNAs, that typically derive from three types of loci with annotated transcripts: the introns of protein coding genes, the exons of non-coding genes, and the introns of non-coding genes (Rodriguez, *et al.,* 2004; Liu, *et al.,* 2008). Alternatively, these genes may derive from intergenic regions. The miRNAs that belong to the class that reside within annotated transcripts are under the direct transcriptional control of their host gene (Rodriguez, *et al.,* 2004; Liu, *et al.,* 2008).

Intergenic miRNA genes, however, are under their own transcriptional control, and in most cases are thought to be transcribed via the action of RNA polymerase II (Fujita and Iba, 2008; Liu, *et al.,* 2008). One exception is a cluster situated on chromosome 19 with numerous surrounding Alu elements that have subsequently been found to be transcribed by RNA polymerase III (Borchert, *et al.,* 2006). Further still, some of these intergenic miRNAs have shown to be regulated by some very important transcription factors such as cNF-κB (Taganov, *et al.,* 2006), c-Myc (O'Donnell, *et al.,* 2005) and p53 (He, *et al.,* 2007).

Interestingly, 36% of the miRNAs within the human genome are found in clusters≤10kB apart (Griffiths-Jones, *et al.,* 2008), and many of these are only 100-1000kB from each other. This has lead to the discovery that many miRNAs are transcribed together as a single transcriptional unit, or polycistron (Fujita and Iba, 2008; Griffiths-Jones, *et al.,* 2008). The function of the multi-miRNA polycistrons are thought to be for the efficient

targeting of a single mRNA transcript, or to target multiple transcripts in a signal molecular pathway.

After transcription, the pri-miRNA forms a stem-loop structure with a dsRNA stem of ~33 nucleotides (Liu, *et al.*, 2008). This dsRNA intermediate is then recognized by the RNaseIII-type enzyme Drosha with its dsRNA-binding partner DGCR8/Pasha (Han, *et al.,* 2004; Kim, *et al.,* 2005; Liu, *et al.,* 2008). This microprocessor than excises the dsRNA stem from the pri-miRNA stem loop, creating the pre-miRNA with a 5' monophosphate and a 3' two-nucleotide overhang (Kim, *et al.,* 2005; Liu, *et al.,* 2008). However, the pri- to pre-miRNA cropping by the microprocessor also utilizes a number of accessory proteins to produce different types, or subsets of miRNAs (Fukuda, *et al.*, 2007; Guil and Caceres, 2007).

After the excision by the microproccesor, the pre-miRNA is bound by the nuclear export factor Exportin-5 and RanGTP. This binding functions to both stabilize the duplex, protecting it from degradation, in addition to transporting the pre-miRNA to the cytoplasm (Kim, *et al.,* 2005; Liu, *et al.,* 2008). Exportin-5 and RanGTP target the duplexes to nuclear pores, whereby the hydrolysis of RanGTP to RanGDP results in the release of the pre-miRNA to the cytoplasm.

However, a very recently discovered microprocessing-independent miRNA pathway has been found in mammals. The mirtron pathway occurs when small, debranched hairpins that derive from small introns fold in such a manner as to produce 5' monophosphate and

3’ nucleotide overhang, mimicking cleavage from the microprocessor (Berezikov, *et al.*, 2007; Ruby, *et al.,* 2007). These structures are then bound by Exportin-5 and RanGTP and exported to the cytoplasm to be further processed as miRNAs.

Once in the cytoplasm, the cytoplasmic RNase-III endonuclease, Dicer, recognizes the 3’ two-nucleotide overhang and binds the pre-RNA. Recognition and binding of Dicer stimulates the cleavage of the pre-miRNA ~22-nucleotides from the 3’-OH, and produces the mature miRNA duplex with 3’ two-nucleotide overhangs (Kim, *et al.*, 2005; Liu, *et al.,* 2008).

This cleavage attracts the Argonaut protein to associate with the Dicer and miRNA:miRNA* duplex. The Argonaut protein then stimulates the dissociation of one of the strands (miRNA*) which is then degraded. Again, although the exact mechanism of strand selection is unknown in humans, it is thought to follow the asymmetry rule (Chapter 2.2.2.1). The Dicer protein than dissociates from the complex, leaving the active machinery for silencing termed the miRNA/AGO ribonucleoprotein (miRNP), also known as the RNA-induced silencing complex (RISC ; Liu, *et al.*, 2008).

The mature miRNAs within the miRNP direct silencing in animals in a sequence-specific manner, using the miRNAs as guides to bind to target mRNAs at miRNA recognition elements (MREs). These MREs are usually found in the 3’ untranslated region (UTR) of the mRNA, however recent evidence for 5’ UTR binding has also been presented (Lytle, *et al.,* 2007). There are two miRNP-mediated mechanisms behind silencing in animals,

albeit one is far more prominent. First, providing the miRNP contains AGO2 (the most common human AGO), and there is near-perfect complementarity between the miRNA and the MRE, AGO2 will direct cleavage of the target transcript (Kim, *et al.*, 2005; Liu, *et al.,* 2008). However, this is a very rare case in animals, and is found much more commonly in plants (Chapter 2.2.2.1).

The second method involves inhibiting the translational machinery, preventing the production of the target mRNAs protein product. This method is mediated by any of the human Argonauts (1-4), and needs little, although specific, MRE complementarity. In this instance, binding is dictated by small regions of perfect complementarity, called seed regions, between the miRNA:transcript duplex. In Drosophila, it was thought that the nucleotides 2 – 8 from the 5' end were involved in targeting recognition, with some miRNAs needing varying degrees of pairing at the 3' end for greater stability (Brennecke, *et al.,* 2005). More recent work in mammals maintains that the importance of the seed site, nucleotides 2 – 7 from the 5' end in mammals, but has outlined several other important pairing observations that boost miRNA:target binding affinity. These include additional pairing at the 8^{th} nucleotide, and/or an adenosine at the first position of the 5' end (Lewis, *et al.,* 2005; Krek, *et al.,* 2005). Further, as in drosophila, binding sites at the 3' region, especially at nucleotides 13 – 16 from the 5' end, can greatly enhance binding affinity (Grimson, *et al.,* 2007).

True, binding affinity affects the degree of translational inhibition, but other factors also seem to affect the efficieny of inhibition. For example, MREs flanked with A-U rich

sequences, and/or MREs that are located on the ends of the 3'UTR at least 15 nucleotides away from the stop codon have greater translational repression ability (Grimson, *et al.*, 2007). Logically, the greatest translational inhibition was found when multiple binding sites, for the same or different miRNA, were found clustered on the 3'UTR (Grimson, *et al.,* 2007).

But how does miRNP binding of the 3' UTR direct translational inhibition? The exact mechanism still eludes silencing investigators, but several working hypothesis have been suggested. The most widely proposed mechanism for translational inhibition in humans occurs at the initiation step of translation. In human cells, *in vivo* and *in vitro,* it has been found that the 5' methyl-7-guanine (m7G) cap is necessary for translational inhibition by the miRNP (Humphreys, *et al.,* 2005; Wakiyama, *et al.,* 2007). Further, it has been found that human AGOs bear similarity to the cap binding protein eIF4, presenting the model that translational inhibition occurs through competition between the miRNP and eIF4E/G complex that promotes translation (Mathonnet, *et al.,* 2007; Liu, *et al.,* 2008).

The fate of mRNAs after target inhibition is somewhat unknown. Emerging evidence suggests that mRNAs that have been translationally repressed are directed to processing-bodies (P-bodies). These P-bodies are subcellular structures containing a number of enzymes involved in deadenylation, decapping, and deregulation of mRNAs, which may ultimately lead to mRNA degradation (Liu, *et al.,* 2008).

Although miRNAs have been shown to be regulated at the transcriptional level, another method of regulating miRNAs has been discovered. The mechanism of RNA editing has recently been shown to effect mammalian miRNA silencing in several ways. RNA editing is the deamination of adenosine (A) to produce inosine (I) nucleotides in dsRNA intermediates by the enzymes Adenosine Deaminase that acts on RNA 1 and 2 (ADAR1/2; Ohman, 2007). Essentially, this inosine acts as guanine when read by translational or splicing machinery, and can form Watson-Crick pairs with uracil, adenine, or cytosine.

pri-miRNAs have been shown to undergo A → I RNA editing, and, depending on the number and locations of the As, can result in secondary structure changes that affects Drosha cleavage (Yang, *et al.*, 2006). Further, as both ADARs and Drosha have dsRNA binding domains, it has been shown that these proteins complete for substrate (Ohman, 2007).

Pre-miRNAs have also been shown to be a substrate for RNA editing, and changes produced at either the pri- or pre-miRNA level in the seed regions of the miRNAs result in miRNAs that target different transcripts (Kawahara, *et al.*, 2007).

The final method for RNA editing to effect silencing is through the A → I changes in the 3'UTRs of targeted transcripts. This has been shown to halt MRE recognition by the miRNP, resulting in the transcript avoiding miRNP regulation (Liang and Landweber, 2007; Ohman, 2007).

2.2.3.2 Functions

2.2.3.2.1 miRNAs in Development

As in plants, small regulatory RNAs play essential roles in normal animal development. As would be expected, mutations to Dicer, Ago2, and Drosha are embryo lethal in mice. It has been hypothesized that one function of miRNA-mediated gene control is through bestowing robustness to developmental programs, allowing for the control of such factors as leaky transcription and maintaining optimal mRNA expression levels (Stefani and Slack, 2008).

Unfortunately, most work on the developmental role of miRNAs in animals has been done in invertebrates and lower vertebrates, but work in mammals is beginning to find its way into the literature. For example, the brain specific miR-124 in mice has been found to play a critical role in neuronal differentiation, down-regulating hundreds of genes to help determine neuronal-specific gene pattern in mice (Sempere, *et al.*, 2004); several miRNAs are involved in correct muscle and heart development, most notably miR-1, while others have been found to be involved in such responses as cardiac hypertrophy (Stefani and Slack, 2008); various stages of T-lymphocyte development is characterized by a distinct miRNA expression pattern (Neilson, *et al.*, 2007), while other sets mediate myeloid lineage development (Fatica, *et al.*, 2006) and macrophage function (Dajlberg, and Lund, 2007). Again, theses are but a few well-studied mechanisms in miRNA and mammalian development, but to cite further examples is far beyond the scope of this review.

2.2.3.2.2 miRNAs in Cancer

Given miRNAs essential roles in development, it would be prudent to assume that they are involved in a number of diseases. Indeed, miRNA deregulation has been found in a number of human diseases, including fragile X syndrome (Plante, *et al.*, 2006), DiGeorge syndrome (Landthaler, *et al.*, 2004), schizophrenia (Hansen, *et al.*, 2007) and, most notably, cancer (Cho, 2007; Croce, 2008). MiRNA deregulation has been found in every type of analyzed cancer (Barbarotto, *et al.*, 2008).

The classic example of miRNAs in cancer development and progression is that of miR-16 and -15 in chronic lymphocytic leukemia (CLL; Calin, *et al.*, 2002; Calin, *et al.*, 2005). These miRNAs were found severely down-regulated or deleted in ~70% of CLLs (Calin *et al.*, 2002). It was later found that miR-16 and -15 were responsible for targeting the anti-apoptotic factor B-cell lymphoma 2 (BCL2; Cimmino, *et al.*, 2005), and this loss of miRNA was one of the root causes of the BCL2 overexpression observed in most CLL cases (Calin, *et al.*, 2005; Cimmino, *et al.*, 2005). Thus, loss of expression of these miRNAs resulted in increased expression of BCL2 which inhibited apoptosis, one of the hallmarks of cancer. In this instance, miR-16 and -15 are miRNAs functioning as a tumour suppressor; however, another classic example of miRNAs in cancer has miRNAs functioning as oncogenes.

The miR-17-92 cluster contains six miRNAs that are transcribed together as a single polycistron. This cluster was first found to be up-regulated in B-cell lymphoma (He, *et al.*, 2005), and then in lung cancer (Hayashita, *et al.*, 2005). These studies demonstrated

that the up-regulation of these miRNAs were correlated to increased levels of cellular proliferation and decreased levels of apoptosis. A model soon arose that the oncogene c-Myc was activating these miRNAs, which in turn, were found to be targeting the E2F1/3 transcription factor. It is believed that this feedback mechanism pushes the cell towards a pro-proliferate state (Mendell, 2005; Cho, 2007). Many other cancers are now showing the up-regulation of these 'oncomirs,' which is logical as c-Myc has been found to be up-regulated or misregulated in most tumours and tumour-types (Hermeking, 2003).

But under what circumstances is miRNA expression becoming deregulated? In the case of the miR-17-92 cluster, it is a case of transcription factor deregulation preceding miRNA deregulation. Indeed, this has been found for p53 (He, *et al.*, 2007) and NF-κB (Taganov, *et al.*, 2006) responsive miRNAs as well. However, it has been found that greater than 50% of the miRNAs found to date reside at or near fragile site loci, which are prone to breakage and rearrangement in cancer cells (Calin, *et al.*, 2005). The deletion or amplification of these loci have been shown to greatly alter miRNA expression (Cho, 2007; Croce, 2008). In fact, the frequent down-regulation of miR-16 and -15 are due to chromosomal deletions or mutations at 13q13.4, a fragile site associated with CLL (Calin, *et al.*, 2002).

Another mechanism for altering miRNA expression patterns in cancers is a result of the hyper- or hypomethylation of CpG islands in the promoters of miRNA genes. The addition or subtraction of methyl groups in the promoter area has long been associated with traditional oncogenes and tumour suppressors (Weber, *et al.*, 2007).

miRNAs have become an ever-increasing interest in cancer biology, as their ability to modulate the expression patterns of many important cellular transcripts provides an interesting focal point for study. Further, they have become important markers in diagnosing particular cancers, as well as giving strong indications of prognosis (Barbarotto, *et al.*, 2008; Croce, 2008). Naturally, the use of non-coding regulatory small RNAs has been implicated in the future of cancer therapy (Izquierdo, 2005).

2.2.3.2.2 miRNAs in Radiation Response

Despite all the different radiation exposures we will potentially receive throughout our lifetime, there is little literature on the effects of this genotoxic stress on miRNA expression patterns. Considering the potential effects changes in the micrRNome may have on the cellular phenotype, this is certainly a field that warrants study.

The first look at miRNA expression patterns in response to γ-radiation found no real expression changes in an immortalized lymphoblast cell line (Marsit, *et al.*, 2006). Conversely, Ishii and Saito (2006) showed profound changes in the microRNome murine embryonic stem cells in response to radiation.

In a pivotal work, bringing the miRNA and radiation response *in vivo,* Weidhaas *et al.* (2007) found numerous miRNAs significantly deregulated in response to γ-radiation in a lung cancer cell line (A549) and *in vivo* in a radiation responsive worm model. Further, this laboratory showed that down-regulation of a particular family of miRNAs conferred

radioresistance the cell line and the worm. This work displayed that miRNAs do play an important role in radiation response.

The only mammalian *in vivo* analysis of miRNA expression in response to direct radiation exposure comes from unpublished data from our laboratory. In this experiment, we looked at the response of different brain tissues to ionizing radiation. We found significant deregulation of the micoRNAome in response to radiation that was both tissue- and sex-specific. Concurrently, the Kovalchuk laboratory has found deregulation of miRNAs in the protected spleen of cranially exposed mice, demonstrating regulation of miRNAs in response to bystander effects (Koturbash, *et al.*, 2007).

3.0 LOCAL AND SYSTEMIC MIRNA REGULATION IN RESPONSE TO BIOTIC AND ABIOTIC STRESS MODULATES AUXIN RESPONSE IN ARABIDOPSIS

3.1 INTRODUCTION

The systemic transmission of stress signals is a well-known phenomenon in plant molecular biology. The local application of biotic or abiotic stresses to plant tissue can lead to global changes in pathogen resistance (Grant and Lamb, 2006), methylation pattern (Boyko, *et al.*, 2007), recombination rate (Kovalchuk, *et al.*, 2003), hormone levels (Schilmiller and Howe, 2006) and gene expression (Truman, *et al.*, 2006). This laboratory has previously shown the existence of a systemic signal that leads to changes in the genome stability of local biotically infected (Kovalchuk, *et al.*, 2003) and local abiotically treated (Filkowski, *et al.*, 2004) leaves. This signal, termed the systemic recombination signal (SRS), has not been further characterized since its principle discoveries.

Auxin is a phytohormone essential to the normal growth and development of plants (Leyser, 2002; Woodward and Bartel, 2005; Teale, *et al*., 2006). Its influence ranges throughout plant development from the earliest stages of embryogenesis (Geldner, *et al.*, 2000; Weijers, and Jürgens, 2005) to the development of sexually mature flowers (Benkova, *et al.*, 2003; Aloni, *et al.*, 2006) as well as having important roles for photo- and gravitropic responses (Friml, *et al.*, 2002; Woodward and Bartel, 2005). Many other phytohormones have been shown to change both locally and systemically in response to stress; for example, jasmonic acid in systemic wound infection (Schilmiller and Howe, 2006) and ethylene production in systemic acquired resistance (Grant and Lamb, 2006).

However, changes in auxin levels in response to pathogen challenge and reactive oxygen producing stresses has not been recently explored.

microRNAs are small, regulatory RNAs with roles in down-regulating target messenger RNAs (mRNA), primarily through mRNA cleavage (see Chapter 2.2.2). Like auxin, they play diverse roles throughout the plant life cycle to help regulate the spatial and/or temporal regulation of important development genes (Jones-Rhoades, *et al.*, 2006). Indeed, a number of small RNAs regulate key factors in the auxin response pathway (Eckardt, NA., 2005). Further, small RNA biogenesis has been shown to be involved in anti-viral defence through the recognition of viral double-stranded (ds)RNA genomes or dsRNA intermediates of viral replication. This foreign dsRNA is cleaved and viral small RNAs (viRNAs) are produced that can be systemically transported throughout the plant as a form of immunization (Voinnet, 2005; Li and Ding, 2006; Ding and Voinnet, 2008).

Naturally, such an effective defence system in plants has had an impact on viral evolution. Nearly every plant virus to date has been ascribed a viral suppressor of RNA silencing (VSR; Li and Ding, 2006; Ding and Voinnet, 2008), which act to suppress directed cleavage of viral genomes. However, the suppression of viral silencing through VSRs may also suppress endogenous pathways of silencing, a mechanism thought to be responsible for much of the symptom progression in viral infected plants (Ruiz, *et al.*, 1998; Ding and Voinnet, 2008).

The goal of this research was to assay the roles of plant miRNAs involved in auxin response in local and systemic leaves of viral infected (O*ilseed rape tobamovirus;* ORMV) and Rose Bengal (RB) treated leaves. Further, changes in the expression of these miRNAs were related to changes seen in *in vivo* auxin signalling using the *DR5::GUS* construct. Overall, we found a decrease in miRNA expression in treated leaves from both stresses, which coincided with a down-regulation of auxin response. MiRNAs were also found to be changed significantly in systemic leaves in both stresses. Further, a large increase in auxin response was found in systemic leaves 24-hours after infection. These changes are discussed in detail, and several mechanisms for their regulation are suggested.

3.2 METHODS

3.2.1 Plant Growth Conditions

For the miRNA Northern blot analyses, *Arabidopsis thaliana* line 15D8 (Columbia ecotype) was planted in 3:1 all-purpose potting soil (Plant Etc., Lethbridge, Canada) to Terra-Lite 2000 fine vermiculite (Peavey Mart, Lethbridge, Canada), stratified for 48 hours at 4^{o}C, and then grown in Enconair growth chambers (Winnipeg, Canada) at 16/8 hours light/dark, at 23^{o}C and 18^{o}C, respectively. At eight days post germination (dpg), plants were transplanted to single pots. Two fully expanded true leaves were treated with various stresses at 32 dpg. 24 hours after stress application, the two treated leaves (24I), as well as two non-treated leaves (24S), were harvested and flash frozen in $N_{2(l)}$. 6 days after treatment, a second set of non-treated leaves (6dS) were harvested and frozen.

For auxin expression analyses, DR5::GUS lines were kindly obtained from Dr. Elizabeth Schultz (University of Lethbridge). Plants were then grown and treated in exactly the same manner as the plants for the miRNA analyses.

3.2.2 Rose Bengal Treatment

A 20 mmol working solution of Rose Bengal (RB; Fisher) was prepared in ddH_2O. 10 µL of the solution was added and spread on the fully expanded 6^{th} and 7^{th} leaves. Treated leaves were rinsed with ddH_2O prior to their harvest.

3.2.3 ORMV Treatment

The 6^{th} and 7^{th} fully expanded leaves were marked and lightly dusted with silicon carbide. 10 µL of 25 ng/µL *Oilseed rape mosaic virus* in buffer (10 mM Phosphate buffer pH 7.0; 82 mg/L Na_2HPO_4, 50 mg/L NaH_2PO_4) or 10 µL of buffer alone (mock) was added to each leaf. The solution was gently rubbed into the leaf tissue with the silicon carbide in order to lightly score the leaves. Treated leaves were rinsed with ddH_2O prior to their harvest.

3.2.4 RNA Extraction, Size Fractionation & Northern Blot Analysis

A total of 10 leaves from five different plants from each treatment were pooled together and homogenized in $N_{2(l)}$. RNA was then extracted from the homogenate using TRIzol (Invitrogen) as per the manufacture's protocol. RNAs of ~200 nt and less were than fractionated from total RNA using Mini RNeasy Kits (Qiagen) and the RNA clean-up protocol. sRNA fractionations (6-10 µg) were roto-vacuumed and the sRNA fractionate

was resuspended in 10 µL of loading dye (95% formamide, 20 mM EDTA pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples were heated to 95^{o}C for two minutes before being loaded onto an 8 M Urea 18% polyacrylamide gel (19:1 acrylamide to bis-acrylamide; BioRad). The gel was run for 90 minutes at 300 V and then electrotransferred to Gene Screen Plus (Perkin Elmer) membranes in 1X TBE at 40 V overnight at 4^{o}C. Northern blot analysis with each probe (Table 3.1) was done as follows. Membranes were pre-hybridized for 2 hours in UltraHyb-Oligo Buffer (Ambion). Probes were prepared by endlabelling DNA oligos (IDT) with [γ^{32}P]-ATP (6000 µCi/mmol; Perkin Elmer) using T4 polynucleotide kinase (NEB), and purified using MicroSpin G-25 columns (Amersham) according to the manufacturer's protocol. Probes were hybridized to membranes in a hybridization chamber set at 35^{o}C and low rotisserie speed for 12-16 hours. The blots were than washed three times with 2XSSC, 0.5% SDS for 30 min at 35^{o}C, and set to develop on Kodak BioMAX MR films at -80^{o}C for 12-24 hours, or until sufficient exposure. Membranes were stripped for repeated hybridization using 80^{o}C 0.1% SDS for one hour, followed by a rinse with 2xSSC for 15 min. Films were analyzed and quantified using ImageJ software (Abramoff, *et al.*, 2004), using the 5S ribosomal sRNAs as loading controls. Statistics were performed in MS Excel 2003 professional.

3.2.5 DR5::GUS Expression and Histochemical Staining

Two treated leaves (24I) and two non-treated leaves (24S) of the DR5::GUS line were harvested 24 hours after treatment from 5 and 10 plants for treatment and controls, respectively. Non-infected tissues six days after treatment was not collected as these leaves were still small and developing, likely providing a large bias in auxin expression.

Harvested 24I and 24S leaves were then immediately placed in GUS staining solution (100 mg of 5-bromo-4-chloro-3-indolyl glucuronide substrate (GBT) in 300 mL of 100 mM phosphate buffer (pH 7.0), 0.05% NaN_3, and 1 mL dimethylformamide) and vacuum infiltrated for 10 minutes. Plants were incubated in the staining solution for 37^{o}C for 48 hours, and then destained with 70% ethanol for 24 hours. The leaves were then moved to 100% ethanol for one hour, and then 30% ethanol for 30 minutes. Leaves were then moved to 50% glycerol for one hour, and then carefully mounted on glass microscope slides in 50% glycerol. The coverslip was applied and then sealed together with an adhesive. Individual leaves were then carefully photographed (Nikon Coolpix 4800 ED), and the intensity and area of leaf showing transgene expression was analyzed using Scion Image (Scion Image Software, Frederick, MD). Leaves that showed complete lack of or very little GUS expression were removed from the analysis. The final numbers displayed as 'GUS expression' represents the intensity of the GUS staining multiplied by the area of leaf that the staining covered. Statistics were performed in MS Excel 2003 professional.

A 35S::GUS transgenic line was then treated with ORMV and RB to confirm that the wounding and RB treatments themselves were not responsible for interference with the GUS substrate and/or staining procedure.

3.3 RESULTS

3.3.1 Northern Blots for Auxin-Related miRNAs

Arabidopsis thaliana plants were treated with then treated with either of the two stresses, *Oilseed rape tobamovirus* (ORMV) and Rose Bengal (RB). The 6th and 7th rosette leaves were treated with the stress, and 24 hours after infection the two treated leaves (24I) and two non-treated leaves, the 8th and 9th rosette leaves (24S), were harvested. Six days following treatment, two additional non-treated leaves were harvested (6dS). A pilot experiment of Northern blots to assay levels of possible stress-responsive miRNAs found that both miR-171b and miR-393 were dramatically changed in infected and systemic tissue. As both of these miRNAs were auxin related, we tested two further auxin-related miRNAs, miR-160 and -167, in an independent repeat that included miR-171b and -393. Thus, miR-171b and miR-393 have included in the statistics a separate independent trail, whilst miR-160 and miR-167 contain only a technical repeat from frozen tissue.

Since ORMV can produce a systemic infection in *Arabidopsis thaliana* (Aguilar, *et al.*, 1996; Whitham, *et al.*, 2003), it was important to show that the 24-hour incubation time on the inoculated leaf was not sufficient to produce a systemic infection in distal tissues. Northern blot analysis of infected and non-infected leaves were performed using a previously published set of probes detecting ORMV-derived viRNAs produced upon compatible infection (Table 3.1; Akbergenov, *et al.*, 2006). This blot contained ORMV infected, mock-infected, and control plants at the 24I, 24S and 6dS time points, as well as a lane containing RNA from systemically infected plants (+). This blot showed that 24 hours after infection no viRNAs were detectable, indicating that this was not sufficient

for the virus to move and replicate in systemic tissue nor time enough for the transport of viRNAs (Figure 3.1).

Representative blots from the ORMV infection experiment can be seen in Figure 3.2, and displayed related to the loading control in Figure 3.3A. Infection of leaves with ORMV resulted in a severe down-regulation of 3 of the 4 miRNAs 24 hours after infection (-1.47, -2.04, and -1.58-fold for miR-160, -171b, and -393, respectively). This significant result was not reflected in the mock treatment, suggesting that it is the viral infection, not wounding, which is responsible for the significant down-regulation.

In non-infected tissue 24-hours after infection, the trend for the majority of the miRNAs appeared to be up-regulation. We did find a significant increase in miR-167 (~1.20-fold), with a concordant decrease in miR-393 (-1.25-fold).

Interestingly, the auxin-related miRNAs have a large reduction in six-day non-infected tissue, with significant down-regulation of miR-171b and -393 (-1.27 and -1.57-fold respectively). This response suggests that the changes in miRNA expression in response to viral infection are persistent.

Rose Bengal treatment showed some very consistent and interesting results (Figure 3.2 and 3.3B). In the treated leaves 24 hours post-treatment, an overall downward trend of ~1.25 is seen for all the miRNAs. In the non-treated leaves 24 hours post-treatment, all the miRNAs show a ~1.35-fold up-regulation. For miR-171b and -393, which contain an

independent repeat, these changes were found to be significantly different than the controls ($p<0.05$). The non-treated leaves 6 days after treatment appear to return to the control levels.

3.3.2 DR5::GUS Expression Analysis

In an attempt to try to correlate the changes in auxin-related miRNA expression to actual auxin response, DR5::GUS lines were subjected to the same experimental design. This line is widely used as a marker to study endogenous auxin distribution, as it provides a common and reliable reporter gene system to study auxin-responsive transcriptional activation (Ulmasov, *et al.*, 1997; Nakamura, *et al.*, 2003).

We found a remarkable correlation between the auxin-related miRNAs and auxin transcriptional activation (Figure 3.4). In the ORMV experiment, *in vivo* auxin response mimicked what was seen in the Northern blot analysis. In the infected leaves, auxin levels dropped off from the control over 1.5-fold, while in the non-treated leaves auxin levels were found to be 1.74-fold up-regulated. Curiously, we found a significant down-regulation in non-treated leaves 24 hours post-infection in the mock-infected leaves. This appears to represent a wound response in the plants; however, although a trend exists, there is no concurrent down-regulation in the auxin-related miRNAs in the mock treatments. Further, if systemic wound response is causing a negative effect on auxin response, and the systemic viral response is having a positive effect, the actual effect of auxin response change in systemic leaves is much larger (>3-fold).

The RB experiments also showed a relationship to miRNA expression patterns, as the treated leaves showed a dramatic 4.5-fold reduction in auxin-inducible expression. However, dramatic decrease in GUS expression could be due to changes in the auxin levels, or the chemical RB interfering with GUS expression. However, preliminary experiments using *35S::GUS* lines with the viral wounding and the RB treatment suggests no deregulation of GUS activity in response to these treatments (data not shown).

Although we found an upward trend in auxin-related miRNA expression in non-treated leaves 24 hours post-treatment, we found no significant changes in these leaves in the DR5::GUS expression analysis.

3.4 DISCUSSION

A systemic response to environmental cues is a common plant occurrence that includes such phenomena as systemic acquired resistance (SAR; Dong, 2001; Grant and and Lamb, 2006), systemic wound signalling (SWS; Pearce, *et al.,* 2001; Browse, 2005), systemic recombination (SRS; Kovalchuk, *et al.,* 2003; Boyko, *et al,* 2007) systemic acquired acclimation to light (Karpinski, *et al.,* 1999), systemic induction of flowering (An, *et al.,* 2004) and systemic post-transcriptional RNA silencing (Waterhouse, *et al.,* 2001; Mlotshwa, *et al.,* 2002). Having been the first laboratory to show the existence of the SRS, we found a particular interest in the local and systemic responses of microRNAs (miRNAs) in response to biotic and abiotic stress. miRNAs are small, non-coding RNAs that work to target mRNAs for degradation or translational inhibition (See Chapter 2.2.2).

Post-transcriptional regulation of gene expression via these non-coding RNAs has become a hot topic in plant biology, and they have been shown to play vital roles in such process as seed germination (Reyes and Chua, 2007), embryogenesis (Lou, *et al.*, 2006), flowering time and flower development (Aukerman and Sakai, 2003), root initiation and development (Gou, *et al.*, 2005), leaf morphogenesis and polarity (Mallory, *et al.*, 2004; Xu, *et al.*, 2007), biotic stress resistance (Navarro, *et al.*, 2006), and abiotic stress resistance (Sunkar, *et al.*, 2007), among others. However, the role of these small RNAs in the systemic stress response in the literature is nearly non-existent.

A pilot experiment analyzing some previously regulted stress-induced miRNAs found that several of the miRNAs were involved in auxin response. Further, of all the miRNAs, the single miRNA that was known to be regulated in biotic stress, miR-393 (Navarro, *et al.*, 2006), did so by targeting one of the most important proteins in auxin response, *transport inhibitor response 1* (*TIR1*). Thus, as both miRNAs and auxin are known to be related to stress response, we tried to relate the two phenomena.

The auxin response has been studied for decades, with large advances occurring within the last several years. For example, the first auxin receptor was just recently discovered (Dharmasiri *et al.*, 2005). The current model suggests that auxin response is modulated through auxin recognition, followed by specific protein degradation, leading to the induction of auxin-inducible genes (Summarized in Figure 3.5). Some of the main players in this response are the auxin response factors (ARFs) which, in the cases of ARF5-8 and 19 (Woodward and Bartel, 2005; Guilfoyle and Hagan, 2007), are directly responsible for

the activation of auxin inducible genes. However, the remaining 18 ARFs, which bind the same auxin response elements (AREs; TGTCTC; Ballas *et al.*, 1993; Woodward and Bartel, 2005) act to repress auxin inducible genes (Guilfoyle and Hagan, 2007). It is currently thought that these negative regulating ARFs have evolved to provide a competitive inhibitory system to further modulate auxin response.

3.4.1 Virus-regulated miRNAs

Early work predicting miRNA targets found that miR-160 and -167 had strong potential for targeting ARFs (Rhodes, *et al.*, 2002). Subsequently, miR-160 has been studied in seed germination and post-embryonic development (Lui, *et al.*, 2007), and extensively in root development (Mallory, *et al.*, 2005; Wang, *et al.*, 2005). Together, these sources show an intimate relationship between miR-160 and the ARF subgroup of ARF10, 16, and 17. Importantly for our study, miR-160 was found to be expressed in mature leaf tissue (Mallory, *et al.*, 2005; Wang, *et al.*, 2005). Our data show that miR-160 is significantly down-regulated in plants exposed to viral infection 24 hours post inoculation, with no significant expression changes in systemic tissue 24 or 6 days after infection. Given the previous association of this miRNA with ARF10, 16, and 17, this suggests an up-regulation of the target ARFs in 24-hour post-infected tissue.

This ARF group has been shown to act as repressors on AREs (Mallory, *et al.*, 2005; Wang, *et al.*, 2005); as such, an increase in ARFs 10, 16, and 17 could potentially sway the intercellular activating-ARF to repressing-ARF ratio to a state that would decrease intercellular auxin response. DR5::GUS staining of infected leaves 24 hours post

infection substantiates this hypothesis. However, further work would have to substantiate whether a small change in a single miRNA gene could account for the loss of auxin-responsive transcription we observed in the DR5::GUS experiments in infected tissue.

miR-167 also changed in response to ORMV infection. Our experiments show up-regulation in non-infected tissue 24 hours after infection and down-regulation in non-infected tissue 6 days post-inoculation. miR-167 has also been studied since the first miRNA targeting ARF predictions, and has been shown to down-regulate the enhancer ARFs 6 and 8 in both Arabidopsis and rice (Wu, *et al.,* 2006; Yang, *et al.,* 2006). In Arabidopsis, ARFs 6 and 8 were found to be critical in floral development and fertility (Wu, *et al.,* 2006; Ru, *et al*., 2006). In fact, the control of miR-167 was postulated to act to repress ectopic expression of their target genes (Wu, *et al.,* 2006), similarly to the miRNA mechanism behind abaxial/adaxial tissue differentiation via the miRNA pair *miR165/166* (Kidner, *et al.,* 2004; Mallory, *et al.,* 2004). This is logical, considering expression of miR-167 was amongst the highest of the miRNAs assayed. Indeed, it has been suggested that miR-167 is inducible by auxin, and given the increase auxin levels found in non-infected tissue 24 hours post-infection, this correlates with our results. However, an increase in miR-167 would presumably down-regulate the activating ARFs it targets, lowering auxin response. What we may be witnessing here is a feedback loop mechanism, whereby higher auxin levels trigger the repression of activating ARFs through miRNA expression.

One way in which cells can manipulate intercellular auxin levels is through auxin conjugation, sequestering the molecule from signalling in the cell. Interestingly, both of these ARF-targeting miRNAs target ARFs with well-established roles in modulating genes involved in auxin conjugation. miR-160 directed cleavage of ARF17 down-regulated several members of the GH3-like auxin conjugating genes while miR-167 cleavage of ARF8 also attenuates several other members of the GH3-family. It has been suggested that both miR160 and miR167 co-ordinately regulate GH3-like mRNA expression, thus controlling free auxin levels in the cell via the auxin conjugation (Mallory, *et al.,* 2004). Again, when one considers the increase in ARF17 expression in response to decreases of miR-160 in 24I tissue, an increase in GH3-family transcripts should result, thus decreasing auxin response as seen.

Given these results, one may think that these miRNAs are auxin responsive themselves. However, there are contradictory publications showing the inducability of these miRNAs to exogenous auxin sources. Mallory, *et al.* (2005), showed that auxin application did not induce miR-160 or -167, nor did levels of ARF17 or ARF17 cleavage products change in response to application. In contrast, Yang *et al.* (2006) found that in rice cell suspensions, miR-167 was strongly induced by application of auxin to the media. Wang *et al.* (2005) found that ARF16 was strongly induced by exogenous auxin, unfortunately, however, they did not coincidentlly measure miR-160 levels. Given these contradictory results and the importance these miRNAs play in auxin response, it is prudent that a thorough experiment settles these questions. Further, the question cannot be answered simply through the application of exogenous auxin sources, as this may function to indirectly

change miRNA expression levels. *In silico* experiments looking at ARF binding elements in the promoters of these miRNA genes, with further confirmed ARF binding through such experiments as chromatin immunoprecipitation, will settle this question.

miR-171, previously referred to as miR-39, has three loci in Arabidopsis, a, b, and c, in which b and c have identical sequences, while a is out-of-frame by three nucleotides. Mir-171b and c, which corresponds to our probe, are perfectly complementary to three genes of the GRAS family of transcription factors, *SCARECROW-LIKE6-II, SCL6-III, and SLC6-IV* (or *SCL6, SCL22, SCL27,* respectively; Llave, *et al.,* 2002; Bolle, 2004). The GRAS family of transcription factors have diverse functions including signal transduction, root/shoot radial patterning, axillary meristem formation, and gametogenesis (Tain, *et al.,* 2004). Unfortunately, these SCLs have no characterized function.

Although miR-171b/c is not known to directly affect auxin levels *in planta,* we looked at its expression for two reasons. Firstly, members of the SCL family have been previously shown to be auxin responsive (Bolle, 2004; Gao, *et al.,* 2004; Sánchez, *et al.,* 2007). Secondly, miR-171b/c has previously been shown to be regulated by abiotic stress (Sunkar and Zhu, 2004; Liu, *et al.,* 2008).

MiR-171b/c is severely down-regulated (~2-fold) in infected tissue and 6-days after infection. If the SCL6s are indeed auxin-induced, the lack of auxin in the 24I leaves could trigger release of suppression by the miRNA pathway. However, given this miRNA

is down-regulated in cold, drought, and salinity stress (Liu, *et al.,* 2008) and in our reactive oxygen stress (Figure 3.3B), it is likely that miR-171 is a common stress-regulator. If so, this warrants a deeper look into the function of the SCL6 transcription factors and the regulation of miR-171, as they may have a universal response to stress in Arabidopsis.

miR-393 is an important miRNA as it has been shown to target *TRANSPORT INHIBITOR1 (TIR1)* and its three functional paralogues *AUXIN SIGNALLING FBOX 1-3 (AFB1-3),* to-date the only known auxin receptors in Arabidopsis (Figure 3.5). MiR-393 has previously been shown to be regulated by abiotic stress (Sunkar and Zhu, 2004) and biotic stress (Navarro, *et al.,* 2006). In response to bacterial challenge, Arabidopsis was found to have increased levels of miR-393, which was correlated to a decrease in auxin signalling. This decrease in auxin signalling was then associated with an increase in bacterial resistance (Navarro, *et al.,* 2006).

The activity of miR-393 in response to viral infection is both dramatic and persistent. In our results, we find a significant decrease in both systemic and infected tissue that extended to 6-days post-infection. Unfortunately, this change in miR-393 expression does not correlate with auxin expression in the 24I leaves. A drop in miR-393 expression should correlate with an increase in auxin response. This can be seen as suggesting that decreased auxin-response found in infected leaves is independent of miRNA expression, and is modulated in another manner. However, in 24S leaves, miR-393 is regulated in such a manner that reflects the auxin signalling found in the *DR5::GUS* experiments.

The expression patterns of the miRNAs that can potentially affect auxin signalling in Arabidopsis do not necessarily reflect the auxin levels found in the treated tissue, except in the instance of miR-160, but especially in the instance of miR-393. This could suggest that auxin response in infected tissue is modified in another manner, independent of miRNA expression. It would be interesting to examine further the method in which auxin signalling is being suppressed in infected tissue (ie, transport, conjugation, catabolism) and what is triggering this response.

However, when one considers the case of the down-regulation of nearly all the miRNAs looked in this study (miR-168 and miR-173 were two additional miRNAs that were down-regulated in infected tissue, data not shown), one could make a case for the action of viral suppressors of RNA silencing (VSRs; See Chapter 2.2.2.2.1.3). Indeed, ORMV has been found to interfere with the HEN1-mediated methylation of endogenous miRNAs in a manner that is proposed to cause miRNA degradation (Akbergenov, *et al.*, 2006; Vogler, *et al.,* 2007). Further, HEN1 inhibition in Arabidopsis has specifically been shown to degrade miR-171 (Boutet, *et al.,* 2003). It is possible that our data suggests that auxin response is important to dismantle in response to biotic stress. Indeed, this was shown by Navarro *et al.* (2006) in the case of *Pseaudomonas syringae* and miR-393. However, in the case of viral infection, an alternative pathway to miRNAs has evolved on the account of VSRs modulating small RNA pathways. Much further research will have to be conducted to show this speculated plant-derived ‘counter-counter’ defence.

In fact, some of the research may have already begun. It has been shown that the *Tobacco mosaic tobamovirus* (TMV), which is of the same genus as ORMV, has a replicase protein that interacts directly with several Aux/IAAs modulating auxin response upon infection (See Figure 3.5; Padmanabhan, *et al.*, 2005; Padmanabhan, *et al.*, 2006) and tomato (Padmanabhan, *et al.*, 2008). Upon further investigation, these authors found that ~30% of all the genes regulated in TMV infection of Arabidopsis have two or more AREs, and are indeed regulated by auxin response (Padmanabhan, *et al.*, 2005). The model these authors suggest is the binding and sequestering of the Aux/IAAs by the TMV replicase protein, which would mimick auxin signalling in the cell, thus driving auxin-stimulated gene expression (Padmanabhan, *et al.*, 2005; Padmanabhan, *et al.*, 2006). Indeed, providing this mechanism is similar in ORMV infection, this could explain the large decrease in auxin response. Auxin response has a number of feedback mechanisms to control its intercellular levels of active auxin (Leyser, 2002; Friml, 2003), and if infection is mimicking high-auxin levels this could result in the evacuation, conjugation, or destruction of free auxin in an attempt to re-regulate gene expression.

But what about the very large increase in auxin in the systemic leaves? It is very unlikely that the free auxin 'missing' from the infected leaves has transported to the systemic leaves, as this would involve the complete relocalization of the PIN-FORMED (PIN) proteins involved in auxin transport. Interestingly, however, it has been shown that high auxin intercellular levels can inhibit viral replication, possibly through ethylene production leading to the expression of resistance genes (Clarke, *et al.*, 1998). Certainly, this scenario makes sense in our model, as we have ~3-fold increase in DR5::GUS

expression in 24S leaves. Further, the miRNA expression patterns that we see in systemic leaves could be attenuated to accommodate high auxin expression levels. However, the systemic signal from the infected to non-infected leaves will remain unclear. It cannot be a case of VIGS, as the systemic leaves were devoid of any pieces of the viral genome (Figure 3.1). It has been postulated that small RNAs can move from the local site of infection to systemic leaves ahead of the virus, but we have no evidence to support this model. It would be interesting to measure some of the other hormones in systemic tissue to see if more mobile phytohormones, such as ethylene or jsamonic acid, are involved in this auxin-modulating and miRNA expression-changing viral infection response.

Interesting is the finding that three of the four miRNAs being significantly different at 6 days post-infection. Further, the single miRNA that is not down-regulated shows a strong trend towards down-regulation. This seems to demonstate that these miRNAs are persistenly regulated after pathogen infection. Further, they seem to be attenuated in such a manner as to represent an up-regulation of auxin signalling. This persistant response to pathogen attack is an important finding that needs to be further investestagted.

3.4.2 ROS-regulated miRNAs and auxin response

The RB-induced changes in miRNA response were very consistent within each other in both treated and systemic tissue (Figure 3.3B); further, this treatment caused a significant reduction in auxin signalling in treated tissue (Figure 3.4). RB ($C_{20}H_2Cl_4I_4Na_2O_5$) produces reactive oxygen species *in planta* through its photoactivation leading to the production of singlet oxygen and superoxide anion (Filkowski, *et al.*, 2004), which can

quickly interchange into other ROS species such as hydrogen peroxide (H_2O_2) and ozone (O_3).

Reactive oxygen species in plants are a very curious set of molecules. They are both genotoxic and cytotoxic, but necessary, as they play crucial roles in signalling, development, and defence (Kwak, *et al.,* 2006). ROS also accumulates in plants under the abiotic stresses of cold, salinity, and drought (Sunkar, *et al.,* 2007), as well as in incompatible, R-mediated resistance to pathogens (Torres, *et al.,* 2006).

Treatment resulted in the uniform down-regulation of miRNAs in 24I leaves, as well as severe down-regulation of auxin signalling. The uniform down-regulation of miRNAs in response to severe ROS stress is a truly novel finding. It seems logical to postulate that the increases in intercellular ROS are interfering with some of the miRNA biogenesis machinery. Interestingly, miRNA regulation of oxidative stress has been shown before. miR-398 targets *CU-ZN SUPEROXIDE DISMUTASE1* and *2* (*CSD1* and *2*), which are up-regulated in response to oxidative stress (Sunkar, *et al.,* 2006). The authors show a coordinate decrease in expression of miR-398 under these oxidative stress conditions, and provide evidence that resistance is mediated through a decrease in miR-398 expression. However, the expression levels of all other miRNAs are not evaluated. If our results are reliable, they would suggest that the mass decrease of many miRNAs occurs under oxidative conditions, and the decrease of miR-398 is a consequence of this down-regulation. Further experiments would have to confirm this speculation. Contrary to this hypothesis is the up-regulation of a number of miRNAs during cold, drought and salt

stress (Sunkar, *et al.*, 2007), but the severity and particulars of the oxidative stress produced by our RB treatment versus the oxidative stress produced by these stresses may be very different.

Interestingly, the RB treatment resulted in a consistent up-regulation of the same miRNAs in systemic tissue. ROS have long been shown to be involved in the generation of systemic signals such as systemic acquired resistance (Grant and Lamb, 2006). Further, our lab has previously shown that the local application of RB results in a clear systemic recombination signal, whereby the homologous recombination frequrncy in systemic tissue increased over 3-fold (Filkowski, *et al.,* 2004). Could this be a case of attenuation of the microRNome by the SRS? Indeed, we saw the systemic attenuation of miRNAs in response to local viral challenge, another stress shown to elicit the SRS (Kovalchuk, *et al.,* 2003). Further investigation will have to be done to help merge the SRS with changing miRNA levels in systemic leaves.

The dramatic change in auxin level in response to the RB treatment proffers several hypotheses. First, an increase in reactive oxygen species in the cell triggers the up-regulation and activation of a number of superoxide dismutases (SODs) and peroxidases (Noctor and Foyer, 1998). Interestingly, it has been shown that increases in peroxidases can trigger the peroxidase-dependent decarboxylation of active auxin (Jameson and Clarke, 2002). Considering the ROS stress applied to the treated leaves, it can be presumed that peroxidase levels in these leaves would be grossly elevated, leading to the decreased levels of auxin seen in these leaves.

Secondly, the cell-cell signalling of ROS has shown to interfere with auxin signalling through a mitogen-activated protein kinase-signalling cascade involving NPK1-RELATED PROTEIN KINASE family members (ANP; Kovtun, *et al.*, 2000). It has been shown that cell-cell ROS signalling activated the ANP kinase cascade and functioned to activate stress-responsive genes while repressing auxin-inducible promoters (Kovtun, *et al.*, 2000). Indeed, given the exogenous application of the ROS producing treatment, this could likely be an explanation for the large decrease in auxin expression in RB-treated leaves.

3.5 CONCLUSIONS

Here we have found the regulation of auxin-related miRNAs in both treated and systemic tissues in response to viral challenge and the oxidative-stress inducing dye Rose Bengal. These changes coincided with the regulation of free auxin as measured by the *DR5::GUS* reporter line. These stresses are similar in that a compatible virus infection does produce ROS; however, this is in the form of a microburst, and not relatable to the high and persistent oxidative stress applied through RB application. We hypothesize that the action of the viral-suppressor of silencing in the ORMV replicase is responsible for the down-regulation of the miRNAs, but this is likely independent of the down-regulation of auxin. Auxin down-regulation in infected leaves could be accounted for through the tobamovirus specific viral interference with Aux/IAA proteins leading to the stimulation of auxin-regulating feedback loops. In RB infected leaves, we saw a similar down-regulation of auxin-related miRNAs. In the instance of miR-171, it has been shown to be down-regulated in Arabidopsis in nearly all stresses tested, here and in the literature,

suggesting that it is commonly down-regulated in stress, regulating universal stress proteins. Again, the miRNA regulation is likely not responsible for the coordinate down-regulation of auxin in RB-treated leaves. It is likely that the combined action of ROS activated peroxidases, which decarboxylate free auxin, and ROS-like cell-cell signalling, which interferes with auxin responsive promoters.

Systemic tissues also produced changes in the auxin-related miRNAs under both stresses. Local application of both stresses has previously been shown to generate the systemic recombination signal within the frame of this experiment. It is interesting to speculate that the SRS that attenuates genome stability is also affecting the microRNome in systemic plant tissues. Further, systemic leaves in the viral treatment had a gross up-regulation of auxin response. Given the regulation of the miRNAs in this tissue, it could suggest that their expression levels could be responsible for this change in auxin signalling. Many experiments need to be created and repeated in order to further characterize the preliminary research presented here.

3.6 Future Work

In order to confirm the changes in miRNA expression in the infected and non-infected leaves, a complete independent trial of this experiment should be conducted. Indeed, this trial has already begun. Further, it has been recently discovered that miR-390 regulates ARF3 and 4 through the ta-siRNA pathway (see 2.2.2.1) and miR-164 regulates the auxin responsive NAC-domain containing genes. Future experiments should include these miRNAs in order to get a more complete picture of the auxin/miRNA response to stress.

It has also been noted that intercellular auxin levels measured through *DR5::GUS* expression are only partially accepted by the scientific community. It would be ideal to further confirm the auxin levels in infected and non-infected tissue using another method such as mass-spectrometry.

As our data may be indicating the loss of auxin-related miRNA expression through the action of viral suppressors of silencing, experiments using the agroinfiltation of the known VSRs or the ORMV replicase protein may show if these treatments alone are responsible for the results we have found.

Finally, the systemic changes in miRNA expression and auxin level in the viral treatment is a curious finding. It would be very interesting to associate these systemic changes with an altered plant response to future stress.

3.7 FIGURES AND TABLES

Table 3.1: Probes used in Northern blot analyses.

Probes	Sequence
as-ath-MIR160	5'-TGG CAT ACA GGG AGC CAG GCA-3'
as-ath-MIR167	5'-TAG ATC ATG CTG GCA GCT TCA-3'
as-ath-MIR171b/c	5'-CGT GAT ATT GGC ACG GCT CAA -3'
as-ath-MIR393a	5'-GAT CAA TGC GAT CCC TTT GGA -3'
as-U6 snoRNA	5'-CTC GAT TTA TGC GTG TCA TCC TTG C-3'
ORMV siRNA	5'-TAA CTA AAA GTG AGA GGT TCG AAT CCT-3'
	5'-ATC ACC TGT TAA CGT ACG CGT GGC GTA-3'
	5'-TTA GAT GAG GCC GTT GCC GAG GTC CAT-3'

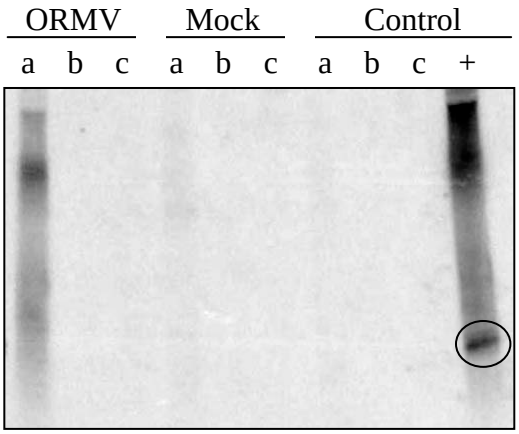


Figure 3.1: Northern blot analysis using probes for anti-sense viRNAs derived from ORMV viral infection (Table 3.1). Lanes = a – 24I, b – 24S, c – 6dS, + – ORMV positive control. Circle in ORMV positive control are ORMV viRNAs (~21-24 nuceotides).

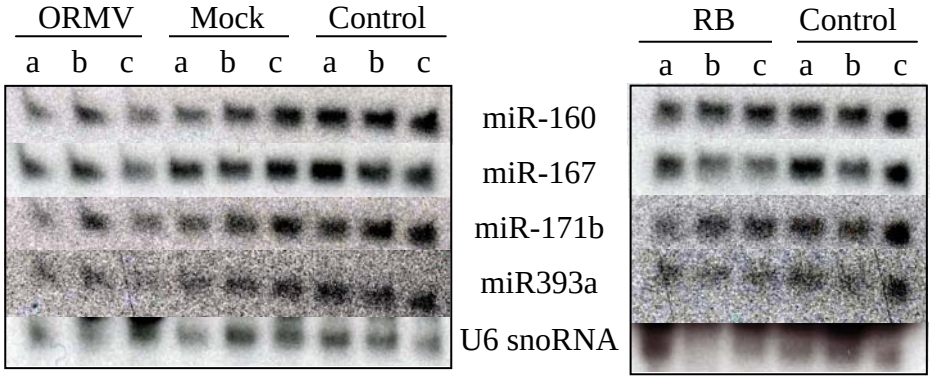


Figure 3.2: Representative Northern blots of auxin-related miRNAs in infected leaves 24 hours after infection (24I; a), non-infected leaves 24 hours after infection (24S; b), and non-infected leaves six days after infection (6dS; c). Pictures represent a single membrane that has been stripped and re-probed. Internal loading control used for comparison was the U6 snoRNA.

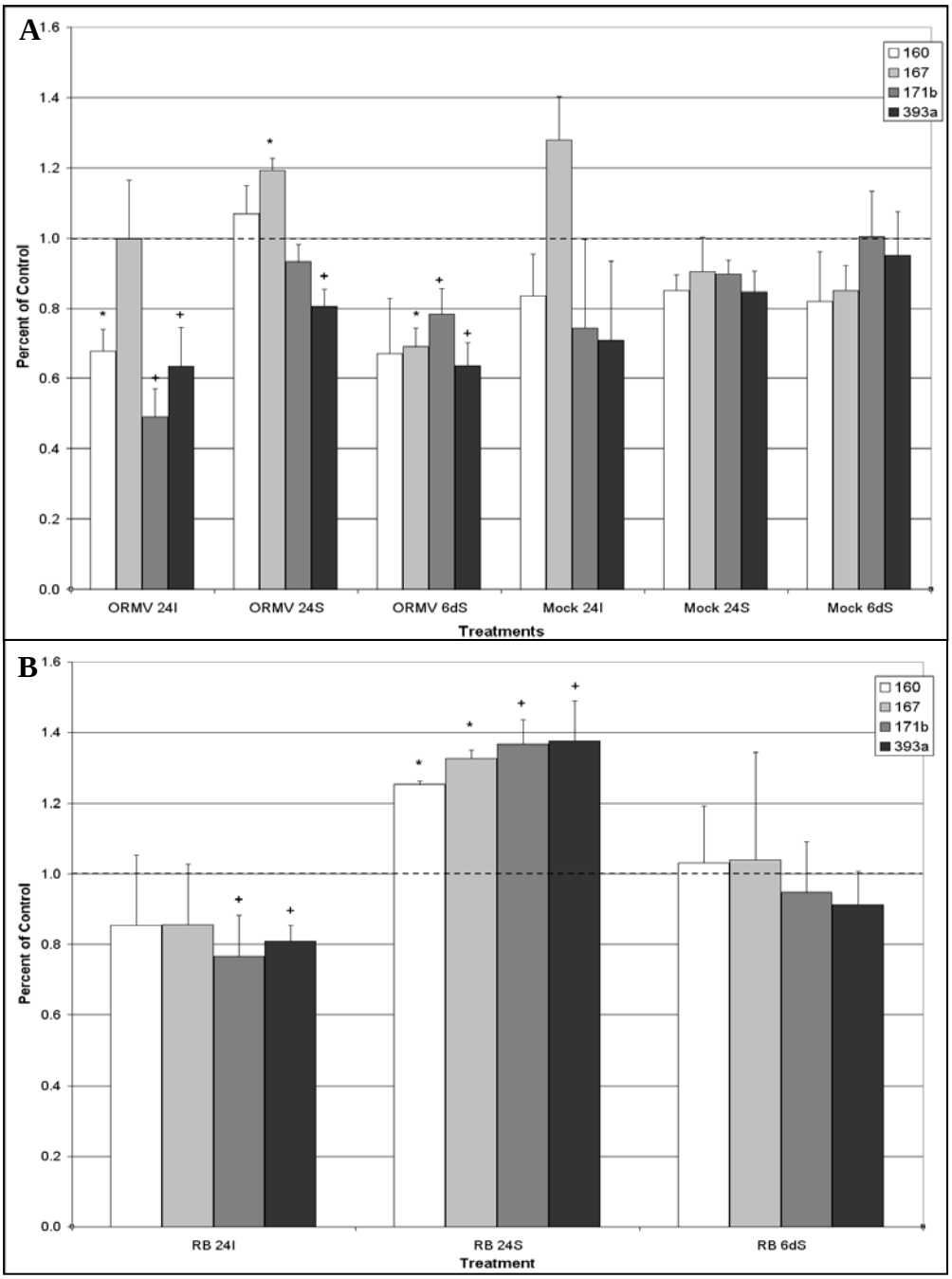


Figure 3.3: *Oilseed rape tobamovirus* infection (A) and Rose Bengal (B) treatment of *Arabidopsis thaliana* in infected and non-infected leaves. The graph represents a Northern blot analysis from a total RNA pooled from 10 leaves from 5 plants for each treatment that has been quantified and the bands normalized to internal controls (see figure 3.2). ORMV – ORMV infected plants; Mock – Mock treated plants; RB – Rose Bengal treated plants; 24I – Treated leaves 24 hours after infection; 24S – non-treated leaves 24 hours after infection; 6dS – Non-treated leaves 6 days after infection. The error bars represent the standard error of a comparison between the technical repeats, except in the case of miR-171b and -393a, which include a separate, independent trial. The asterisks (*) represent significant differences from 1.0 as tested via a one-sample T-test ($p<0.05$) between replicates. The pluses (+) represent significant differences from 1.0 (one-sample T-test, $p<0.05$) when testing the data against an independent trial

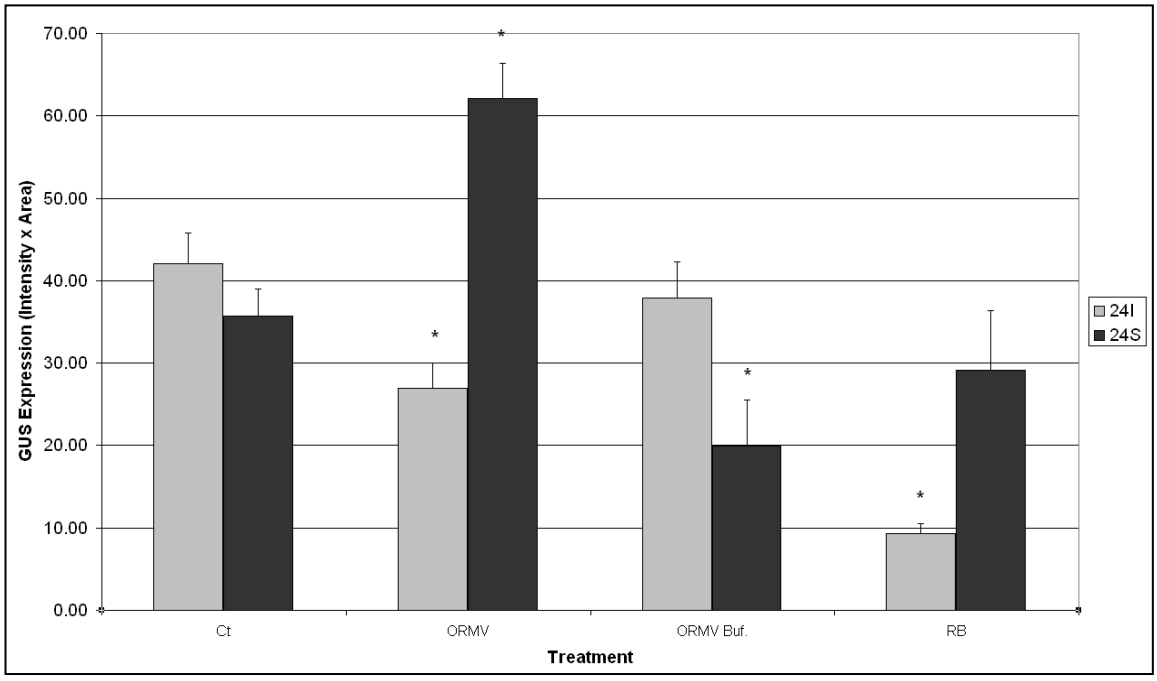
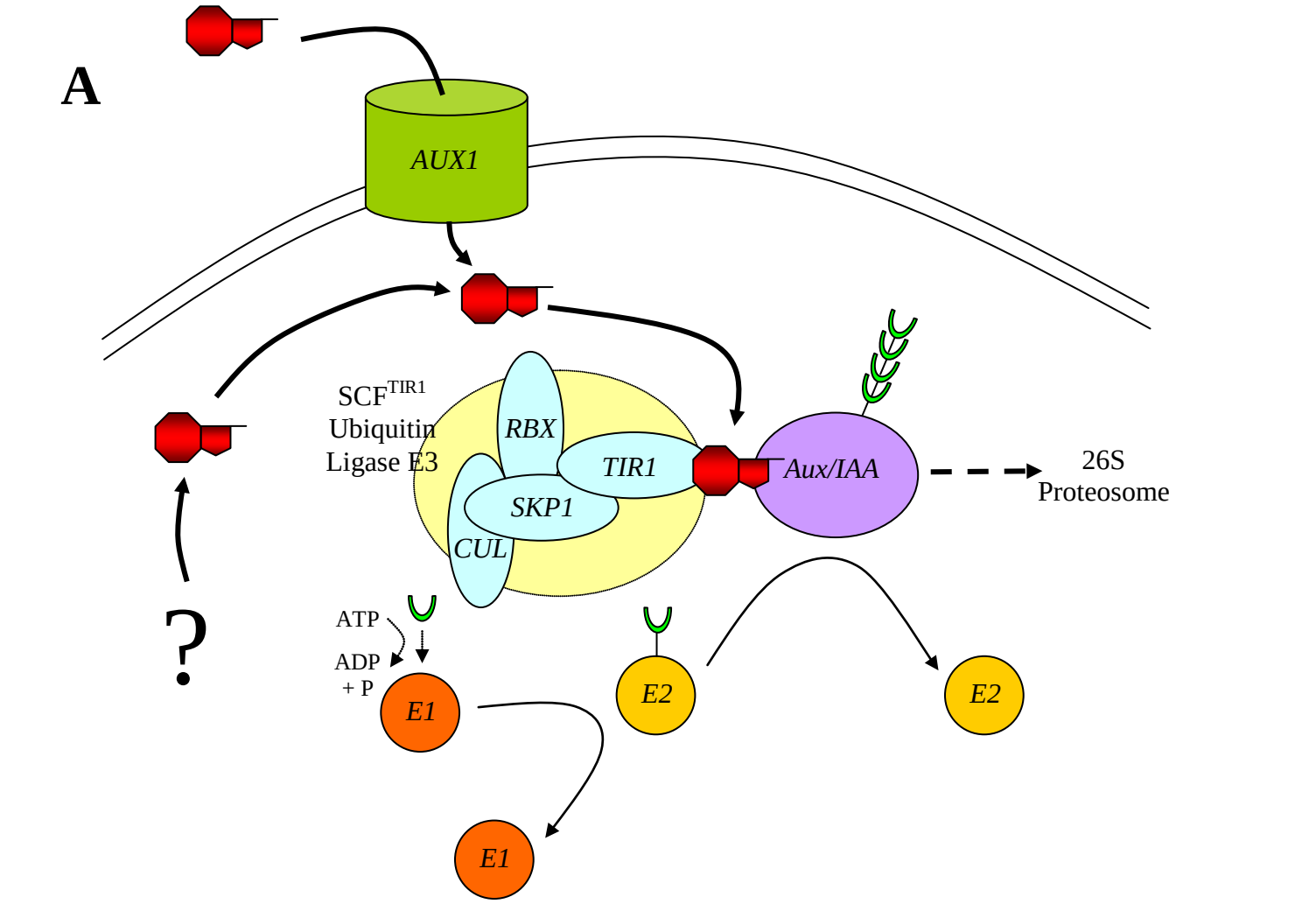


Figure 3.4 – Results of *Oilseed Rape Mosiac Virus* infection and Rose Bengal treatment of *Arabidopsis thaliana* DR5::GUS in infected and non-infected leaves. The graph represents the histochemical staining of 10 leaves from 5 plants for each treatment, and 20 leaves from 10 plants for the controls. The GUS expression represents the quantified transgene expression as determined by the intensity of GUS staining multiplied by the percent area of the leaf stained. ORMV – ORMV infected plants; Mock – Mock treated plants; RB – Rose Bengal treated plants; Ct – control plants; 24I – Treated leaves 24 hours after infection; 24S – non-treated leaves 24 hours after infection. The error bars represent the standard error, and the asterisks (*) represent significant differences when compared to the repective controls (Two-tailed T-Test, $p>0.05$).


A
AUX1
SCF^TIR1
Ubiquitin
Ligase E3
RBX
TIR1
SKP1
CUL
Aux/IAA
26S
Proteosome
?
ATP
ADP
+ P
E1
E2
E2
E1

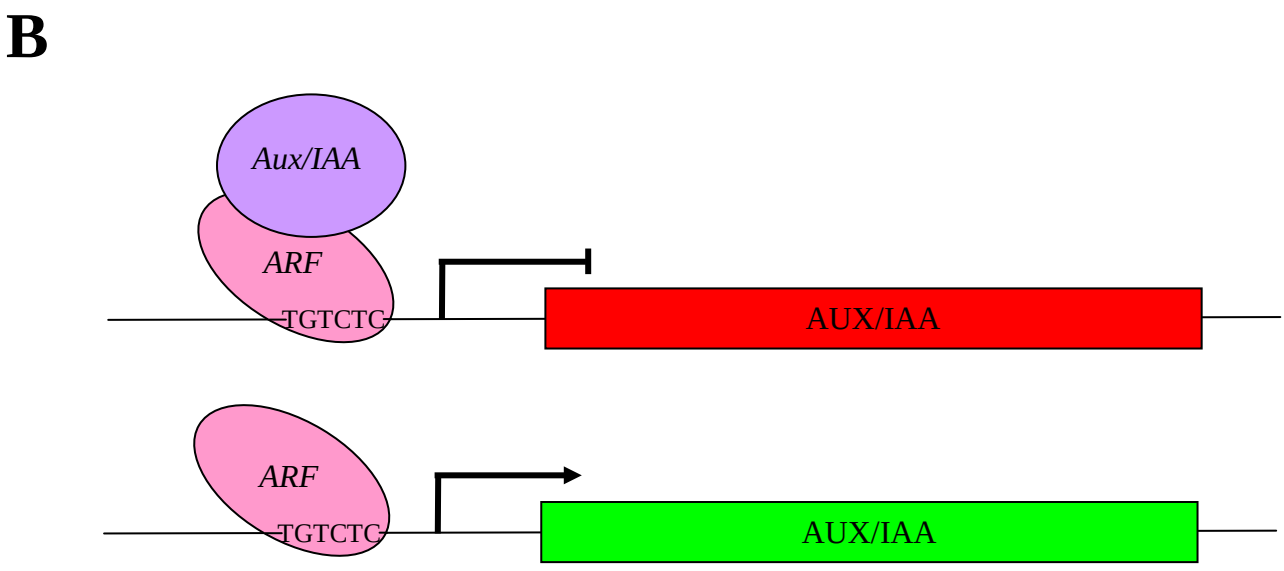

B
Aux/IAA
ARF
TGTCTC
AUX/IAA
ARF
TGTCTC
AUX/IAA

Figure 3.5 (previous page): The Auxin Response. **A -** Ubiquitin is activated in an ATP-dependent manner by E1, and subsequently passed to the ubiquitin conjugating enzyme E2. *TIR1* is an F-box protein that confers protein specificity to target proteins for ubiquitination within the E3 ubiquitin ligase complex. In the absence of auxin, there is no ubiquitination of the target *AUX/IAA* proteins. However, when auxin is perceived and bound to *TIR1* the affinity for the target proteins increases dramatically, allowing for the E3 complex to complete the ubiquitination of the target. Ubiquitination of these proteins leads to protein degradation via the 26S proteosome. **B –** The consequence of the loss of the *AUX/IAA* proteins is the release of translational repression of certain auxin response factors (*ARFs)*. These ARFs are transcription factors bound to auxin response elements (AREs; TGTCTC), that may activate transcription in the absence of the *AUX/IAAs.* Figure adapted from Leyser, 2002 and Guilfoyle, 2007.

4.0 INTER-PLANT COMMUNICATION OF GENOME INSTABILITY IN RADIATION EXPOSED ARABIDOPSIS

4.1 INTRODUCTION

Plant-plant communication was established several decades ago with the observation that both wounded and neighbouring plants exhibited similar defensive responses. Since, numerous examples of plant-plant communication have been discovered and studied (Baldwin, *et al.,* 2006). Plants are also capable of intraplant signals, such as systemic acquired resistance (SAR) and systemic response to wounding (SRW). In these scenarios, the non-infected leaves of pathogen infected plants (SAR) and the non-wounded leaves of wounded plants (SRW) turn on defence related mechanisms. Interestingly, common to both plant-plant communication and systemic signalling are some volatile phytohormones.

Homologous recombination in plants plays dual roles as both a double-strand break repair mechanism as well as the mechanism responsible for increased genetic diversity during meiosis (Putcha, *et al.,* 1996; Boyko, *et al.,* 2007). In our laboratory and others, it has been shown that plants treated with ionizing radiation have increased levels of homologous recombination (Ries, *et al.,* 2000; Filkowski, *et al.* 2004; Molinier, *et al.*, 2005; Boyko, *et al.*, 2006a; Molinier, *et al.,* 2006). Further experiments have even shown that the local leaf treatment with UV-C leads to increased recombination rates in both the local and systemic leaves (Filkowski, *et al.,* 2004). This systemic recombination signal (SRS) was also found in pathogen- (Kovalchuk, *et al.,* 2003) and reactive oxygen stress-treated plants (Filkowski, *et al.,* 2004), and may or may not constitute similar mechanisms.

It is possible that the SRS, as well as some of the other systemic signals, are transmitted not through the plant vasculature, which is the common perception, but through the immediate gaseous environment of the plant. If this was indeed the case, plants exposed to ionizing radiation with increased recombination rates may transmit the signal to neighbouring plants in a closed environment. This chapter shows that the whole plant exposure to ionizing radiation results in a media-derived aqueous signal and plant-derived volatile signal that increases the recombination rates of neighbouring, unexposed plants. The ramifications of this discovery are discussed.

4.2 METHODS

4.2.1 Plant Lines and Growth Conditions

For the experiments on liquid media, sterilized (Boyko, *et al.,* 2006) *Arabidopsis thaliana* (cv. 24) line 11 seeds were spread on sterile Whatman No. 1 filter paper in a divided (Fisher) or undivided (Fisher) Petri plates. A total of 4 mL of liquid MS media (Murashige and Skoog**,** 1962) was added to the plates (2 x 2 mL in divided plates). In the solid media experiments, the same sterilized seeds were spread on divided or undivided Petri plates containing 25mL of solid MS media (liquid media recipe plus 8 g/L agar (Sigma)). The plates were stratified for 48 hours at 4^{o}C, and then grown in Enconair (Winnepeg, Canada) growth chambers at 16/8 hours light/dark, at 23^{o}C and 18^{o}C, respectively.

Arabidopsis line 11 is a transgenic line that contains a homologous recombination (HR) reporter gene. This reporter line has two, non-functional, overlapping, truncated copies of

the *β-glucaronidase* gene (GUS; Boyko, *et al.,* 2006; Swoboda, *et al.,* 1994). If a strand break occurs anywhere in the region of homology in one of the two truncated *GUS* genes, the HR double-strand break repair pathway could repair it using the other strand as a template, potentially restoring the function of the GUS transgene (Figure 4.1A; Boyko, *et al.,* 2006). In this case, sectors of blue will result after histochemical staining.

4.2.2 Experimental Set-up

Ten-day old Arabidopsis line 11 plants were subjected to either X-Ray (XR) or UV-C (UV) irradiation while growing on either solid or liquid MS media in either undivided or divided Petri dishes. Half of the plates were covered with a shielding that protected the plants underneath, and the plants were irradiated. Seven days post-irradiation the plants were harvested separately as either irradiated (IT; not covered) or bystander (BS; covered) plants. An additional treatment was added, half-bystander (½BS), in which the plates were covered as above, but only media was irradiated (Figure 4.1B). 75-150 plants were counted per treatment, and the averages taken to represent the homolgous recombination frequency for that experiment. The experiment was independently repeated three times, and statistics were performed on those three averages in MS Excel 2003.

4.2.3 Radiation Treatments

Plants were prepared for irradiation at 10 days post-germination. In the instance of UV-C irradiation, the Petri lids were removed and half of the plate was completely covered in tinfoil. The entire plate was than irradiated with 7000 ergs (~60 ergs/second) of UV-C. A

set of control plates was completely covered with tinfoil and irradiated under the same conditions to ensure there was sufficient UV protection. X-ray irradiation was done in a similar manner. Half of the Petri dish was covered with a 2.5 mm thick medical grade lead shield, and whole plates received 20 gray of X-rays (90 kV, 5 mA, ~18 mGy/sec); Figure 4.1B). Again, a set of control plates was irradiated while completely covered with the lead shielding to ensure sufficient protection.

4.2.4 Histochemical Staining

Whole plants were harvested seven days post-irradiation. These plants were then immediately placed in GUS staining solution (100 mg of 5-bromo-4-chloro-3-indolyl glucuronide substrate (GBT) in 300 mL of 100 mM phosphate buffer (pH 7.0), 0.05% NaN_3, and 1 mL dimethylformamide) and vacuum infiltrated for 10 min. Plants were incubated in the staining solution for 37^{o}C for 48 hours, and then destained with 70% ethanol for 24 hours. The number of event per plant were than counted under a dissecting microscope, and the number of spots/plant homologous recombination frequency was calculated.

4.3 RESULTS

4.3.1 Liquid Media

The liquid media showed the most interesting results. The irradiation treatment (IT), which represents directly irradiated plants, shows a significant increase in homologous recombination frequency (HRF) in both divided and undivided plates for X-Ray treatment (~1.8 fold) and a significant increase in the divided plates for the UV-treatment

(1.9-fold; $p<0.05$). The undivided plates follow these results with a trend of 1.7-fold increase (Figure 4.2).

These increases in recombination rate in IT plants were mirrored by the shielded bystander plants (BS), with significant ($p<0.05$) increases in HRF in both X-ray (~2.0-fold) and UV (~1.9-fold) on both divided and undivided plates. The half-bystander treatments (½BS) only showed significant increases in the undivided plates for X-Ray (~2.1-fold) and UV (~1.9-fold), with divided plates showing HFRs similar to that of the control (Figure 4.2).

4.3.1 Solid Media

The solid media experiments showed very similar trends to that of the liquid media experiments, but these trends were not as defined as those seen in the liquid media. Interestingly, the only significant increase in IT plants was found in UV-irradiated divided plants (2.4-fold), although trends of increased HFR are seen throughout the treatments (~1.5-fold). BS plants exhibited significant increases in both divided and undivided plates for XR (~1.5-fold) and for divided plates for UV (1.9-fold; $p<0.05$), with an increasing 1.3-fold trend for undivided UV plates. The ½BS plates showed no significant increases in HFR, although they tend to be elevated in the undivided plates and unchanged in the divided plates.

4.4 Discussion

Homologous recombination frequency (HRF) increases are a result of increases in the homologous recombination double-strand break repair pathway, and has been shown

numerous times to increase in response to direct ionizing radiation (Ries, *et al.*, 2000; Filkowski, *et al.* 2004; Molinier, *et al.*, 2005; Boyko, *et al.*, 2006a; Molinier, *et al.*, 2006). This increase in HFR is a measure of genome instability, which generally refers to the susceptibility of the genome to mutations, rearrangements, and activation of mobile elements (Boyko, *et al.*, 2007).

Elevated levels of HFR are generally accountable through two mechanisms, the increase in the amount of double strand breaks, and/or the increase in the activity of homologous recombination repair machinery (Boyko, *et al.*, 2006b). In the case of ionizing radiation, it is not surprising that increased HFR occurs upon exposure, as ionizing radiation is well known to induce double-strand breaks in eukaryotic cells, most commonly through the production of genotoxic intercellular reactive oxygen species (Chatgilialoglu and O'Neill, 2001; Filkowski, *et al.*, 2004).

However, here our data suggests that direct UV-C and X-ray exposure cause increases in HFR that are more pronounced in liquid than solid media. This is an interesting finding, as it would suggest that plants in liquid media are more susceptible to total plant irradiation. However, if one considers the framework of the experiment, the plants as well as the media are being irradiated. It may be possible that the irradiation of the media is creating free radicals that are affecting the plants global HFR. It is well documented that the radiolysis (X-ray) and photolysis (UV) of water produces substantial quantities of hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2; LaVerne, 2000; Azarague, *et al.*, 2005). It is possible that irradiation of the media is producing these reactive oxygen

species (ROS), which are then being taken in by the plant. Further, as the solid media is a semi-solid gel, it is possible that the ROS produced in solid media are less mobile; resulting in less uptake by the plant and causing less damage. These less-mobile ROS also explains the less dramatic increase in HFR in bystander plants in solid media. However, the background HFR in the solid versus liquid media may be accounting for the changes we are seeing when we irradiate these plants. Further investigation into the different background HRFs in the two media should be performed in order to promote more solid conclusions.

This ROS produced by the irradiation of media could explain the bystander-like effect in the non-divided plates. It is likely that the ROS created by the radio- or hydrolysis of the media on the irradiated side of the plates can diffuse to the bystander side, causing similar effects in these plants. This is supported by the ½BS treatments where only media is irradiated resulting in similar increases in HFR in undivided bystander plants. Further, this increase is completely abolished in the ½BS divided plates, suggesting the physical barrier separating the media is capable of halting the signal. This evidence supports the media diffusible signal hypothesis and removes any speculation of media produced gaseous signals.

Interestingly, however, is that BS plants on divided plates that do contain IT plants do produce an elevated HFR. This would suggest that in addition to the media produced ROS causing elevated HFRs in BS plants, the directly irradiated plants are producing a gaseous signal causing increased genome instability in neighbouring, unexposed plants.

This is a truly phenomenal and novel finding. It is curious, however, why there is not an additive result in the increase in HFR in the bystander undivided plates. In this instance, the BS plants are receiving both the putative aqueous ROS as well as the IT-derived volatile signal. It may be possible that there is a certain level of saturation in this system, and HFR rates produced in this manner are regulated to a maximal level. Alternatively, as the IT plants covered much of the surface of the media, it could be the scenario that the plants are absorbing most of the dose and the media is receiving little radiation, resulting in the generation of only one prominent signal.

Plants have long been known to communicate to one another via diffusible signals, or volatile organic compounds (VOCs). This phenomenon was first discovered several decades ago when it was observed that non-herbivore attacked (bystander) plants residing next to herbivore-attacked plants were emitting the same defensive signals and activating the same defensive machinery as the attacked plants (Baldwin, *et al.,* 2006). The reason for this signalling, or eavesdropping as some would put it, has a number of purposes including the priming of defences before the arrival of a herbivore, the readying of pathogen defences before the arrival of a pathogen, or the release of attractants of predators or parasitoids of the stress-inducing organism (Jiménez-Martínez, *et al.,* 2004; Baldwin, *et al.,* 2006).

A number of volatile organics have been studied over the years in an attempt to characterize these plant-plant interactions. Small highly volatile compounds, such as ethylene, methanol, isoprene, acrolein and some monoterpenes, can diffuse in the

surrounding environment rapidly, limiting signalling to systemic leaves or very close neighbours (Baldwin, *et al.,* 2006). Heavier volatile compounds, such as methyl jasmonate (MeJA) and methyl salicylate (MeSA), have been suggested to function over longer distances, with a slow dispersal that allows for the establishment of denser, turbulence-resistant plumes (Baldwin, *et al.,* 2006). In our case, since we are working in a limited air volume of a sealed Petri dish, the signalling of either type of volatile could be possible.

MeSA and MeJA has previously been implicated in intraplant stress responses and systemic signalling, with examples in systemic acquired resistance (SAR; Grant and Lamb, 2006; Park, *et al.,* 2007) and systemic response to wounding (SRW; Schilmiller and Howe, 2005). Interestingly, both the plant-plant signalling of wounding (Baldwin, *et al.,* 2006) and pathogen infection have been shown before (Shulaev, *et al.,* 1997).

Similar to these systemic effects is the systemic recombination signal (SRS) discovered in this laboratory several years ago (Kovalchuk, *et al.,* 2003; Filkowski, *et al.,* 2004). In this model, the local application of virus, UV-C or rose Bengal (a ROS producing compound) resulted in the local and systemic induction of increased recombination rates. Indeed, this systemic induction of increased HFR may be similar to the plant-plant induction of HFR we show here.

The biological relevance of such a signal between plants is indeed quite perplexing. It has been postulated that the SRS generated in systemic leaves in plants helps drive increasing

genetic diversity in plants, as their sessile nature forces them to adapt to survive. However, overwhelming rates of recombination are not ‘geno-healthy’ either, as homologous recombination can cause the loss of heterozygosity at herterozygous loci. It is possible that this putative plant-plant signal evolved as a systemic signal as opposed to an interplant signal, and it is only in the context of this experiment where close proximately plants in a small gaseous environment are exhibiting this plant-plant-like phenomenon.

3.5 Conclusions

Here we demonstrate the existence of a plant-plant signal in Arabidopsis that communicates genome instability as measured through increased levels of homologous recombination. We show that both an aqueous media-derived and volatile plant-derived signal may be produced by plants receiving genotoxic levels of ionizing radiation. The discovery of this signal is very interesting as it may lead to clues to the nature of the systemic recombination signal (SRS) which was previously thought to be an internal signal. Further work into the nature and generation of this signal is warranted to gather a better understanding of plant-plant and possibly systemic signalling.

3.6 Future Work

Much work on this phenomenon needs to be completed in order to elucidate the intricacies of this plant-plant signalling of genome instability. Preliminary experiments irradiating media alone, with or without radical scavengers, and applying this to plants

has provided some credence to the hypothesis of media produced free radicals. However, these experiments need to be repeated and refined before they can be presented.

It would also be very intriguing to use some phytohomone synthesis or reception defective mutants such a *jasmonate insensitive* (*jin1*), *salicyclic acid insensitive1* (*sai1*), *ethylene receptor1-1* (*etr1-1*) or *jasmonate methyltransferase* (*jmt*), to see if these plants are impaired in either producing or receiving the instability signal.

Further, experiments applying local stresses, such as rose Bengal and viruses, to plants on one side of the Petri dish and examining HFR in untreated plants on the other might also elicit increases in HFR.

However, most importantly, these experiments need to be carefully scrutinized to make sure that future experimenters are not chasing artefacts. For example, an alternative explanation for divided BS plants with increased HFR while divided ½BS plants do not show the increase could be due to the reflection of UV or X-rays from the IT plants. Experiments looking into this 'scatter-dose' should be implemented in the future.

3.6 Figures and Tables

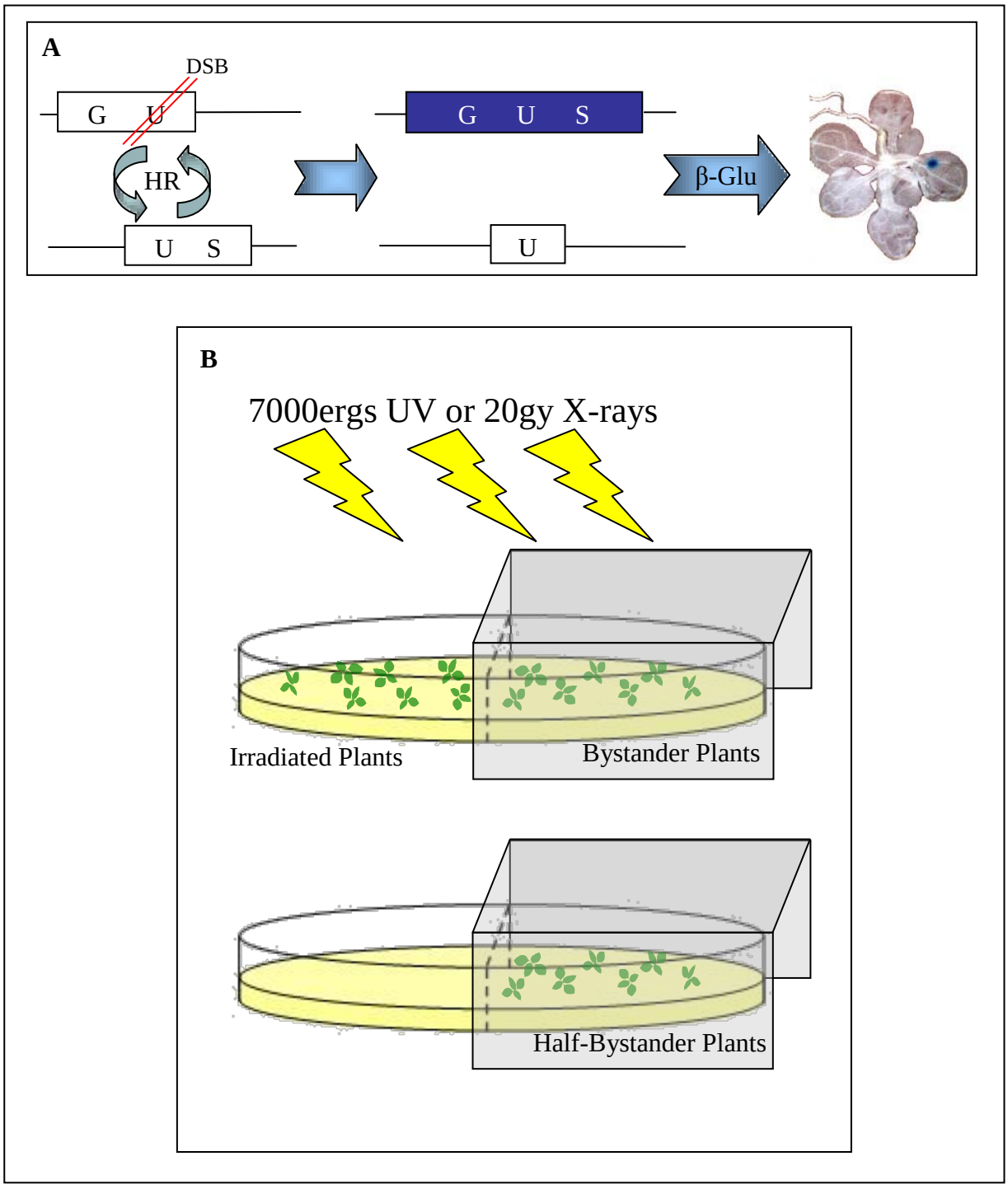


Figure 4.1 A: Representation of homologous recombination reporter line 11 in Arabidopsis (HR - homologous recombination; DSB – double strand break; β-Glu – GUS staining solution). **B**: Experimental set-up. Dotted line represents area where partition in Petri plates occurs in divided plates.

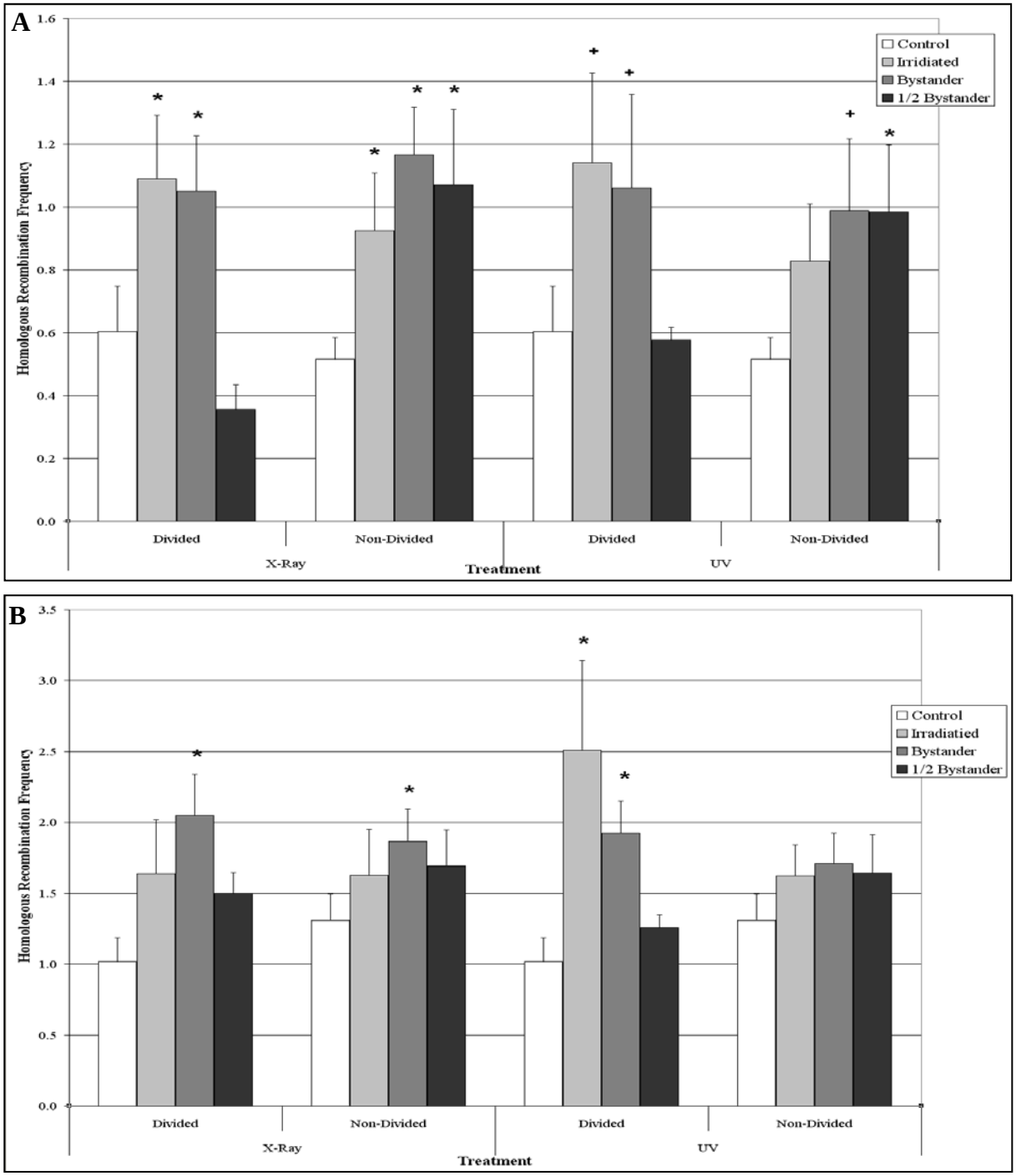


Figure 4.2: Ten-day old Arabidopsis line 11 plants were a subject to either 20 gy of X-Ray (A) or 7000ergs of UV-C (B) irradiation while growing on either solid or liquid MS media in either undivided or divided Petri dishes. Half of the plates were covered with a shielding that protected the plants beneath from the radiation (Bystander), while the other half was uncovered (IT). A treatment of plates with only exposed media and protected plants was also performed (1/2 Bystander). Seven days post-irradiation the plants were harvested and stained to assay the recombination rate for each treatment. The error bars represent standard error of three independent experiments. Black asterisks (*) represent significant differences (Student's T-Test, $p<0.05$) and pluses (+) represent significant differences (Student's T-Test, $p<0.10$).

MICRORNAOME CHANGES IN BYSTANDER 3D HUMAN TISSUE MODELS SUGGESTS 'PRIMING' OF TRAIL-MEDIATED APOPOTOSIS: AN IN SILICO MODEL

5.1 INTRODUCTION

Bystander effects are non-targeted effects of radiation, whereby unexposed cells exhibit the molecular symptoms of stress exposure when adjacent or nearby cells are traversed by ionizing radiation. To date, a variety of radiation-induced bystander effect studies have been performed using cell culture models (Mothersill, *et al*., 2001; Zhou, *et a.l*, 2002a; Zhou, *et al.*, 2002b; Suzuki, *et al*., 2004; Suzuki and Tsuruoka, 2004; Maguire, *et al*., 2005; Yang, *et al*., 2005; Zhou, *et al*., 2005; Lyng, *et al*., 2006a, b; Gaugler, *et al*., 2007; Maguire, *et al*., 2007). These experiments utilized immortalized cell lines in a single monolayer, making the extrapolation of the results to human exposure somewhat difficult. However, recent work in tissue explants *(*Belyakov, *et al.*, 2002; Belyakov, *et al*., 2003; Belyakov, *et al*., 2006; Mothersill, *et al*., 2002), spheroids (Persaud, *et al*., 2005), and in artificially reconstructed human tissue (Belyakov, *et al.*, 2005; Sedelnikova, *et al.*, 2007), has suggested that the cell-cell bystander effect operates *in vivo* in human tissue in a three dimensional fashion. Thus, bystander effects remain an important phenomenon in radiobiology.

Overall, bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations, and amplifications (Zhou, *et al*., 2000; Huo, *et al*., 2001; Lorimore, *et al*., 2001; Zhou, *et al*., 2002a; Zhou, *et al*., 2002b; Suzuki, *et al*., 2003; Klokov, *et al*., 2004; Lorimore, *et al*., 2005; Smilenov, *et al*., 2006; Hamada, *et al*., 2007). They influence gene expression, cellular proliferation, senescence, and cell death (Sawant, *et al*.,

2001; Sawant, *et al*., 2002; Liu, *et al*., 2006; Lyng, *et al*., 2006b; Sedelnikova, *et al.*, 2007) and are believed to be linked to radiation-induced genome instability (Morgan, 2003a; Huang, *et al*., 2007; Kovalchuk and Baulch, 2008). However, while a great deal of data has been accumulated on the existence and manifestation of bystander effects in cultured cells and 3D tissues, the mechanisms of this phenomena remain to be uncovered.

The high frequency of induction and persistence of bystander responses suggests an epigenetic background (Lorimore, *et al*., 2003; Morgan, 2003a; Morgan, 2003b; Nagar, *et al*., 2003; Kaup, *et al*., 2006, Wright and Coates, 2006; Sedelnikova et al., 2007). Epigenetic changes are stable alterations in gene expression that include DNA methylation, histone modification, and RNA-associated silencing (Jaenisch and Bird, 2003). MicroRNAs (miRNAs) are important componenets of the RNA-associated silencing machinery. MiRNAs are small regulatory molecules known to target mRNA transcripts for translational inhibition or, rarely, degradation in humans (See Chapter 2.2.3.1). Since their discovery, miRNAs have been found to play essential roles in development, regulating such processes as terminal differentiation (Foshay and Gallicano, 2007), cell cycle (Carleton, *et al.*, 2007), apoptosis (Jovanovic and Hengartner, 2006), and DNA methylation (Fabbri, *et al.*, 2007). Deregulation of these miRNAs has been associated with a number of diseases, including cancer. Further, genotoxic stress exposure was shown to deregulate cellular microRNA expression.

Given the importance of miRNAs in the regulation of various cellular processes, and the fact they have been shown to be regulated in *in vivo* abscobal bystander responses (Koturbash, *et al.*, 2007), we decided to look at the microRNome changes in bystander tissues after α-

particle microbeam irradiation of three-dimensional artificial human tissue using microRNA microarrays. The results of the microarray analysis indicate that some of the molecular endpoints previously associated with this bystander model could potentially be regulated by miRNAs. Our data suggest that miRNA regulation in concert with c-Myc activation in bystander tissue may be sensitizing cells to TRAIL-mediated apoptosis.

5.2 METHODS

5.2.1 Tissue systems and culture

These experiments utilized a novel human 3D tissue culture system (MatTek Corp, Ashland, MA). These artificial tissues (MatTek Corp, Ashland, MA) reconstruct the normal tissue microarchitecture and preserve the *in vivo* differentiation pattern. Further, they are mitotically and metabolically active, release the relevant cytokines, and demonstrate the presence of gap junctions (Monteria-Riviere, *et al.*, 1997; Zhao, *et al.*, 1999; Boelsma, *et al.*, 2000). Artificial tissues are very stable and allow a high degree of experimental reproducibility (Sedelnikova, *et al.*, 2007).

We used the EpiAirway Tissue System (Air-100) model, which consists of normal, human-derived tracheal/bronchial epithelial cells that have been cultured to form a highly differentiated model that closely resembles the epithelial tissue of the respiratory tract (Sedelnikova, *et al.*, 2007). Figure 5.1 displays tissue dimensions.

The artificial tissues were cultured according to a recommended protocol, using an air-liquid interface tissue culture technique. The tissues were grown on a semi-permeable membrane,

fed with serum-free medium from below, and cultivated in Millicell-CM culture inserts (Millipore: Billerica, MA) using a 25 μm-thick hydrophilic membrane. The surfaces of the tissues were exposed to the air, which stimulates differentiation (Sedelnikova, *et al.*, 2007).

5.2.2 Culture Irradiation

In order to produce direct radiation damage to specific cells within the 3D tissue without directly irradiating surrounding cells, the Columbia University single-cell α-particles microbeam was used.

The irradiation procedure was conducted as previously described by Sedelnikova, *et al.* (2007). In brief, Columbia University uses a microbeam of 7.0 MeV ^{4}He ions produced by the 5-MV Singletron of the Radiological Research Accelerator Facility of Columbia University (Randers-Pehrson, *et al.*, 2001). The beam size was restricted to a 1 to 2 nuclei width along the line of irradiation. A single plane of tissue was irradiated from below through the 20-μm-thick membrane that forms the base of the culture insert. The insert was positioned in a custom-designed holder attached to a microbeam stage. The ^{4}He ion energy at the surface of the tissue was ~4.6 MeV and the range in tissue was ~31 μm. Because the samples were thicker than the particle range, the linear energy transfer (LET) varied from ~100 keV/μm at the membrane/tissue interface to >200 keV/μm as the ions slowed. The average number of particles for 5 μm of translation (~1 cell nuclear diameter) was ~2, with a total spread of approximately ~10%. The statistical uncertainty for the number of particles is 1.6%. For a broad beam irradiation, the dose delivered by 0.076 ion/μm^2 (1.9 particles in a 5 x5 μm^2 area) with a LET of 100 keV/μm is 3.2 Gy.

After irradiation, each tissue was returned to a multi-well dish filled with fresh medium and incubated at 37°C in a humidified atmosphere with 5% CO_2. Tissue was removed and flash frozen in $N_{2(l)}$ at 0.3 (8 hours; 3 samples), 1 (2 samples) 2 (2 samples), 3 (2 samples), 5 (3 samples), and 7 (3 samples) days post-irradiation . Time matched mock-treated controls were available for the 8 hours (3 samples), 3 (5 samples), and 7 days (3 samples) post-mock irradiation. 0 hours post-irradiation (4 samples) represented a no irradiation or mock control, as well as establishing a base-line expression level.

5.2.3 Tissue Microdissection and RNA Extraction

Frozen tissue was placed on $N_{2(l)}$ chilled glass plates, and held in place by forceps. Scalpels (BP, Rib-back #12) were used to cut ~1-1.5 mm slabs from the center of the tissue model and removed to microfuge tubes (See Figure 5.1). These portions represented irradiated and bystander tissue. The remaining tissue on either side of the slab was removed and pooled to a single microfuge tube. These samples represented purely bystander tissue.

Tissues were than immersed in TRIzol (500µL), and samples vortexed for 5-10 seconds to remove the tissues from the membrane. RNA extraction was carried out as per the manufacturer's protocol (TRIzol, Invitrogen).

5.2.4 miRNA microarrays

The miRNA microarray analysis was performed by LC Sciences (Houston, TX; www.lcsceinces.com). The assay started from 2 to 5 µg total RNA, which was size fractionated using a YM-100 Microcon centrifugal filter (from Millipore) and the small

RNAs (<300 nt) isolated were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was performed overnight on a µParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, http://microrna.sanger.ac.uk/sequences/; human set version 9.0) or other RNA (control) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100µL 6xSSPE buffer (0.90M NaCl, 60mM Na_2HPO_4, 6mM EDTA, pH 6.8) containing 25% formamide at 34°C. After hybridization, detection used fluorescence labeling using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two color experiments, the ratio of the two sets of detected signals (log2 transformed, balanced) and p-values of the t-test were calculated.

5.2.5 Promoter analysis

It has been suggested that no single promoter prediction program (PPP) can achieve a sensitivity and positive predictive value of over 65% (Bajic, *et al.,* 2004). Further, each program has strengths and weaknesses. Thus, *in silico* analysis of the promoter region of the miR-106a cluster was performed using a suite of promoter prediction programs (PPPs) freely available on-line. The PPPs used were Dragon Gene-Start Finder (Bajic and Seah, 2003), First Exon (Ramana, *et al.,* 2001), Web Promoter Scan Service (Prestridge, 1995), Neural Network Promoter Prediction v2.2 (Reese, 2001), and McPromoter (Ohler, *et al.,* 2001). Searches were performed in the program's default setting.

CpG island prediction was performed using freeware provided by the European Bioinformatics Institute (www.ebi.ac.uk/). The search was performed using a minimal observed CpG/expected CpG ratio of 0.6, and a minimal GC content of 50%, with a minimum length of 500bp.

5.3 RESULTS

5.3.1 Individual Microarray analysis

The miRNA expression profiles of bystander tissues was compared to the time matched mock controls at 8 hours, 3 days and 7 days post-irradiation (dpi). MiRNAs were considered for comparisons only if their average expression levels were over 1000 arbitrary units of fluorescence (AUF). The reasons for this were three-fold. First, miRNAs with very low expression levels, <1000AUF, are analyzed with less sensitivity in our microarray methods. Secondly, downstream studies involving qRT-PCR methods are limited to miRNAs with expression patterns of >1000AUF. Finally, fold inductions of

miRNAs with low expression patterns seem less biologically significant than those of higher expression levels. For example, a 1.5-fold change of a miRNA expressed at 100 versus 1000AUF would coincide with a change of 50 and 500 units, respectively.

At the 8 hour time point (8hpi), four miRNAs significantly ($p<0.05$) changed over the time matched controls. These were miR-22, -141, and -16, which were 2.0-, 1.8-, and 1.2-fold up-regulated, and miR-183 which was 1.3-fold down-regulated (Table 5.1).

At 3 dpi, six miRNAs were found to be significantly different from the mock control. miR-29a, -29c, -30a-5p, and -20a were found to be 2.45-, 1.62-, 1.47-, and 1.25-fold up-regulated, respectively, while miR-146a and -125b where found to be 1.26-, and 1.18-fold down-regulated, respectively (Table 5.1).

The final time point, 7 dpi, found only two significantly regulated miRNAs, corresponding to miR-181a and -181b, which were 1.5- and 1.2-fold down-regulated (Table 5.1).

Despite not having any time-matched controls for the 1-, 2-, and 5-day time points, we can look at the levels of those miRNAs at those time points to establish an expression trend for the experimental period.

Although only significantly up-regulated at 8 hpi, miR-22, -141, and -16 show a trend of up-regulation until 7 dpi, where they return to the mock level of expression. Conversely, mir-183 displays a trend of down-regulation until the 7 dpi (Figure 5.2A).

The 3 dpi miRNA expression patterns do not show as consistent trends as the 8 hpi. Only miR-146a shows persistent down-regulation until the 7 dpi point. Interestingly, miR-29a and miR-29c show expression patterns that follow the mock data points until the 2–5 dpi, where a separation in expression occurs (Figure 5.2B).

MiR-181b, although having only a significant difference at the 7dpi point, has persistent separation from the mock expression pattern starting at 2dpi. Further, in addition to the significant 1.2-fold down-regulation at 7 dpi, at 3 dpi there is a 1.4-fold down-regulation ($p<0.10$; Figure 5.2C).

5.3.2 Group Microarray Analysis

An ANOVA analysis of the expression patterns from all the treatments can be seen in the heat map ($p<0.05$; Figure 5.3). These miRNAs were clustered together according to expression pattern. Viewing the array in this manner revealed a very intriguing group of inter-related miRNAs.

This group included miRNAs 106a, 106b, 17-5p, 20a, and 19b, that all belong to the same family, miR-17. Interestingly, this family has members that reside in three separate polycistrons on chromosomes 13 (the mir-17 cluster; 13: 90800860-90801646 [+]), 7,

(the miR-106b cluster; X: 133131074-133131974 [-]), and X (the 106a cluster; 7: 99529119 -99529633 [-]). The miR-17 cluster consists of miR-17, -18a, -19a, -20a, 19b-1, and 92-1, while cluster 106a has members miR-106a, -20b, -19b-2, -92-2, and -363. Both clusters 17 and 106a are intergenic, while cluster 106b, which consists of miR-106b, -93, and -25, resides in the 11th intron of the MCM7 gene. The regulation of this group consisted of an initial increase at 8 hours that persisted until the 7-day mark, where it returned to the control levels (Figure 5.4).

Given the like regulation of these miRNAs situated on different chromosomes, it is possible that they have similar regulatory elements. In fact, both the miR-17 and -106b promoters have been previously characterized, and have been shown to be regulated by c-Myc. To predict the promoter sequence for the miR-106a cluster, we used five PPPs which all located a promoter within a 1Kb region ~2Kb upstream of the 106b pre-miRNA sequence. Further, CpG analysis found a CpG island corresponding to this area. A search for c-Myc binding E-boxes around this site found three within 1 Kb of the putative promoter area (Figure 5.5).

The regulation of miR-29a and -29b also held much interest, as they are similarly regulated, with miR-29a being significantly different from the mock treatement at 3dpi. Further, when adding the other family member into the group, miR-29c, which is also significantly regulated at 3 dpi, the entire family follows the same overall expression trends. Thus, we can suggest that these family members are likely co-regulated to some degree.

The miR-29 family members are situated at two separate intergenic loci, miR-29a and miR-29b-1 on chromosome 7 (7: 130212046-130212838 [-]) and miR-29c and miR-29b-2 on chromosome 1 (1: 206041820-206042491 [-]). However, considering the genomic organization of these miRNAs, it seems that the co-regulation in response to bystander effect is separate than that which is occurring at a basal level. The expression level of miR-29a is very high, higher than miR-29b, which has two loci. The miR-29c locus, although showing similar patterns of expression, has a much lower expression level. This would seem to indicate that either miR-29a is transcribed separately from miR-29b-1, or there is a degree of post-transcriptional regulation of miRNA maturation from these polycistronic miRNAs.

Promoter analysis using our *in silico* methods revealed no putative regulatory regions within 10Kb of the pre-miRNA sequence. Thus, as there is no previous data on the regulation of these miRNAs, and their promoter regions are unknown, inferences in to putative regulation of these clusters cannot be drawn.

5.4 Discussion

In this study, we analyzed the changes in the microRNAome of bystander tissue in an '*in vivo'* human tissue model. This model has been previously shown to exhibit profound cellular and molecular bystander changes (Seldelnikova, *et al.,* 2007). Specifically, the previous experiment showed increases in apoptosis, double-strand breaks, micronuclei formation, and senescent cells, as well as a decrease in global genome methylation. Given that miRNAs are known to regulate some of these processes, we investigated the roles miRNAs may play in the bystander response.

At 8 hours post-irradiation (hpi), we found a significant change in regulation in four miRNAs. miR-22, -141, and -16 were up-regulated, and miR-183 down-regulated (Table 5.1). Unfortunately, in the previous experiment using this bystander model system, changes in the tissue at this time point were minimal. Of the end-points assayed, only double-strand breaks were found to be elevated (~25%) this early in the bystander response.

Of the four miRNAs regulated at 8hpi, only miR-16 has been previously characterized. The miR-15/16 family has a notable role in the carcinogenesis, being amongst the first discovered cancer deregulated miRNAs. Its most notorious members are the polycistronic miR-15a and -16, which are severely down-regulated or deleted in ~70% of chronic lymphocytic leukemia patients (CLL: Calin *et al.*, 2002) due to chromosomal deletions or mutations at the 13q13.4 loci were they are situated.

It has been shown that miR-16 targets Bcl2, a known anti-apoptotic protein (Calin *et al.*, 2002), and it is the overexpression of Bcl2 that largely contributes to the malignant phenotype in CLL. Here we show that miR-16, which is strongly expressed in this tissue, is up-regulated, suggesting cellular Bcl2 levels would be lower. Despite the absence of large increased rates of apoptosis in the earlier experiments at this time-point, the elevated levels of Bcl2, if experimentally confirmed, could be pushing the cellular equilibrium towards a pro-apoptotic state.

MiR-16 has also been found to be involved in modulating G_0/G_1 cell cycle progression. Linsley, *et al.,* (2007) found that cell lines transfected with miR-16 caused the down-regulation of a number of genes involved in cell cycle progression. The transfected cells were found to be arrested in G_0/G_1. Therefore, the observed early up-regulation of miR-16 may suggest that the bystander cells are beginning to become delayed in the cell cycle. This has been previously shown in bystander 3D-cultures of human fibroblasts (Pinto, *et al.,* 2006), and could be attributed to the increase in double-strand breaks at this time point.

In the previous experiment, it seems logical that there are no large changes in molecular events at the 8-hour time point, as it takes time for these processes to manifest. Indeed, it also takes time for the bystander signal to propagate through the cells. However, finding these changes in miRNA expression at early time-points, before the manifestation of bystander symptoms, could suggest that miRNA regulation is upstream of some of the bystander responses. Further work characterizing some of the other regulated miRNAs at this time-point needs to be accomplished.

The miRNAs regulated at the 3 days post-irradiation (dpi) proved to be the most interesting, especially when considering them in association with the previous work done using this model. Here we found four miRNAs significantly up-regulated, miR-29a, -29c, -30a-5p, and -20a, and two miRNAs significantly down-regulated, miR-146a and -125b (Table 5.1).

MiRNAs 29a and 29c are members of the miR-29 family, along with, miR-29b, which also is 2.5-fold up-regulated in our array, but with less confidence ($p<0.10$). The miR-29 family has been well characterised in humans, and has been found to have some very interesting and relevant targets.

MiR-29a is the most highly regulated miRNA in the array. Even though its fold induction is not amongst the highest observed, it has the largest absolute change in expression, from ~12,000AUF in mock to ~20,000AUF in bystander cells. Considering the competition for miRNA machinery in the cell, it is likely that a change of this magnitude is more biologically significant than a higher fold induction in a low expressed miRNA; for example, a 5-fold change of 50AUF to 250AUF.

The miR-29 family has been found to target Mcl-1, the tightly controlled Bcl2 family member important in regulating tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis (Mott, *et al.*, 2007). Mcl-1 functions as a pro-survival protein by binding the pro-apoptosis Bcl2 BH3-only family members Bim, Bid, Bik, Noxa, and Puma (Chen, *et al.*, 2005). Although the exact mechanisms behind how Bcl2 family members mediate apoptosis is unknown, it has been shown that Mcl-1 binding of Bid and Bim protects against TRAIL-induced cell death (Willis and Adams, 2005; Mott, *et al.*, 2007). TRAIL-induced apoptosis is a cell-cell signalling mechanism of apoptosis mediated by the TRAIL ligand, a member of the tumour necrosis factor (TNF) family of cytokines (Sulliman, *et al.*, 2001; Willis and Adams, 2005). Further, TRAIL and other death cytokines are well known to be released by cells insulted with ionizing radiation

(Petit-Frère, *et al.,* 2000; Shareef, *et al.,* 2007). Thus, this method of cell-cell death signalling could be very important in bystander studies.

Interestingly, it has been shown that TRAIL-mediates cell-cell apoptotic bystander effects in response to cancer therapeutics (Huang *et al.,* 2003). Further still, Shareef, *et al.,* (2007) showed that the TRAIL-ligand was extensively released by irradiated cells *in vitro,* and media from these irradiated cells dramatically dropped bystander cell survival. When this media was treated with an anti-TRAIL antibody, the cell killing effect in bystander cells diminished. Together, these reports strongly implicate this TNF-mediated cytokine signalling in bystander effects, with possible regulation through miR-29 family members.

Mott *et al.,* (2007), showed that transfection of human cell lines with miR-29 family members' down-regulated Mcl-1 levels, and sensitized the cells to TRAIL-mediated apoptosis. One could presume that the large up-regulation of the miR-29 family in our bystander cells may be sensitizing or priming bystander cells for TRAIL-mediated apoptosis. Relating this to our previous experiment with this bystander model, apoptotic levels reached a maximum in 3 and 4 dpi, before returning to basal levels at 7 dpi. These increases in apoptosis over the experimental period closely mirror the expression level of the miR-29 family over the same experimental period, further suggesting the important role of the miR-29s in this effect.

The miR-29 family also targets the *de novo* DNA methyltransferases DNMT3a and DNMT3b. Fabbri, *et al* (2007) has shown that the miR-29 family targets the methyltransferases and have shown that miR-29 restoration in lung cancer cell lines restores aberrant methylation patterns. This paper showing that miRNA expression can directly affect methylation patterns is a truly novel finding. Even more interesting is the fact that DNA hypomethylation levels in our previous experiments were found to peak at 3 dpi, and tail-off at 7 dpi. Again, it appears that the expression of these miRNAs is very well correlated with the DNA methylation levels that it may be regulating.

MiR-20a is also up-significantly regulated at 3dpi. It derives from an important group of miRNAs, the miR-17 family. The miR-17 family was another of the first miRNA groups to be discovered to be deregulated in a number of cancers, including lymphomas (O'Donell, *et al.,* 2005), leukemias (Venturini, *et al.,* 2007), and lung (Hayashita, *et al.*, 2005), breast (Hossain, *et al.*, 2006), and testicular (Novotny, *et al.*, 2007) cancers, among others.

Specifically, miR-20a has been found to target transforming growth factor, beta receptor II (TGFBR2; Volinia, *et al.,* 2006). TGFBR2 is a tumour suppressor, and simply put, signals to inhibit cell cycle progression by phoshorylating a number of downstream targets (Xu and Pasche, 2007). Down-regulation of this transcript through up-regulation of miR-20a has not been shown to directly impact cell proliferation, making the purpose of its regulation in bystander response elusive. However, one could speculate that a decrease in the receptor would result in a decrease in signalling ability, pushing the cell to increasing proliferation.

Although only miR-20a is significantly ($p<0.05$) up-regulated at 3 dpi, both miR-106a and miR-106b, two other miR-17 family miRNAs, show a strong similar trend towards up-regulation ($p<0.10$). Interestingly, the mir-106 miRNAs have been shown to target the retinoblastoma protein (Rb1), another oft deregulated cancer transcript (Volinia, *et al.*, 2006). Rb1 has long been studied in oncology and is a known suppressor of cellular proliferation (Leiderman,*et al.,* 2007). Interestingly, the up-regulation in the miR-106 genes suggests a down-regulation of Rb1, again tilting the cells towards proliferation.

However, the most well known mechanism surrounding the miR-17 family is the regulation of the E2F transcription factors by both miR-20a and miR-17-5p, and possibly other miRNAs of this cluster. In this model, it has been shown that the proto-oncogene c-Myc transcription factor binds the mi-17 cluster's promoter region, inducing these miRNAs to target the E2F transcripts (O'Donnel, *et al,.* 2005; Sylvestre, *et al.,* 2007). Interestingly, the E2F transcription factors are also induced by c-Myc and, conversely, c-Myc is induced by the E2Fs (Matsumura, *et al.,* 2003). It is currently thought that the miR-17 cluster works as a regulator of the positive feedback loop between c-Myc and the E2F transcription factors, otherwise their reciprocal positive regulation could result in 'runaway' regulation (Coller, *et al.,* 2007). The up-regulation of the miR-17 cluster via c-Myc to produce an increase in expression of miR-17 and -20a are thought to indicate the interpretation of environmental signals to switch the cell to a proliferative state (Coller, *et al.*, 2007).

Given that miR-20a is a member of a polycistron, it is curious why the rest of the members of this polycistron were not also regulated in a similar fashion. Indeed, looking

at the expression patterns of the rest of the members of the polycistron, a similar expression pattern does emerge; however, the absolute abundance of the mature miRNA transcripts varies quite considerably. However, a number of factors could account for these discrepancies, including the regulation of maturation or nuclear export, as well as specific mature miRNA degradation (Lee, *et al.,* 2002; Mott, *et al.,* 2007). Further, the process of RNA editing could be modulating the sequence of the pri-miRNA, halting Drosha recognition. Editing can also affect the mature sequence itself, whereby it would no longer be detected by the microarray (Yang, *et al.,* 2006; Ohman, 2007).

Interestingly, we discovered very similar regulation occurring between family members located on different polycistrons (Figure 5.3, 5.4), suggesting that all of these polycistrons are regulated by the same factors. Research into the regulation of the 106a and 106b polycistrons found that the 106b cluster was within the 11th intron of MCM7. As miRNAs are most often transcribed with their host gene (Rodriguez, *et al.*, 2004) it is likely that the activation of MCM7, a gene involved in promoting cellular proliferation, is up-regulated in this bystander tissue. Further, it has been shown that MCM7 is transcriptionally regulated by c-Myc *in vivo* (Suzuki, *et al.,* 1998).

Given the previous two clusters were regulated by c-Myc, it follows that the intergenic 106a cluster is also regulated by c-Myc activity. To investigate the regulation of this cluster, we used a number of promoter prediction programs (PPPs) to find candidate areas for regulation upstream of this polycistron. All five of the PPPs predicated a promoter within a 1kB region ~2kB upstream of the 106a pre-miRNA sequence. Further, as it is

known that more than 70% of human promoters co-localize with a CpG island (Saxonov, *et al.,* 2006), this area was searched for CpG islands. Indeed, a large CpG island was found overlapping the promoter area (Figure 5.5).

Given we have a putative transcription start site for the 106a cluster, we searched the surrounding areas for c-Myc binding sites. c-Myc, with its partner Max, is known to bind to E-boxes (5'-GACGTG-3') to activate transcription (Blackwood and Eisenman, 1991; Knoepfler, 2007). We found three putative E-boxes within the 1kB of the putative promoter region (Figure 5.5). Taken together, these findings suggest that the entire miR-17 family is regulated by c-Myc.

c-Myc is a very widely studied gene, involved in the control of the cell size, cell proliferation, and apoptosis (Knoepler, 2007). Finding the regulation of this miRNA cluster to be additionally regulated by c-Myc is not surprising, as c-Myc has estimated to regulate 10-15% of the all genes in the human genome (Zeller, *et al.,* 2006; Knoepfler, 2007). However, looking at the expression patterns of the significantly regulated miR-17 family (ANOVA $p<0.05$), the up-regulation of these miRNAs by c-Myc endures for nearly the entire course of the experiment (Figure 5.4). It is likely that this indicates the up-regulation or increased activation of c-Myc in bystander cells, which is a novel finding.

It is interesting to speculate the function of c-Myc activity in bystander tissue. Increased levels of c-Myc in the cell are most often associated with increased cellular proliferation;

however, at the same time, it is thought that while promoting proliferation, c-Myc primes the cell for mitochondrial apoptosis (Nieminen, *et al.,* 2007a; Nieminen, *et al.,* 2007b). For example, c-Myc induction is associated with a decrease in anti-apoptotic Bcl2, and increases in pro-apoptotic Bax and Bim proteins (Nieminen, *et al.*, 2007a), as well as activation of the pro-apoptotic Bak (Nieminen, *et al.*, 20007b). Further, c-Myc has been found to play a notable role activating Death Receptor 5 (DR5), one of the TRAIL ligand receptors, and up-regulation of DR4 by c-Myc has also been shown to sensitize cells to TRAIL-induced apoptosis (Sheikh, 2004).

c-Myc and Bcl2 have long been shown to have an intimate relationship in cancer cells, whereby it is usually the overexpression of both that leads to the cancer phenotype. The result of this cooperation is that Bcl2 suppresses c-Myc driven apoptosis (Bissonnette, *et al.,* 1992; Fandi, *et al.,* 1992; Nieminen, *et al.,* 2007a). However, our miRNA expression patterns also lead us to believe that Bcl2 is down-regulated via the action of miR-16. MiR-16 is significantly up-regulated at 8hpi, with a strong trend towards up-regulation at 3dpi (Figure 5.2A). This decrease in cellular Bcl2 is likely to accent c-Myc directed priming of apoptosis, and increase the efficiency of TRAIL-mediated apoptosis.

Our experimental time-period extended to 7 dpi, however it appeared that much of the miRNAome of the bystander cells returned to basal expression levels by this point. However, there were two miRNAs, miR-181b and -181c, that were down-regulated at the 7 dpi. These miRNAs may be very important as they represent a persistent effect to the bystander response, and could be involved in transmission of the genome instability or

increasing transformation rates, two bystander phenomena widely studied in bystander effects. However, the functions of these miRNAs have not been characterized in a context that may elucidate their roles in bystander tissue. Future work characterizing how and who these miRNAs are regulating could prove important to understanding the persistent effects of the bystander response.

5.5 Summary and Conclusions

Here we show that miRNA regulation in bystander tissue is the most dramatically changed at the 3 dpi. Interestingly, this corresponds to the maximum apoptotic and hypomethylation levels seen in a previous bystander experiment under the same conditions (Sedelnikova, *et al.,* 2007). The miR-29 family may be playing a dual role in this tissue, promoting both global hypomethylation through the regulation of the *de novo* methyltransferases DNMT3A/3B, as well as preparing the cell for TRAIL-mediated apoptosis through the repression of the anti-apoptotic Mcl-1.

We further found that a fraction of the miR-17 miRNAs are co-regulated in bystander tissue. The up-regulation of these miRNAs is associated with pro-survival signalling, seeming in contrast to the pro-apoptotic state that may be established by miR-29 and miR-16. However, looking in to the regulation of this family it appears that all three clusters are regulated by c-Myc, suggesting c-Myc's up-regulation in bystander tissue. Interestingly, c-Myc has also been shown to 'prime' cells for TRAIL-mediated apoptosis by up-regulating death receptors and inhibiting anti-apoptotic proteins. Further, with the

regulation of miR-16 further down-regulating Bcl2, c-Myc mediated apoptosis should be elevated.

Taken together, our data may be suggesting that some of the miRNAs regulated in bystander tissue may be 'priming' cells to TRAIL-mediated apoptosis, preparing the cells for an impending death signal. Indeed, this is a novel notion, despite the TRAIL ligand being previously implicated in bystander effects. Interestingly, it is thought that TRAIL-mediated apoptosis is ineffective in cells not 'primed' for its response by accessory factors such as c-Myc (Nieminen, *et al.*, 2007a, Nieminen, *et al.*, 2007b), making these observations all the more logical.

Considering the above putative model in bystander cells, it implicates a two-signal mechanism. A preliminary signal would have to be sent to instigate the initial regulation of the 'primed' cell state, in order for the TRAIL-ligand to be signalling apoptosis in bystander cells. Of course, much work confirming this putative pathway needs to be performed.

5.6 Future Work

This model is based on the *in silico* and bioinformatic analysis, with the potential for many interesting experiments that can be performed to confirm this system.

Firstly, the miRNA expression levels should be independently confirmed via RT-PCR or *in situ* hybridization. Additionally, protein levels for the important miRNA targets

implicated above should be assayed; most importantly of which being c-Myc, as well as Mcl-1, Bcl2, and DNMT3A and 3B. A very interesting experiment would be to use fluorescently labelled anti-TRAIL antibodies to investigate the *in situ* levels of TRAIL over the experimental time course. Further works could utilize TRAIL-inhibitors in bystander tissue to assay apoptotic levels after radiation exposure.

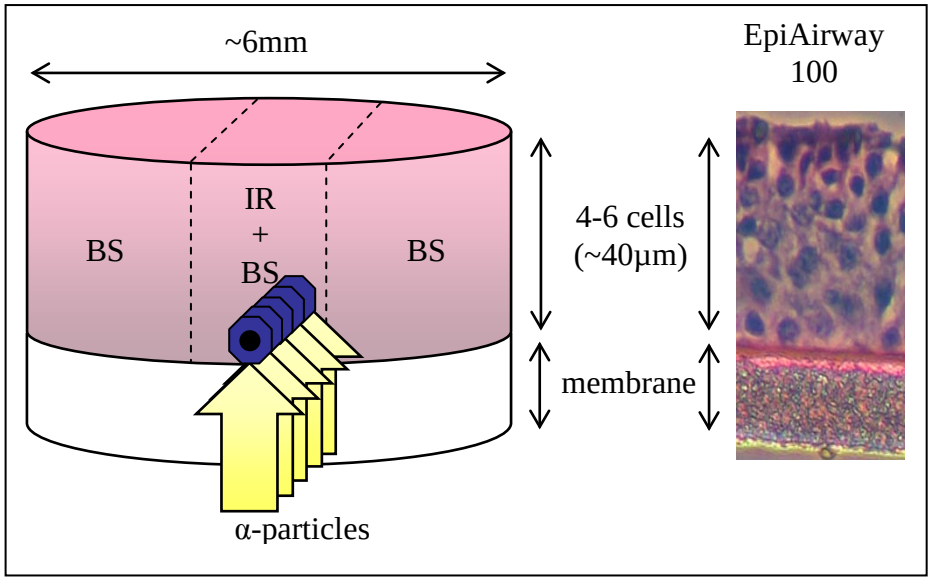


Figure 5.1: Tissue dimensions and irradiation set-up. The α-particle microbeam irradiated the Air-100 tissue through the membrane to deliver ~2 particles to each cell's nuclei in a single plane across the tissue base. Bystander tissue (BS) was harvested on either side of the irradiation plane for the microarray experiments.

Table 5.1: Significantly regualted miRNAs in bystander tissue when compared to the time mathched controls compared to their time-matched controls.

Days Post Irradiation	miRNA	Fold Induction
8 hours	hsa-miR-22	1.95
	hsa-miR-141	1.79
	hsa-miR-16	1.20
	hsa-miR-183	-1.31
3 days	hsa-miR-29c	2.45
	hsa-miR-29a	1.62
	hsa-miR-30a-5p	1.47
	hsa-miR-20a	1.25
	hsa-miR-146a	-1.26
	hsa-miR-125b	-1.18
7 days	hsa-miR-181a	-1.50
	hsa-miR-181b	-1.14

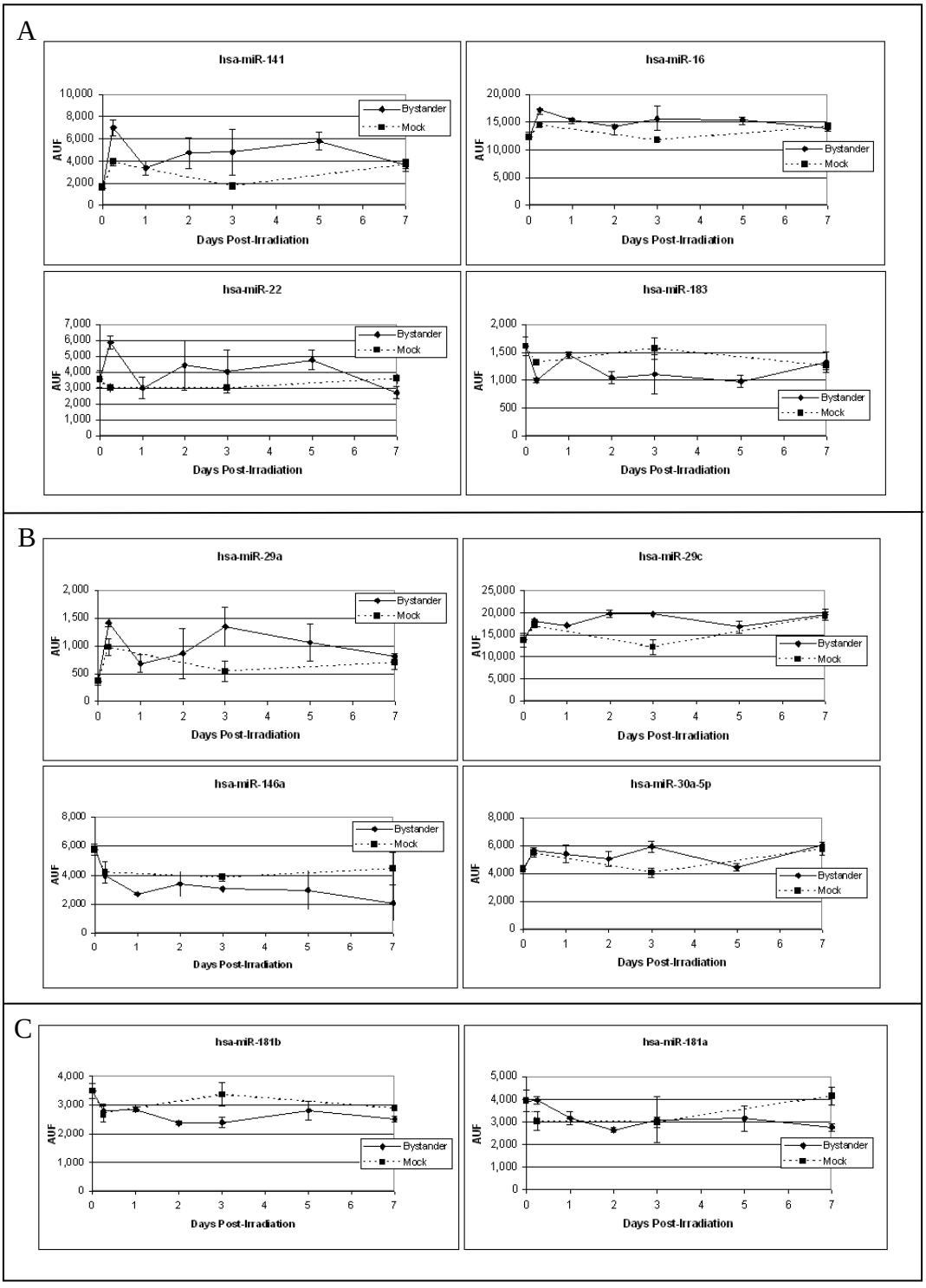


Figure 5.2: Significantly regulated miRNAs at 8 hours (A), 3 days (B), and 7 days (C) post irradiation. These graphs show the expression level of bystander miRNAs at 0, 0.25, 1, 2, 3, 5, and 7 days post-irradiation, thus displaying miRNA expression trends from bystander tissue.

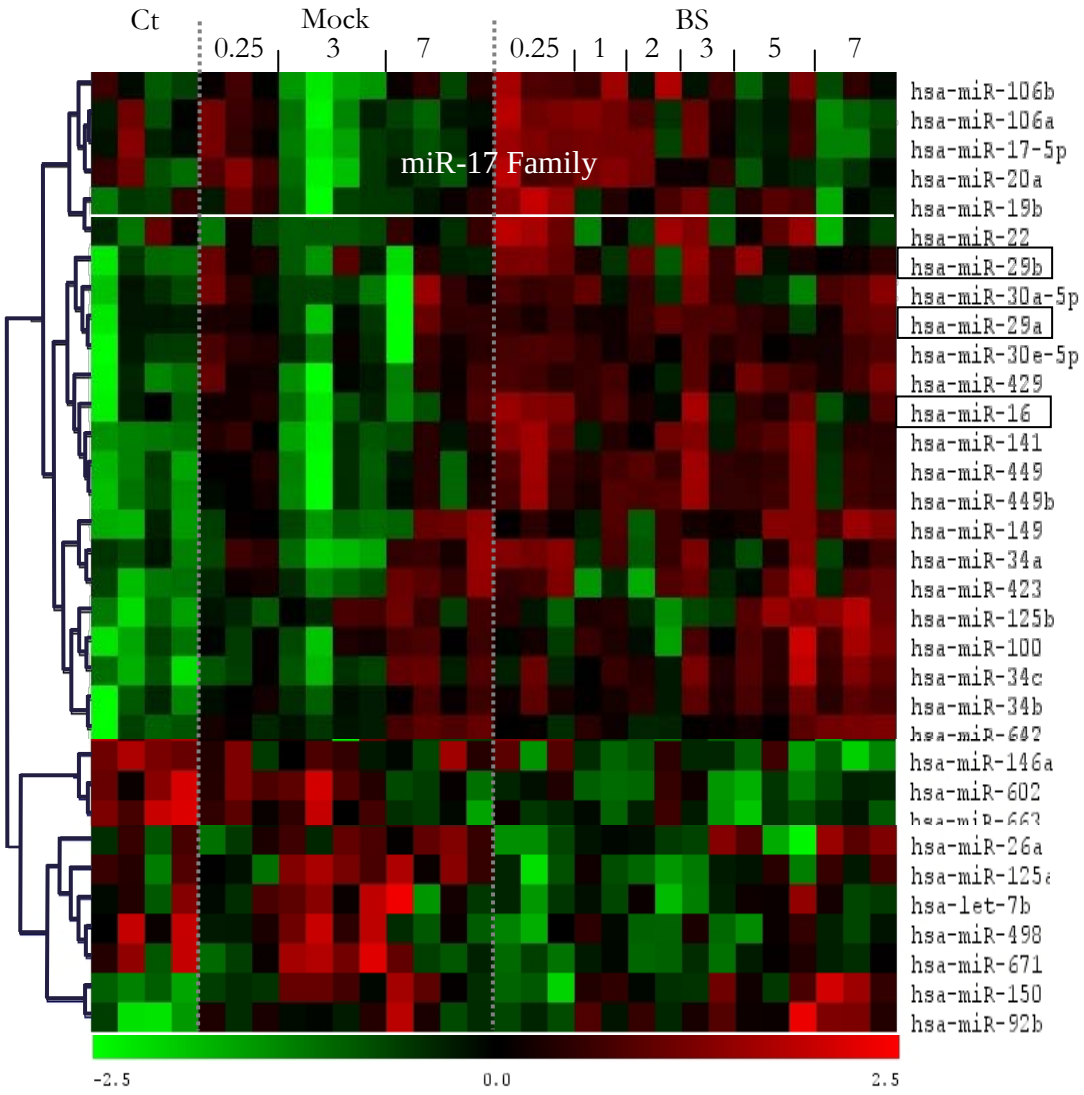


Figure 5.3: Heat map of ANOVA ($p<0.05$) expression analysis for all the treatments. White box includes co-regualted fraction of the miR-17 family. Black boxes highlight important miRNAs in the array.

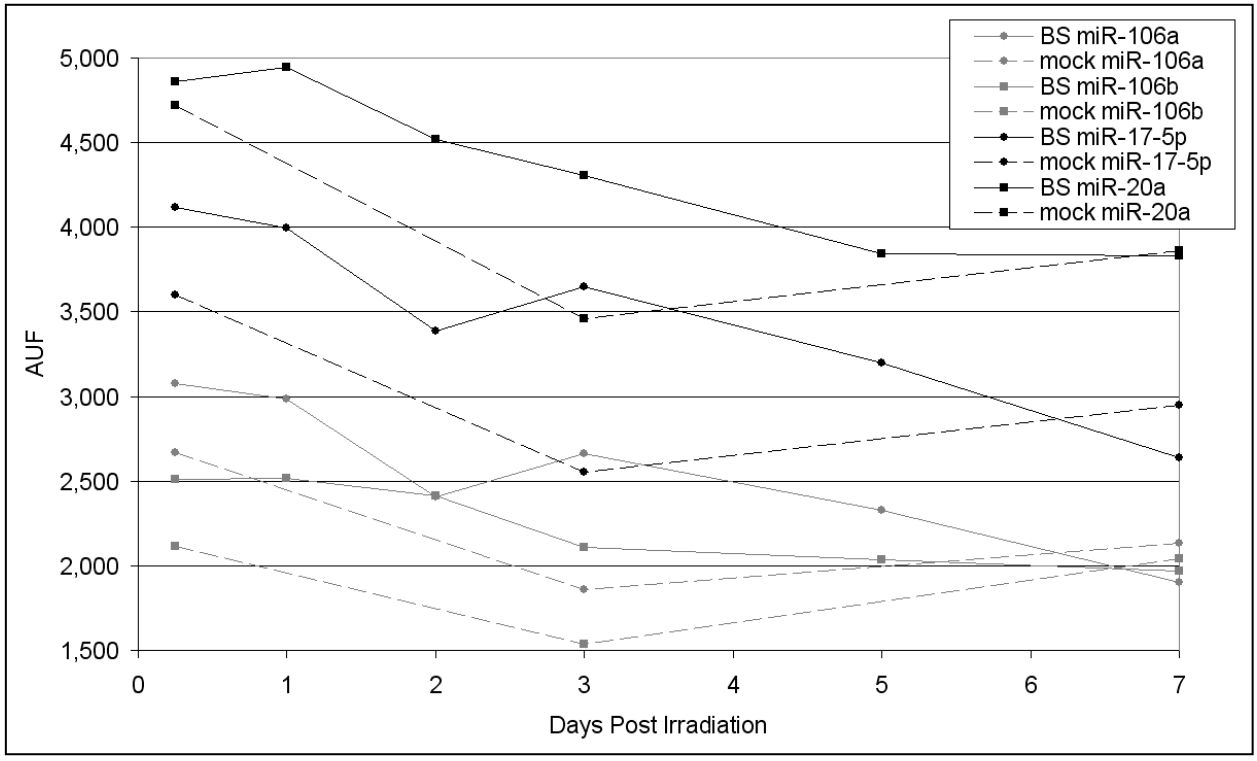


Figure 5.4: Like regulation of miR-17 family members located at different loci (miR-17-5p and 20a, 13q31.3; miR-106a, Xq26.2; miR-106b, 7q22.1).

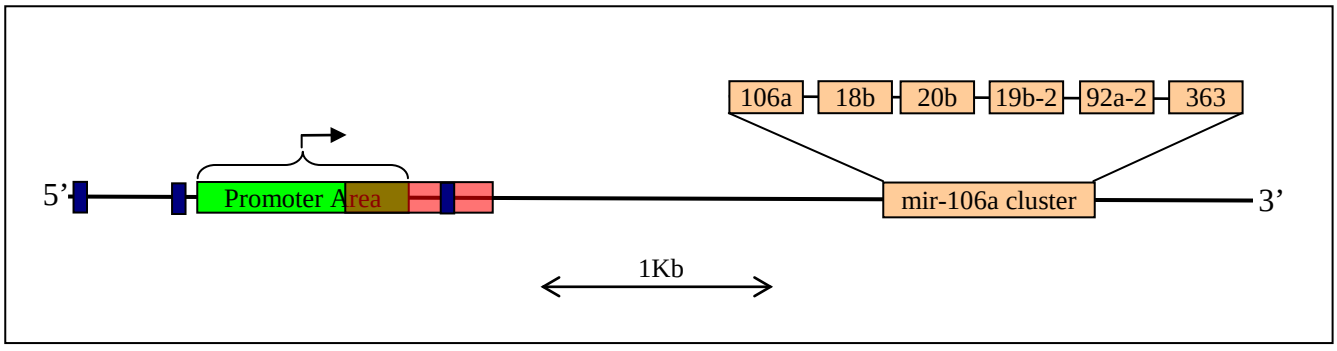


Figure 5.5: Putative promoter prediction for the miR-106a cluster. Green box indicates the predicated transcription start site by all five programs. Red box indicates a >500bp CpG island, and blue boxes represent putative c-Myc binding E-boxes (5'-GACGTG-3').

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