The effect of the viral suppressor protein p38 on anthocyanin biosynthesis in *Arabidopsis thaliana*

Eline Rustad Gaustad

Hedmark University College

Thesis for the Degree Master of Science

Department of Education and Natural Science

HEDMARK UNIVERSITY COLLEGE

2010

Table of Contents

TABLE OF CONTENTS **2**

ACKNOWLEDGEMENTS **4**

ABSTRACT **5**

NORWEGIAN ABSTRACT **7**

1. INTRODUCTION **9**

1.1 *ARABIDOPSIS THALIANA* AS A MODEL ORGANISM 9

1.2 *CHLAMYDOMONAS REINHARDTII* AS A MODEL ORGANISM 9

1.3 RNA SILENCING 10

1.3.1 Core components of RNA silencing pathways *12*

1.3.2 siRNAs *14*

1.3.3 miRNAs *15*

1.3.4 Example of a RNA silencing mechanism *16*

1.3.5 RNA silencing as a tool *18*

1.4 VIRAL SUPPRESSORS OF RNA SILENCING 20

1.5 THE FLAVONOID BIOSYNTHETIC PATHWAY IN *ARABIDOPSIS THALIANA* 21

1.5.1 Flavonoid biosynthesis *22*

1.5.2 Regulation of the flavonoid biosynthetic pathway *24*

2. AIMS OF STUDY **25**

3. MATERIALS AND METHODS **27**

3.1 PLANT METHODS 27

3.1.1 Plant lines *27*

3.1.2 Surface sterilization of seeds and growth conditions *27*

3.2 QUANTITATIVE REAL-TIME PCR ANALYSIS 28

3.2.1 Total RNA isolation *28*

3.2.2 cDNA synthesis optimalization *29*

3.2.3 Primer verification *29*

3.2.4 Sequencing *30*

3.2.5 Quantitative real-time PCR analyses *30*

3.3 ALGAL METHODS 31

3.3.1 Strains and culture conditions *31*

3.3.2 Artificial miRNA design *32*

3.3.3 Artificial miRNA dsDNA oligonucleotide cloning *32*

3.3.4 Transformation of Chlamydomonas *34*

3.3.5 Algal colony PCR of putative transformed cells 35
3.4 STANDARD DNA TECHNIQUES 35
3.4.1 Agarose gel electrophoresis 35
3.4.2 Quantification of RNA 36
3.5 BIOINFORMATICS 36
4. RESULTS 37
4.1 RNA ISOLATION 37
4.2 CDNA SYNTHESIS 38
4.3 PRIMER VERIFICATION AND SEQUENCING RESULTS 38
4.4 THE EFFECT OF P38 EXPRESSION ON THE TRANSCRIPT LEVELS OF FLAVONOID BIOSYNTHESIS GENES IN TRANSGENIC AT RCHSP38 PLANTS 39
4.4.1 Effect of transgenic constructs on the transcript levels of flavonoid biosynthesis genes 39
4.4.2 Effect of stress exposure with high sucrose treatment and high intensity light treatment on the transcript levels of flavonoid biosynthesis genes 43
4.5 THE EFFECT OF P38 EXPRESSION ON *ARABIDOPSIS* PHENOTYPE 47
4.6 *CHLAMYDOMONAS* TRANSFORMATION AND GROWTH 49
5. DISCUSSION 51
5.1 THE EFFECT OF P38 EXPRESSION ON THE TRANSCRIPT LEVELS OF FLAVONOID BIOSYNTHESIS GENES IN TRANSGENIC AT RCHSP38 PLANTS 51
5.1.1 Effect of transgenic constructs on the transcript levels of flavonoid biosynthesis genes 51
5.1.2 Effect of stress exposure with high sucrose concentration treatment and high intensity light treatment on the transcript levels of flavonoid biosynthesis genes 54
5.1.3 The phenotypic effects of p38 expression in Arabidopsis 57
5.1.4 Further studies 58
5.2 THE EFFECT OF P38 EXPRESSION IN *CHLAMYDOMONAS* 59
5.2.1 Other methods that could have been tried to ensure transformation of Chlamydomonas 60
5.2.2 Further studies 61
REFERENCES 63
ABBREVATIONS 71
APPENDIX 73

Acknowledgements

The work presented in this thesis was carried out at the Department of Education and Natural Science, Hedmark University College, in the period between January 2008 and March 2010. Supervision has been provided by Associate Professor Wenche Johansen and Associate Professor Robert C. Wilson (co-supervisor).

First I would like to thank Associate Professor Wenche Johansen for excellent and enthusiastic supervision provided during this project, and for assisting me throughout the writing process. I have learned a lot from being your student and I am very grateful for all the help you have given me.

I would also thank Associate Professor Robert C. Wilson for help and critical reading of this thesis and to fellow students, PhD-students and staff at the biotechnology group for their support and help throughout this project.

I want to thank my friends and family for their support and especially my dear husband André for all his support and encouragement. A special thanks to you that have critically read through this thesis. And finally my daughter Dina deserves a huge hug for giving me something else to think about besides this thesis.

Hamar, March 3th 2010

Eline Rustad Gaustad

Abstract

In this project, the effect of expressing p38, a viral suppressor of RNA silencing encoded by *Turnip Crinckle Virus* (TCV), in *Arabidopsis* and *Chlamydomonas* was investigated.

Transgenic *Arabidopsis* At RCHSp38 plants, which co-express an IR-transgene designed to induce RNA silencing of the endogenous *chalcone synthase* (*CHS*) gene and the RNA silencing suppressor protein p38, are known to accumulate high levels of anthocyanins. Our hypothesis was that the increased levels of anthocyanin in At RCHSp38 plants was a consequence of p38 expression leading to increased levels of the transcription factor *MYB75/PAP1,* as a result of p38-induced impaired RNA silencing control of *MYB75/PAP1*. To investigate our hypothesis, transcript levels of the flavonoid biosynthesis genes *phenylalanine ammonia lyase* (*PAL1*), *CHS*, *dihydroflavonolreductase* (*DFR*), *glutathione S-transferase* (*GST*), and *MYB75/PAP1,* were determined using quantitative real-time PCR in selected At RCHSp38 lines and compared to the same transcript levels in wild-type and At PAP1 plants, which over-expresses *MYB75/PAP1*. The effect of stress exposure with high sucrose concentration and high intensity light treatment on At RCHSp38 plants compared to wild-type and At PAP1 plants was also studied, to investigate if stress could elucidate differences in the transcript levels of the investigated genes among the different plant lines. Finally, phenotypic investigations of leaves and flowers in At RCHSp38 plants were undertaken.

The results show that the transcript levels of *MYB75/PAP1, PAL1, CHS, DFR,* and *GST* are up-regulated both in transgenic At RCHSp38 seedlings and adult plants compared to wild-type plants grown under the same experimental conditions, and in At RCHSp38 seedlings and plants exposed to stress. At RCHSp38 seedlings and adult plants also showed an altered phenotype with a more intense purple colouration compared to wild-type plants. The elevated level of *MYB75/PAP1* in At RCHSp38 plants indicate that this transcription factor is under RNA silencing control, which becomes impaired under p38-expression. A tasiRNA targeting *MYB75/PAP1* has been identified in previous studies and further analyses to confirm tasiRNA regulation of *MYB75/PAP1* and whether p38-expression interferes with tasiRNA biogenesis should be performed.

Low level of transgene expression is a problem in *Chlamydomonas*. If low transgene expression is the result of transgene silencing, a possible solution is to use suppressors of

RNA silencing in vector constructs to insulate your favourite transgene from silencing. We wanted to investigate if expression of the p38 protein in *Chlamydomonas* could suppress RNA silencing, more specifically if p38 could suppress RNA silencing of the endogenous *phytoene synthase* (*PSY*) gene induced by artificial microRNAs (amiRNAs). After successful induction of RNA silencing in transformed cells, we wanted to introduce p38 to investigate if the effect of RNA silencing of *PSY* was reduced or abolished. However, we were not able to successfully transform *Chlamydomonas* cells with the amiRNAs designed to silence *PSY*. Further studies to successfully transform *Chlamydomonas* cells, both with amiRNAs designed to silence *PSY* and with the p38-expression construct, are needed to answer the question whether p38 expression is able to reduce or abolish RNA silencing induced by amiRNAs.

Norwegian abstract

I dette prosjektet har effekten av å uttrykke p38, et ’RNA silencing’ suppressor protein fra *Turnip Crinckle Virus* (TCV), i *Arabidopsis* og *Chlamydomonas* blitt undersøkt.

Tidligere studier har vist at transgene *Arabidopsis* At RCHSp38 planter som uttrykker både et IR-transgen konstruert for å indusere ‘RNA silencing’ av det endogene *chalcone synthase* (*CHS*) genet, og det virale suppressor proteinet p38, akkumulerer høye nivåer av antocyaniner. Vår hypotese var at det høye nivået av antocyaniner i At RCHSp38 planter skyltes uttrykk av p38 som igjen fører til induksjon av transkripsjonsfaktoren *MYB75/PAP1*, et resultat av at p38 reduserer ‘RNA silencing’ kontroll av *MYB75/PAP1*. For å undersøke vår hypotese ble transkripsjonsnivå av følgende flavonoid biosyntese gener analysert ved bruk av kvantitativ real-time PCR i utvalgte At RCHSp38 plantelinjer, og sammenlignet med de samme transkripsjonsnivå i villtype og At PAP1 planter, som overuttrykker *MYB75/PAP1*: *phenylalanine ammonia lyase* (*PAL1*), *CHS*, *dihydroflavonolreductase* (*DFR*), *glutathione S-transferase* (*GST*) og *MYB75/PAP1*. Effekten av å utsette At RCHSp38 plantene for stress ved å dyrke plantene på agar medium med høy sukrose konsentrasjon og ved å inkubere plantene med høy lys intensitet, ble studert for å undersøke om stress kunne tydeliggjøre forskjeller i transkripsjonsnivå hos de undersøkte genene blant de forskjellige plantelinjene. Det ble også utført fenotypiske analyser av At RCHSp38 plantenes blad og blomster.

Resultatene viser at transkripsjonsnivå av *MYB75/PAP1*, *PAL1*, *CHS*, *DFR*, og *GST* er oppregulert i både transgene At RCHSp38 kimplanter og voksne planter sammenlignet med villtype planter dyrket under de samme forsøksbetingelsene og i At RCHSp38 planter utsatt for stress. At RCHSp38 kimplanter og voksne planter viser en forandret fenotype med en mer intens lilla farge i forhold til villtype planter. Det økte nivået av *MYB75/PAP1* i At RCHSp38 planter er en indikasjon på at denne transkripsjonsfaktoren er under ‘RNA silencing’ kontroll, hvorpå denne mekanismen forstyrres som følge av p38 uttrykk. I tidligere studier har et tasiRNA med *MYB75/PAP1* som mål blitt identifisert, og videre undersøkelser for å bekrefte tasiRNA regulering av *MYB75/PAP1* og hvorvidt p38 uttrykk forstyrrer tasiRNA biogenese bør utføres.

Lavt nivå av transgent uttrykk er et problem i *Chlamydomonas*. Hvis det lave transgene uttrykket kommer av transgen inaktivering er en mulig løsning å bruke ‘RNA silencing’

suppressor proteiner i vektorkonstrukter for å isolere transgenet fra inaktivering. Vi ville undersøke om uttrykk av p38 proteinet i *Chlamydomonas* kunne undertrykke 'RNA silencing', mer spesifikt om p38 kunne undertrykke 'RNA silencing' av det endogene *phytoene synthase* (*PSY*) genet indusert ved bruk av kunstige microRNAer (amiRNAer). Etter å ha indusert 'RNA silencing' i transformerte celler, ville vi introdusere p38 for å undersøke om effekten av 'RNA silencing' av *PSY* ble redusert eller opphevet. Våre forsøk på å transformere *Chlamydomonas* celler med amiRNAer var ikke vellykkede. For å svare på spørsmålet om hvorvidt utrykk av p38 kan undertrykke eller oppheve 'RNA silencing' indusert av amiRNAer, må videre studier utføres for å lykkes med å transformere *Chlamydomonas* celler, både med amiRNAer laget for å undertrykke *PSY* og med p38 uttrykkskonstruktet.

1. Introduction

1.1 *Arabidopsis thaliana* as a model organism

Arabidopsis thaliana is a dicotyledonous plant belonging to the mustard family (*Brassicaceae*). This plant is one of the best studied model organisms in plant biology, and has been used extensively in developmental, genetic and physiological studies (Leonelli, 2007). *Arabidopsis* has several advantages as a model organism; this includes a small and fully sequenced genome, short generation time, small plant size, and the plant produces a large number of offspring per generation. In addition, *Arabidopsis* is easy to transform and since the plant is self-pollinating, homozygous transgenic lines can be quickly generated. Because of its small size, large number of *Arabidopsis* plants can be grown in a greenhouse or indoor growth chamber in a relatively restricted place. The plant has a rapid life cycle; it takes only about 6-8 weeks from seed germination to the production of a new generation of seeds.

The *Arabidopsis* genome is 125Mbp and is organized into five chromosomes. Sequencing of the *Arabidopsis* genome was completed in 2000 by the Arabidopsis Genome Initiative (Arabidopsis Genome, 2000). The last genome release by The Arabidopsis Information Resource (TAIR9; http://arabidopsis.org/doc/news/breaking_news/140) includes 27 379 protein coding genes, 4827 pseudogenes or transposable elements and 1312 ncRNAs (33 518 genes in all, 39 640 gene models). In TAIR9, a total of 282 new loci and 739 new gene models were added.

1.2 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas reinhardtii is a unicellular chlorophyte algae (Harris, 2001). It is about 10 µm in diameter, though this varies significantly through the life cycle. Normally, the cells have two anterior flagella. *Chlamydomonas* has a cell-wall that consists primarily of hydroxyproline-rich glycoproteins. They have two contractile vacuoles at the anterior end of the cell and mitochondria dispersed throughout the cytosol. The chloroplast is cup-shaped and covers the basal two thirds of the cell. The pyrenoid is a distinct body within the chloroplast and is the centre of carbon-dioxide fixation.

As a model organism, *Chlamydomonas* has been used to study different biological processes including eukaryotic photosynthesis, flagellar structure and motility, cell-to-cell recognition and cell cycle control. Several features make *Chlamydomonas* a popular research target. It can easily be grown in liquid or agar media containing inorganic salts, at neutral pH. Wild-type *Chlamydomonas* carry out oxygenic photosynthesis to provide energy, but can also grow in the dark if acetate is added to the medium as an energy- and carbon source. This heterotrophic growth in the dark enables mutants blocked in photosynthesis to be viable as well. Under normal growth conditions *Chlamydomonas* is haploid, and effects of mutations can therefore be seen immediately without further crosses. Sexually competent gametes develop under nitrogen starvation, and the two mating types ((mt)+ and (mt)-) can fuse and form a diploid zygote. The diploid zygote can remain viable in soil for many years and under favourable conditions, four or as many as eight flagellated haploid cells can be released by meiosis.

In 2007 the *Chlamydomonas* genome sequence was completed and this revealed many previously unknown genes (Merchant et al., 2007). *Chlamydomonas* nuclear genome is 121Mbp and consists of 17 chromosomes (Merchant et al., 2007).

1.3 RNA silencing

RNA silencing is a common term used to describe several cellular pathways where small RNA molecules (sRNAs) regulate the expression of genes (reviewed by Carthew and Sontheimer, 2009). Both chromosome structure, chromosome segregation, transcription, RNA processing, RNA stability and RNA translation can be regulated by RNA silencing. Initially, RNA silencing pathways were named RNA interference (RNAi) in animals (Fire et al., 1998), post-transcriptional gene silencing (PTGS) in plants (Waterhouse et al., 1999) and quelling in fungi (Cogoni et al., 1996). The biological functions of RNA silencing range from antiviral defence in plants, regulation of endogenous gene expression in a diverse range of eukaryotic organisms, transposon taming, heterochromatin formation and chromosome segregation in *Saccharomyces cerevisiae*, and other types of genome regulation (Bartel, 2004; Lippman and Martienssen, 2004; Jones-Rhoades et al., 2006; Camblong et al., 2009; Dunoyer, 2009). In brief, as shown in Figure 1, RNA silencing is induced by double-stranded (ds) RNA molecules of various origins that are hydrolyzed by Dicer enzymes into sRNAs approximately 20-30 nt in length. The sRNAs are incorporated into Argonaute (Ago)

containing multiprotein complexes known as RNA Induced Silencing Complex (RISC) (Hammond et al., 2000). The sRNAs serve as specificity factors within RISC, and function to direct RISC to target complementary nucleic acid molecules via base paring interactions (Hammond et al., 2000; Zamore et al., 2000). Depending on various factors (discussed later) this base paring interactions between target nucleic acid and sRNA result in sequence-specific gene silencing at the DNA or mRNA level (Figure 1). Thus, although distinct, RNA silencing pathways share three common core features: 1) Dicer or Dicer-like enzymes, 2) regulatory sRNAs (20-30 nucleotides long) and 3) effector complexes (RISC) containing a protein from the Argonaute protein family. In the following, these three core components will be discussed in more detail.

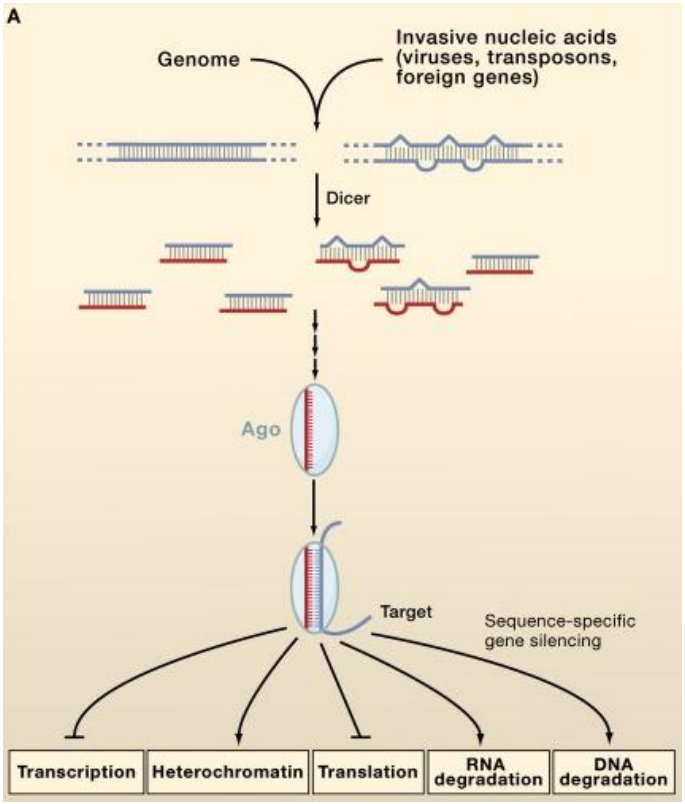


Figure 1: Core features of RNA silencing. dsRNA or ssRNA with a partial dsRNA structure, triggers the RNA silencing pathway. Dicer or dicer-like enzymes produce small RNAs (sRNAs) from the longer dsRNA precursors. The sRNAs serve as specificity factors within Argonaute protein containing effector complexes, called RNA inducing silencing complexes (RISC). The sRNA guides RISC to target nucleic acids and sequence-specific gene silencing occurs. The figure is from Carthew and Sontheimer, 2009.

1.3.1 Core components of RNA silencing pathways

Dicer-enzymes

The Dicer or Dicer-like enzymes are RNase III-type ribonucleases which produce sRNAs (Bernstein et al., 2001). The sRNAs are produced from longer perfect dsRNA (dsRNA with no mismatches and bulges), near perfect dsRNA precursors, or from single-stranded RNA (ssRNA) molecules adopting a partial dsRNA structure. These long dsRNA molecules originate from both endogenous and exogenous sources (see below for further description of sRNAs, siRNAs and miRNAs). The number of Dicers in different organisms varies, from one in mammals and nematodes, two in *Drosophila melanogaster* and four in *Arabidopsis thaliana* (reviewed by Carthew and Sontheimer, 2009). *Chlamydomonas* contains three Dicer-like enzymes (Casas-Mollano et al., 2008). Dicer-enzymes contain several functional domains in a specific order, from the amino- to carboxy terminus, including an ATPase domain, a DUF283 domain with unknown function, a PAZ domain, two tandem RNase III domains, and a dsRNA-binding domain (dsRBD) (reviewed by Carthew and Sontheimer, 2009). Dicer cuts dsRNA preferentially from the ends of dsRNA molecules (Zhang et al., 2004; MacRae et al., 2006). The dsRNA ends are bound by the PAZ domain, which especially binds duplex ends with short, ~2 nt, 3'overhangs (MacRae et al., 2006). The dsRNA substrate coils around the Dicer enzyme in approximately two helical turns before it reaches a processing centre involving the RNase III domains. The substrate is then cleaved by the two RNaseIII active sites, creating sRNAs with 5'-phosphate and 3'-hydroxyl ends, and with 2 nt overhang at the 3' end. For some species, for example *Drosophila*, the ATPase domain promotes dsRNA processing (Tomari and Zamore, 2005). For others, for example in humans, ATP is dispensable (Tomari and Zamore, 2005).

Argonaute proteins

Proteins of the Argonaute super family are the core component of the effector complex RISC. After dsRNA substrates have been cleaved into 20-30 nt sRNAs by Dicer, they enter into a RISC assembly pathway where the duplex sRNA unwind and one of the two strands, the guide strand, associates with the Argonaute protein resulting in stabilization of the guide strand (Tomari and Zamore, 2005). The other strand, the passenger strand is discharged and degraded. Which strand becomes incorporated into RISC is determined by the relative thermodynamic stability of the 5' ends of sRNAs. The strand with its 5' terminus at the less stably base-paired end will be the guide strand. The function of the guide strand is to direct RISC to target complementary nucleic acids. Argonaute proteins have four domains: the

PAZ domain, the PIWI domain and the N and Mid domains (reviewed by Carthew and Sontheimer, 2009). The PAZ domain has RNA 3' terminus binding activity which is used in guide strand binding (Yan et al., 2003). The PIWI domain can make a guide-strand-dependent endonucleotic cut in a base-paired target by adopting an RNase H-like fold catalyzing the cleavage (Parker et al., 2004; Song et al., 2004). The Argonaute protein super family can be divided into three sub-families: the Piwi clade binds piRNAs, the Ago clade associates with miRNAs and siRNAs, and a third clade only described in nematodes (Yigit et al., 2006). Most species contain several Ago proteins, for example *Arabidopsis* contains 10 (Voinnet, 2009) and *Chlamydomonas* 3 Argonaute-like proteins (Casas-Mollano et al., 2008).

sRNAs

Small RNAs can be subdivided into different classes based on their origin, the proteins required for their biogenesis, the Argonaute containing complex executing their regulatory function, and their biological function (reviewed by Ghildiyal and Zamore, 2009). The different sRNA classes from plants and metazoan animals are shown in Table 1. New classes and new sRNAs within existing classes are constantly discovered. As more knowledge is gathered, the distinctions between the classes become more blurred, but there are still some main differences. There are three main classes of small RNAs: short interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs). siRNA and miRNA are the most abundant both phylogenetic and physiological, and their precursors are perfect dsRNAs or RNAs adopting partial double stranded structure. Historically, the view of miRNA and siRNA has been that miRNA are of endogenous origin and siRNA are derived primarily from exogenous sources, typically from viruses, transposable elements or transgenes (reviewed by Carthew and Sontheimer, 2009). piRNA was formerly named repeat-associated siRNA (rasiRNA) (Aravin et al., 2001; Vagin et al., 2006). piRNA precursors appear to be single stranded, but they are not well understood. piRNA are primarily found in animals and exert their functions most clearly in the germline.

Table 1: Overview of different types of small silencing RNAs. From Ghildiyal and Zamore, 2009.

Name	Organism	Length (nt)	Proteins	Source of trigger	Function
miRNA	Plants, algae, animals, viruses, protists	20–25	Drosha (animals only) and Dicer	Pol II transcription (pri-miRNAs)	Regulation of mRNA stability, translation
casiRNA	Plants	24	DCL3	Transposons, repeats	Chromatin modification
tasiRNA	Plants	21	DCL4	miRNA-cleaved RNAs from the *TAS* loci	Post-transcriptional regulation
natsiRNA	Plants	22	DCL1	Bidirectional transcripts induced by stress	Regulation of stress-response genes
		24	DCL2		
		21	DCL1 and DCL2		
Exo-siRNA	Animals, fungi, protists	~21	Dicer	Transgenic, viral or other exogenous dsRNA	Post-transcriptional regulation, antiviral defense
	Plants	21 and 24			
Endo-siRNA	Plants, algae, animals, fungi, protists	~21	Dicer (except secondary siRNAs in *C. elegans*, which are products of RdRP transcription, and are therefore not technically siRNAs)	Structured loci, convergent and bidirectional transcription, mRNAs paired to antisense pseudogene transcripts	Post-transcriptional regulation of transcripts and transposons; transcriptional gene silencing
piRNA	Metazoans excluding *Trichoplax adhaerens*	24–30	Dicer-independent	Long, primary transcripts?	Transposon regulation, unknown functions
piRNA-like (soma)	*Drosophila melanogaster*	24–30	Dicer-independent	In *ago2* mutants in *Drosophila*	Unknown
21U-RNA piRNAs	*Caenorhabditis elegans*	21	Dicer-independent	Individual transcription of each piRNA?	Transposon regulation, unknown functions
26G RNA	*Caenorhabditis elegans*	26	RdRP?	Enriched in sperm	Unknown

1.3.2 siRNAs

siRNAs are produced by Dicer from dsRNA or dsRNA-like precursors, typically derived from exogenous sources such as viruses, transposable elements, nucleic acids taken up from the environment, or introduced experimentally or clinically (reviewed by Carthew and Sontheimer, 2009). Biological functions of exogenous derived siRNA are protecting genome integrity and in antiviral defence. siRNA duplexes consist of two 21 nt or 24 nt strands, with 5’ phosphate and 3’ hydroxyl group, and a 2 nt overhang at the 3’ ends. The guide strand from the siRNA duplex is incorporated into RISC by association with the Argonaute protein. The guide strand directs RISC to complementary RNA targets. If the siRNA guide strand is perfectly complementary to the RNA targets, degradation of the targets can occur as a result of the ‘slicer’ activity of the PIWI domain of the Argonaute protein (Liu et al., 2004; Song et al., 2004). The PIWI domain cleaves the phosphodiester linkage between target nucleotides base paired to the siRNA residue 10 and 11 (Tomari and Zamore, 2005). Once the cut is performed, cellular exonucleases complete the degradation process (Orban and Izaurralde,

2005). If the complementarity between the guide strand and the target RNA is not 100 %, or if the target RNA is recognized by an endonuclease-inactive siRISC, the target RNA can be silenced at a post-transcriptional level (Chiu and Rana, 2003). This can involve translational repression or exonucleolytic degradation. Heterochromatin formation can also be directed by siRISC. siRNA from genomic sources, so called endo-siRNA are derived from structured loci, convergent and bidirectional transcription or mRNAs paired to antisense pseudogene transcripts. As shown in Table 1, there are several different endo-siRNAs in plants; *cis*-acting siRNA (casiRNA) which are derived from transposons, repetitive elements and tandem repeats (Xie et al., 2004). CasiRNAs directs DNA methylation and histone modification at the loci from which they originate, hence promoting heterochromatin formation. *Trans*-acting siRNA (tasiRNA) includes elements from both the miRNA and siRNA pathway and functions in developmental timing and leaf polarity (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005; Ghildiyal and Zamore, 2009). TasiRNAs are derived from non-coding single stranded transcripts, called pri-tasiRNAs, which are transcribed from tasiRNA generating loci, that are converted into dsRNA by RNA directed RNA Polymerase 6 (RdRP6) (Peragine et al., 2004; Vazquez et al., 2004). Pri-tasiRNAs contain a binding site for a miRNA that guides cleavage at a defined point. This cleavage point triggers RdRP6-directed complementary strand synthesis, followed by a DCL4-dependent processing reaction which produces mature tasiRNAs (Allen et al., 2005). Natural antisense transcript-derived siRNA (natsiRNA) originates from a pair of convergent transcribed RNAs, in response to stress in plants (Borsani et al., 2005).

1.3.3 miRNAs

miRNA are excised from endogenous non-coding transcripts which fold into a stem-loop structure (reviewed by Brodersen and Voinnet, 2006). miRNAs are encoded in the genome, by the so called *MIR* genes. Animal *MIR* genes are found mostly within introns or exons, and are often genomically clustered and co transcribed as polycistronic RNAs (Kim, 2005). Plant *MIR* genes are mostly not clustered and are often found between protein-coding genes (Voinnet, 2009). miRNA repress gene expression post-transcriptionally by translation inhibition and/or mRNA cleavage. The biological functions of miRNA are thought to be in regulating gene expression in diverse developmental and physiological processes (Bartel and Chen, 2004; Stefani and Slack, 2008). The biogenesis of both plant and animal miRNA starts with the transcription of precursor transcripts called primary-miRNAs (pri-miRNAs) from

MIR genes by RNA Polymerase II (Lee et al., 2004). In animals the pri-miRNA is first processed by Drosha, an RNase III endonuclease (Lee et al., 2003), and its dsRNA-binding domain (dsRBD) partner, called DGCR8 in mammals and Pasha in flies. Pri-miRNAs are cut at the base of the stem-loop releasing a hair-pined structure called pre-miRNA, which contain a 2 nt 3' overhang and a 5' phosphate (Basyuk et al., 2003; Lee et al., 2003). The pre-miRNA is then transported to the cytoplasm by the nuclear export protein Exportin 5 and Ran, a GTPase that moves RNA and protein through the nuclear pore (Yi et al., 2003). Dicer, with help from its dsRBD partner protein (TRBP in mammals, LOQS in flies), cut the pre-miRNA in the cytoplasm (Kim, 2005). The resulting duplex consists of a miRNA and a miRNA* strand, corresponding to guide and passenger strand, respectively. In plants, DCL1 and its dsRBD partner HYL1, process the pri-miRNA to miRNA-miRNA* duplex in the nucleus. Some miRNAs are shown to be dependent on DCL4 rather than DCL1 in *Arabidopsis* (Rajagopalan et al., 2006). HASTY, an Exportin-5 homolog, exports the miRNA-miRNA* duplex to the cytoplasm (Park et al., 2005). The S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1) methylates plant miRNAs at their 3' ends in order to protect miRNAs from degradation, and this occur probably before miRNAs are loaded into AGO1 (Yu et al., 2005). When the miRNA duplex associates with the Ago-protein in RISC, it unwinds. The retained strand is usually the miRNA strand with the 5' terminus at the less stably base-paired end of the duplex (Kim, 2005). The miRNA strand then guides miRISC to recognize complementary mRNAs. The miRNA target sites are usually in the 3'UTR region of animal mRNAs, and the miRNA binds with several mismatches and bulges. This in contrast to plant mRNAs where the miRNA target sites usually are found in the coding region of the mRNA and the miRNA binds with almost perfect complementarity. It seems that both animals and plants have translational repression as the default mechanism by which miRNAs repress gene expression (Brodersen et al., 2008). In plants, perfect complementarity also allows Ago-catalyzed cleavage of the mRNA strand followed by degradation (Baumberger and Baulcombe, 2005).

1.3.4 Example of a RNA silencing mechanism

In the following, the miRNA pathway in plants is described in more detail, as shown in Figure 2. In plants, RNA polymerase II transcribes most pri-miRNA from *MIR* genes located between protein-coding genes (Voinnet, 2009). The pri-miRNA is both capped and polyadenylated and presumably stabilized by the RNA-binding protein DAWDLE (DDL)

(Yu et al., 2008). To convert pri-miRNA to pre-miRNA, DCL1, the double stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), and the C2H2-zinc finger protein SERRATE (SE) are needed. SE and HYL1 interact with DCL1 in nuclear processing centers called D-bodies (Fang and Spector, 2007). The pre-miRNA has a stem-loop structure. Pre-miRNAs, or mature miRNAs produced by DCL1, are transported to the cytoplasm. HASTY, the plant Exportin-5 homolog, exports some of the miRNAs from the nucleus to the cytoplasm, but *hasty* mutants show only decreased accumulation in some miRNAs in the cytoplasm, indicating the existence of a HASTY-independent miRNA export system (Park et al., 2005). HEN1 stabilizes mature miRNA duplexes by methylating the 3' terminal nucleotides of each strand to prevent uridylation and hence degradation (Li et al., 2005). The methylation by HEN1 protects miRNAs from SMALL RNA DEGRADING NUCLEASE (SDN). The guide miRNA strand is loaded into Ago-proteins to guide the RISC effector complex to target complementary mRNAs.

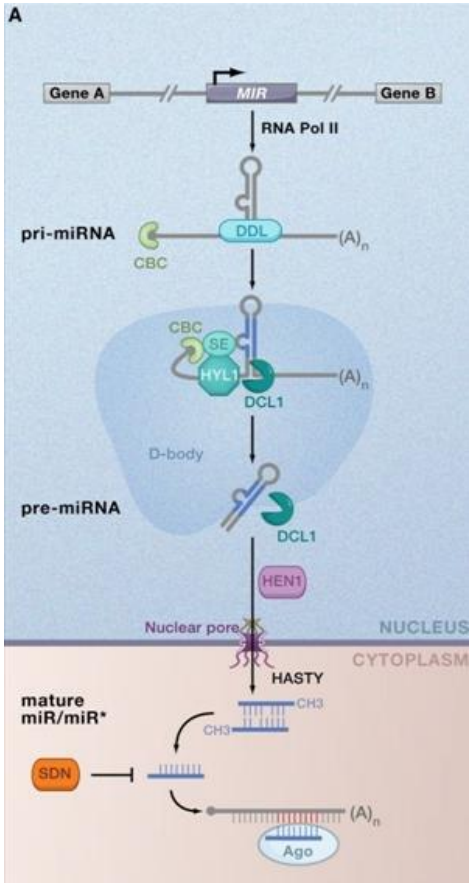


Figure 2: miRNA biogenesis in plants. MIR-genes are transcribed by RNA polymerase II into pri-miRNAs which are capped and polyadenylated, and presumably stabilized by the RNA-binding protein DAWDLE (DDL). DCL1, HYL1 and SE participate in the conversion of pri-miRNA to pre-miRNA in processing centers called D-bodies. HEN1 methylates miRNAs, methylated miRNAs are protected from SDN, an exonuclease that degrade miRNA single strands. HASTY is an Exportin 5 homolog that transports miRNAs from the nucleus to the cytoplasm. The figure is from Voinnet, 2009.

miRISC effector complexes can repress gene silencing by translation inhibition, slicing or both. But the exact mechanisms remain unclear. Most plant miRNA involves a combination of translation inhibition and slicing (Brodersen et al., 2008)

1.3.5 RNA silencing as a tool

RNA silencing is an important tool in basic research, biotechnology and medicine. RNA silencing can be exploited to specifically down-regulate the expression of any gene of interest. Experimentally, RNA silencing is induced by a silencing trigger delivered into the cell or organism. This trigger can either be synthetic siRNA, dsDNA, a transgene DNA construct designed to transcribe either long dsRNA molecules or stem loop precursors, or alternatively, viral vectors can be used to deliver the silencing trigger. In *Caenorhabditis*

elegans, RNA silencing, or more specifically RNA interference (RNAi), has been used to down-regulate the expression of about 86 % of its genes to facilitate analysis of the connection between gene sequence, chromosomal location and gene function (Kamath et al., 2003). In *Drosophila melanogaster*, the functions of 91 % of the genes predicted to participate in growth and viability were identified in a genome-wide RNAi analysis (Boutros et al., 2004). After several successful pre-clinical trials in small animals, RNAi is already being used therapeutically in human clinical trials (Castanotto and Rossi, 2009). Some of the diseases that hopefully can be treated with RNAi-based tools are HIV and other virus infections, different types of cancer and age-related macular degeneration (reviewed by Grimm, 2009).

Inverted repeat (IR) transgene constructs have been the method of choice to silence genes in plants, and this method has formed the basis of the RNA silencing experimental approach in plants (reviewed by Brodersen and Voinnet, 2006). Upon transcription of the IR-transgene, a perfect dsRNA molecule is produced that is hydrolyzed by Dicer resulting in a pool of siRNA. The siRNA then guide RISC to target mRNA molecules complementary to the siRNAs resulting in mRNA degradation. Even though this method has proven as an efficient way to down-regulate endogenous plant gene expression (Waterhouse and Helliwell, 2003), several studies have reported variable levels of target transcript reduction among independent RNA silencing lines transformed with the same IR-transgene construct (Kerschen et al., 2004; Johansen et al., manuscript in preparation). The effectiveness has been shown to be strictly and positively correlated to the accumulation level of siRNA (Johansen et al., manuscript in preparation). This is probably the result of variable levels of transgene dsRNA production, and as a consequence, variable levels of siRNA production, in different independent transgenic lines (Johansen et al., manuscript in preparation). A more recent developed method for RNA silencing in plants, more specifically in *Arabidopsis*, is the use of artificial microRNAs, amiRNAs (Schwab et al., 2006). A DNA construct designed to produce an artificial miRNA precursor is transformed into the plant. Upon transcription, the artificial miRNA precursor is recognized by the organisms miRNA pathway, resulting in the release of a 21-nt fully functionally ssRNA molecule that mimic miRNA molecules. This miRNA, known as amiRNA, then guide RISC to target complementary mRNA molecules, resulting in a sequence specific down-regulation of the target gene. The construction of the amiRNA sequence is critical and needs to be designed carefully in order to specifically silence the gene of interest. A web based tool (Web MicroRNA Designer, WMD3,

http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) has been developed to enable this design. The amiRNA approach offers several advantages over the IR method. Expression of long dsRNA, produced by the IR-transgenes, can be problematic because of self silencing of the IR-transgenes through the transcriptional silencing mechanism (Rohr et al., 2004). Off-target effects are also possible because of the generation of different siRNA molecules from the long dsRNA precursors, where some siRNAs may be able to target mRNAs unrelated to the target gene (Xu et al., 2006). By using amiRNAs, both the self-silencing problems and off-target effects of siRNAs can be overcome, since an amiRNA precursor gives rise to only a single small RNA species that can be optimized to avoid off-target effects (Molnar et al., 2009). Recently, the amiRNA approach has also been used in *Chlamydomonas reinhardtii* as a highly specific, high-througput silencing system (Molnar et al., 2009). The use of amiRNAs provides a relatively easy method for specific gene silencing and has been used in this project to silence the *phytoene synthase* (*PSY*) gene in *Chlamydomonas reinhardtii*.

1.4 Viral suppressors of RNA silencing

RNA silencing functions as an antiviral defence mechanism in plants and possibly also other organisms (Obbard et al., 2009). To protect themselves from the antiviral RNA silencing mechanism, many viruses have evolved suppressors of RNA silencing (VSRs) (Li and Ding, 2006; Wu et al., 2009). In addition to their function in the normal lifecycle of the virus, VSRs have evolved the ability to suppress RNA silencing. By 2006 more than 50 VSRs from over 30 viral genomes have been identified (Li and Ding, 2006). VSRs are encoded by both plant and animal-infecting RNA viruses and some DNA viruses as well. Most of the VSRs are proteins with dsRNA-binding activity. It is also suspected that some of the VSRs are RNAs that function by binding RNA silencing components (Li and Ding, 2006). Different VSRs target different components of the RNA silencing machinery (Ding and Voinnet, 2007). VSRs show no amino acid sequence similarity, indicating no structural similarity. In addition to functioning as a counter defensive strategy to the RNA silencing response, VSRs have been shown to interfere with transgene-induced and virus-induced PTGS of transgenes or endogenous genes in plants (Voinnet et al., 1999) as well as endogenous RNA silencing pathways such as the miRNA (Chapman et al., 2004) and tasi-RNA pathways (Moissiard et al., 2007). In general, VSRs may interfere with different steps in the RNA silencing pathway (Burgyan, 2006):

- Preventing the generation of siRNAs
- Inhibiting the incorporation of siRNAs into the effector complex
- Interfering with the antiviral effector complex
- Interfere with transitivity silencing leading to signal amplification and systemic spread

P19, encoded by *Tomato bushy stunt virus* (TBSV), is one of the best mechanistically studied VSRs. In the normal life cycles of the virus, this protein is required for short and long distance virus movement, in addition to symptom production (Canto et al., 2006). As a suppressor of RNA silencing, p19 functions to bind siRNA thus preventing the incorporation of siRNA into RISC (Silhavy et al., 2002). Another VSR is the p38 capsid protein encoded by *Turnip Crinkle Virus* (TCV). TCV infects many plant species, including *Arabidopsis thaliana*. P38 is a strong suppressor of RNA silencing that blocks PTGS induced by sense, antisense and dsRNA (Qu et al., 2003). It does not interfere with miRNA function (Dunoyer et al., 2004) but it does interfere with tasiRNA accumulation (Moissiard et al., 2007), transitivity and systemic silencing (Qu et al., 2003). The exact mechanism by which p38 exerts its effect on RNA silencing is unknown, but the protein is known to suppress DCL4 activity (Deleris et al., 2006).

1.5 The flavonoid biosynthetic pathway in *Arabidopsis thaliana*

Flavonoids consist of a diverse family of aromatic molecules derived from phenylalanine and malonyl-coenzyme A (Winkel-Shirley, 2001b). In most of higher plants, six major subgroups of flavonoids are found; the chalcones, flavones, flavonols, flavandiols, condensed tannins and anthocyanins. The aurones comprise a seventh group which is found in many higher plants, but it is not ubiquitous. In addition to these subgroups, some plant species synthesize isoflavonoids, phlobaphenes and stilbenes. Flavonoids are responsible for the red, blue and purple pigments in plants. Flavonoids serve many biological functions in plants, including protecting them from environmental stress, signalling between plants and microbes, male fertility of some species, defence against antimicrobial agents, feeding deterrents, UV protection, recruitment of pollinators and seed dispersers (Winkel-Shirley, 2001b).

1.5.1 Flavonoid biosynthesis

Flavonoids are synthesized via the phenylpropanoid biosynthetic pathway (Winkel-Shirley, 2001b). The first enzyme in the general phenylpropanoid pathway is phenylalanine ammonia lyase (PAL), as shown in Figure 3 (from Winkel-Shirley, 2001a). The first committed step in the flavonoid biosynthesis is catalyzed by chalcone synthase (CHS), which uses malonyl CoA and 4-coumaroyl CoA from the general phenylpropanoid pathway as substrates. The resulting chalcones can be further modified into for example aurones, flavones, isoflavonoids and flavonols. From dihydroflavonols, dihydroflavonol reductase (DFR) produces leucoanthocyanidin that can be further modified into condensed tannins, also called proanthocyanidins, or become anthocyanins. DFR is specific for the anthocyanins branch of the pathway in *Arabidopsis* (Borevitz et al., 2000). Anthocyanins are the subgroup of the flavonoids which is responsible for red, purple and blue pigmentation. They are water soluble vacuolar pigments that appear in all tissues of higher plants. In the central flavonoid pathway in *Arabidopsis* all of the enzymes, except one, are encoded by single genes (Winkel-Shirley, 2001a). The exception is flavonol synthase (FLS), which may be encoded by six genes (Winkel-Shirley, 2001b). Mutations in genes in this pathway thus affect expression in all tissues and under all environmental conditions in *Arabidopsis*. Because of this, *Arabidopsis* is useful to reveal the effect of the flavonoid enzymes.

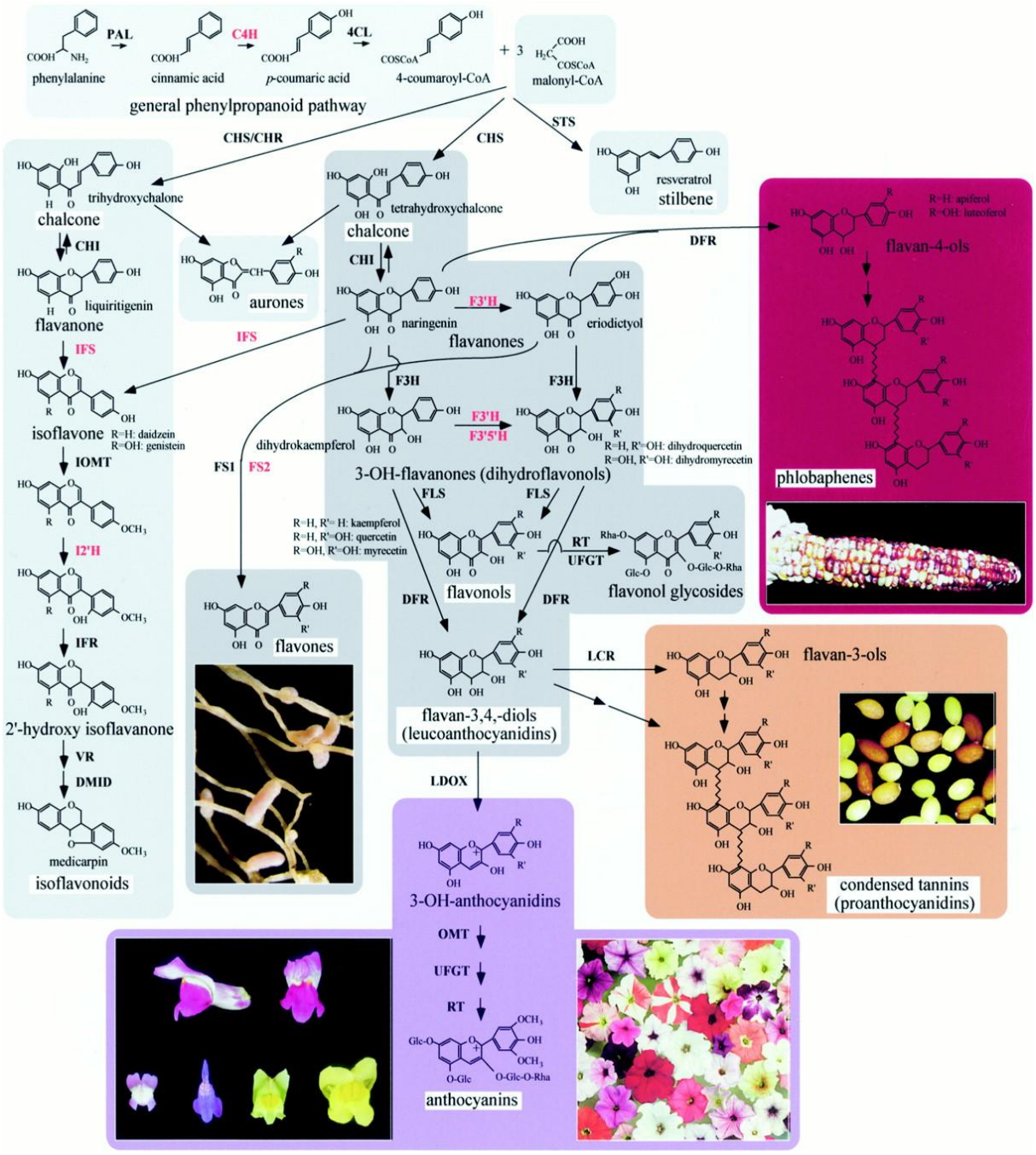


Figure 3: Schematic drawing of the major branch pathways of the flavonoid biosynthesis, starting with general phenylpropanoid metabolism and leading to the major subgroups. Only a few examples of the enormous variety of end products are shown. The colourless subgroups are shown in grey boxes; the chalcones, aurones, isoflavonoids, flavones, flavonols and flavandiols. Coloured boxes show the subgroups responsible for pigmentation; the anthocyanins, proanthocyanins and phlobaphenes. Enzyme names are abbreviated as follows: cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase (DMID), flavanone 3-hydroxylase (F3H), flavones synthase (FSI and FSII), flavonoid 3'hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H),

isoflavone O-methyltransferase (IOMT), isoflavone reductase (IFR), isoflavone 2'-hydroxylase (I2'H), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), O-methyltransferase (OMT), Phe ammonia-lyase (PAL1), rhamnosyl transferase (RT), stilbene synthase (STS), UDPG-flavonoid glucosyl transferase (UFGT), and vestitone reductase (VR). From Winkel-Shirley, 2001a.

1.5.2 Regulation of the flavonoid biosynthetic pathway

Both biotic and abiotic stress influences the flavonoid biosynthetic pathway. Many transcription factors are involved in the regulation of the flavonoid biosynthetic pathway, including members of Myb, bHLH and WD-repeat families (Gonzalez et al., 2008). Members of these families can form complexes, called MBW complexes, involved in regulating the transcription of genes involved in anthocyanin and proanthocyanin biosynthesis (Baudry et al., 2004; Gonzalez et al., 2008). Different combinations of transcription factors can specify which flavonoid pigment class is produced, where it will be produced, what stimulus will turn on the production, and whether transcriptional regulation of structural genes is positive or negative (Aharoni et al., 2001; Winkel-Shirley, 2001b; Baudry et al., 2004; Solfanelli et al., 2006). The flavonoid biosynthetic pathway in *Arabidopsis* can be subdivided into early and late steps where the early steps are not dependent of the MBW complexes, but the late steps are (Gonzalez et al., 2008). *CHS* are among the early genes, while *DFR* are among the late genes. *PAL* is in the general phenylpropanoid pathway, preceding the flavonoid pathway. Examples of members from the Myb-family that regulates flavonoid biosynthesis in *Arabidopsis* are the MYB genes *MYB75/PAP1* and *PAP2* (Borevitz et al., 2000). The analysis of loss-of-function mutants suggested that MBW-complexes involving MYB75/PAP1 mainly targets late anthocyanin biosynthetic genes (Cominelli et al., 2008; Gonzalez et al., 2008). Examples of abiotic stress treatment to induce anthocyanin production in *Arabidopsis* are sucrose and excess light. Sucrose induces anthocyanin production in *Arabidopsis* in a concentration dependent manner (Tsukaya et al., 1991; Ohto et al., 2001; Teng et al., 2005; Solfanelli et al., 2006), and the *MYB75/PAP1* gene is required for sucrose to induce anthocyanin production (Teng et al., 2005). Light is also a strong inducer of anthocyanin regulatory genes in *Arabidopsis*, and high intensity light treatment induces accumulation of anthocyanin pigments (Feinbaum and Ausubel, 1988; Jenkins, 1997; Cominelli et al., 2008). Exposure to fungi is an example of biotic stress influencing anthocyanin biosynthesis (Lo and Nicholson, 1998).

2. Aims of study

The aims of the present study were to investigate the effect of expressing p38, a viral suppressor protein of RNA silencing encoded by *Turnip Crinckle Virus,* on the anthocyanin synthesis in *Arabidopsis*, and to investigate if p38 is able to suppress RNA silencing in *Chlamydomonas*.

Previously studies have shown that transgenic *Arabidopsis* plants (At RCHSp38) co-expressing an IR-transgene designed to induce RNA silencing of the endogenous *chalcone synthase* (*CHS*) gene and the viral suppressor protein p38 encoded by *Turnip Crinckle Virus,* accumulate high levels of anthocyanins (a flavonoid subgroup) in leaves and stems at late developmental stages (Johansen and Wilson, 2008). Other studies has shown that p38 suppresses DCL4 activity (Deleris et al., 2006) and that DCL4 mutants (*dcl4-2*) shows increased levels of anthocyanins (Nakazawa et al., 2007). Taken together, these observations indicate that loss of DCL4 activity leads to over-accumulation of anthocyanins. MYB75/PAP1 is a key regulator of anthocyanin synthesis in *Arabidopsis* (Borevitz et al., 2000; Teng et al., 2005; Gonzalez et al., 2008). Accordingly, in transgenic plants (At PAP1) over-expressing the transcription factor *MYB75/PAP1,* the transcription of the flavonoid biosynthetic genes *PAL1, CHS, DFR* and *GST* are induced (Borevitz et al., 2000). A tasiRNA targeting *MYB75/PAP1* has been identified (Rajagopalan et al., 2006; Hsieh et al., 2009), indicating that *MYB75/PAP1* is under RNA silencing control via the tasiRNA pathway. We speculate that this control keeps the level of *MYB75/PAP1* low, and thus the anthocyanin level normal in wild-type plants. Our hypothesis is that the increased levels of anthocyanin in At RCHSp38 plants is a consequence of p38 expression that leads to increased levels of *MYB75/PAP1* as a result of p38-induced impaired RNA silencing control of the *MYB75/PAP1* transcription factor. In order to test this hypothesis, transcript levels of selected genes coding for enzymes involved in the flavonoid biosynthetic pathway and regulation were analysed. Thus, the transcript levels of *phenylalanine ammonia lyase* (*PAL1*), *chalcone synthase* (*CHS*), *dihydroflavonolreductase* (*DFR*), *glutathione S-transferase* (*GST*), and the transcription factor *MYB75/PAP1* were determined by quantitative real-time PCR in selected At RCHSp38 lines and compared to the same transcript levels in wild-type. For comparison, the same transcript levels were investigated in At PAP1 plants. We also wanted to investigate the effect of stress exposure on At RCHSp38

plants compared to wild-type and At PAP1 plants, to see if stress could elucidate differences in flavonoid biosynthetic gene expression among the different plant lines.

The p38 protein is a well-known suppressor of RNA silencing in plants. We wanted to investigate if this protein is also able to suppress RNA silencing in the model organism *Chlamydomonas*. The rationality for this investigation is two-folded. First, studies using suppressors of RNA silencing can result in important knowledge regarding basic aspects of RNA silencing in *Chlamydomonas*. Secondly, in *Chlamydomonas*, the exploitation of transgene technology (specifically, nuclear transformation) are often found to be hampered by low level of transgene expression, in some cases a result of transgene silencing (Fuhrmann et al., 1999; Neupert et al., 2008; Eichler-Stahlberg et al., 2009). Therefore, strategies to overcome the silencing mechanisms are attractive. One possible solution would be to use suppressors of RNA silencing in vector constructs to insulate your favourite transgene from silencing. In order to investigate if expression of the p38 protein in *Chlamydomonas* could suppress RNA silencing, we wanted to induce RNA silencing of the endogenous *phytoene synthase* (*PSY*) gene by using artificial microRNAs. Then, after successful induction of RNA silencing in transformed cells, we wanted to introduce p38 to investigate if the effect of RNA silencing of *PSY* was reduced or abolished.

3. Materials and Methods

3.1 Plant methods

3.1.1 Plant lines

In this project wild-type *Arabidopsis thaliana* (At) ecotype Columbia-0 plants were used in addition to the transgenic lines At RCHSp38 and At PAP1. The At RCHSp38 lines were obtained from Wenche Johansen, HUC. At RCHSp38 lines, which are transformed with the pRCHSp38 DNA vector, co-express an IR-transgene, designed to induce RNA silencing of the endogenous chalcone synthase (*CHS*) gene, and the viral suppressor protein p38 encoded by *Turnip Crinckle Virus* (Johansen and Wilson, 2008). In these lines, the IR-transgene is controlled by the 35S promoter, while the p38 viral suppressor gene is controlled by the promoter CmpC from *Cestrum yellow Curling Virus* (CmYLCV) (Stavolone et al., 2003). Four different At RCHSp38 lines (lines 1, 4, 27 and 37) were selected for this study because these lines express p38 transcripts at high levels (Johansen and Wilson, 2008). At PAP1 seeds were obtained from the European Arabidopsis Stock Centre (NASC, Code N3884). At PAP1 has a bright-purple phenotype caused by over expression of the MYB transcription factor *MYB75/PAP1* resulting from the activation tagging by *Agrobacterium*-mediated transformation with a T-DNA that carries the 35S enhancer (35Se) sequence at the right border (Borevitz et al., 2000).

3.1.2 Surface sterilization of seeds and growth conditions

Arabidopsis thaliana seeds were surface-sterilized in 75 % EtOH for 5 min at room temperature and with careful stirring. EtOH was removed and 10 % chlorine was added and the seeds were further incubated for 20 min at room temperature and careful stirring. The seeds were then washed 4 times with sterile water and finally kept in 250 µl sterile water before plated on 0.5 x Murashige & Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 1.5 % agar and 1 % (w/v) sucrose and added 0.75 % H_2O-agar. For some experiments 3 % (w/v) sucrose was added to the MS-medium. 10 mg/l glufosinate ammonium (trade name BASTA, Riedel-de Haën) were added to the medium as a selective marker and used to plate out transgenic seeds. Approximately 100 seeds were plated for each line. The plates were sealed with surgical tape and placed at 4 ˚C for about 48 hours, and

then incubated at 23 ˚C in continuous white light (100 µE/m^2s) and 60-65 % relative humidity. After approximately two weeks the seedlings were transferred to autoclaved compost soil (Herbia, Nordic Garden AS) and further cultivated at 23 ˚C in continuous white light (100 µE/m^2s) and 60-65 % relative humidity. High intensity light treatment was performed on 3.5 week old plants illuminated by a sodium lamp (1000 µE/m^2s) for 48 hours and with adequate watering.

3.2 Quantitative real-time PCR analysis

3.2.1 Total RNA isolation

RNeasy Plant Mini Kit

Total RNA was isolated from approximately 100 mg leaf tissue using RNeasy Plant Mini Kit (QIAGEN). The procedure was performed according to the manufacturer's instructions. Tissue was collected and disrupted with the MiniBeadbeater (Biospec Products Inc.) for 1.5 min using 3 mm stainless steel beads, and then lysed under denaturing conditions. The plant lysate was centrifuged through a QIAshredder Mini Spin Column (QIAGEN) to remove insoluble material. Ethanol was added to provide selective binding of RNA to the silica-gel membrane. The sample was applied to an RNeasy Mini Spin Column (QIAGEN) where total RNA bound to the membrane. Finally, RNA was eluted in 50 µl RNase free water. After isolation, RNA was treated with DNase I (DNase I Amplification Grade; AMPD1, Sigma) or Deoxyribonuclease I, Amplification Grade (Invitrogen) according to the manufacturer's instructions to remove contaminating DNA.

Trizol reagent

Total RNA was isolated with TRIZOL®Reagent (Invitrogen) from approximately 100 mg plant tissue, according to the manufacturer's instructions. Tissue was collected and homogenized with 1 ml Trizol reagent and 3 mm stainless steel beads using the MiniBeadbeater (Biospec Products Inc.) for 1.5 min, followed by 5 min incubation at room temperature. 200 µl chloroform was added, the tube was shaken vigorously by hand for 15 sec, then left for 3 min incubation in room temperature, followed by 15 min centrifugation at 12 000 x g, 4 ˚C (Sigma 112, B. Braun Biotech International). The aqueous phase was transferred to a fresh tube, 500 µl isopropyl alcohol was added, followed by 30 min incubation in room temperature and 10 min centrifugation at 12 000 x g, 4 ˚C (Sigma 112, B. Braun Biotech International), to precipitate the RNA. The supernatant was removed and the

pellet was washed with 1 ml 75 % ethanol by vortexing followed by 5 min centrifugation at 7500 x g at 4 ˚C. The RNA pellet was dried and resuspended in RNase-free water. After isolation, RNA was treated with DNase I (DNase I Amplification Grade; AMPD1, Sigma) or Deoxyribonuclease I, Amplification Grade (Invitrogen) according to the manufacturer's instructions to remove contaminating DNA.

3.2.2 cDNA synthesis optimalization

First strand cDNA was synthesized from total RNA using SuperScript™ III RNase H$^-$ reverse transcriptase (RT-enzyme) (Invitrogen). In order to optimize the cDNA synthesis reaction for down-stream applications (i.e. q-PCR), four different test reactions were carried out using 260 ng total RNA in each reaction. The parameters varied were the use of either 200 U or 400 U of RT-enzyme, in combination with either 0.5 µg/µl oligo(dT) primers or 0.5 µg/µl oligo(dT) and 0.275 µg/µl random hexamer primers. The finale (optimal) cDNA synthesis reaction conditions selected was the following: 1 µg total RNA was reverse transcribed using 200 U of SuperScript™ III RNase H$^-$ reverse transcriptase (Invitrogen), 0.5 µg/µl oligo(dT) primers, 10 mM dNTP Mix, 5 x First Strand Buffer, 0.1 M DTT and 40 U RNaseOUT (Invitrogen) in a total reaction volume of 20 µl. The RT-reaction was incubated for 60 min at 50 ˚C, and for 15 min at 70 ˚C to inactivate the enzyme.

3.2.3 Primer verification

To verify that the primers designed for the amplification of the different flavonoid biosynthesis genes (Table 1, Appendix) resulted in the expected amplification product, and to determine the optimal annealing temperature for the q-PCR reaction, a thermal gradient PCR reaction followed by sequencing of the amplification products were conducted. The PCR reaction contained 1x PCR buffer (150 mM Tris-HCl, 500 mM KCl pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM forward and reverse primers (Table 1, Appendix) and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) using genomic DNA from *Arabidopsis* as template. The amplification conditions were: 95 ˚C for 10 min, then 30 cycles with 95 ˚C for 30 sec, 54-60 ˚C for 30 sec and 72 ˚C for 30 sec, then 72 ˚C for 5 min. A negative control, omitting template DNA from the PCR reaction was included. The samples were analysed on 1.5 % agarose gel.

3.2.4 Sequencing

The PCR amplification product was verified by sequencing using BigDye®Terminator v3.1 Sequencing Kit (Applied Biosystems). 2 µl of the PCR reaction was treated with 4 U ExoI (NEB) in 1 x BigDye Sequencing buffer in a 10 µl reaction volume in order to digest excess, unincorporated PCR primers. The samples were incubated for 1 hour at 37 ˚C, followed by incubation at 85 ˚C at 15 min to inactivate the enzyme. 5 µl of the sample reaction was then added 0.5 µl BigDye Terminator mix v3.1, 2 µl 5 x BigDye Sequencing buffer, 3.2 pmol gene specific primers forward and reverse (Table 1, Appendix) in a 10 µl reaction volume. Cycling conditions used were 96 ˚C for 1 min, then 15 cycles with 96 ˚C for 10 sec, 50 ˚C for 5 sec, 60 ˚C for 1.15 min, followed by 5 cycles with 96 ˚C for 10 sec, 50 ˚C for 5 sec, 60 ˚C for 1.30 min and finally 5 cycles with 96 ˚C for 10 sec, 50 ˚C for 5 sec, 60 ˚C for 2 min. The cycling conditions are described in "Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol" (Platt et al., 2007). DNA was precipitated with 3 M sodium acetate, 125 mM EDTA and 96 % EtOH in a total volume of 28 µl. DNA was pelleted with centrifugation at 3000 x g, 4 ˚C, for 30 min (Allegra™25R Centrifuge, BeckmanCoulter™), and washed with 35 µl 70 % EtOH. After centrifugation at 1650 x g for 15 min, invert centrifugation at 150 x g for 1 min (Centra CL3, International Equipment Company) was performed to remove the supernatant. The samples were air dried for 15 min and then finally dissolved in 10 µl Hi-Di™Formamide (Applied Biosystems). DNA sequencing was performed in the 3130xl Genetic Analyzer (Applied Biosystems) with Sequencing analysis 5.3.1 software. The sequences were analysed in CLC main workbench.

3.2.5 Quantitative real-time PCR analyses

The endogenous transcript levels of the following genes were determined using quantitative real-time PCR (q-PCR) (7500 RealTime PCR System, Applied Biosystems): *chalcone synthase* (*CHS*, At5g13930), *phenylalanine ammonia lyase 1* (*PAL1*, At2g37040), *dihydroflavonolreductase* (*DFR*, At5g42800), *glutathione S-transferase* (*GST*, At5g17220), and *MYB75/PAP1* (At1g56650). *Actin* (*ACT*, At3g18780) was used as a reference gene. Reverse transcribed cDNA samples were used as template in the q-PCR reactions and SYBR green was used to monitor dsDNA synthesis. The q-PCR reaction contained 1 x Power SYBR®Green PCR Master Mix (Applied Biosystems), 0.8 µM each of sense and antisense gene-specific primers (Table 1, Appendix) and 1 µl 10-fold diluted cDNA in a total reaction volume of 25 µl. The reactions were performed in optical 96-well plates. Cycling conditions

used for the amplifications were 40 cycles consisting of 95 ˚C for 30 sec, 60 ˚C for 45 sec and 72 ˚C for 34 sec. Prior to the amplification step, an initial denaturing and activation step at 50 ˚C for 2 min and 95 ˚C for 10 min, respectively, was included. Dissociation curve analysis was always included after the final amplification step. Data was analysed and evaluated by the SDS 1.3.1 software (Applied Biosystems). Relative mRNA levels were determined using the Pfaffl method (Pfaffl, 2001). The following equation was used to calculate the effect of different growth conditions (3 % sucrose compared to 1 % sucrose, and high intensity light treatment compared to normal light intensity treatment) on transcript level of flavonoid biosynthetic genes in At RCHSp38, wild-type and At PAP1 lines: fold change = $(E_{target})^{\Delta Ct\ (control\text{-}treated)}/(E_{ref})^{\Delta Ct\ (control\text{-}treated)}$. ΔCt was the change in Ct values in control versus treated. Control samples were cDNAs from either seedlings grown on 1 % sucrose or plants not exposed to high intensity light, and treated samples were cDNAs from either seedlings grown on 3 % sucrose or plants exposed to high intensity light. E_{target} and E_{ref} was the PCR efficiency to the target gene (*CHS, PAL1, DFR, GST* or *MYB75/PAP1*) and the reference gene (*ACT*) respectively. The PCR efficiencies (E) were determined using dilution curves and the equation $E = 10^{[-1/slope]}$ (Pfaffl, 2001). To calculate what effect the transgene constructs in At RCHSp38 and At PAP1 lines had on the transcript level of the different flavonoid biosynthetic genes, the following equation was used: fold change = $(E_{target})^{\Delta Ct\ (wild\text{-}type\text{-}transgenic)}/(E_{ref})^{\Delta Ct\ (wild\text{-}type\text{-}transgenic)}$, with all calculations performed within the same treatment (seedlings grown on 1 % sucrose or plants not exposed to high intensity light). ΔCt was the change in Ct values in wild-type versus transgenic plants (At RCHSp38 or At PAP1), grown under the same conditions. E_{target} and E_{ref} was the PCR efficiency to the target gene (*CHS, PAL1, DFR, GST,* or *MYB75/PAP1*) and the reference gene (*ACT*) respectively. Control samples containing no template cDNA, and samples prepared without the RT enzyme were always included in the q-PCR assay.

3.3 Algal methods

3.3.1 Strains and culture conditions

The *Chlamydomonas* strain CC-1618, which carries a mutation in the arginine biosynthesis *arg7* gene and the cell wall component *cw-15* gene, and the plasmid pChlamiRNA2 (Molnar et al., 2009) were obtained from the Chlamydomonas center (Chlamy center; http://www.chlamy.org/index.html). CC-1618 cells were plated on High Salt medium (HS)

(Sueoka, 1960) containing 1.5 % agar, supplemented with 50 mg/l L-arginine and grown under continuous illumination at 22 ˚C.

3.3.2 Artificial miRNA design

Artificial miRNAs (amiRNAs) were designed by using the Web MicroRNA Designer platform (Web MicroRNA Designer, WMD 3, http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) as described in Protocol S1 by Molnar et al, 2009. In brief, a BLAST search was performed with the mRNA sequence of *phytoene synthase* (*PSY,* XM_001701140) to obtain the accompanying FASTA format definition line. Database chosen was Chlamydomonas reinhardtii JGI transcript release Chlre3.1. Then the *PSY*-gene sequence was pasted together with the resulting FASTA format definition line into the DESIGNER. The output amiRNA sequences from the DESIGNER were ranked by efficiency and specificity using empirical data. The following criteria were used to select two different amiRNA sequences (Table 2, Appendix), one targeting the coding region (nt 819-839 of target gene 5'-3') and one targeting the 3' untranslated region (nt 1437-1457 of target gene 5'-3') of *PSY:* absolute hybridization energy between -35 and -38 kcal/mole, no mismatch between positions 2 and 12, and one or two mismatches at the amiRNA 3' end. Two 90 nucleotide long DNA oligonucleotides, based on the selected amiRNAs, were designed using the Oligo Design tool and ordered from Invitrogen (Table 2, Appendix). The oligos were designed to contain *Spe*I compatible ends both 5' and 3'end to the molecule.

3.3.3 Artificial miRNA dsDNA oligonucleotide cloning

The DNA oligos from the Oligo Design tool step (see section 3.3.2) were processed according to the description in Molnar et al., 2009, Protocol S2: "Detailed protocol of double-stranded DNA oligo-nucleotide cloning", with minor modifications. 1 nmol forward and reverse ssDNA oligo were mixed with 20 µl of annealing buffer (20 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl; pH 8.0) and boiled in hot water bath for 5 min and left to cool gradually overnight at room temperature. QIAquick PCR clean up kit (QIAGEN) was used to purify the resulting dsDNA oligo according to the manufacturer's instructions. Approximately 0.8 µg dsDNA oligo was phosphorylated in a 10 µl reaction volume using 1 µl 10 x T4 DNA ligase buffer (New England Biolabs) and 10 U T4 polynucleotide kinase (Invitrogen). The reaction was incubated at 37 ˚C for 30 min and then for 20 min at 65 ˚C to inactivate the kinase enzyme. The phosphorylated dsDNA oligo was then cloned into

pChlamRNA2 in the following manner. First, approximately 2 µg pChlamRNA2 was digested with 5 U *Spe*I (New England Biolabs), and then dephosphorylated using 20 U CIP (New England Biolabs). Then 70 ng phosphorylated dsDNA oligo was ligated to 30 ng *Spe*I linearized and dephosphorylated pChlamRNA2 vector using 40 U T4 DNA ligase in 1 x ligation buffer and in a total volume of 10 µl. One Shot TOP10 F' Chemically Competent Cells (Invitrogen) were transformed using 2.5 µl ligation mixture according to the manufacturer's instruction. Transformed TOP10 cells were selected on Luria Bertani (LB) plates (1 % tryptone, 0.5 % yeast extract, 0.17 M NaCl and 1.5 % agar) supplemented with 50 µg/ml ampicillin. In order to select for clones harbouring the dsDNA insert in the right orientation, colony PCR was performed. The 10 µl colony PCR reaction contained 1 x PCR buffer (150 mM Tris-HCl, 500 mM KCl pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM each of primers $AmiRNAprec_{for}$ and $Spacer_{rev}$ (Table 3, Appendix), 0.5 U Amplitaq Gold DNA Polymerase (Applied Biosystems). Cycling conditions used were 95 ˚C for 2 min, and 35 cycles with 95 ˚C for 30 sec, 65 ˚C for 30 sec and 72 ˚C for 30 sec, then 72 ˚C for 7 min. The PCR reactions were analysed on a 2 % agarose gel. The expected PCR product from colonies with the insert in the right orientation was 182 bp. Plasmid DNAs from positive colonies were extracted from 10 ml LB_{amp50} overnight culture using E.Z.N.A. Plasmid Mini Kit I (Omega Bio-tek) according to the manufacturer's instructions. To verify a single copy insertion of the dsDNA oligonucleotide, restriction enzyme analyses were performed using *Spe*I and *EcoR*I. The resulting enzyme digests were analysed on a 2 % agarose/0.5 x TBE gels. Final verification of the putative clones was performed by sequencing. The sequencing reaction contained about 0.1-0.2 µg DNA, 3.2 pmol $AmiRNAprec_{for}$ primer (Table 3, Appendix), 2 µl 5 x BigDye Sequencing buffer (BigDye®Terminator v3.1 Sequencing Kit, Applied Biosystems), 1 µl BigDye Terminator mix v3.1 and 0.5 µl DMSO in a 10 µl reaction volume. Cycling conditions used were 96 ˚C for 45 sec, then 25 cycles with 96 ˚C for 10 sec and 60 ˚C for 4 min. DNA was precipitated with BigDye®XTerminator™ Purification Kit (Applied Biosystems) according to the manufacturer's instructions. DNA sequencing was performed in the 3130xl Genetic Analyzer (Applied Biosystems) with Sequencing analysis 5.3.1 software. The sequences were analysed in CLC main workbench 5 (CLC bio).

3.3.4 Transformation of *Chlamydomonas*

Transformation of *Chlamydomonas reinhardtii* strain CC-1618 was performed with the glass beads method (Kindle, 1990). Prior to transformation, the pChlamRNA2 empty vector, pChlamRNA2 with the amiRNA insert targeting the coding region (CDS) of *PSY*, and pChlamRNA2 with the amiRNA insert targeting the 3' untranslated region (3'UTR) of *PSY* were linearized with *Hind*III, and the restriction enzyme inactivated according to the manufacturer's instructions. To confirm complete digestion by *Hind*III, an aliquot of the restriction digests in addition to undigested empty vector were analysed on a 0.7 % agarose gel. CC-1618 cells were grown in High salt (HS) medium (Sueoka, 1960), supplemented with 50 mg/l arginine, under continuous illumination at 22 ˚C. The cells were grown until the culture reached a density of about 1.8 x 10^6 cells/ml, measured by cell counting using a Bürkerchamber. The cells were harvested at 5000 rpm for 5 min (Allegra™25R Centrifuge, BeckmanCoulter™), then washed twice in HS medium to remove residual arginine, and finally resuspended in 1/100 volume the original cell culture in HS medium containing 2.5 g/l Ka-acetate. DNA used for transformation was precipitated in the following way: 1 volume DNA was added 0.1 volume 3M sodium acetate pH 5.5 and 2 volumes absolute ethanol. After 30 min incubation on ice, the samples were centrifuged at 12 000 x g, 4 ˚C (Sigma 112, B. Braun Biotech International) washed with 70 % ethanol, and finally resuspended in 10 µl sterile H_2O. *Chlamydomonas* was transformed by mixing 300 µl cells, 100 µl 20 % polyethylene glycol (m.w. 8000, USB™), 1-2 µg linearized precipitated DNA, and 300 mg autoclaved acid-washed glass beads (425-600 µM, Sigma), and vortexed for 30 sec. The cells were plated on HS medium containing 2.5 g/l Ka-acetate and 1.5 % agar. Plates were placed at 22 ˚C under dim light for 24 hours before they were sealed with parafilm and incubated in dark at 22 ˚C and inspected weekly for colonies with pale green phenotype over a period of several weeks. The *Chlamydomonas PSY* mutant is grown in dark because of absence of protective carotenoids (McCarthy et al., 2004). *PSY* catalyses the first step in the carotenoid biosynthesis pathway and mutants in *PSY* have an altered pigmentation including lack of coloured carotenoids and reduced levels of chlorophylls. An experiment with liquid growth of transformation product was also performed. After transformation, the transformed cells were transferred to 100 ml liquid HS medium containing 2.5 g/l Ka-acetate and grown at 22 ˚C under dim light for 24 hours, then grown in dark. 100 µl cell cultures were plated out after 8 and 13 days on HS medium containing 2.5 g/l Ka-acetate and 1.5 % agar. After 14 days, dilution series with 100 µl undiluted – 10^4

diluted cell cultures were plated out on HS medium containing 2.5 g/l Ka-acetate, 50 µg/ml ampicillin, and 1.5 % agar, and incubated in dark at 22 ˚C and inspected weekly for colonies with pale green phenotype over a period of several weeks.

3.3.5 Algal colony PCR of putative transformed cells

To confirm successful transformation of *Chlamydomonas* cells, putative transformed colonies were analysed with colony PCR. Individual putative transformed colonies were treated with 50 µl 10 mM EDTA, according to *Chlamydomonas* colony PCR procedure (Cao et al., 2009). The solution was vortexed for 10 sec, incubated for 10 min at 100 ˚C, then put on ice for 1 min before being vortexed at 10 sec and centrifuged at 13000 rpm for 1 min (Sigma 112, B. Braun Biotech International). 1 µl of the supernatant was applied to the PCR reaction. The PCR reaction contained 1 x PCR buffer (150 mM Tris-HCl, 500 mM KCl pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM forward and reverse primers (Table 4, Appendix) and 0.5 U AmpliTaq Gold DNA polymerase (Applied biosystems). The amplification conditions were: 95 ˚C for 10 min, then 30 cycles with 95 ˚C for 30 sec, 55 ˚C for 30 sec and 72 ˚C for 30 sec, then 72 ˚C for 4 min. Positive controls with plasmid DNA templates pChlamRNA2, and pChlamRNA2 with inserted amiRNA oligo targeting 3'UTR were included, as well as a negative control omitting DNA from the reaction. The PCR reactions were analysed on a 1 % agarose gel.

3.4 Standard DNA techniques

3.4.1 Agarose gel electrophoresis

Separation and identification of DNA fragments according to size was performed by agarose gel electrophoresis (Sambrook and Russel, 2001). 1-2 % agarose gels in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) stained with ethidium bromide (EtBr) (final concentration 0.1 µg/ml) were used for analysing the DNA fragments. Samples were loaded with loading buffer (0.25 % Brom Phenol Blue, 0.25 % Xylene cyanol FF, 30 % Glycerol) in the appropriate volume and run at 80-100 V for 40-60 min. 1 kb DNA ladder (Thermo Scientific) or 100 bp ladder (Superladder mid 100 bp ladder, Thermo Scientific) were used for size determination of the DNA fragments.

3.4.2 Quantification of RNA

RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (Saveen Werner).

3.5 Bioinformatics

Throughout the project, bioinformatics tools have been used for primer designs, sequence alignments and to search for gene sequences. To search for gene sequences, the universal Basic Local Alignment Search Tool (BLAST) engine at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) was used. To design primers, primer-BLAST (NCBI, primer-BLAST, http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) was used. CLC Main Workbench 5 (CLC bio) was used to analyse sequencing results and to align sequences.

4. Results

In this project, the effect of expressing p38, a viral suppressor of RNA silencing encoded by TCV, in *Arabidopsis* and *Chlamydomonas* was investigated.

Specifically, in *Arabidopsis*, the effect of p38 expression on the transcript levels of different flavonoid biosynthetic pathway genes was investigated by analysing transgenic At RCHSp38 plants. These transcript levels were then compared to the expression level of the same genes in wild type plants and in the transgenic At PAP1 plants, which, as a consequence of activation tagging, over-express the transcription factor *MYB75/PAP1* again leading to the induction of several flavonoid biosynthetic genes (Borevitz et al., 2000). The *Arabidopsis* plants, both wild-type and transgenic lines, were also exposed to different treatments known to induce flavonoid biosynthetic pathway genes, in order to investigate the effect of these treatments on plants expressing p38. In order to perform this investigation, different RNA isolation methods and cDNA synthesis reaction conditions were tested to optimize the analysis. Finally, phenotypic investigations of leaves and flowers in plants expressing p38 were undertaken.

With respect to *Chlamydomonas*, artificial microRNAs (amiRNAs) constructs were produced and attempted transformed into *Chlamydomonas* cells in order to induce RNA silencing of the *phytoene synthase* (*PSY*) gene. A construct harbouring the p38 gene were intended transformed into transformed *Chlamydomonas* cells with down-regulated *PSY* expression, to investigate if p38 expression could suppress RNA silencing of *PSY* in this pathway. Establishing *Chlamydomonas* as a model organism in our lab has also been a part of the project.

4.1 RNA isolation

Different methods for total RNA isolation from *Arabidopsis* plant material were tested in order to find the method giving the best yield and being the best suited for this project. RNA was isolated in two parallels from equal amounts of *Arabidopsis* rosette leaves with RNeasy Plant Mini Kit (QIAGEN) and TRIZOL®Reagent (Invitrogen), as described in Materials and Methods. RNA was quantified using a NanoDrop® ND-1000 Spectrophotometer, and RNA isolation with RNeasy Plant Mini Kit showed the best yield per mg tissue and the highest

purity (assessed by $Abs_{260/280}$ and $Abs_{260/230}$) of the two methods employed (data not shown). In addition, quantitative real-time PCR (q-PCR) was performed on the same RNA samples to further investigate which RNA isolation method were the best. Equal total RNA amounts of each sample (1 µg) were taken into cDNA synthesis, and cDNA was synthesized as described in Materials and Methods. Q-PCR was then performed with Actin primers as described in Materials and Methods. Results from q-PCR show that RNA isolated with TRIZOL®Reagent yielded the lowest Ct values compared to RNA isolated with RNeasy Plant Mini Kit (data not shown). Since the differences in yield and purity between the two RNA isolation methods assayed evidently did not influence down stream applications, TRIZOL®Reagent was used to isolate RNA in this project.

4.2 cDNA synthesis

For optimalization of cDNA synthesis reaction conditions, two different parallels with total RNA from *Arabidopsis* plant material were investigated, one using oligo(dT) primers alone and one using oligo(dT) primers together with random hexamer primers. In the two parallels, test of whether 200 U or 400 U of SuperScript™ III RNase H^- reverse transcriptase (Invitrogen) gave the best cDNA yield was also included. The different conditions were evaluated by q-PCR using Actin primers, as described in Materials and Methods. There were very little difference in q-PCR values from the samples prepared with different reaction conditions (results not shown), but a slight increase in relative cDNA yield was obtained using both oligo(dT) and random hexamer primers together with 400 U of SuperScript RT. Since the difference were so small it was decided to use oligo(dT) alone and 200 U of SuperScript RT in the following cDNA synthesis in this project.

4.3 Primer verification and Sequencing results

The PCR reactions with primers designed to target flavonoid biosynthetic genes (Materials and Methods, 3.2.3, Table 1, Appendix) yielded the expected size products, and the results from sequencing (Materials and Methods, 3.2.4) revealed the expected sequences (data not shown).

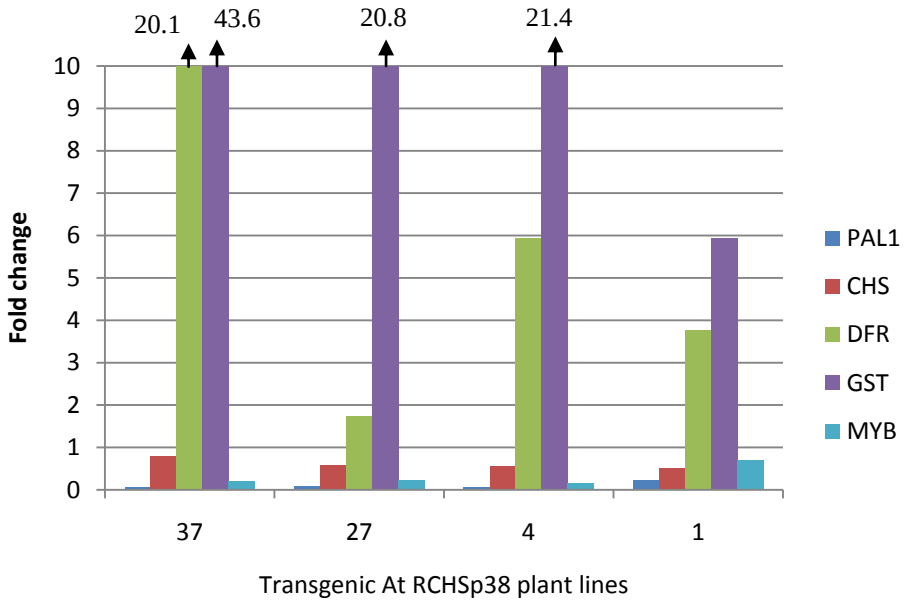
4.4 The effect of p38 expression on the transcript levels of flavonoid biosynthesis genes in transgenic At RCHSp38 plants

4.4.1 Effect of transgenic constructs on the transcript levels of flavonoid biosynthesis genes

Wild-type plants and transgenic At RCHSp38 and At PAP1 lines were grown as described in Materials and Methods. After 14 days of growth on MS-plates (containing 1 % sucrose) 10 random seedlings were selected from each plant line, the seedlings from each individual line pooled and RNA isolated. Seedlings were also transferred to soil and after 3.5 week of growth, 3 individual leaves were selected from each of 3 plants from each plant line, the leaves from each individual plant pooled and RNA isolated. The steady-state level of mRNA corresponding to *PAL1, CHS, DFR, GST* and *MYB75/PAP1* transcripts in transgenic lines were then compared to the same transcript levels in wild-type plants grown under the same experimental conditions, and the fold change in each comparison was calculated as described in Materials and Methods. The results of these experiments are shown in Figure 4 and Figure 5 for seedlings and adult plants, respectively.

In seedlings grown on 1 % sucrose (Figure 4), all of the genes analysed in the At RCHSp38 lines were up-regulated compared to wild-type seedlings grown under the same experimental conditions. *GST* was the most up-regulated gene, with a fold change increase between 6.0-43.6 times in individual lines compared to wild-type seedlings. *DFR* showed a fold change increase between 1.7-20.1 times the wild-type level. *CHS* had a more modest increase in fold change (0.5-0.8 times the wild-type level), and *MYB75/PAP1* followed by *PAL1* showed the smallest increase with a fold change between 0.2-0.7 and 0.05-0.2 times wild-type levels, respectively. The At PAP1 line had the largest increase of all the genes analysed in seedlings grown on 1 % sucrose. *DFR* was most up-regulated with fold change 642.8 times the wild-type level, followed by *GST* and *MYB75/PAP1* with fold change 556.1 and 345.3 times wild-type levels, respectively. *PAL1* and *CHS* showed the least increase in fold change, 1.4 and 2.7 times wild-type levels, respectively.

A)



B)

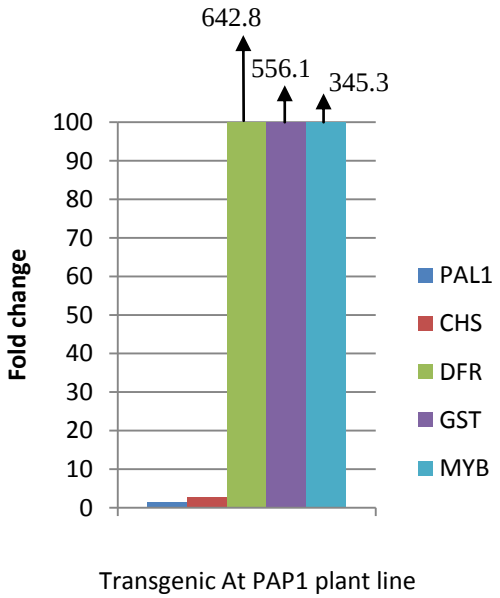
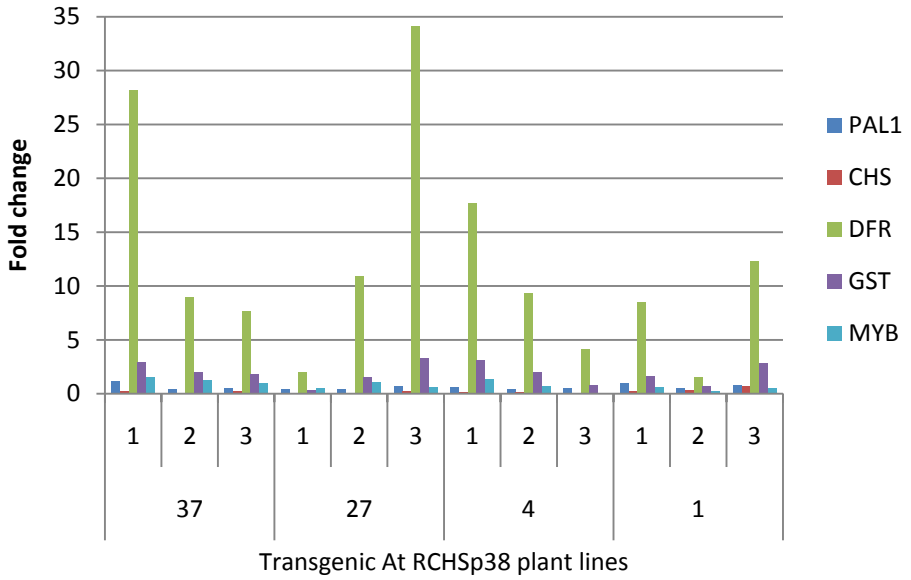


Figure 4: Quantitative PCR analysis in A) transgenic At RCHSp38 and B) transgenic At PAP1 seedlings showing the effect of their respective genetic modifications on flavonoid biosynthesis genes in Arabidopsis seedlings. *Seedlings were grown for 14 days on MS-medium containing 1 % sucrose before 10 random seedlings were selected from each plant line (wild-type, At PAP1 and At RCHSp38), the seedlings from each individual line pooled and RNA isolated. cDNA was synthesized and q-PCR using primers specific for the different target genes (Table 1, Appendix) was performed. Fold change in the transgenic plants were calculated compared to wild-type as described in Materials and Methods. The arrows shows fold change values beyond the y-axis scale, and these values are written above each arrow.*

In adult plants, At RCHSp38 also showed an increase in all the genes analysed compared to wild-type plants grown under the same experimental conditions (Figure 5). *DFR* was most up-regulated with a fold change increase between 1.6-34.1 times in individual lines compared to wild-type plants, followed by *GST* with fold change increase between 0.3-3.3 times the wild-type level. *MYB75/PAP1* showed a fold change between 0.05-1.5 times the wild-type level, *PAL1* and *CHS* 0.4-1.1 and 0.007-0.7 times wild-type levels, respectively. In At PAP1 lines, *DFR* was also most up-regulated with fold change between 890.8-2604.2 times the wild-type level, followed by *GST* (191.7-245.7) and *MYB75/PAP1* (69.5-185.2). *CHS* and *PAL1* were up-regulated with a fold change between 9.8-11.8 and 6.2-13.1 times wild-type levels, respectively.

In summary, both in seedlings and in adult plants, all the flavonoid biosynthesis genes transcript levels analysed were up-regulated both in the At RCHSp38 lines, and to a larger extent in the At PAP1 lines compared to wild-type plants. In the At RCHSp38 lines, *GST*, followed by *DFR*, was most up-regulated in seedlings compared to adult plants where *DFR*, followed by *GST* was most up-regulated. In At PAP1, *DFR* was the most up-regulated gene followed by *GST* both in seedlings and plants.

A)



B)

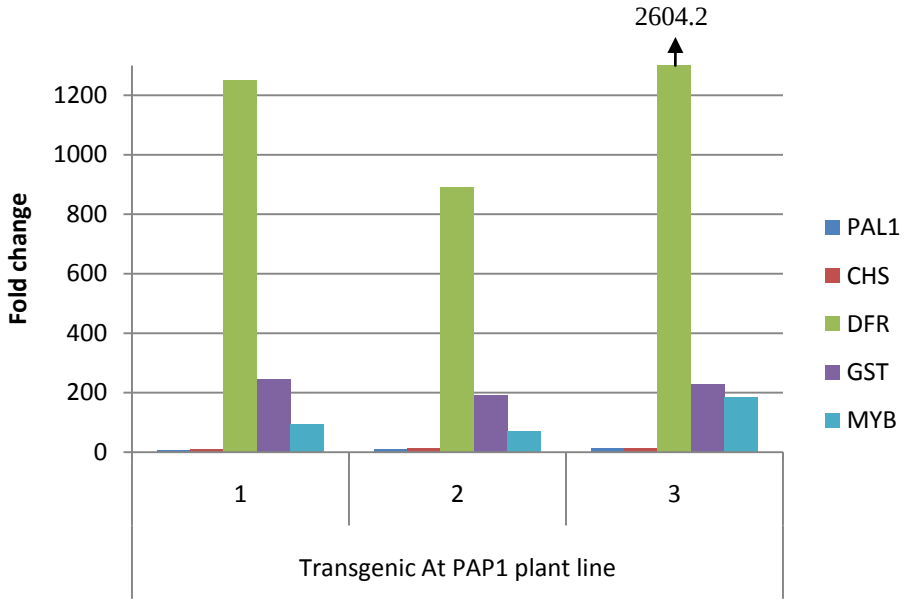


Figure 5: Quantitative PCR analysis in A) transgenic At RCHSp38 and B) transgenic At PAP1 plants showing the effect of their respective genetic modifications on flavonoid biosynthesis genes in adult Arabidopsis plants. *RNA was isolated from 3.5 week old wild-type, At PAP1 and At RCHSp38 plants. cDNA was synthesized and q-PCR using primers specific for the different target genes (Table 1, Appendix) was performed. Fold change of transcript levels in the transgenic plants were calculated compared to wild-type as described in Materials and Methods. The numbers 1-3 in the figure is the result from three individual plants from the same plant line. The arrow shows a fold change value beyond the y-axis scale, and this value is written above the arrow.*

4.4.2 Effect of stress exposure with high sucrose treatment and high intensity light treatment on the transcript levels of flavonoid biosynthesis genes

High concentrations of sucrose in the growth medium of *Arabidopsis* and illumination of plants with high intensity light are known to induce anthocyanin biosynthesis in this organism (Ohto et al., 2001; Solfanelli et al, 2006; Teng et al, 2005; Tsukaya et al, 1991; Cominelli et al., 2008; Feinbaum & Ausubel, 1988; Jenkins, 1997). To investigate the effect of these two growth conditions on the transcript levels of *PAL1*, *CHS*, *DFR*, *GST* and *MYB75/PAP1*, and thereby indirectly the effect on anthocyanin biosynthesis in At RCHSp38 lines, RNA was isolated from seedlings grown on 3 % sucrose and from 3.5 week old plants exposed to high intensity light. Wild-type plants and transgenic At RCHSp38 and At PAP1 lines were grown as described in Materials and Methods. After 14 days of growth on MS-plates (containing 3 % sucrose) 10 random seedlings were selected from each plant line, the seedlings from each individual line pooled and RNA isolated. Seedlings (grown on MS-plates containing 1 % sucrose) were also transferred to soil and 3.5 week old plants were exposed to high intensity light for 48 hours prior to RNA isolation. 3 individual leaves were selected from each of 3 plants from each plant line, the leaves from each individual plant pooled and RNA isolated. The steady-state level of mRNA corresponding to *PAL1, CHS, DFR, GST* and *MYB75/PAP1* transcripts in stress exposed transgenic and wild-type lines were analysed by q-PCR, and fold change were calculated as described in Materials and Methods.

In order to visualize the direct effect of high sucrose treatment or illumination with high intensity light, the transcript levels of the different genes can be compared in the same type of plants (e.g. wild-type, At RCHSp38 or At PAP1 plants) exposed to stress treatment and not exposed to stress treatment. In this way, the effect of these treatments on the expression levels of the selected flavonoid biosynthesis genes in wild-type, At RCHSp38 and At PAP1 can be compared. The results are presented in Figure 6 and Figure 7, for sucrose treatment and high intensity light treatment, respectively.

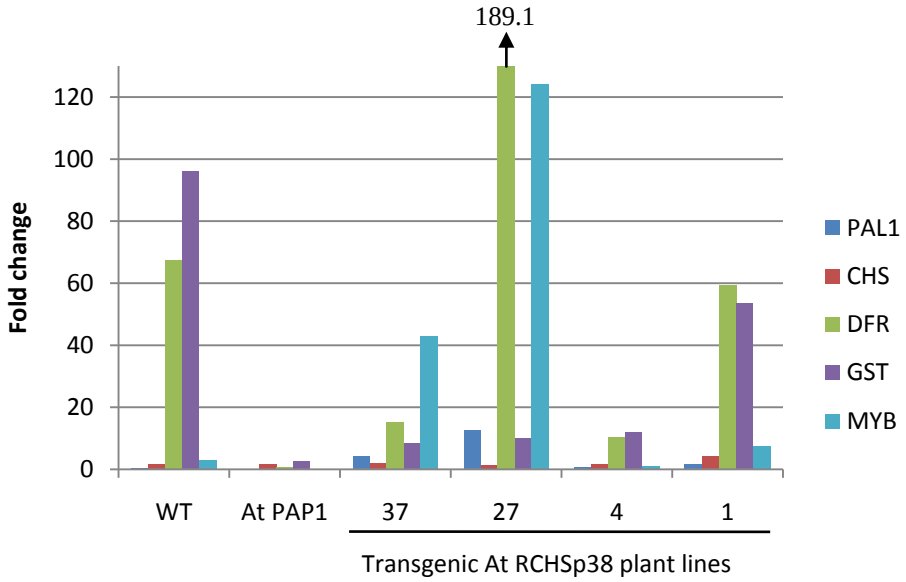


Figure 6: Quantitative PCR analysis in wild-type seedlings and transgenic At PAP1 and At RCHSp38 seedlings showing the effect of stress exposure with high sucrose concentration on flavonoid biosynthesis genes in Arabidopsis plant seedlings. *Seedlings were grown for 14 days on MS-medium containing 3 % sucrose before 10 random seedlings were selected from each plant line, the seedlings from each individual line pooled and RNA isolated. cDNA was synthesized and q-PCR using primers specific for the different target genes (Table 1, Appendix) was performed. The transcript levels were compared to the same transcript levels in wild-type and transgenic seedlings grown under the same experimental conditions, but not exposed to high sucrose concentrations, and fold change were calculated as described in Materials and Methods. The arrow shows a fold change value beyond the y-axis scale, and this value is written above the arrow.*

In At RCHSp38 lines seedlings grown on 3 % sucrose showed an increase in the transcript levels of all genes analysed compared to seedlings grown on 1 % sucrose (Figure 6). *DFR* showed a fold change increase between 10.2-189.1 times in the individual lines compared to At RCHSp38 seedlings grown in 1 % sucrose, *MYB75/PAP1* 1.0-124.0, GST 8.4-53.5, *PAL1* 0.6-12.5 and *CHS* 1.4-4.2. At PAP1 also showed an increase in the transcript levels of all the genes analysed, but to a lesser extent than At RCHSp38 lines. In At PAP1, *GST* was most up-regulated with a fold change of 2.8 times compared to 1 % sucrose-grown seedlings, followed by *CHS* (1.6), *DFR* (0.7), *PAL1* (0.08) and *MYB75/PAP1* (0.03), all values representing the fold change in 3 % sucrose-grown seedlings compared to 1 % sucrose-grown seedlings. Wild-type plants showed a large increase in *GST* expression (96.2 times) compared to 1 % sucrose-grown seedlings, followed by *DFR* (67.4), *MYB75/PAP1* (2.9),

CHS (1.7), and *PAL1* (0.3), all values representing the fold change in 3 % sucrose-grown seedlings compared to 1 % sucrose-grown seedlings.

In summary, seedlings grown on 3 % sucrose had an increase in the transcript levels of all genes analysed compared to the 1 % sucrose grown seedlings (Figure 6). The increase was largest in *DFR* and *GST* in all lines, in addition to *MYB75/PAP1* in some of the At RCHSp38 lines. *CHS* and *PAL1* showed the least increase in gene expression in all lines. At PAP1 had the lowest overall increase in the genes analysed.

In Figure 7, data from the different plant lines exposed to high intensity light are compared to data from the same plants not exposed to high intensity light. Thus, the resulting figure describes the effect of high intensity light treatment on gene expression of the selected flavonoid biosynthesis genes in wild-type, At RCHSp38 and At PAP1.

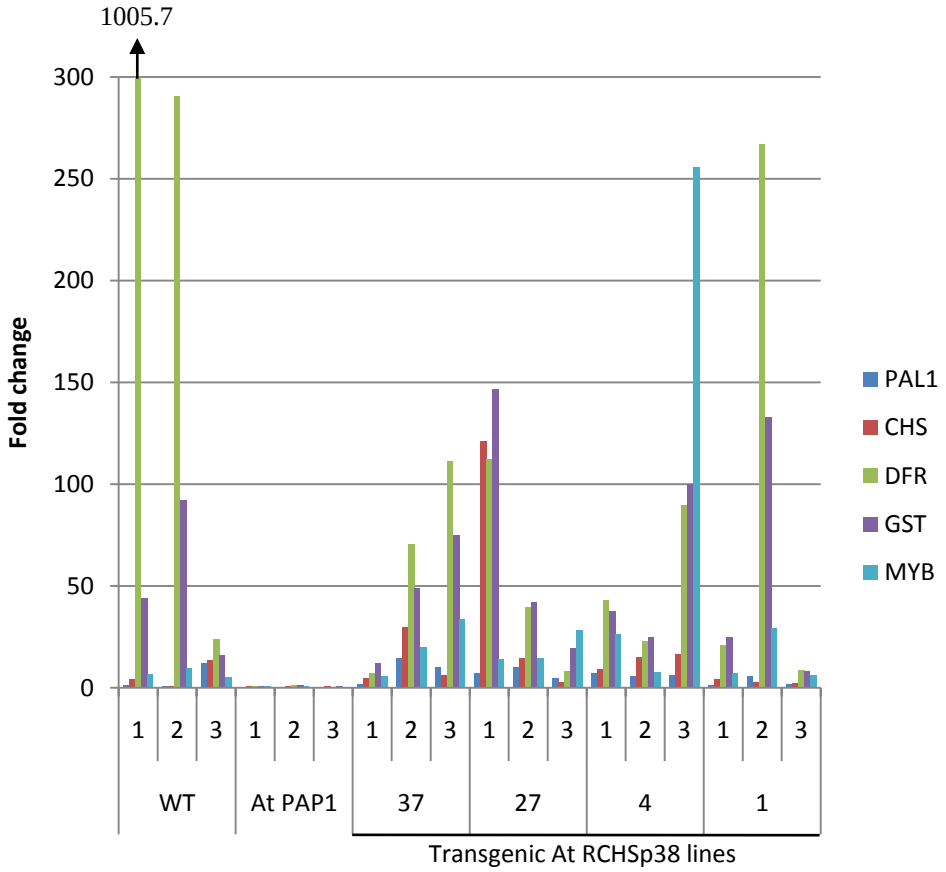


Figure 7: Quantitative PCR analysis in wild-type plants and transgenic At PAP1 and At RCHSp38 plants showing the effect of stress exposure with high intensity light treatment on flavonoid biosynthesis genes in adult Arabidopsis plants. *3.5 week old plants were exposed to high intensity light treatment for 48 hours prior to RNA isolation. cDNA was synthesized and q-PCR using primers specific for the different target genes (Table 1, Appendix) was performed. The transcript levels were compared to the same transcript levels in wild-type and transgenic plants grown under the same experimental conditions, but not exposed to high intensity light, and fold change was calculated as described in Materials and Methods. The numbers 1-3 in the figure is the result from three individual plants from the same plant line. The arrow shows a fold change value beyond the y-axis scale, and this value is written above the arrow.*

In the high intensity light treated plants, At RCHSp38 lines showed an increase in the transcript levels of all genes analysed compared to the plants not treated with high intensity light (Figure 7). *DFR* showed a fold change increase between 7.0-266.8 times in the individual lines compared to At RCHSp38 plants not exposed to high intensity light treatment, *GST* 12.0-133.0, *MYB75/PAP1* 5.6-255.6, *CHS* 2.2-121.1, and *PAL1* 1.3-14.6. Wild-type also showed an increase in the transcript levels of all the genes analysed, *DFR* had a fold change increase between 24.0-1005.7 times compared to wild-type plants not exposed

to high intensity light treatment, *GST* 16.2-92.2, *MYB75/PAP1* 5.2-9.5, *CHS* 0.8-13.7, and *PAL1* 0.6-11.8. At PAP1 was the plant line with least increase in the transcript level in all the genes analysed for plants exposed to high intensity light compared to plants not exposed to high intensity light. *DFR* showed a fold change increases between 0.2-1.2 compared to At PAP1 plants not exposed to high intensity light treatment, *GST* 0.8-1.4, *MYB75/PAP1* 0.3-0.8, *CHS* 0.7-0.9, and *PAL1* 0.3-0.5.

When investigating the effect of high intensity light treatment in the different plant lines, At RCHSp38 lines showed a large increase in *DFR* and *GST* expression, but not as large as in wild-type plants (Figure 7). On the other hand, *MYB75/PAP1* in the At RCHSp38 lines were overall more up-regulated than *MYB75/PAP1* in wild-type plants. The At PAP1 plants showed a very limited increase in all genes analysed.

4.5 The effect of p38 expression on *Arabidopsis* phenotype

Whether p38 expression also affects seedling and plant phenotypes was examined. In Figure 8, representative seedling phenotypes of wild-type At RCHSp38 and At PAP1 lines grown on MS agar containing 3 % sucrose are shown. The At PAP1 seedling is clearly purple compared to wild-type plants, probably due to an increased level of anthocyanin pigments in the plants. At RCHSp38 seedlings also showed a more intense purple leaf colouration than wild-type, though not as intense as the At PAP1 seedling. The At RCHSp38 seedlings showed an accumulation of purple colour in their leaf veins, while the leaves from the At PAP1 seedling had an overall purple colouration.

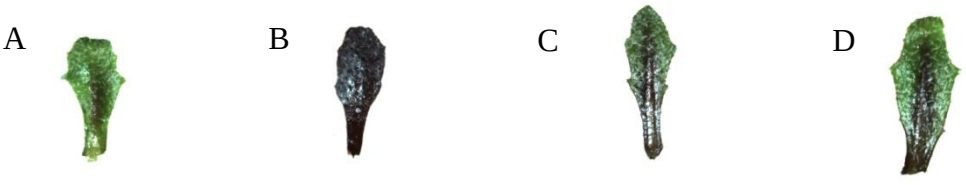


Figure 8: Arabidopsis leaf phenotype of seedlings grown on 3 % sucrose containing MS agar. A) Wild-type line B) At PAP1 line C) and D) At RCHSp38 line

Plants grown under normal growth conditions were also examined for pigment accumulation, as shown in Figure 9. At PAP1 plants clearly showed a more intense purple colouration than wild-type plants, both in stem and leaves. The At RCHSp38 plants also

showed an increase in purple colouration, but to a much lesser extent than the At PAP1 plants.

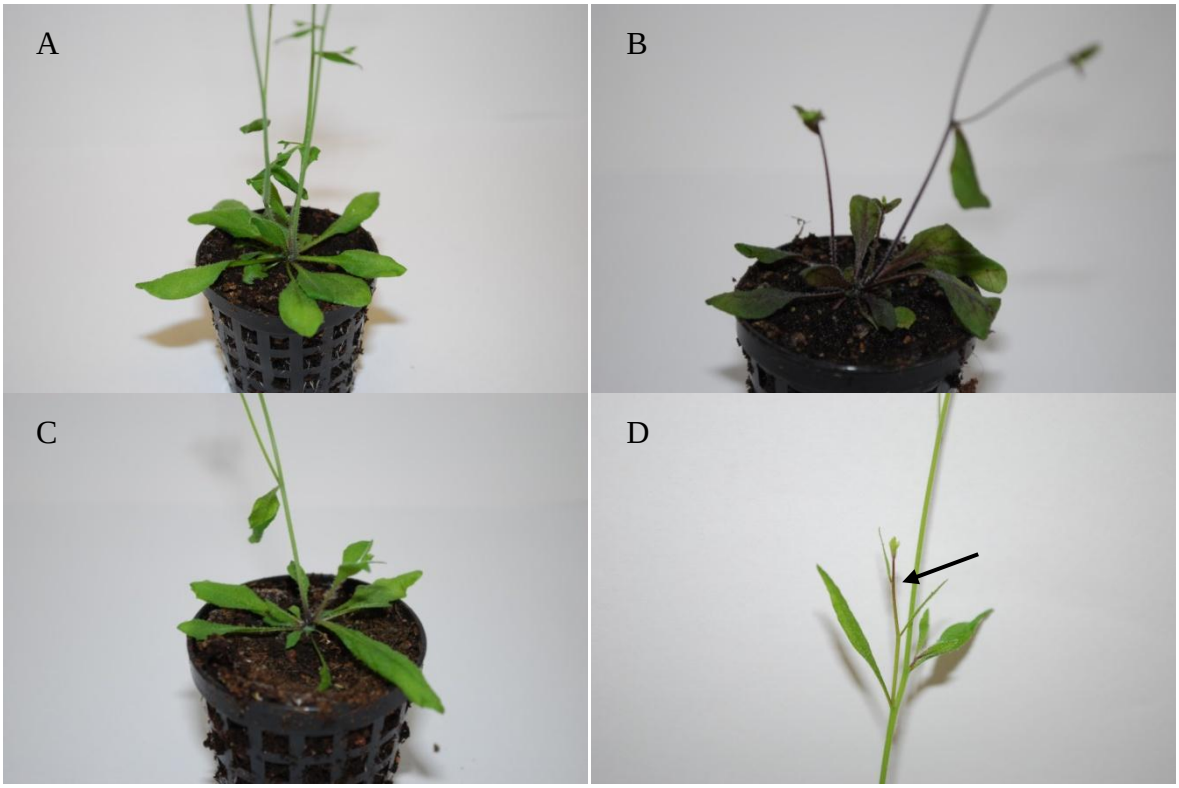


Figure 9: Phenotype of 4.5 week old Arabidopsis plants. A) Wild-type line B) At PAP1 line C) and D) At RCHSp38 line. The arrow points to a purple region of the stem.

The phenotypes of leaves harvested from wild-type, At PAP1 and At RCHSp38 plants were examined, as shown in Figure 10. At RCHSp38 leaves showed a more jagged morphology than wild-type and At PAP1 leaves. At RCHSp38 plants were generally more slender than wild-type and At PAP1 plants, and they produced much fewer seeds. Therefore, the flowers of At RCHSp38 plants were investigated and compared to the flower phenotype of wild-type and At PAP1 plants, as shown in Figure 11.

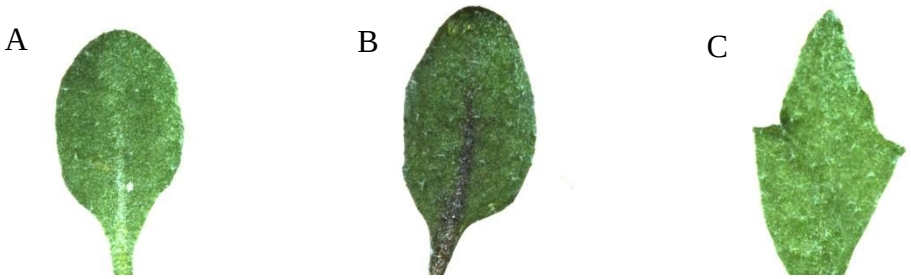


Figure 10: Leaf phenotype of 4.5 week old Arabidopsis plants. A) Wild-type line B) At PAP1 line C) At RCHSp38 line

While wild-type and At PAP1 flowers had normal length of stamens, the At RCHSp38 flowers showed considerably shorter stamens not reaching up to the carpel.

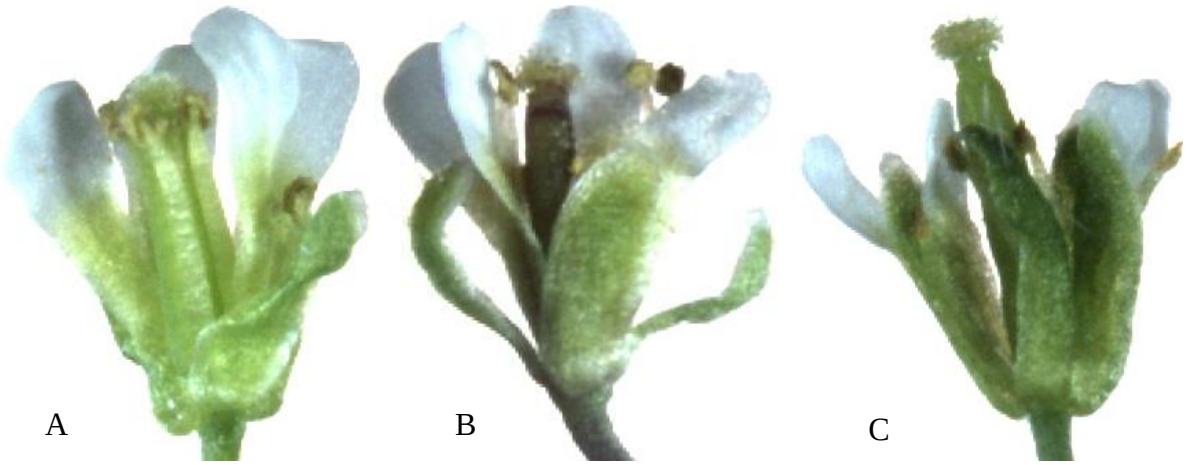


Figure 11: Flower phenotype of 4.5 week old Arabidopsis plants. A) Wild-type line B) At PAP1 line C) At RCHSp38 line

4.6 *Chlamydomonas* transformation and growth

In this study, the ability of p38 to suppress RNA silencing in *Chlamydomonas* was investigated, more specificially, p38's ability to suppress RNA silencing induced by an artificial miRNA designed to target an endogenous gene in *Chlamydomonas*. Recent experiments have shown that artificial miRNAs can be used to achieve highly specific gene silencing in *Chlamydomonas* (Molnar et al., 2009). Our experimental approach was to transform *Chlamydomonas* with an amiRNA construct designed to target the expression of *phytoene synthase (PSY)*. A null mutation in this gene has previously been shown to result in an albino or pale green algal phenotype (McCarthy et al., 2004). *Chlamydomonas* transformants which showed down regulated *PSY* expression was intended transformed with a DNA construct harbouring a p38 expression cassette. If p38 expression in *Chlamydomonas* could interfere with RNA silencing of *PSY*, then the original dark green algal phenotype would be expected to be restored.

Several repeated transformation attempts were performed, but were not successful. Putative transformed *Chlamydomonas* cells were analysed with colony PCR (Materials and Methods, 3.3.5). Positive controls revealed the expected PCR product, but none of the putative transformed cells gave the expected product (results not shown).

When the transformed cells were plated on selective medium (without arginine) and allowed to grow for up to 10 weeks, it was difficult to see growth of individual arginine-independent colonies. Therefore an experiment with liquid growth culture, to enrich for transformed cells, was performed. Cells were taken from the “transformed” culture 8, 13 and 14 days after transformation and plated as before. However, this approach was not successful either. A further discussion of the problems with *Chlamydomonas* can be found in section 5.2.

5. Discussion

5.1 The effect of p38 expression on the transcript levels of flavonoid biosynthesis genes in transgenic At RCHSp38 plants

5.1.1 Effect of transgenic constructs on the transcript levels of flavonoid biosynthesis genes

The results presented in this study show that the transcript levels of *PAL1, CHS, DFR, GST* and *MYB75/PAP1* are up-regulated in transgenic At RCHSp38 seedlings and adult plants compared to wild-type plants grown under the same experimental conditions. The result of the present study also clearly verify previous findings by Johansen and Wilson (2008), that At RCHSp38 plants accumulate higher than wild-type levels of anthocyanins in stems and leaves at late developmental stages. Thus, properties of the inserted gene construct pRCHSp38 in At RCHSp38 plants is likely the cause of the elevated levels of *PAL1, CHS, DFR, GST* and *MYB75/PAP1* and thus, as a consequence, higher levels of anthocyanins in At RCHSp38 plants compared to wild-type plants.

In At PAP1 plants, *MYB75/PAP1* is over-expressed due to activation tagging, causing massively enhanced expression of the flavonoid biosynthetic genes *PAL1, CHS,* and in particular *DFR* and *GST* (Borevitz et al., 2000), as verified in this study. The Myb transcription factor *MYB75/PAP1* interacts with bHLH proteins and WD-repeats to form MBW complexes involved in controlling mainly late flavonoid biosynthetic genes (Cominelli et al., 2008; Gonzalez et al., 2008). In this study we show that *MYB75/PAP1* is slightly over-expressed in At RCHSp38 seedlings and plants (Figure 4 and Figure 5) when compared to wild-type plants. The late flavonoid biosynthesis genes specific for the anthocyanin branch, *DFR* and *GST,* are most up regulated, compared to the early flavonoid biosynthesis gene *CHS,* and the general phenylpropanpoid biosynthetic pathway gene *PAL1*. Thus our results are in correspondence with the Cominelli et al. (2008), and the Gonzalez et al. (2008) studies showing that elevated *MYB75/PAP1* transcript levels result in stronger up regulation of late flavonoid biosynthetic genes than the early flavonoid biosynthesis genes. However, the overall transcript levels of the investigated flavonoid biosynthesis genes in At PAP1 plants are much higher than in At RCHSp38 plants, probably a result of the massively enhanced over-expression of *MYB75/PAP1* in At PAP1 plants.

Theoretically, the increased levels of anthocyanins in At RCHSp38 plants could be the consequence of four different properties of the inserted pRCHSp38 gene construct: 1) stress reaction from inserting a gene construct, 2) the IR-transgene designed to target *CHS*, 3) the kanamycin resistance gene used for selection, or 4) p38 expression or stress caused by the integration of this gene into the plant genome. Of these four possibilities, p38 expression is the most likely explanation for the increased anthocyanin levels in At RCHSp38 plants. Plants transformed with the same IR-transgene targeting *CHS*, including the kanamycin resistance gene (At RCHS plants), resulted in transgenic plants with highly reduced levels of anthocyanin (Johansen et al., manuscript in preparation). Plants transformed with the same IR-transgene construct targeting *CHS*, including the kanamycin resistance gene, but harbouring other viral suppressors of RNA silencing (p25, T2b or C2b) instead of p38 gave plants with normal levels of anthocyanin (Johansen and Wilson, 2008). This leaves transcription of p38 as the most likely cause of the increased levels of anthocyanin in At RCHSp38 plants. From this we can hypothesize that p38 expression in *Arabidopsis* leads to elevated levels of *MYB75/PAP1* which again induce the flavonoid biosynthesis genes resulting in anthocyanin biosynthesis and accumulation. The question then to be asked is how p38 expression can lead to higher levels of *MYB75/PAP1* transcripts in At RCHSp38 plants? This can be explained if *MYB75/PAP1* expression is under RNA silencing control.

The p38 protein suppresses DCL4 activity by an unknown mechanism, resulting in the loss of 21 nt siRNA species (Deleris et al., 2006). In addition to its enrolment as an essential antiviral component, DCL4 is also involved in the tasiRNA pathway (Yoshikawa et al., 2005), and it has been shown that p38 interferes with tasiRNA biogenesis resulting in decreased levels of specific tasiRNAs (Moissiard et al., 2007). A tasiRNA targeting *MYB75/PAP1* has been identified (Rajagopalan et al., 2006; Hsieh et al., 2009). This might indicate that *MYB75/PAP1* is under RNA silencing control via the tasiRNA pathway. TasiRNA precursors can be processed by DCL2 and DCL3 in the absence of DCL4 (Gasciolli et al., 2005). DCL2 is also an alternative to process hpRNA and replicating viral RNA when DCL4 activity is lost (Fusaro et al., 2006). However, it has been suggested that p38 functions in a concentration-dependent manner, and that a high enough concentration of p38 can completely block RNA silencing (Deleris et al., 2006; Johansen and Wilson, 2008). Also, plants carrying a null-mutation in DCL4 (*dcl4-2*) over-accumulate anthocyanins at late development stages (Nakazawa et al., 2007). Thus, it is indicated that loss of DCL4 activity,

either as a result of viral protein suppression or as a consequence of an inactive protein, leads to over-accumulation of anthocyanins.

A tasiRNA targeting *MYB75/PAP1* has been identified, called *TAS4*-siR81(-). *TAS4*-siR81(-) is cleaved from pri-*TAS4*-siR81(-) transcripts, originating from the *TAS4* loci, by the microRNA miR828 (Rajagopalan et al., 2006; Hsieh et al., 2009). Since we know that p38 suppresses DCL4 activity and that DCL4 is involved in the tasiRNA pathway, we can speculate that p38 expression leads to reduced levels of *TAS4*-siR81(-), resulting in the loss or reduced silencing control of *MYB75/PAP1*. Hsieh et al., (2009) has shown that in *tas4* and in *mir828* mutants, the level of *MYB75/PAP1* is elevated compared to the wild-type levels, and that anthocyanin accumulation in these mutants are also increased compared to wild-type levels. The accumulation level of anthocyanins in the *tas4* and *mir828* mutants has been shown to be intermediate of those found in wild-type and At PAP1 plants (Hsieh et al., 2009). At PAP1 plants showed a higher accumulation level of both *miR828* and *TAS4*-siR81(-) compared to wild-type plants (Hsieh et al., 2009). Presuming that p38 expression leads to lower level of *TAS4*-siR81(-), it might be possible to draw a comparison between the *tas4* and *mir828* mutants and that of the At RCHSp38 lines; the phenotypic observation in this study reveal that the anthocyanin levels in At RCHSp38 plants were in between anthocyanin levels in wild-type and At PAP1 plants.

Hsieh et al., (2009) suggests an autoregulation of *MYB75/PAP1* in which *MYB75/PAP1* can positively regulate *MIR828* and/or *TAS4* genes by activating their expression directly or indirectly or by suppressing a negative regulator of *MIR828* and/or *TAS4* via a different feedback loop. Their research is concentrated on the stress response given by phosphate deficiency in *Arabidopsis*, but they suggest that the autoregulation of *MYB75/PAP1* also could apply to other stress conditions in which anthocyanins are accumulated. Thus, according to our hypothesis, At RCHSp38 plants could have an impaired or nonexistent autoregulative *MYB75/PAP1* mechanism, because p38 suppressed DCL4-activity renders the plants incapable of restoring normal levels of *MYB75/PAP1* through up-regulation of *TAS4*-siR81(-).

In summary, given the result presented in this study that the steady-state level of *MYB75/PAP1* transcripts in transgenic At RCHSp38 plants is higher than in wild-type plants combined with the following knowledge: 1) the key regulator of flavonoid genes specific for the anthocyanin branch is the transcription factor *MYB75/PAP1*, 2) a tasiRNA targeting

MYB75/PAP1 is known, 3) DCL4 is involved in the tasiRNA pathway, 4) loss of DCL4 leads to over-accumulation of anthocyanins, and 5) p38 suppresses DCL4 activity, we hypothesize that the increased levels of anthocyanin in At RCHSp38 plants is a consequence of p38 expression that leads to increased levels of *MYB75/PAP1* as a result of p38-induced impaired RNA silencing control of the *MYB75/PAP1* transcription factor.

5.1.2 Effect of stress exposure with high sucrose concentration treatment and high intensity light treatment on the transcript levels of flavonoid biosynthesis genes

Different stress treatments are known to induce anthocyanin production in *Arabidopsis*. Sucrose is known to induce anthocyanin production in a concentration-dependent manner (Tsukaya et al., 1991; Ohto et al., 2001; Teng et al., 2005; Solfanelli et al., 2006) and sucrose induction of the anthocyanin biosynthesis requires *MYB75/PAP1*. High intensity light treatment is also known to induce anthocyanin production (Feinbaum and Ausubel, 1988; Jenkins, 1997; Cominelli et al., 2008), and earlier studies have shown that the *MYB75/PAP1* gene is strongly induced by light and may have a key role in the light induction of anthocyanin biosynthesis (Cominelli et al., 2008).

When studying the data from the stress-exposed plants and comparing these to the data from the same plant lines not exposed to stress (Figure 6; comparing 3 % sucrose grown seedlings with 1 % sucrose grown seedlings, and Figure 7; comparing light exposed plants with not light exposed plants), we can investigate the effect of stress treatments on the transcript levels of flavonoid biosynthesis genes in the different plant lines, including wild-type. At RCHSp38 seedlings and plants exposed to stress show a large increase in the transcript levels of both *MYB75/PAP1* and the other flavonoid biosynthesis genes, especially *DFR* and *GST*, compared to seedlings and plants not exposed to stress (Figure 6 and Figure 7). The transcript level of *MYB75/PAP1* in wild-type seedlings and adult plants was also found to be up-regulated, however to a slighter extent than in At RCHSp38 plants. The At PAP1 plants showed very low increase in the transcript levels of all the genes analysed. The reason for the low increase in target gene transcript levels in the stress-treated At PAP1 seedlings and plants compared to At PAP1 seedlings and plants not exposed to stress might be that the over-expression of *MYB75/PAP1* is already so extensive that stress treatment does not increase the expression much further.

However, the results from the stress-induced plants are generally difficult to interpret because the calculated fold change values for the specific transcripts among the individual plant lines show a large spread. The variable transcript level of the flavonoid biosynthesis genes measured in the same plant line is most likely due to the fact that the different leaves used for RNA extraction were exposed differently to light. It is difficult to get the exact same amount of light to every individual leaf, given the plant morphology. Another important factor influencing the anthocyanin levels in leaves is their age and developmental stage. Although leaves were harvested from plants grown for the same amount of time, and that the same type of leaves (rosette) were used, this, together with different light exposure to each leaf, may be an explanation for the inconsistencies in calculated fold change values for the specific transcripts among the same plant lines observed in this study. In addition, the levels of *CHS* transcripts in the At RCHSp38 lines are difficult to interpret because they are also influenced by the effect of the IR-transgene designed to silence *CHS* expression.

Given our assumption that autoregulation of *MYB75/PAP1* in At RCHSp38 plants are impaired or lost due to p38 expression, we could expect higher *MYB75/PAP1* levels in stress induced At RCHSp38 plants then in stress induced wild-type plants. This is actually what we observe.

Cominelli et al (2008) shows that in wild-type plants, *MYB75/PAP1* transcripts accumulate before *CHS* and *DFR* transcripts in response to light treatment. They also show that *DFR* transcripts accumulate to higher levels than *CHS* transcripts in response to light treatment. Sucrose has been shown to induce late flavonoid biosynthesis genes (from *DFR* and down-stream) and *MYB75/PAP1* to a larger extent than the genes up-stream from *DFR*, namely *PAL1* and *CHS* (Solfanelli et al., 2006). These findings are in accordance with our results which show that in At RCHSp38 and wild-type plants, both sucrose-treated seedlings and adult plants exposed for high intensity light illumination were found to have higher steady-state levels of *DFR*, *MYB75/PAP1* and *GST* than the steady-state levels of *PAL1* and *CHS* transcript (Figure 6 and Figure 7). In this respect, it should be noted that the steady-state level of *CHS* transcripts in At RCHSp38 plants are also influenced by the IR-transgene designed to target *CHS* transcripts.

A conspicuous result from the stress treatment experiments is that even though *MYB75/PAP1* in wild-type seedlings and adult plants is less up-regulated than in At RCHSp38 lines, the results show that the transcript levels of *GST* and *DFR* in wild-type

seedlings and plants, respectively, are more up-regulated than in the At RCHSp38 lines. According to our hypothesis we expected that in plants where the steady-state level of *MYB75/PAP1* was highest, the transcript levels of the other flavonoid biosynthesis genes, especially the late ones such as *DFR* and *GST,* would also be highest. This is not, however, always the case as can be seen in the At RCHSp38 seedlings and plants. It may be possible that p38 expression also interferes with other components controlling the expression of the flavonoid biosynthesis genes, either directly or indirectly. Another explanation is that the IR-transgene targeting *CHS* in At RCHSp38 may influence the results. However, to our knowledge it is not known whether *CHS* levels can influence gene expression in the remaining flavonoid pathway by feedback regulation. Yet another explanation can be that when *MYB75/PAP1* is up-regulated so are the flavonoid biosynthesis genes especially the late ones *DFR* and *GST*, but how much *MYB75/PAP1* is required to achieve this up-regulation is unknown. It is difficult to rationally explain these results and a rational explanation must await further studies.

When comparing the effect of high sucrose concentration (Figure 6) and high intensity light exposure (Figure 7) with regard to the increase in transcript levels of the selected flavonoid biosynthesis genes, the effect of high intensity light exposure is overall larger than the effect of high sucrose concentration. Light is a very important environmental stimulus regulating expression of the flavonoid structural genes (Cominelli et al., 2008). It is not un-expected that plants react stronger to light than to sucrose when taking into account which of these two factors one would expect to be most important in the plants natural habitat. We would presume this to be light, since sucrose concentrations in the soil normally do not vary to the same degree as light conditions.

Another question is whether stress or gene modification has the greatest stimulatory effect on anthocyanin biosynthesis. In an attempt to answer this question, data in Figure 4 and Figure 5 (effect of transgenic constructs) can be compared to data in Figure 6 and Figure 7 (effect of stress treatment). This will not give a totally clear answer since the data in Figure 4 and Figure 5 results from comparing transgenic lines with a wild-type line exposed to the same growth conditions, and data in Figure 6 and Figure 7 results from comparing the same plant line exposed to different growth conditions. However, when comparing these results, the fold change values in the investigated transcripts in At RCHSp38 lines are higher for the effect of stress treatment, compared to the effect of transgene constructs. This indicates that in At RCHSp38 plants, the effect of stress treatment has more stimulatory effect on

anthocyanin synthesis than p38 expression. In At PAP1 plants, the effect of over-expressing *MYB75/PAP1* clearly exceeds the effect of stress treatment.

5.1.3 The phenotypic effects of p38 expression in *Arabidopsis*

The phenotypic effects of p38 expression in *Arabidopsis* were also examined. At RCHSp38 seedlings and adult plants showed an altered phenotype compared to wild-type plants, with a more intense purple colouration, in accordance with earlier results (Johansen and Wilson, 2008). However, the At RCHSp38 plants were not as purple as the At PAP1 seedlings and plants (Figure 8 and Figure 9). In fact, the purple colouration of the At RCHSp38 plants was found to be intermediate to that in wild-type and At PAP1 plants. Given the fact that the purple colouration is the result of increased anthocyanin accumulation in *Arabidopsis*, we would anticipate that the steady-state levels of the flavonoid transcripts *PAL1, CHS, DFR* and *GST* in At RCHSp38 to be intermediate to those in wild-type and At PAP1 plants. This is shown to be correct (Figure 4 and Figure 5).

The At RCHSp38 leaves and flowers also showed morphological defects (Figure 8 and Figure 9), the leaves having a jagged morphology and the flowers having shorter stamens compared to wild-type and At PAP1 plant lines. The morphological defects detected in transgenic At RCHSp38 plants are similar to defects typically associated with the expression of viral proteins were miRNA function is compromised (Chapman et al., 2004; Dunoyer et al., 2004). Previously studies have shown that p38 expression in *Arabidopsis* is not correlated with developmental defects or altered miRNA function (Dunoyer et al., 2004). However, as discussed previously, p38 interferes with DCL4-dependent tasiRNA biogenesis, resulting in decreased levels of tasiRNA and increased level of target mRNA (Moissiard et al., 2007). Accordingly, *Arabidopsis* dcl4 mutants show altered tasiRNA levels and function, and phenotypic defects (Gasciolli et al., 2005). Thus the morphological defects observed in At RCHSp38 plants could be attributed to a disturbance in the general tasiRNA function as a result of suppression, by p38, of DCL4 activity. The constitutive promoter used to drive expression of p38 in At RCHSp38 plants, the CmpC promoter, is even stronger than the 35S promoter previously used to drive expression of p38 in *Arabidopsis* (Dunoyer et al., 2004). Thus, it is possible that p38 is transcribed in At RCHSp38 plants at levels high enough to completely suppress DCL4 activity, resulting in a phenotype comparable to the null dcl4 mutant.

5.1.4 Further studies

Further studies to investigate if *MYB75/PAP1* is under RNA silencing control and that expression of p38 in *Arabidopsis* disrupts the regulation of *MYB75/PAP1* as a consequence of suppressed DCL4 activity should be undertaken. The most ideal plant line to investigate such an effect would be transgenic *Arabidopsis* plants expressing p38 alone, without the *CHS* IR-construct. However, generation of such transgenic lines has proven difficult, both in our lab and in the Voinnet group (personal communication with Dr. Angélique Déléris). It might be that such a plant line is not viable, especially when plants are made homozygous for p38. However, in the present study we have shown that *MYB75/PAP1* is up-regulated in At RCHSp38 plant lines and these plants should then be suited to further study the effect of p38 expression on the transcript levels of *MYB75/PAP1*. One obvious analysis would be to determine the accumulation levels of the small RNA species *TAS4*-siR81(-) and *miR828* in transgenic p38-expressing plants, and compare these levels to the same accumulation level in wild-type plants. The accumulations level of small RNA molecules can be examined by isolating small RNA molecules and then perform Northern hybridization to detect specific RNA molecules. If p38 indeed interferes with the regulation of *MYB75/PAP1*, one might expect that the accumulation level of *TAS4*-siR81(-) in p38-expressing plants would be lower than in wild-type plants since these small RNA molecules are the product of DCL4 activity which, as a consequence of suppression of p38, has lowered activity. Such an analysis is best performed in plants in which transcription of *MYB75/PAP1* is induced, such as in light-stressed plants. Otherwise, the accumulation level of *TAS4*-siR81(-) might be too low to be detected by conventional Northern hybridization experiments. It could also be interesting to see if there is a correlation between the level of p38 expression and the transcript level of *MYB75/PAP1*. If our hypothesis about *MYB75/PAP1* being under RNA silencing control is correct, we would expect a correlation between high p38 expression and high transcript levels of *MYB75/PAP1*.

As previously noted, loss of DCL4 activity, either as a consequence of suppression by viral proteins or in mutants defective in DCL4 (*dcl4-2*), is correlated with high anthocyanin accumulation in *Arabidopsis* (Nakazawa et al., 2007; Johansen and Wilson, 2008). It should therefore also be interesting to measure the transcript levels of *MYB75/PAP1* and the accumulation level of *TAS4*-siR81(-) and *miR828* in *dcl4* mutants. The *dcl4* mutant line was

planned to be included in the present study, but this mutant line was not available from the stock centre when the study was initiated.

5.2 The effect of p38 expression in *Chlamydomonas*

Poor level of transgene expression from the *Chlamydomonas* nuclear genome is a problem in this model organism (Fuhrmann et al., 1999; Schroda et al., 2000; Neupert et al., 2008). Why this is a problem is not well-understood, but a possible explanation is silencing of the transgene after integration into the nuclear genome. Different methods have been applied to overcome transgene silencing in this organism. In one study, mutant strains of *Chlamydomonas* were generated that efficiently express nuclear transgenes (Neupert et al., 2008). They developed a genetic screen that search for mutants where the transgene suppression mechanism was defective. Another possible strategy is to incorporate genes in vector constructs that upon transcription in transformed cells produce proteins known to suppress transgene silencing, specifically RNA silencing. Given that RNA silencing can account for some of the transgene silencing phenomenon observed in *Chlamydomonas,* viral suppressors of RNA silencing, such as p38, could be used to protect the transgene from silencing by inhibiting the silencing pathway.

We wanted to explore the second strategy, more specifically to investigate if p38 is able to suppress RNA silencing in *Chlamydomonas*. Using the amiRNA approach we intended to induce RNA silencing of the endogenous phytoene synthase (*PSY)* gene in *Chlamydomonas*. Down-regulation of *PSY* leads to transformed cells with a pale green algal phenotype, as shown by Molnar et al., (2009). Using cells with RNA-induced down-regulated *PSY* expression, we intended to study the effect of expressing the viral suppressor protein p38 in order to investigate if p38 was able to suppress RNA silencing in *Chlamydomonas*. Successful suppression by p38 in these transformed cells would then restore the normal dark green algal phenotype. However, we were not able to successfully detect transformed *Chlamydomonas* cells even after several transformation attempts. Therefore we did not attempt to transform cells further with the p38-expression gene construct.

We cannot rationally explain why we were not able to successfully transform *Chlamydomonas* cells in our lab. However, we did run into several problems that can help to explain why we were not successful: 1) Microbial contamination on the selective plates used to select for transformants. After transformation acetate had to be added to the medium in

order to ensure growth of transformants deficient in photosynthesis. Supplementation of the growth medium of *Chlamydomonas* with acetate is known to be problematic because of the risk of contaminating micro organisms. Strict aseptic procedures are therefore very important when handling *Chlamydomonas* cultures grown in acetate-containing medium. 2) It was difficult to detect pale green colonies on the agar plates used to select for transformed cells because the cell density after transformation was very high. 3) To identify "the best" suited medium to ensure optimal growth and satisfied cells was challenging. Successful transformation of *Chlamydomonas* is highly dependent on "happy" cells.

To solve these problems we tried different approaches. To avoid contamination during the transformation procedure, sterile technique was used at all stages, and DNA was precipitated with 3M sodium acetate and absolute ethanol and then dissolved in sterile buffer, before transformation into *Chlamydomonas* cells. To insure cells were given enough time to integrate DNA into the genome, and at the same time to insure optimal growth conditions for of the cells, transformed cells were incubated under dim light for 24 hours before they were transferred to growth in dark. Since *Chlamydomonas* was a new model organism in our lab, knowledge about growth conditions and mediums, and transformation procedures etc. had to be gathered. We used different growth mediums, including 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-acetate-phosphate (TAP), Sagar and Granick II medium, and High salt (HS) medium, all found at the Chlamydomonas center web page (Chlamy center, http://www.chlamy.org/media.html). After personal communication with Associate Professor Uwe Klein, Department of Molecular Biosciences, University of Oslo, we decided to use High salt medium for growth, as described in Materials and Methods, section 3.3.1.

5.2.1 Other methods that could have been tried to ensure transformation of *Chlamydomonas*

Transformation with the glass bead method can yield up to 1 x 10^3 transformants per µg DNA (Kindle, 1990). With the use of electroporation as a transformation method, a much higher transformation rate has been reported, up to 1.9 x 10^5 transformants per µg DNA (Shimogawara et al., 1998). To improve plating efficiency of cell wall deficient *Chlamydomonas* strains, a starch embedding method has been developed and used after transformation with electroporation (Shimogawara et al., 1998). We used the glass bead method for transformation, because we believed the transformation rate was high enough.

Since several attempts with the glass bead method were unsuccessful, the electroporation method in combination with starch embedding should be the next method of choice.

Chlamydomonas cells could also have been treated with autolysin prior to transformation. Autolysin is composed of proteolytic enzymes synthesized at characteristic points during the developmental cycles of the alga, with strong specificity towards the cell wall structure (Jaenicke et al., 1987). The *Chlamydomonas* strain we used (CC-1618) carries a cell wall mutation, *cw-15,* leading to absent or greatly reduced cell wall quantity compared to the wild-type cells. Therefore we did not treat the cells with autolysin. However, it has been reported improved transformation rate of *cw-15* mutants when treating them with autolysin prior to transformation (Kindle, Chlamydomonas center web page, http://www.chlamy.org/methods/beads.html). Another approach could also be to use another *Chlamydomonas* strain for transformation, for example the CC-325 strain which was used by Molnar et al. (2009). The CC-325 strain, like the CC-1618 strain, carries a mutation in the argininosuccinate lyase (*ASL*) gene and also the cell wall component *cw-15,* and should therefore be well suited for the experimental set-up used in this study.

5.2.2 Further studies

Further studies to investigate the effect of p38 expression in *Chlamydomonas* should be focused on successfully completing the transformation and obtaining *Chlamydomonas* cells with down-regulated *PSY* expression, and then to transform these cells with the p38 expressing construct. To verify that transformed cells show down-regulated *PSY* expression, total RNA should be isolated and then determining the levels of *PSY* transcript, using q-PCR, in cells transformed with the amiRNA containing construct and that of the empty vector. The expression level of *PSY* in cells harbouring the amiRNA construct is anticipated to be much lower than in cells harbouring the empty vector. In addition, an RNA blot to verify the presence of expressed amiRNAs should be conducted. Then, after transforming the *PSY* down-regulated *Chlamydomonas* transformants with an expression cassette for p38, total RNA should be isolated and the transcript level of *PSY* re-analysed. If p38 is able to suppress amiRNA-based RNA silencing of the endogenous *PSY* gene in *Chlamydomonas,* we would then expect higher transcript levels of *PSY* since the p38 expression would reduce or abolish the effect of RNA silencing. It should also be verified that p38 is efficiently expressed at the protein level, e.g. with western blot and anti-p38-antibody-based immune-

detection. This is especially important given the fact that codon usage in *Chlamydomonas* is atypical.

References

Aharoni, A., De Vos, C.H.R., Wein, M., Sun, Z.K., Greco, R., Kroon, A., Mol, J.N.M., and O'Connell, A.P. (2001). The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant Journal **28,** 319-332.

Allen, E., Xie, Z.X., Gustafson, A.M., and Carrington, J.C. (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121,** 207-221.

Arabidopsis Genome, I. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408,** 796.

Aravin, A.A., Naumova, N.M., Tulin, A.V., Vagin, V.V., Rozovsky, Y.M., and Gvozdev, V.A. (2001). Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D-melanogaster germline. Current Biology **11,** 1017-1027.

Bartel, D.P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell **116,** 281-297.

Bartel, D.P., and Chen, C.Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nature Reviews Genetics **5,** 396-400.

Basyuk, E., Suavet, F., Doglio, A., Bordonne, R., and Bertrand, E. (2003). Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res. **31,** 6593–6597.

Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B., and Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana. Plant Journal **39,** 366-380.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits rnicroRNAs and short interfering RNAs. Proceedings of the National Academy of Sciences of the United States of America **102,** 11928-11933.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409,** 363-366.

Borevitz, J.O., Xia, Y.J., Blount, J., Dixon, R.A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12,** 2383-2393.

Borsani, O., Zhu, J.H., Verslues, P.E., Sunkar, R., and Zhu, J.K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123,** 1279-1291.

Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Paro, R., and Perrimon, N. (2004). Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science **303,** 832-835.

Brodersen, P., and Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. Trends in Genetics **22,** 268-280.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. Science **320,** 1185-1190.

Burgyan, J. (2006). Virus induced RNA silencing and suppression: Defence and counter defence. J. Plant Pathol. **88,** 233-244.

Camblong, J., Beyrouthy, N., Guffanti, E., Schlaepfer, G., Steinmetz, L.M., and Stutz, F. (2009). Trans-acting antisense RNAs mediate transcriptional gene cosuppression in S. cerevisiae. Genes & Development **23,** 1534-1545.

Canto, T., Uhrig, J.F., Swanson, M., Wright, K.M., and MacFarlane, S.A. (2006). Translocation of Tomato bushy stunt virus P19 protein into the nucleus by ALY proteins compromises its silencing suppressor activity. J. Virol. **80,** 9064-9072.

Cao, M.Q., Fu, Y., Guo, Y., and Pan, J.M. (2009). Chlamydomonas (Chlorophyceae) colony PCR. Protoplasma **235,** 107-110.

Carthew, R.W., and Sontheimer, E.J. (2009). Origins and Mechanisms of miRNAs and siRNAs. Cell **136,** 642-655.

Casas-Mollano, J.A., Rohr, J., Kim, E.J., Balassa, E., van Dijk, K., and Cerutti, H. (2008). Diversification of the core RNA interference machinery in Chlamydomonas reinhardtii and the role of DCL1 in transposon silencing. Genetics **179,** 69-81.

Castanotto, D., and Rossi, J.J. (2009). The promises and pitfalls of RNA-interference-based therapeutics. Nature **457,** 426-433.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V., and Carrington, J.C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes & Development **18,** 1179-1186.

Chiu, Y.L., and Rana, T.M. (2003). siRNA function in RNAi: A chemical modification analysis. Rna-a Publication of the Rna Society **9,** 1034-1048.

Cogoni, C., Irelan, J.T., Schumacher, M., Schmidhauser, T.J., Selker, E.U., and Macino, G. (1996). Transgene silencing of the al-1 gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. Embo Journal **15,** 3153-3163.

Cominelli, E., Gusmaroli, G., Allegra, D., Galbiati, M., Wade, H.K., Jenkins, G.I., and Tonelli, C. (2008). Expression analysis of anthocyanin regulatory genes in response to different light qualities in Arabidopsis thaliana. Journal of Plant Physiology **165,** 886-894.

Deleris, A., Gallego-Bartolome, J., Bao, J.S., Kasschau, K.D., Carrington, J.C., and Voinnet, O. (2006). Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science **313,** 68-71.

Ding, S.W., and Voinnet, O. (2007). Antiviral immunity directed by small RNAs. Cell **130,** 413-426.

Dunoyer, P. (2009). The battle of Silence : action and inhibition of RNA silencing during plant/virus interactions. M S-Med. Sci. **25,** 505-511.

Dunoyer, P., Lecellier, C.H., Parizotto, E.A., Himber, C., and Voinnet, O. (2004). Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell **16,** 1235-1250.

Eichler-Stahlberg, A., Weisheit, W., Ruecker, O., and Heitzer, M. (2009). Strategies to facilitate transgene expression in Chlamydomonas reinhardtii. Planta **229,** 873-883.

Fang, Y.D., and Spector, D.L. (2007). Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants. Current Biology **17,** 818-823.

Feinbaum, R.L., and Ausubel, F.M. (1988). TRANSCRIPTIONAL REGULATION OF THE ARABIDOPSIS-THALIANA CHALCONE SYNTHASE GENE. Molecular and Cellular Biology **8,** 1985-1992.

Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391,** 806-811.

Fuhrmann, M., Oertel, W., and Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in Chlamydomonas reinhardtii. Plant Journal **19,** 353-361.

Fusaro, A.F., Matthew, L., Smith, N.A., Curtin, S.J., Dedic-Hagan, J., Ellacott, G.A., Watson, J.M., Wang, M.B., Brosnan, C., Carroll, B.J., and Waterhouse, P.M. (2006). RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. EMBO Rep. **7,** 1168-1175.

Gasciolli, V., Mallory, A.C., Bartel, D.P., and Vaucheret, H. (2005). Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Current Biology **15,** 1494-1500.

Ghildiyal, M., and Zamore, P.D. (2009). Small silencing RNAs: an expanding universe. Nature Reviews Genetics **10,** 94-108.

Gonzalez, A., Zhao, M., Leavitt, J.M., and Lloyd, A.M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant Journal **53,** 814-827.

Grimm, D. (2009). Small silencing RNAs: State-of-the-art. Advanced Drug Delivery Reviews **61,** 672-703.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature **404,** 293-296.

Harris, E.H. (2001). Chlamydomonas as a model organism. Annual Review of Plant Physiology and Plant Molecular Biology **52,** 363-406.

Hsieh, L., Lin, S., Shih, A.C., Chen, J., Lin, W., Tseng, C., Li, W., and Chiou, T. (2009). Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiology **151,** 2120-2132.

Jaenicke, L., Kuhne, W., Spessert, R., Wahle, U., and Waffenschmidt, S. (1987). CELL-WALL LYTIC ENZYMES (AUTOLYSINS) OF CHLAMYDOMONAS-REINHARDTII ARE (HYDROXY)PROLINE-SPECIFIC PROTEASES. European Journal of Biochemistry **170,** 485-491.

Jenkins, G.I. (1997). UV and blue light signal transduction in Arabidopsis. Plant Cell Environ. **20,** 773-778.

Johansen, W., and Wilson, R.C. (2008). Viral suppressor proteins show varying abilities and effectiveness to suppress transgene-induced post-transcriptional gene silencing of endogenous Chalcone synthase in transgenic Arabidopsis. Plant Cell Reports **27,** 911-921.

Johansen, W., Nestestog, R., Lillehaug, J.R., and Wilson, R.C. (manuscript in preparation). Biological variation in hpRNA-induced PTGS of *Chalcone synthase* in *Arabidopsis* and quantitative correlation with hpRNA expression levels. In preparation.

Jones-Rhoades, M.W., Bartel, D.P., and Bartel, B. (2006). MicroRNAs and their regulatory roles in plants. Annu. Rev. Plant Biol. **57,** 19-53.

Kamath, R.S., Fraser, A.G., Dong, Y., Poulin, G., Durbin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D.P., Zipperlen, P., and Ahringer, J. (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature **421,** 231-237.

Kerschen, A., Napoli, C.A., Jorgensen, R.A., and Muller, A.E. (2004). Effectiveness of RNA interference in transgenic plants. FEBS Lett. **566,** 223-228.

Kim, V.N. (2005). MicroRNA biogenesis: Coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. **6,** 376-385.

Kindle, K.L. (1990). HIGH-FREQUENCY NUCLEAR TRANSFORMATION OF CHLAMYDOMONAS-REINHARDTII. Proceedings of the National Academy of Sciences of the United States of America **87,** 1228-1232.

Lee, Y., Kim, M., Han, J.J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. Embo Journal **23,** 4051-4060.

Lee, Y., Ahn, C., Han, J.J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature **425,** 415-419.

Leonelli, S. (2007). Arabidopsis, the botanical Drosophilia: from mouse cress to model organism. Endeavour **31,** 34-38.

Li, F., and Ding, S.W. (2006). Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. Annual Review of Microbiology **60,** 503-531.

Li, J.J., Yang, Z.Y., Yu, B., Liu, J., and Chen, X.M. (2005). Methylation protects miRNAs and siRNAs from a 3 '-end uridylation activity in Arabildopsis. Current Biology **15,** 1501-1507.

Lippman, Z., and Martienssen, R. (2004). The role of RNA interference in heterochromatic silencing. Nature **431,** 364-370.

Liu, J.D., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science **305,** 1437-1441.

Lo, S.C.C., and Nicholson, R.L. (1998). Reduction of light-induced anthocyanin accumulation in inoculated sorghum mesocotyls - Implications for a compensatory role in the defense response. Plant Physiology **116,** 979-989.

MacRae, I.J., Zhou, K.H., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., and Doudna, J.A. (2006). Structural basis for double-stranded RNA processing by dicer. Science **311,** 195-198.

McCarthy, S.S., Kobayashi, M.C., and Niyogi, K.K. (2004). White mutants of Chlamydomonas reinhardtii are defective in phytoene synthase. Genetics **168,** 1249-1257.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L., Marshall, W.F., Qu, L.H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q.H., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C.L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Balle, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P.A., Lemaire, S.D., Lobanov, A.V., Lohr, M., Manuell, A., Meir, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J.V., Moseley, J., Napoli, C., Nedelcu, A.M., Niyogi, K., Novoselov, S.V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J.P., Riano-Pachon, D.M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S.L., Allmer, J., Balk, J., Bisova, K., Chen, C.J., Elias, M., Gendler, K., Hauser, C., Lamb, M.R., Ledford, H., Long, J.C., Minagawa, J., Page, M.D., Pan, J.M., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A.M., Yang, P.F., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V.N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y.W., Jhaveri, J., Luo, Y.G., Martinez, D., Ngau, W.C.A., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K.M., Grigoriev, I.V., Rokhsar, D.S., and Grossman, A.R. (2007). The Chlamydomonas genome reveals the evolution of key animal and plant functions. Science **318,** 245-251.

Moissiard, G., Parizotto, E.A., Himber, C., and Voinnet, O. (2007). Transitivity in Arabidopsis can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral-encoded suppressor proteins. Rna-a Publication of the Rna Society **13,** 1268-1278.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., and Baulcombe, D. (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga Chlamydomonas reinhardtii. Plant Journal **58,** 165-174.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. . Physiol Plant **15,** 473-497.

Nakazawa, Y., Hiraguri, A., Moriyama, H., and Fukuhara, T. (2007). The dsRNA-binding protein DRB4 interacts with the Dicer-like protein DCL4 in vivo and functions in the trans-acting siRNA pathway. Plant Molecular Biology **63,** 777-785.

Neupert, J., Karcher, D., and Bock, R. (2008). Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. Plant Journal **57,** 1140-1150.

Obbard, D.J., Gordon, K.H.J., Buck, A.H., and Jiggins, F.M. (2009). The evolution of RNAi as a defence against viruses and transposable elements. Philosophical Transactions of the Royal Society B-Biological Sciences **364,** 99-115.

Ohto, M., Onai, K., Furukawa, Y., Aoki, E., Araki, T., and Nakamura, K. (2001). Effects of sugar on vegetative development and floral transition in arabidopsis. Plant Physiology **127,** 252-261.

Orban, T.I., and Izaurralde, E. (2005). Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. Rna-a Publication of the Rna Society **11,** 459-469.

Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R.S. (2005). Nuclear processing and export of microRNAs in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **102,** 3691-3696.

Parker, J.S., Roe, S.M., and Barford, D. (2004). Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. Embo Journal **23,** 4727-4737.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H.L., and Poethig, R.S. (2004). SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. Genes & Development **18,** 2368-2379.

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

Platt, A.R., Woodhall, R.W., and George, A.L. (2007). Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. Biotechniques **43,** 58-+.

Qu, F., Ren, T., and Morris, T.J. (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. J. Virol. **77,** 511-522.

Rajagopalan, R., Vaucheret, H., Trejo, J., and Bartel, D.P. (2006). A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. Genes & Development **20,** 3407-3425.

Rohr, J., Sarkar, N., Balenger, S., Jeong, B.R., and Cerutti, H. (2004). Tandem inverted repeat system for selection of effective transgenic RNAi strains in Chlamydomonas. Plant Journal **40,** 611-621.

Sambrook, J., and Russel, D.W. (2001). A laboratory manual. Molecular Cloning.

Schroda, M., Blocker, D., and Beck, C.F. (2000). The HSP70A promoter as a tool for the improved expression of transgenes in Chlamydomonas. Plant Journal **21,** 121-131.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell **18,** 1121-1133.

Shimogawara, K., Fujiwara, S., Grossman, A., and Usuda, H. (1998). High-efficiency transformation of Chlamydomonas reinhardtii by electroporation. Genetics **148,** 1821-1828.

Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M., and Burgyan, J. (2002). A viral protein suppresses RNA silencing and binds silencing-generated, 21-to 25-nucleotide double-stranded RNAs. Embo Journal **21,** 3070-3080.

Solfanelli, C., Poggi, A., Loreti, E., Alpi, A., and Perata, P. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiology **140,** 637-646.

Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004). Crystal structure of argonaute and its implications for RISC slicer activity. Science **305,** 1434-1437.

Stavolone, L., Kononova, M., Pauli, S., Ragozzino, A., de Haan, P., Milligan, S., Lawton, K., and Hohn, T. (2003). Cestrum yellow leaf curling virus (CmYLCV) promoter: a new strong constitutive promoter for heterologous gene expression in a wide variety of crops. Plant Molecular Biology **53,** 703-713.

Stefani, G., and Slack, F.J. (2008). Small non-coding RNAs in animal development. Nat. Rev. Mol. Cell Biol. **9,** 219-230.

Sueoka, N. (1960). Proc. Natl. Acad. Sci. USA **46,** 83-91.

Teng, S., Keurentjes, J., Bentsink, L., Koornneef, M., and Smeekens, S. (2005). Sucrose-specific induction of anthocyanin biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. Plant Physiology **139,** 1840-1852.

Tomari, Y., and Zamore, P.D. (2005). Perspective: machines for RNAi. Genes & Development **19,** 517-529.

Tsukaya, H., Ohshima, T., Naito, S., Chino, M., and Komeda, Y. (1991). SUGAR-DEPENDENT EXPRESSION OF THE CHS-A GENE FOR CHALCONE SYNTHASE FROM PETUNIA IN TRANSGENIC ARABIDOPSIS. Plant Physiology **97,** 1414-1421.

Vagin, V.V., Sigova, A., Li, C.J., Seitz, H., Gvozdev, V., and Zamore, P.D. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. Science **313,** 320-324.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A.C., Hilbert, J.L., Bartel, D.P., and Crete, P. (2004). Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. Mol. Cell **16,** 69-79.

Voinnet, O. (2009). Origin, Biogenesis, and Activity of Plant MicroRNAs. Cell **136,** 669-687.

Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proceedings of the National Academy of Sciences of the United States of America **96,** 14147-14152.

Waterhouse, P.M., and Helliwell, C.A. (2003). Exploring plant genomes by RNA-induced gene silencing. Nature Reviews Genetics **4,** 29-38.

Waterhouse, P.M., Smith, N.A., and Wang, M.B. (1999). Virus resistance and gene silencing: killing the messenger. Trends Plant Sci. **4,** 452-457.

Winkel-Shirley, B. (2001a). It takes a garden. How work on diverse plant species has contributed to an understanding of flavonoid metabolism. Plant Physiology **127,** 1399-1404.

Winkel-Shirley, B. (2001b). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiology **126,** 485-493.

Wu, Z.T., Zhu, Y.L., Bisaro, D.M., and Parris, D.S. (2009). Herpes Simplex Virus Type 1 Suppresses RNA-Induced Gene Silencing in Mammalian Cells. J. Virol. **83,** 6652-6663.

Xie, Z.X., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. Plos Biology **2,** 642-652.

Xu, P., Zhang, Y.J., Kang, L., Roossinck, M.J., and Mysore, K.S. (2006). Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiology **142,** 429-440.

Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., and Zhou, M.M. (2003). Structure and conserved RNA binding of the PAZ domain. Nature **426,** 469-474.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes & Development **17,** 3011-3016.

Yigit, E., Batista, P.J., Bei, Y.X., Pang, K.M., Chen, C.C.G., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C-elegans argonaute family reveals that distinct argonautes act sequentially during RNAi. Cell **127,** 747-757.

Yoshikawa, M., Peragine, A., Park, M.Y., and Poethig, R.S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. Genes & Development **19,** 2164-2175.

Yu, B., Yang, Z.Y., Li, J.J., Minakhina, S., Yang, M.C., Padgett, R.W., Steward, R., and Chen, X.M. (2005). Methylation as a crucial step in plant microRNA biogenesis. Science **307,** 932-935.

Yu, B., Bi, L., Zheng, B.L., Ji, L.J., Chevalier, D., Agarwal, M., Ramachandran, V., Li, W.X., Lagrange, T., Walker, J.C., and Chen, X.M. (2008). The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. Proceedings of the National Academy of Sciences of the United States of America **105,** 10073-10078.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101,** 25-33.

Zhang, H.D., Kolb, F.A., Jaskiewicz, L., Westhof, E., and Filipowicz, W. (2004). Single processing center models for human dicer and bacterial RNase III. Cell **118,** 57-68.

Abbrevations

35S	Cauliflower mosaic virus 35S promoter
Abs	absorbance
Agrobacterium	*Agrobacterium tumefaciens*
amiRNA	artificial microRNA
Arabidopsis	*Arabidopsis thaliana*
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
bp	base pair
CC	Chlamydomonas genetics center strain
cDNA	complementary DNA
CDS	coding region
Chlamydomonas	*Chlamydomonas reinhardtii*
Ct	cycle threshold
cw	cell-wall
D-bodies	dicing bodies
DCL	Dicer like
DNA	deoxyriboducleic acid
DTT	dithiothreitol
Endo-siRNA	endogenous small interfering RNA
EtOH	ethanol
HUC	Hedmark University College
hpRNA	hairpin RNA
HYL1	Hyponastic leaves 1
LB_{amp50}	Luria Bertani medium supplemented with 50 mg/mL ampicillin
Mbp	Mega base pair

min	minute
mRNA	messenger RNA
MIR genes	microRNA genes
miRNA	micro RNA
mt	mating type
ncRNAs	non-coding RNA
nt	nucleotide
PAZ	Piwi Argonaute Zwille
PCR	polymerase chain reaction
piRNA	piwi-interacting RNA
PIWI	P-element induced wimpy testis
pri-miRNA	primary miRNA
pri-tasiRNA	primary tasiRNA
q-PCR	quantitative real time PCR
RNA	ribonucleic acid
RNase	ribonuclease
ss	single stranded
sec	second
sRNA	small RNA
siRNA	small interfering RNA
TAE	Tris base, acetic acid and EDTA
TBE	Tris/Borate/EDTA
T-DNA	transfer DNA
U	Units
UTR	un-translated region
VSR	viral suppressor of RNA silencing
w/v	weight/volume

Appendix

Table 1: Primer sequences for analysis of Arabidopsis thaliana flavonoid biosynthesis genes.

Used for	Primer	Sequence
Primer verification and sequencing to confirm correct amplification product, and real time quantitative PCR.	Actin2-F	5’- GCTGGTTTTGCTGGTGATGATG -3’
	Actin2-R	5’- TAGAACTGGGTGCTCCTCAGGG -3’
	MYB75-F	5’- CAATGCCCCACCAAAAGTTGACG -3’
	MYB75-R	5’- GTTGTCGTCGCTTCAGGAACCAAAAT -3’
	PAL1-F	5’- TTGTAGCGCAACGTACCCGT -3’
	PAL1-R	5’- TCCTGTTCGGGATAGCCGAT -3’
	DFR-F	5’- AGCTGACGGACATGGGGTTT -3’
	DFR-R	5’- TCGGTTCTCTCGCCGGTTAT -3’
	GST-F	5’- CTACCAAGTTCGCGGACCAA -3’
	GST-R	5’- GCCAAAAACCGGTTCGAAGA -3’

Table 2: Artificial microRNA oligonucleotide sequences.

	3’ UTR	Coding sequence
Artificial microRNA	5’-TATCTCTTTAAGTACAAGCGC-3’	5’-TTTTCCGAAATGCCGTACTCG-3’
Forward oligo	5’-CTAGTGCGCTTGTACTTAAAGTGATATCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTATATCTCTTTAAGTACAAGCGCG-3’	5’-CTAGTCGAGTACGGCATTTCGCAAAATCTCGCTGATCGGCACCATGGGGGTGGTGGTGATCAGCGCTATTTTCCGAAATGCCGTACTCGG-3’
Reverse oligo	5’-CTAGCGCGCTTGTACTTAAAGAGATATAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGATATCACTTTAAGTACAAGCGCA-3’	5’-CTAGCCGAGTACGGCATTTCGGAAAATAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGATTTTGCGAAATGCCGTACTCGA-3’

Table 3: Primer sequences for Chlamydomonas amiRNA preparation.

Used for	Primer	Sequence
Colony PCR to search for clones with insert in right orientation, and sequencing to verify correct cloning	$AmiRNAprec_{for}$	5’-GGTGTTGGGTCGGTGTTTTTG-3’
Colony PCR to search for clones with insert in right orientation	$Spacer_{rev}$	5’-TAGCGCTGATCACCACCACCC-3’

Table 4: Primer sequences for Chlamydomonas colony PCR screen for amiRNA transformants.

Primer	Sequence
Chlamy-F	5’-TTGCTCACCCAGAAACGCTG-3’
Chlamy-R	5’-GCCATCCGTAAGATGCTTTTCTGT-3’