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TRANSCRIPTIONAL CHANGES IN *NICOTIANA BENTHAMIANA* INDUCED BY TOBAMOVIRAL TRANSFECTION

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I dedicate my dissertation to all whom I love.

ABSTRACT

This research has been dedicated to the development of a system to study the utility of plant viral vector technology for metabolic engineering and functional genomic applications. Potato cDNA microarrays developed by The Institute for Genomic Research (TIGR) were employed to determine gene expression changes in transfected *Nicotiana benthamiana* plants. Several challenges were addressed in developing the system, such as selection of appropriate controls; subtracting out effects of virus infection on plant transcription; and determining the effectiveness of using heterologous microarrays for *solanaceous* species.

Regulation of carotenoid biosynthesis was investigated by inoculating plants with TTU51/CTP-*CrtB*-RZ carrying a phytoene synthase cDNA derived from *Erwinia herbicola*. Microarray analysis showed that the expression of genes encoding enzymes in leaf carotenogenesis was upregulated and that transcripts both upstream (isopentenyl diphosphate isomerase) and downstream (β-carotene hydroxylase) of the targeted enzyme accumulated at ten days post-inoculation (dpi). Quantitative real-time PCR (QRT-PCR) data validated an elevation of endogenous phytoene synthase and phytoene desaturase (*pds*) mRNAs. Plants transfected with a TTO1/*PDS*⁻ viral vector carrying a partial *pds* antisense construct, showed a 5-fold decrease in transcript levels of a putative 9-cis-epoxycarotenoid dioxygenase (NCED) and a 78-fold decrease in *pds* mRNA compared to controls. These data demonstrate that *tobamoviral* vectors are valuable tools in the

metabolic engineering of plant pathways. Accumulation of colorless phytoene in both transfections does not appear to play a role in pathway regulation.

In a reverse genetics approach, a time course analysis was conducted for *N. benthamiana* transfected with 740AT#120 viral vector carrying an antisense *Arabidopsis* ADP-ribosylation factor-1 (*ARF-1*). Cytoplasmic inhibition of gene expression produced a progressively stunted phenotype. A Western analysis showed that ARF protein levels gradually decline and that all members of this multigene family are knocked down by 20 dpi. QRT-PCR shows endogenous *ARF* transcripts are 2.4x lower than control levels. A Welch ANOVA of microarray data identified sixteen genes for further characterization that have a potential role in the secretory or G-protein signaling pathways. Overall, these data suggest that combining viral vector and transcriptional profiling technologies is a useful strategy to rapidly filter gene expression data to assess gene function in plants.

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ABBREVIATIONS

ABA	Abscisic Acid
ABRC	*Arabidopsis* Biological Resource Center
ARF	ADP Ribosylation Factor
cDNA	Complementary DNA
crtB	Carotenoid B (*Erwinia uredovora* phytoene synthase)
crtI	Carotenoid I (*Erwinia uredovora* phytoene desaturase)
dpi	Days post-inoculation
ds	Double-stranded
GFP	green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
GTP	Guanosine triphophate
GTPase	Guanosine triphophate hydrolyzing enzyme
GUS	beta-glucuronidase
IPP	Isopentenyl diphosphate
IRES	Internal ribosome entry sequence
NCBI	National Center for Biotechnology Information
NSF	National Science Foundation
PCR	Polymerase Chain Reaction
PDS	Phytoene desaturase
PSY	Phytoene synthase
PTGS	Post-transcriptional gene silencing
QRT-PCR	Quantitative Real-Time PCR
RdRP	RNA dependent RNA Polymerase
RNAi	RNA interference
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
ss	Single-stranded
TIGR	The Institute for Genomic Research
TMV	*Tobacco mosaic virus*
ToMV	*Tomato mosaic virus*
VIGS	Virus induced gene silencing

CHAPTER 1. BACKGROUND AND RESEARCH OBJECTIVES

FOREWORD

The following sections highlight the major applications of plant viral vector technology, focusing on pharmaceutical production, gene silencing investigations, and gene function studies. Several RNA plant viruses have been engineered to deliver genes derived from various sources into plant host cells through a process known as transfection. As the virus replicates and moves through the plant, the gene transcript associated with the vector accumulates in the cytoplasm, without integrating into the host plant genome.

The first section is a copyrighted, peer-reviewed article that was published in the *Encyclopedia of Plant and Crop Sciences,* Ed. Robert M. Goodman, New York: Marcel Dekker, Inc., 2004. In addition to the use of tobamoviruses in gene expression studies, this paper summarizes and highlights other areas of utility, including their use in pharmaceutical production and metabolic engineering of compounds that are not naturally found in plants. Permission has been granted from Marcel Dekker Publisher (CRC Press; MWolff@crcpress.com) for this paper to appear as a chapter in my thesis dissertation. Bibliographic citations have been modified to conform to the style of the dissertation. A review of the literature regarding transcriptional profiling in transfected plant systems follows. Finally, my research objectives are outlined, providing information about the pathways under investigation, as well as the microarray and quantitative real time PCR assays used in these experiments.

1.1 TOBAMOVIRAL VECTORS: DEVELOPING A PRODUCTION SYSTEM FOR PHARMACEUTICALS IN TRANSFECTED PLANTS

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1.1.1 Introduction

Transgenic crops have played a predominant role in the production of pharmaceuticals and other valuable biological molecules. A more efficient strategy has involved inoculating non-transgenic plants with virus-based vectors that carry foreign genes. With the development of infectious cDNA clones, single-stranded RNA plant viruses have become key players in gene function discovery, metabolic engineering, and biomanufacturing. Viral expression vectors provide epigenetic expression of foreign sequences throughout infected plants, leading to gain- or loss-of-function phenotypes due to overexpression or cytoplasmic inhibition of gene expression.

Plant viruses are powerful transfection tools in molecular farming, producing pure, properly folded and glycosylated proteins in plants faster and more economically than other expression systems. They are a highly desirable alternative to transgenic systems that require protracted periods to transform and regenerate whole plants, and that have variation in the expression levels of heterologous proteins. In transgenic systems, once a particular construct is inserted into the plant genome, it may take several crosses

to establish a stable line in an elite cultivar. In contrast, plant viral vectors employed in the large-scale production of therapeutic drugs in greenhouse and field-grown crops directly yield high levels of foreign protein due to the rapid rate of viral replication. In plants transfected with a recombinant tobacco mosaic virus (TMV) *tobamovirus*, alpha-trichosanthin, a potential anti-AIDS drug, accumulated to approximately 2% of total soluble protein.

Therapeutic compounds stably produced in transfected plants are numerous, and include anti-viral drugs such as human interferon-alpha 2 as well as vaccines, proteins, and secondary metabolites. Plant-derived anti-cancer vaccines have been produced for treatment of human papillomavirus-induced cancer by expressing recombinant E7 fusion oncoproteins in *N. benthamiana*. The HIV p24 nucleocapsid protein, used as an antigen in the development of HIV vaccines, has been produced in plant protoplasts using tomato bushy stunt virus (TBSV) *tombusvirus* vector. For viruses that cannot be grown in tissue culture, such as hepatitis C (HCV), tobamoviral vectors are under development to produce a plant-derived vaccine. Recombinant proteins for use in diagnostics have also been expressed in plants. Full-length recombinant monoclonal antibodies (rAbs) directed against a colon cancer antigen and recombinant allergens have been expressed in *N. benthamiana* leaves using a TMV vector. The binding of IgE from sera from birch pollen- and latex-allergic patients suggests that the plant-produced allergens are properly folded.

1.1.2 Tobacco Mosaic Virus Vectors

Several virus groups have been under investigation for design as recombinant plant virus vectors including geminiviruses; potyviruses; potexviruses; comoviruses; tombusviruses; tobraviruses; alfamoviruses; and hordeiviruses. Members of the tobamovirus group (Fitzmaurice *et al.*, 2002; Kumagai *et al.*, 2000; Nemchinov *et al.*, 2000), are the most widely studied; the autonomously replicating RNA viral vectors based on the *tobacco mosaic virus* (TMV) genome have been particularly successful as research and commercial tools.

TMV possesses a positive-sense, single-stranded genome of 6396 nucleotides, which encodes replicase enzymes, and movement and coat proteins. Viral genes are expressed through the production of both genomic and subgenomic RNA. Essentially designed as cDNA plasmids, TMV vectors are modified to contain a foreign gene sequence. Originally vectors were constructed with the gene of interest replacing the capsid protein, until it was recognized that these viral vectors do not move efficiently. Presently, TMV vectors are hybrid versions of several different strains of tobamoviruses (Kumagai *et al.*, 1995; Kumagai *et al.*, 1993) that include all essential viral genes, a bacterial origin of replication (ori) and an antibiotic resistance marker. Dual subgenomic promoters from related tobamoviruses have enhanced stability, while an internal ribosome entry site sequence (IRES) (Toth *et al.*, 2001) has been incorporated into the design to enable expression of multiple proteins.

The transfection process involves mechanically inoculating recombinant *in vitro* RNA transcripts from viral cDNA clones onto plants. Recombinant virions are assembled in the plant and move systemically by associating with plasmodesmata and intercellular cytoplasmic channels, producing foreign protein as they travel. One to two weeks after inoculation, recombinant proteins can be isolated from transfected plants. Interstitial fluid containing the desired product can be quickly separated from other cellular proteins by vacuum infiltration and gentle centrifugation. For large agronomic applications, virions can be purified from transfected plants and used for subsequent inoculations using high-pressure sprayers in the field.

1.1.2.1 Viral Vector Design Construct

For vaccine production, TMV vectors have been developed as coat protein fusions (Turpen *et al.*, 1995), with the viral coat providing a flexible framework for the recombinant protein. TMV CP fusions include human immunodeficiency virus type I (HIV-I) peptide, influenza virus hemagglutinin epitope, malaria parasite peptide, and hepatitis C virus peptide (Nemchinov *et al.*, 2000). A key technical advance in design has enabled TMV vectors to produce "free" proteins that are not fused to the coat protein. Instead, genes encoding bioactive compounds are fused to signal peptide sequences that cause the translated protein to be processed via the endoplasmic reticulum and Golgi complex and to be targeted for cellular secretion. It is recognized that the faithful and efficient expression of such heterologous proteins is influenced by the choice of the signal peptide.

One of the most highly efficient TMV vector constructs fuses the gene of interest to a sequence encoding a rice α-amylase signal peptide adjacent to a 5' untranslated leader (Kumagai *et al.*, 2000). Viral vectors containing the rice α-amylase signal peptide ORF have been used to express a wide variety of heterologous proteins including mammalian peptides, blood products, glycoproteins and cytokines. TMV vectors that incorporate this signal peptide have been used successfully to secrete single chain variable fragment (Fv) antibodies for the treatment of Non-Hodgkin's Lymphoma (NHL). Currently in Phase II/III clinical trials, these highly customized patient-specific vaccines act as anti-tumor agents (McCormick *et al.*, 1999).

The rice alpha-amylase 5' untranslated leader sequence may help to enhance translation of the heterologous protein. The highly expressed viral coat subgenomic RNA has a 5' cap (m7GpppN) and terminates with a tRNA-like structure instead of a poly (A) tail. The 3' untranslated region (UTR) has two domains, which contain five RNA pseudoknots. It is possible that interactions between the 34 base pair 5' leader of α-amylase and the 3' UTR may cause a synergistic regulation of translation in transfected plants. Significantly, the rice α-amylase signal peptide has been recognized and processed in other transformed organisms, including *Escherichia coli*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica*, as well as transgenic rice cell suspension. Subtle differences in the size, source, and sequence of the alpha-amylase signal peptide can greatly affect the secretion process.

Striving for higher protein yields, researchers are collaborating to develop improvements in vector design for increased production. It is recognized that inclusion of foreign genes into TMV vectors reduces efficiencies of replication and movement compared to the wild type; these studies are aimed at improving the ability of these vectors to move and to replicate through "gene shuffling" of the 30K movement gene (Rabindran and Dawson, 2001). Visible markers for heterologous gene expression in plants are also under development. Tobacco mosaic viral (TMV) vectors have been engineered to overexpress an enzyme involved in carotenoid biosynthesis in *N. benthamiana* and other solaneaceous plants (Kumagai *et al.*, 1995). As the viral genome is translated, the encoded enzyme, phytoene synthase (*psy*), is targeted to the chloroplast causing an accumulation of phytoene, a colorless compound. However, transfected plants show a characteristic orange phenotype in the leaves and flower sepals, as early as 4 days post-inoculation (Figure 1.1). If fused to a heterologous sequence in a recombinant vector, *psy* may serve as a useful marker for gene expression, particularly in field applications.

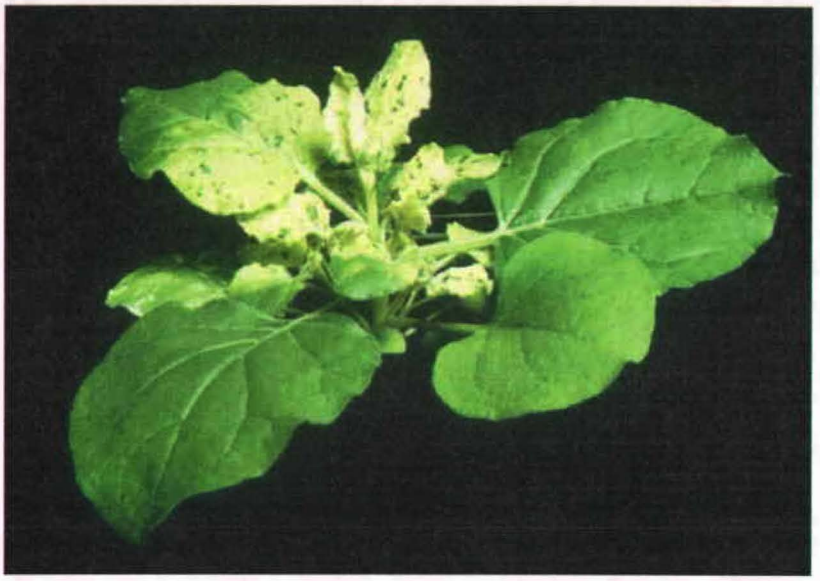

Figure 1.1. Overexpression of phytoene synthase (crtB) in leaves of *Nicotiana benthamiana* produces an orange phenotype.

1.1.2.2 Metabolic Engineering

While plant viruses that are engineered to produce pharmaceutically relevant proteins have proved to be powerful gene expression tools, they are also valuable tools for use in gene discovery and in the metabolic engineering of existing pathways in plants. The biosynthesis of leaf carotenoids in transfected *N. benthamiana* was altered by forced re-routing of the pathway, resulting in the synthesis of capsanthin, a non-native chromoplast-specific xanthophyll. The ectopic expression of capsanthin-capsorubin synthase (Ccs) cDNA caused the plant to develop an orange phenotype and accumulate high levels of capsanthin, up to 36% total carotenoids. By redirecting the existing pathways of plants that produce biologically active compounds, plant virus expression systems can potentially be used to alter or produce novel enzymes, or cause the accumulation of non-native bioactive compound (Kumagai *et al.*, 1998).

1.1.3 Conclusion

Plant RNA viral vectors have become intensively utilized in several different plant species for large-scale production of high-value therapeutic proteins (Franconi *et al.*, 2002; Verch *et al.*, 1998; Zhang *et al.*, 2000) and secondary metabolites (Kumagai *et al.*, 1993). The United States Food and Drug Administration (FDA) has developed a guidance document on "plant-derived biologics" and has strengthened field-testing controls for permits on those bioengineered traits that are not intended for commodity uses, such as pharmaceuticals, veterinary biologics, or certain industrial products. The human safety of TMV-based expression systems has been documented; plant viruses are not pathogenic to humans. TMV is only transmitted to other plants through mechanical means; proper cleaning of tools and machinery with bleach contains the virus and TMV-based vectors In addition, the demands for recombinant product purity by the FDA are rigorous.

To date, at least nine separate field trials using viral-based vector systems have been conducted in three separate states for the production of biologics. Concerns regarding the spread of engineered TMV and persistence of recombinant viruses have been addressed in these studies, and a recent report indicates that recombinant viruses generally delete the foreign gene, have reduced vigor, and are less competitive and pathogenic than the indigenous TMV (Rabindran and Dawson, 2001). Plant viral vectors are also effective tools in metabolic engineering as well as gene function discovery. Design modifications will lead to improvement of desirable vector traits so that the

potential of plant virus vector gene expression systems is fully realized (Fitzmaurice *et al.*, 2002).

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1.2 COMBINING TECHNOLOGIES: TRANSCRIPTIONAL PROFILING OF VIRUS-TRANFECTED SYSTEMS

1.2.1 Use of Plant Viral Vectors to Understand the Mechanisms of Gene Silencing

In research applications, recombinant plant viral vectors have been used to suppress endogenous plant gene transcripts in order to investigate the mechanism of gene silencing. This requires the cloning of homologous gene fragments into the virus and was first demonstrated with heterologous sequences in *tobamoviral* vectors (Kumagai *et al.*, 1995). It is generally held that plant viruses are initiators and targets of gene silencing, which occurs following transcription in the cytoplasm (Voinnet *et al.*, 1999). Plants infected with such vectors respond by activating an RNA-mediated antiviral defense mechanism, leading to a degradation of endogenous mRNA transcripts. Many viruses, including potyviruses, potexviruses and cucumoviruses, possess genes that encode pathogenicity determinants that interfere with the plant's ability to launch a virus induced gene silencing (VIGS) attack (Jones *et al.*, 1999; Llave *et al.*, 2000; Marathe *et al.*, 2000; Voinnet *et al.*, 1999). There is evidence that *tobamoviruses* use their replicase protein to suppress the RNA silencing machinery (Kubota *et al.*, 2003).

VIGS studies using *tobacco mosaic virus* vectors have been highly successful in *N. benthamiana*, a plant that has become recognized as a highly useful model for the *Solanaceae*, whose members share similar genomes with respect to gene content and genome organization. The underlying mechanism of VIGS has been the subject of numerous investigations, typically involving the silencing of transgenic beta-

glucuronidase (GUS) (Llave *et al.*, 2000) or green fluorescent protein (GFP) plants (Voinnet *et al.*, 1999). There is a high degree of sequence similarity among the genes involved in RNA silencing in various eukaryotic organisms, indicating that the cellular components of the RNA silencing machinery and its regulation are highly conserved (Agrawal *et al.*, 2003; Susi *et al.*, 2004).

1.2.2 Use of Plant Viral Vectors for Genomic Analysis Studies

VIGS is the subject of numerous reviews (Burch-Smith *et al.*, 2004; Fagard and Vaucheret, 2000; Lu *et al.*, 2003; Robertson, 2004), that address the advantages of using this technology for analysis of gene function and in high-throughput functional genomics studies. With the completion of DNA sequencing projects for *Arabidopsis thaliana* and rice, reverse genetic approaches in which young plants are inoculated with recombinant plant virus vectors, have been utilized to associate genes and phenotypes in plant systems. This has provided a particularly important complement to existing functional genomics tools.

Functional genomic studies strive to elucidate basic mechanisms of gene regulation, and to link gene identity with protein function. In plant systems, these studies continue to be challenging, however, as biochemical and other assays have fallen short of associating genes and the pathways in which they participate. A "whole-systems" approach to the identification of gene function, and the expansion of the role of

bioinformatics are recently adopted strategies in the plant research community (www.nsf.gov/pubs/202/bio0202/functional.htm).

Tobamoviral vectors cause a transient suppression of gene expression, and this technology holds many advantages over the classical forward genetics approach. Transfection results in rapid and dramatic phenotypic changes in plant pigmentation, growth, and development. For example, phenotypes that are typically lethal due to insertional mutagenesis are often non-lethal in young plants transfected with viral vectors. Also, insertional mutagenesis can potentially yield no loss-of-function when there is a functional redundancy of genes.

The NSF Potato Genome Project is using a VIGS approach in *N. benthamiana* to develop a comprehensive database of gene expression patterns for the *Solanaceae*. Virus-induced gene silencing (VIGS) technology has become recognized as an important tool for understanding plant defense against pathogens, metabolic pathways, and plant development (Burch-Smith *et al.*, 2004). For example, gene function studies employing VIGS has established roles for *SGT1* in the *R*-mediated resistance to bacteria and viruses (Liu *et al.*, 2002b), and roles for *EDS1* and *NPR1/NIM1* for *N*-mediated resistance to *TMV* (Liu *et al.*, 2002a).

Metabolic pathways in *Nicotiana* species have also been studied using the VIGS approach. The plant sterol synthesis pathway has been studied using VIGS, including to elucidate the function of sterol 4-alpha-methyl oxidases (SMOs) (Darnet and Rahier,

2004). To understand the molecular basis of plant-insect interactions, the jasmonate pathway has been studied. VIGS was used to silence the expression of two jasmonate-induced genes encoding nicotine and proteinase inhibitors (Saedler and Baldwin, 2004).

1.2.3 Investigations in Transfected Plants

Microarray studies are used to examine the expression profiles of large subsets of genes in given tissue under specific physiologic and environmental conditions. In plant systems, expression analyses have been used to discover novel floral fragrance-related genes (Guterman *et al.*, 2002); genes involved in strawberry flavor (Aharoni *et al.*, 2000); and genes involved in regulation of plant defense responses (Schenk *et al.*, 2003; Schenk *et al.*, 2000). Other microarray studies have focused on diagnostic aspects of plant viral pathogens, seeking to design new techniques for identification purposes (Boonham *et al.*, 2003; Bystricka *et al.*, 2003; Lee *et al.*, 2003).

Researchers involved in the National Science Foundation (NSF) Potato Genome Project (PGP) are also using a VIGS approach in *Nicotiana benthamiana*. Expression profiling is being conducted with cDNA potato microarrays developed by TIGR to develop a comprehensive functional genomics resource for solanaceous plants. In contrast to the investigations described in the following chapters, the NSF project will use *Agrobacterium tumafaciens* Ti-plasmid binary vectors to introduce a virus-based silencing vector to the plant.

In animal systems, transcriptional studies have examined the effects of virus transfection to assess the efficacy of viral vectors to deliver antigens to elicit protective immune responses for gene therapy. In addition, information has been obtained regarding (1) the toxicity of vectors; and (2) the impact of knock-down constructs, that carry genes in the antisense orientation. For example, one study examined the changes in cellular transcription resulting from infection with HIV-based vectors. They established that HIV-vector or HIV-1 infection has little effect on cellular transcription, and that that gene therapy with HIV-based vectors should not be particularly toxic to cells (Mitchell *et al.*, 2003) . As of this writing, there are no published reports on the use of microarrays to study the impact of viral transfection in plants.

1.2.4 Research Methodology

Several technologies were combined in this research investigation. First, transfection of *N. benthamiana* was accomplished with *tobamoviral* vectors that were previously constructed. In some cases, new constructs were developed for ease of manipulation (TTU51/CTP-*CrtB*-RZ) or for use as controls (TTOSA1/ARF1+). For the carotenoid biosynthesis studies, the first viral vector encodes an enzyme (*CrtB*) derived from *Erwinia herbicola* that causes overexpression of phytoene synthase. The second vector encodes a partial cDNA derived from tomato that will cause a knock-down of phytoene desaturase (*pds*$^-$). At a single time point, RNA from each of these plants was isolated from transfected plants and hybridized separately to cDNA potato microarrays, using uninfected wild type or GFP-transfected plants as controls. A third viral vector,

740 AT #120 contains an *ADP-Ribosylation Factor*-1 (*ARF*-1) that is derived from *Arabidopsis*. Plants transfected with this vector were used in a time-course analysis.

Several steps were involved in establishing a system to conduct transcriptional profiling of transfected plants. For the microarray studies, conditions and technical parameters had to be determined for RNA extraction, cDNA synthesis, fluorescent labeling of the cDNA, hybridization, and scanning of the slides. Signal data transformation and analysis, and data management also had to be established. The following steps were taken to initiate the microarray studies of carotenoid biosynthesis, and modifications to protocols for continued investigations are noted:

1. Development of viral vector construct to incorporate a ribozyme TMV CTP crtB RZ and development of TTOSA1 ARF1+ for use as a control in the ARF studies.

2. *In vitro* transcription (SP6 or T7) of the infectious clones and inoculation of plants with *in vitro* transcripts (infectious RNA).

3. Isolation of RNA from transfected *N. benthamiana*, cyanine-dye labeling of cDNA, and hybridization to potato microarray.

- Total RNA was isolated from *N. benthamiana* leaves overexpressing *crtB* (phytoene synthase) or from *pds* knock-down plants using Qiagen RNeasy kit plant or TRIzol® protocols.
- Microarray experiments require differential hybridization using a two-color system. Total RNA (25 μg) was required to reverse transcribe and label single stranded cDNA. Cyanine-3 and cyanine-5 dyes (Amersham) were used to fluorescently label the cDNA using Fluoroscript and Superscript III kits from Invitrogen
- A hybridization protocol recommended by TIGR was tested and modified to optimize conditions.

4. Identification of appropriate control plants

- Initial studies used cy3-labeled *crtB* and cy5-labeled wild type *Nicotiana benthamiana*. Subsequent hybridizations used cy5-labled TMV-GFP inoculated plant leaf material in an effort to subtract or minimize the effects of the virus infection

5. Determination of clones on the microarray to address experimental questions

6. Identification of software for signal intensity capture

   - TIGR Spotfinder was used initially to capture signal data. Affymetrix Jaguar® software was subsequently used in conjunction with an Affymetrix scanner. BioRad VersArrayer Chip Reader was determined to be the most user-friendly.

7. Establish Technical Parameters of Scanner and Software

8. Determine Appropriate Analysis Software

   - GeneSpring analysis software (Agilent, formerly Silicon Genetics) is an expression analysis tool . Free demonstration software was downloaded to analyze preliminary data, and later purchased. Training Workshops Levels I, II, and III were attended in Houston, Texas.

9. Normalization and Statistical Analysis

   - Normalization using LOWESS (locally weighted scatterplot smoothing) was used for all microarray experiments.
   - Data was filtered to determine up- or down-regulated genes, t-test p-value < 0.05 was tested. Generation of gene lists, clustering of genes, determining genes with similar expression patterns, and analysis of variance (1-way ANOVA) functions were also used.
   - Gene ontology (GO) functions were identified based on the categories of Biological Process, Cellular Component, and Molecular Function. Real-time PCR will be employed to verify observations of transcriptional profiling.

10. Identification of Web-based Databases

    - Munich Information Center for Protein Sequences (http://mips.gsf.de/)
    - **G**enome **A**nalysis of the Plant **Bi**ological System (GABI) (http://www.gabi.de/)
    - RIKEN Arabidopsis and Genome Encyclopedia (http://rarge.gsc.riken.go.jp/db_home.pl)
    - GCG SeqWeb (http://uhunix2.its.hawaii.edu:8080/gcg-bin/seqweb.cgi)

- Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/tdnaexpress)
- The Institute for Genomic Research (http://www.tigr.org/)
- Kyoto Encyclopedia KEGG Pathways (ftp://ftp.genome.ad.jp/pub/kegg/pathways/)

11. Quantitative Real Time PCR Assays

- QRT-PCR assays involved primer design, cDNA synthesis, preparation of dilution series for standard curves and use of controls

1.3 RESEARCH OBJECTIVES

1.3.1 Transcriptional Profiling Investigations in Transfected *Nicotiana benthamiana*

Research Objective 1	Establish a microarray system to study transfected *Nicotiana benthamiana*
Hypothesis 1	Nucleotide sequence homology among solanaceous plants provides a platform to extract gene expression data for *Nicotiana benthamiana*

1.3.1.1 *Nicotiana benthamiana* as a model system

Tobamovirus vectors are widely utilized to study phenomena in *Nicotiana benthamiana*. We have established a multinational research community of academic, government, and industry laboratories that utilizes this plant to study gene expression systems, virus-induced gene silencing, post-transcriptional gene silencing (PTGS) and its suppression, plant pathogens and plant defense. This plant offers important advantages for basic research in genetics and molecular biology. A member of the *Solanaceae* family, *N. benthamiana* is a relative of tobacco that possesses a small genome with only 38 chromosomes, and a rapid life cycle, making it an ideal candidate for advancing our knowledge in the areas of genomics and bioinformatics.

Although *N. benthamiana* has served as a model organism for gene silencing studies, there is limited information relating to its genome. However, numerous sequences for this plant have recently been deposited into the NCBI public database, and as of this writing, The Institute of Genomic Research (TIGR) has generated 18,822 ESTs from a normalized, full-length-enriched cDNA library constructed from *N. benthamiana*

mixed tissue (heat and cold treated leaves, pathogen challenged leaves, healthy roots and callus). All publicly available *N. benthamiana* ESTs, as well as *N. benthamiana* transcripts from GenBank have been assembled into contigs and entered into the *N. benthamiana* Gene Index (NbGI) (http://www.tigr.org/tdb/tgi/plant.shtml). In addition to these databases, cDNA sequences encoding the carotenoid biosynthesis enzymes, phytoene synthase (*psy*) and phytoene desaturase (*pds*) are found in published patents (Fitzmaurice *et al.*, 1996; Kumagai *et al.*, 2004; Kumagai *et al.*, 1999).

1.3.1.2 Use of heterologous cDNA Microarrays

In order to study global gene expression patterns in transfected *N. benthamiana,* hybridizations to glass microarrays was first tested. Affymetrix GeneChips® were not commercially available for this plant, and creating custom arrays was cost prohibitive. Spotting our own slides is a possibility at some point, because we have two *N. benthamiana* libraries, leaf and root, constructed by visiting scientist, Dr. Ling Hong. The feasibility of using a heterologous 10K cDNA potato microarray developed by the Institute for Genomic Research (TIGR) was studied. Can reliable signal data be obtained if a heterologous cDNA microarray hybridization is employed? Can the effects of the tobamoviral vector be subtracted away from the effects of the construct in question? To determine hybridization conditions, *N. benthamiana* plants were inoculated with *tobamoviral* vectors that will perturb the carotenoid biosynthesis pathway (Figure 1.2).

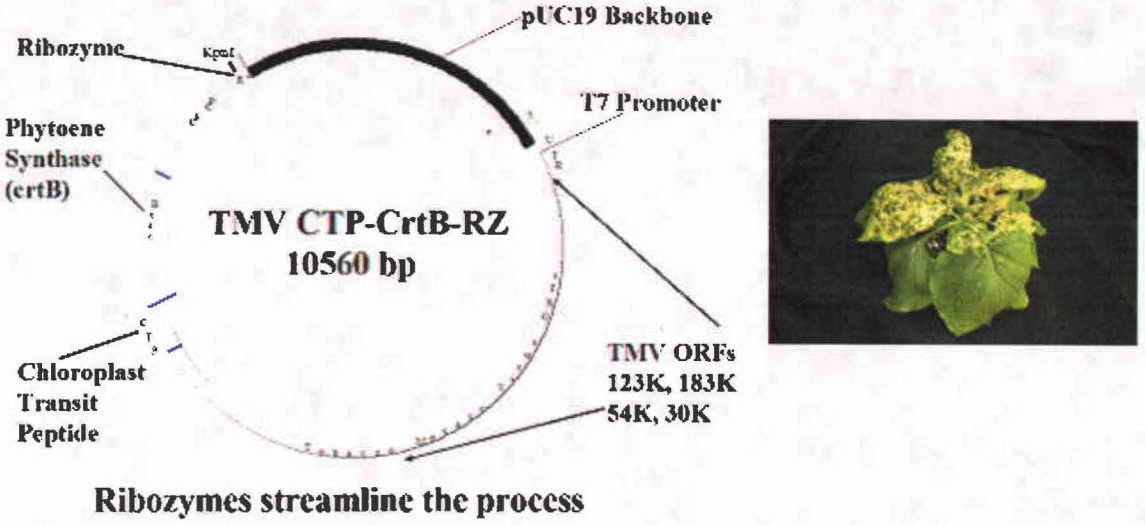

Figure 1.2. *Tobacco mosaic virus* vector with *CrtB* gene and ribozyme. Phenotype due to *CrtB* overexpression, 10 dpi.

As part of a National Science Foundation (NSF)-funded consortium, the Institute for Genomic Research (TIGR) has developed 10K cDNA microarrays for studying solanaceous plants. To select the elements arrayed on the NSF Potato Microarray, TIGR has constructed a set of non-redundant cDNA clones from the StGI. The GenePix Array List (GAL) files for potato 10,000 cDNA clone array (versions 1-3) are available for downloading, and the microarrays are available for distribution to the public research community on a cost-recovery basis. Clones on the array are amplified PCR products derived from various potato tissues. Elements on the microarray include cDNAs derived from healthy potato stolon, root, microtuber, dormant tuber, germinating eye, leaves, shoots, as well as *Phytophthora infestans*-challenged libraries (incompatible and compatible). Ten thousand clones on the microarray (http://www.tigr.org) have been sequenced and validated at TIGR. The control clones include potato cDNA, tomato

cDNA, and human spiking control clones, including genes involved in housekeeping functions, photosynthesis, and defense responses. There are also a few clones from the fungal pathogen *P. infestans*, as controls for pathogen infection. Through differential hybridization, studies using cDNA microarrays enable analysis of expression levels of large numbers of genes, providing insight into the manner in which genes are coordinately regulated.

In a review of gene expression analysis by transcript profiling, Donson *et al.* report on several groups that have performed analyses on cross hybridization to heterologous cDNAs in plant systems. Girke *et al.* hybridized arrays in a series of experiments with probes derived from seeds, leaves, and roots of *Arabidopsis*. They examined three different forms of an *FAD2* gene from *Arabidopsis* that was identical in length and GC content, but had nucleotide similarities of 100%, 90%, and 80%. No detectable hybridization occurred for the 80% fragment. In addition, four ferredoxin and three acyl-ACP-desaturase sequences showed cross hybridization between 60-70%. They concluded that cross-hybridization was estimated to occur if closely related genes have 70% to 80% sequence identity, and suggest that *Arabidopsis* arrays would be useful to hybridization studies for other genera such as *Brassica* (Girke *et al.*, 2000).

The issue of cross-hybridization existed for the studies presented in the following chapters because sequences from a variety of species converged *in vivo* as well as *in vitro*. Cross-hybridization is actually a double-edged sword. While the desired outcome is hybridization of *N. benthamiana* to the potato clones, it will be necessary to analyze

whether the viral vector inserts, in addition to endogenous transcripts, will also cross hybridize and cause a spurious elevation in the microarray analyses. The issue will be addressed by comparing sequences of potato, tomato, *Erwinia* and *N. benthamiana* for the carotenoid study, and potato, *Arabidopsis* and *N. benthamiana* for the ARF study. In addition to analyzing the level of homology, primer sequences for QRT-PCR will be designed, compared in all subject species, and selected for optimum results. QRT-PCR will be used as a method to discern hybridization of viral vector from that of endogenous plant genes.

1.3.1.3 Identification of Appropriate Controls

Because plant viruses will be utilized to introduce genes into *N. benthamiana*, it is important to understand the effects of viruses themselves on gene expression and to able to subtract out or at least recognize the effects of the viruses. One study has been conducted to investigate the effects of viruses on plant gene expression. Using microarray hybridization, Whitham *et al.* demonstrated in a time-course analysis that diverse plant viruses induced common sets of host genes in *Arabidopsis*, and found that *tobamoviruses* specifically and rapidly induce genes encoding heat shock and defense-associated proteins (Whitham *et al.*, 2003).

For the following microarray studies involving transfected plants, several options were considered regarding selection of appropriate controls: 1) plants transfected with an "empty" vector; 2) wild type non-infected plants; and 3) plants transfected with green

fluorescent protein (GFP). Previous studies have demonstrated that transfection with an "empty vector", or viral vector with no insert, causes a more virulent phenotype compared to a vector carrying an inserted sequence. The 5' leader sequence (Ω) and the 3' pseudoknot of the viral genome interact to regulate and enhance translation. Insertion of a sequence between the movement and coat proteins causes a physical separation of these regions, resulting in decreased symptom production by the plant. Therefore, an "empty vector" control was not adopted and wild type uninfected plants were selected for initial studies.

Microarray analysis of initial transfection experiments using wild type uninfected plants as controls showed an increase in heat shock proteins, consistent with Whitham's data (Whitham *et al.*, 2003). Therefore, a virus-infected plant expressing green fluorescent protein (GFP) was selected as a control in an attempt to understand the specific impact of the *crtB*, *pds* and *ARF1* insert sequences in the treatment groups, and to eliminate or minimize changes in gene expression that occur because of virus infection. If both plants have changes in gene expression due to the virus, the viral effects will be subtracted out because microarray data is expressed as a ratio of the experimental and control groups. An assumption was made that GFP expression does not interfere with endogenous gene expression.

1.3.2 Case Study of a Metabolic Pathway: Carotenoid Biosynthesis

Research Objective 2	To investigate the effects of *tobamoviral* transfection on transcript abundance of carotenogenic genes in *N. benthamiana*
Hypothesis 2	Viral vector transfection will cause perturbations in the pathway, demonstrating their utility for metabolic engineering studies

1.3.2.1 Metabolic Engineering of Carotenogenesis

Carotenoid biosynthesis is a metabolic pathway (Figure 1.3) that has been extensively studied in the *Solanaceae* due to the high economic and nutritional value of carotenoids and because of the potential to identify enzymes in the pathway as herbicide targets. Carotenoids, both carotenes and xanthophylls (Busch *et al.*, 2002), protect the plant from excessive light energy and participate in light harvesting in the photosynthetic membranes.

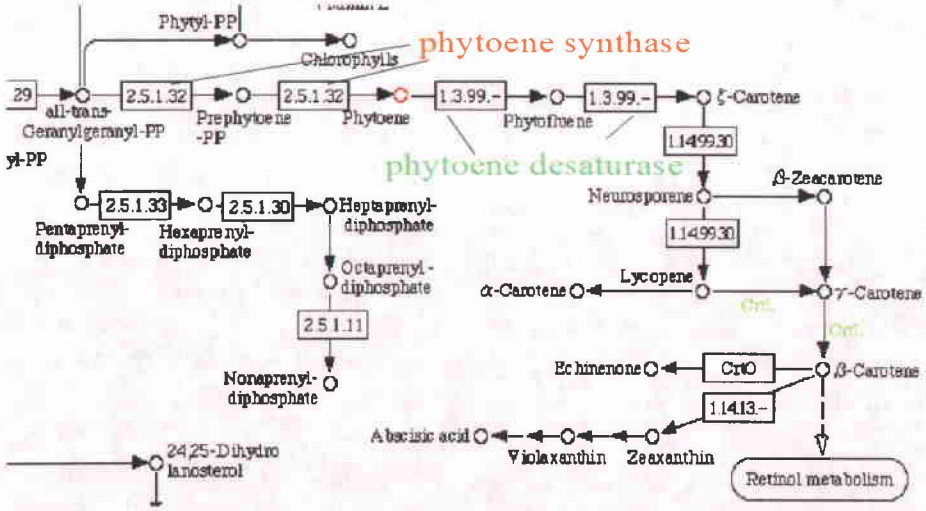

Figure 1.3. Carotenoid Biosynthesis Pathway depicting phytoene synthase and phytoene desaturase enzymes (Source: www.genome.ad.jp)

Carotenoids are built from a 5-carbon compound isopentenyl diphosphate (IPP), via the 1-deoxy-D-xylulose-5-phophate (DOXP) pathway or non-mevalonate pathway

(Bramley, 2002;Hirschberg, 2001). Table 1.1 shows the major enzymes of the pathway and the products resulting from their action on substrates. Isomerization of IPP to dimethylallyl diphosphate (DMAPP) is catalyzed by IPP isomerase. DMAPP is the activated substrate for the formation of geranylgeranylpyrophosphate (GGPP). Two molecules of GGPP condense to form 15-*cis* phytoene in a reaction catalyzed by phytoene synthase (*psy*). Phytoene desaturase (*pds*) and ζ–carotene desaturase (*zds*) are involved in subsequent desaturation reactions to form lycopene. Lycopene cyclase is involved in the cyclization of lycopene, leading to the formation of β-carotene, α-carotene and ε-carotene. β-carotene hydroxylase catalyzes the formation of

CAROTENOGENIC ENZYME	PRODUCT/SUBSTRATE
IPP Isomerase	DMAPP
GGPP Synthase	GGPP
Phytoene Synthase	Phytoene
Phytoene Desaturase	ζ-carotene
ζ-carotene desaturase	Lycopene
Lycopene cyclase	β-carotene
β-carotene hydoxylase	xanthophylls
9-cis-epoxycarotenoid dioxygenase (NCED)	Abscisic acid (ABA)

Table 1.1. Major enzymes of the carotenoid biosynthesis pathway.

xanthophylls, which are formed by the oxygenation of carotenes by the addition of hydroxyl, epoxy, or keto groups to β-carotene. NCED catalyzes the cleavage of 9-*cis* epoxycarotenoids, a key regulatory step in the abscisic acid (ABA) biosynthesis pathway

of higher plants. ABA plays a major role in the adaptation of plants to environmental stress, plant growth and development (Han *et al.*, 2004), and it is increased in response to attack by a variety of pathogens.

Metabolic engineering of carotenoid biosynthesis has been pursued in a variety of organisms for industrial, nutritional, and biomedical applications (Lee and Schmidt-Dannert, 2002;Rohlin *et al.*, 2001;Sandmann, 2001). Numerous genes in the pathway have been cloned from a variety of microorganisms and plants, and their functions identified. With the goals of increasing carotenoid content and creating novel compounds, investigations have focused on carotenoid production in transgenic microorganisms and plants, and have consequently shed light upon the regulatory mechanisms influencing the pathway .

In higher plants, genes encoding carotenogenic enzymes have been cloned from flowers, fruits, and leaves. For example, cDNAs involved in carotenogenesis in flowers that have been cloned from the medicinal plant, *Gentiana lutea*, include geranylgeranylpyrophosphate (GGPP) synthase, phytoene synthase (*psy*), phytoene desaturase (*pds*), and ζ-carotene desaturase (*zds*), and their functions were established by heterologous complementation in *E. coli* (Zhu *et al.*, 2002;Zhu *et al.*, 2003). Two genes encoding phytoene synthase were cloned from tomato, *psy1* which is active in fruit and *psy2* which is active in leaf tissue (Bartley and Scolnik, 1993).

In microorganisms, the *crt* gene clusters that direct the biosynthesis of carotenoids such as lycopene, beta-carotene and astaxanthin have been isolated from carotenogenic bacteria such as *Erwinia* species and the marine bacterium *Agrobacterium aurantiacum.* Recombinant strains of noncarotenogenic bacteria such as *E. coli* and *Zymomonas mobilis* can also express *crt* genes and accumulate carotenoids (Misawa and Shimada, *J Biotech,* 1997). Genetic modification of the astaxanthin pathway has been studied in non-carotenogenic yeast, *Xanthophyllomyces dendrorhous* (Visser et al, FEMS Yeast Research, 2003; Verdoes *et al, Appl Environ Microbiol*, 2003) in an attempt to increase production of astaxanthin for use as a nutraceutical for the food and feed industries. The non-carotenogenic yeast strains, *Candida utilis* (Shimada *et al, J Biotech,* 1998) and *Saccharomyces cerevisiae* (Yamano *et al*, Biosci Biotechnol Biochem, 1994) have been shown to accumulate carotenoids if genes are placed under the control of yeast-derived promoters and terminators.

Several studies have been published that demonstrate that carotenoid accumulation can be modified through genetic manipulation in tomato. Metabolic engineering in tomato leads to an increase of beta-carotene and lycopene content in ripening fruit (Dharmapuri *et al.*, 2002;Rosati *et al.*, 2000). Fruit-specific expression in transgenic tomato plants engineered with an additional phytoene synthase causes an increase in total fruit carotenoids (Fraser *et al.*, 2002). In other studies, novel and non-native carotenoid pigments have been produced in *Nicotiana* species, demonstrating the potential for altering pigmentation of fruits and flowers of horticultural and floricultural importance. Astaxanthin has been produced in transgenic tobacco (Mann *et al.*, 2000).

Metabolic engineering of the carotenoid pathway has also been demonstrated in *Nicotiana benthamiana* using tobamoviral vectors. Capsanthin, a non-native chromoplast-specific xanthophyll, was produced by transfecting plants with viral vectors engineered to express capsanthin-capsorubin synthase, and shown to be functionally integrated into the chloroplast membranes (Kumagai *et al.*, 1998). By introducing genes derived from *Narcissus pseudonarcissus* and *Erwinia uredovora*, a carotenogenic pathway was engineered into rice endosperm tissue that is completely devoid of carotenoids. Beyer *et al* produced a high beta-carotene 'golden rice' grain with the hope of providing an improved human nutritional benefit and to address vitamin A deficiencies (Beyer *et al.*, 2002).

My preliminary work using microarrays to investigate carotenoid biosynthesis is included in Chapter 2. In transfected plants, phytoene accumulates due to overexpression of *crtB*, a bacterial gene encoding phytoene synthase, or due to the knock-down of phytoene desaturase. Using potato cDNA potato microarrays for hybridization, transcriptional profiling of these plants at a single time point revealed an unexpected upregulation of endogenous phytoene synthase. It suggested a possible role for phytoene as a positive feedback mechanism. It also indicated that the heterologous system could provide useful information for functional genomic studies. An additional benefit to these hybridization studies is the insight provided into the genetics of *N. benthamiana*. Because the genomes are highly similar with respect to gene content and genome organization, the potato microarray could used to leverage a significant amount of information about its solanaceous relative.

A more comprehensive analysis of the carotenoid pathway is presented in Chapter 3, including an examination of all carotenoid genes that were available for study on the microarray, followed by relative quantitation using real-time PCR.

1.3.3 Case Study of a Signaling Pathway Involving a Multigene Family: ADP Ribosylation Factor-1 (ARF-1)

Research Objective 3	To conduct a time-course analysis of *ARF-1*$_{as}$-transfected *N. benthamiana* using transcriptional profiling technologies
Hypothesis 3	A subset of genes relating to G-protein signaling can be filtered from the microarray data for further characterization by analyzing gene expression patterns across the time course

1.3.3.1 ARF Gene Function and Regulation

In 1999, the original patent on cytoplasmic inhibition of endogenous plant gene expression by viral RNA was issued (Kumagai, 1999). This patent describes the use of plant viruses to introduce and study the effects of an antisense construct on endogenous plant gene expression. It demonstrated that an episomal RNA viral vector can be used to deliberately manipulate a major, eukaryotic biosynthetic pathway. Subsequently, Baulcombe coined the phrase "virus-induced gene silencing" or VIGS, to describe this phenomenon (Baulcombe, 1999).

Kumagai *et al.* secured another patent that describes how plant viral vectors can be used to conduct a functional genomic screen using genes encoding GTP-binding proteins. This particular invention exemplified that genes encoding GTP binding proteins in one plant can silence endogenous gene expression in an unrelated plant. It was demonstrated that antisense constructs of ADP-ribosylation factors (ARFs) derived

from *Arabidopsis thaliana* caused severe stunting in *N. benthamiana* when introduced using TMV vectors (Kumagai, 2002b).

ARFs are a multigene family of GTP-binding proteins belonging to the Ras superfamily, which is comprised of over 100 small G proteins identified in eukaryotes, from yeast to humans. There are five families in this superfamily, based on structural classification, that include ARF, (intracellular vesicle trafficking); Ran (nucleocytoplasmic transport); Ras (regulation of gene expression); Rho (cytoskeletal organization/gene expression); Rac (Pathogen Defense); and Rab (intracellular vesicle trafficking).

ARFs are monomeric, ranging in size from 20-40 kDa. They share similarity with the alpha subunit of heterotrimeric G proteins, but unlike these proteins, they require a GTPase-activating protein (GAP) to accelerate their intrinsic GTPase activity. They are present in all eukaryotes examined to date (Randazzo *et al.*, 2000), and are best described for their role in membrane traffic (protein secretion, cell migration, and signal transduction) and intracellular signaling systems.

ARFs are highly conserved at the nucleotide and amino acid levels, and in addition to ARFs, other family members include the ARF-like ARLs and the SARs (secretion-associated and RAS-related), which are associated with COPII vesicle transport from the endoplasmic reticulum to form the cis-face of the Golgi apparatus. They have been cloned from a number of species and have been highly studied in

mammals and in yeast, and functional conservation of ARF has been demonstrated between yeast and humans (Kahn *et al.*, 1991). An interspecies comparison of ARFs demonstrated an evolutionary conservation of nucleotide sequences of both the untranslated as well as the coding regions. Indeed, an unusually high degree of conservation exhibited by the untranslated regions implicates these proteins to possess a regulatory role (Price *et al.*, 1996). Less is known about the function of ARF in plants, although its role in the secretory pathway has been well described.

In cases of heterotrimeric G proteins, knock-out of the alpha subunit causes dwarfing in rice, and interest in the regulatory aspects in plants has been generated because semi-dwarfs produce high levels of heterologous proteins (Sasaki *et al.*, 2002). ARFs are allosteric activators of the NAD:arginine ADP-ribosyltransferase activity of cholera toxin. Cholera toxins, like many bacterial toxins, are ADP ribosyltransferase enzymes that commonly use GTP binding proteins as substrates. The ADP ribosylation reaction is a covalent chemical modification that is catalyzed by ADP-ribosyltransferase, which transfers the ADP-ribose moiety of NAD to a target protein with the release of nicotinamide.

In humans, ARF acts as a tumor suppressor; it is a positive regulator of p53, the gene most often disrupted in cancers. ARF1 is also a target of the Myc transcription factor, promoting malignant transformation. A number of cancer labs in the United States—National Cancer Institute in Bethesda; Emory University in Atlanta; Sloan-Kettering Cancer Center in New York; and University of Bristol in the United

Kingdom—study ARFs. Paul Randazzo at Lab of Cellular Oncology at the NCI, NIH is particularly interested in its regulation and has found that ARFs regulate membrane traffic at multiple sites and may serve as a point of integration (Randazzo *et al.*, 1992).

ARF function requires the regulated shuttling between a GTP-bound state active state and a GDP-bound inactive state. The GDP/GTP cycle of ARF is regulated by GTP exchange factors (GEFs). GTP-activating proteins (GAPs) activate GTPase activity of ARFs. Due to myristoylation at the amino terminus, ARF GTPases exhibit weak association with membranes. Much of the research into ARF GTPase function has focused upon their roles in coat protein recruitment during vesicle formation (Szafer *et al.*, 2001). Although the molecular machinery of membrane traffic has been identified (Figure 1.4) regulatory mechanisms are not understood.

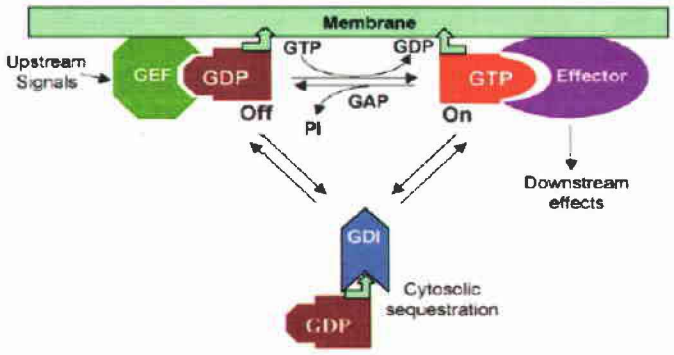

Figure 1.4. Conformational changes in the GDP/GTP cycle of GTP-binding proteins

ARFs play a significant role in the secretion of proteins. Protein sorting by the secretory pathway is in many cases dependent upon the budding of coated vesicles from the Golgi apparatus, which then fuse with their target membranes. In the secretory pathway, this could be the plasma membrane, the storage vacuole or the endoplasmic

reticulum. In the standard model for coat assembly, upon activation and nucleotide exchange, myristoylated ARF-GTPs bind to receptors on the Golgi membrane. They facilitate the formation of transport vesicles by recruiting clathrin and non-clathrin coatomer coats through a direct binding interaction to form coat protomer (COPI). At this point, protein cargo is captured and the coat self-assembles, polymerizes, and leads to deformation of the membrane. This forms a bud that pinches off and moves to some target.

Repression of ARF-1 in transgenic potato has been shown to cause 14-3-3 gene activation (Zuk *et al.*, 2003), that encode signaling proteins that are critical players in cell division, apoptosis, and cell cycle regulation. Plant 14-3-3 proteins are phosphoserine-binding proteins (29-33 kDa), the majority of which in animal cells target proteins involved in signal transduction and transcription. In plants, 14-3-3 proteins play a role in the regulation of the plasma membrane H(+)-ATPase and enzymes of carbon and nitrogen metabolism, as well as in plant development and stress responses (Roberts, 2003).

Chapter 4 of this dissertation summarizes investigations of the ARF multigene family. The ARF-1 that was used in this study was originally identified in a screen that was developed to examine nucleotide sequences in transfected plants by systemically knocking down endogenous gene expression using an antisense mechanism (Kumagai, 2002b). In this case, sequences from *Arabidopsis thaliana*, a plant with a well-

characterized genome, were introduced to *N. benthamiana*, a plant that has yet to be characterized, using tobamoviral vectors. Subsequent sequencing of the cDNA clone that caused severe stunting in plants revealed ARF-1 (Kumagai *et al.*, 2002). To build upon these findings, a time-course transcriptional profiling analysis of an ARF-1 knock-down was conducted in order to associate phenotype with gene function. Gene expression analysis focused on the identification and characterization of genes involved in the G-signaling pathway.

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CHAPTER 2. PRODUCTION OF RABBIT NP1 DEFENSIN IN TRANSFECTED PLANTS BY AN RNA VIRAL VECTOR USING AN ORANGE VISIBLE MARKER

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FOREWORD

This manuscript was submitted for publication to *The Plant Journal* in November 2004. Bibliographic citations have been modified to conform to the style of the dissertation. The contributions that I made to this work include the construction of a tobacco mosaic viral vector construct that contained the phytoene synthase (*crtB*) gene derived from *Erwinia herbicola* together with a ribozyme, and the subsequent microarray investigation of the overexpression effects of *crtB* on the carotenoid biosynthesis pathway in *Nicotiana benthamiana.* Phenotypically, this correlated with a plant that exhibits a bright yellow color change in the leaves and flowers.

Because an appropriate microarray was not commercially available, it was necessary to investigate a system that would be instrumental in understanding the gene expression profile. Additional requirements included the development of an experimental design with appropriate controls; establishing effective hybridization and scanning protocols; and selection and mastery of data analysis software. Potato microarrays developed at The Institute of Genomic Research (TIGR) enabled me to determine the utility of a heterologous system to acquire data, and to study the gene expression changes in the carotenoid pathway. In effect, RNA derived from *Nicotiana benthamiana* plants treated with TMV-*ctrB* was hybridized to potato cDNA microarrays, followed by an analysis using GeneSpring (Agilent, formerly Silicon Genetics). The high degree of homology that exists among solanaceous plant species sufficiently enabled the cDNA hybridization of *N. benthamiana* to *Solanum tuberosum.* In the initial

hybridizations, endogenous phytoene synthase and phytoene desaturase were tracked for changes in gene expression at ten days post-inoculation (dpi), using GFP-transfected plants as controls. These early investigations also provided insight into the genome of *N. benthamiana*, which has yet to be fully sequenced.

2.1 ABSTRACT

We have developed a visible marker to study plant viruses and to produce heterologous proteins. A cDNA encoding a fusion of a chloroplast transit peptide and *Erwinia herbicola* phytoene synthase was placed under the transcriptional control of a tobamovirus subgenomic promoter. One week after inoculation, systemically infected *Nicotiana benthamiana* plants were analyzed for enzyme activity. Leaves from transfected plants expressing phytoene synthase in the chloroplast developed a bright orange phenotype. This visible marker can be used to monitor gene activity, viral replication, and movement. Analysis of the carotenoid content indicated that there was an accumulation (up to 3-fold) of β-carotene, lutein, phytoene, and phytofluene. Transcriptional profiling revealed that the expression of endogenous phytoene synthase and phytoene desaturase was triggered under these conditions. Phytoene synthase can tolerate C-terminal fusions. Systemically infected tissue containing a chimeric gene encoding phytoene synthase and an antimicrobial peptide (defensin) developed an orange phenotype and the hybrid protein cross-reacted to anti-phytoene synthase and anti-defensin antibodies. This system may be used to target biologically active peptides to the plant chloroplasts.

2.2 INTRODUCTION

In leaves the chloroplast membranes have almost exclusively the same carotenoid composition and maintain a specific carotenoid to chlorophyll or protein ratio. Imbalance between the different pigment components induces compensatory adjustment as shown in lutein-deficient mutants of *Arabidopsis* (Pogson et al., 1996) and in xanthophyll epoxide deficient mutants of *Nicotiana plumbaginifolia* (Marin et al., 1996) where the zeaxanthin content is increased to compensate the deficiency. The same trend has been observed in tobacco (Misawa et al., 1994) and tomato (Romer et al., 2000) leaves, following the introduction of the bacterial phytoene desaturase *crtI* gene under the control of the constitutive CaMV 35S promoter.

The molecular basis underlying this tight control of leaf carotenogenesis is unknown and contrasts markedly with that operating in sink tissue such as tubers, flowers, fruits and seeds. Data gained from these non-photosynthetic tissues, reveals that phytoene synthase which dimerizes two geranylgeranyl backbones to phytoene (Dogbo et al., 1988), plays a prominent role in increasing the basal levels of carotenoids. For instance, expression of bacterial phytoene synthase gene resulted in a 50-fold and a 43-fold increase of the carotenoid content in the seeds of canola (Shewmaker et al., 1999) and *Arabidopsis* (Lindgren et al., 2003), respectively. These data contrast with those reported for the expression of an additional phytoene synthase gene in tomato (Fray et al., 1995) and tobacco leaves (Busch et al., 2002) indicating a modest accumulation of lycopene paralleled by a marked dwarfism of the plants.

To further explore the regulatory and phenotypic effects of increasing phytoene synthase in leaves, we used an RNA-based viral vector system (Kumagai et al., 1995) (Kumagai et al., 1998) to manipulate carotenoid biosynthesis in the chloroplast and to study its potential as a new visible marker in various solanaceous species. In our experiments, a bacterial phytoene synthase gene from *Erwinia herbicola* (Armstrong et al., 1990) was used to avoid endogenous gene silencing (Kumagai et al., 1995). Here we report the boosting effect of overexpressing phytoene synthase and display the associated transcriptional profiling. Furthermore, based on the color phenotype and the flexibility of phytoene synthase to tolerate C-terminal fusions, we demonstrate the potential use of this system to overexpress antimicrobial peptides.

2.3 RESULTS

2.3.1 Expression of phytoene synthase in plant chloroplast (+/- CTP)

In this study, we have developed a new viral vector, TTU51, consisting of tobacco mosaic virus strain U1 (TMV-U1) (Goelet, 1982), and tobacco mild green mosaic virus (TMGMV; U5 strain) (Solis, 1990). The open reading frame (ORF) for *Erwinia herbicola* phytoene synthase (*CrtB*) was placed under the control of the tobacco mosaic virus (TMV) coat protein subgenomic promoter in the vector TTU51. This construct also contained the gene encoding the chloroplast transit peptide (CTP) for the small subunit of rubisco and was called TTU51 CTP *CrtB* (Fig. 2.1). Infectious RNA was prepared by *in*

vitro transcription using SP6 DNA-dependent RNA polymerase and was used to mechanically inoculate *N. benthamiana*. The hybrid virus spread throughout all the non-inoculated upper leaves and was verified by local lesion infectivity assay and polymerase chain reaction (PCR) amplification (result not shown).

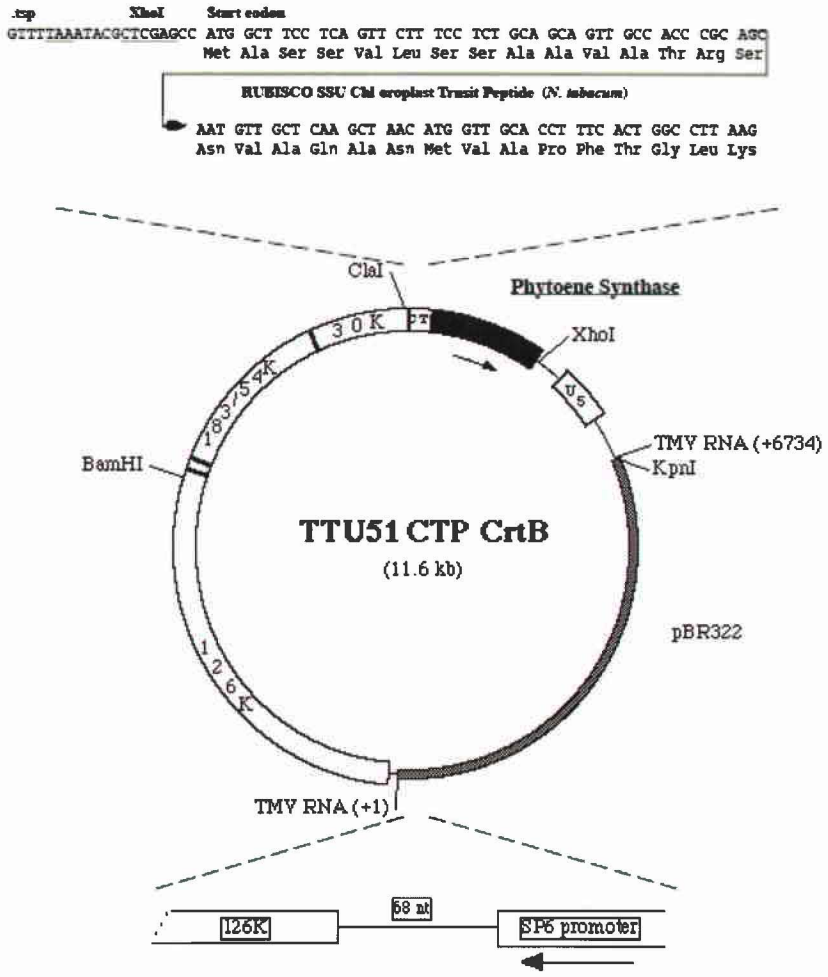

Figure 2.1. Phytoene expression vector TTU51 CTP *CrtB*. This plasmid contains the TMV-U1 126-, 183-, and 30-kDa ORFs, the TMV-U5 coat protein gene, the SP6 promoter, the *Nicotiana tabacum* gene encoding the chloroplast targeting peptide of the small subunit of ribulose-1,5-biphosphate carboxylase, the *Erwinia herbicola* phytoene

synthase gene, and part of the pBR322 plasmid. The TAA stop codon in the 30-kDa ORF is underlined. The TMV-U1 subgenomic promoter located within the minus strand of the 30-kDa ORF controls the expression of phytoene synthase. The putative transcription start point (tsp) of the subgenomic RNA is indicated with a period (.).

The leaves from plants transfected with TTU51 CTP *CrtB* developed an orange pigmentation (Fig. 2.2b) that spread systemically during plant growth and viral replication (Fig. 2.2c). There was a dramatic visual difference if one compares an uninfected plant (Fig. 2.2a) to one week after transfection (Fig. 2.2b). As a control, the sequence encoding the chloroplast transit peptide was removed, creating plasmid TTU51 *CrtB*. One to two week post inoculation, plants transfected with TTU51 *CrtB* did not produce the orange phenotype (result not shown). This result suggests that overexpression of phytoene synthase in the plant chloroplast is required for the phenotypic change.

Figure 2.2. Transfected *N. benthamiana plants*. (a) Uninfected (b) *N. benthamiana* transfected with TTU51 CTP *CrtB* (c) Time course of *N. benthamiana transfected* with TTU51 CTP *CrtB*. Left to right, 1 day, 7 days, and 14 days.

2.3.2 Phenotypic analysis of transfected plants expressing CrtB

Virions were isolated from *N. benthamiana* plants transfected with TTU51 CTP *CrtB* and directly applied to *N. benthamiana*, *N. clevelandii*, *N. tabacum*, and *Solanum tuberosum*. Two to four weeks after inoculation, infected plants were visually monitored. Orange symptoms were observed in the inoculated leaves of the four plant species (Fig. 2.3), while orange systemic symptoms were only observed in *N. benthamiana* and *N. clevelandii*. This result suggests that the phytoene synthase gene may be used to screen various viral vectors for their ability to retain the CTP *CrtB* insert on a large number of different host plants.

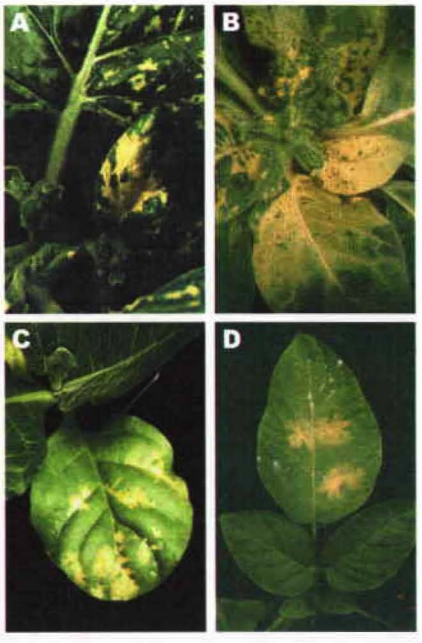

Figure 2.3. Plants transfected with TTU51 CTP *CrtB*. (a) *N. benthamiana* (b) *N. clevelandii* (c) *N. tabacum* (d) *Solanum tuberosum.*

2.3.3 Enzymatic and pigment analysis of transfected TTU51 CTP CrtB plants

Leaves from plants transfected with TTU51 CTP *CrtB* had a decrease in chlorophyll content (result not shown) that exceeded the slight reduction that is usually observed during viral infection (Kumagai et al., 1998). Since previous studies have indicated that the pathways of carotenoid and chlorophyll biosynthesis are interconnected (Susek, 1993), we compared the rate of synthesis of phytoene to chlorophyll. Two weeks post inoculation, chloroplasts from plants infected with TTU51 CTP *CrtB* transcripts were isolated and assayed for enzyme activity. The ratio of phytoene synthesis to chlorophyll synthesis was 0.55 in transfected plants and 0.033 in non-inoculated plants (control). Phytoene synthase activity from plants transfected with TTU51 CTP *CrtB* was assayed using isolated chloroplasts and labeled geranylgeranyl PP. Under these conditions, there was a large increase in phytoene and an unidentified C_{40} alcohol in the CrtB plants, probably corresponding to a prephytoene alcohol derivative (Fig. 2.4a).

We analyzed the pigment content of control and transfected plants by TLC and HPLC. The TLC profile (Fig. 2.4b) revealed a marked increase of the carotene fraction isolated from leaves transfected with TTU51 CTP *CrtB* compared to that of control (Fig. 2.5). This trend was further confirmed by HPLC analysis (Fig. 2.5a-d), which revealed that the orange fraction contained mainly β-carotene (Fig. 2.5b) and the two UV-absorbing carotenoids, phytofluene (Fig. 2.5c) and phytoene (Fig. 2.5d) and the absence of esterified carotenoids. The pigment content of control plants and plants transfected with TTU51 CTP *CrtB* was measured.

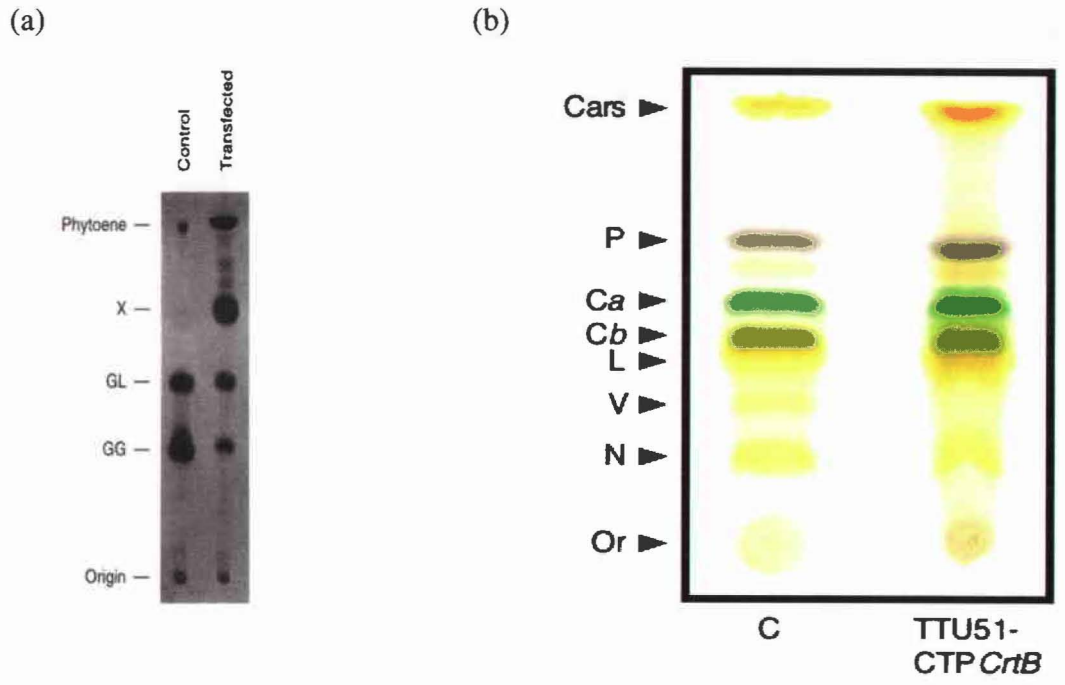

Figure 2.4. Enzymatic assays and TLC analysis of pigments. (a) Autoradiograph from phytoene synthase and chlorophyll synthetase assay from non-inoculated *N. benthamiana* (control) and plants transfected with TTU51 CTP *CrtB*. The products from the reaction were separated by thin layer chromatography on silica gel plate using benzene/ethyl acetate (90 /10, v/v) as a developing solvent. From left to right extracts from: non-inoculated plants and TTU51 CTP *CrtB* transfected plants. The different abbreviations refer to the compounds (X), unidentified C_{40} alcohol, (GL), geranyllinalool, (GG), geranylgeraniol, and the origin (O). (b) Thin layer chromatography of control and transfected *N. benthamiana*. From left to right : pigment extract from non-inoculated plant (C) and from transfected plant using TTU51 CTP *CrtB*. Pigments were separated as described under "Experimental procedures". Abbreviations refer to Cars, carotenes; P, pheophytine; Ca, chlorophyll *a*; Cb, chlorophyll *b*; L, lutein; V, violaxanthin; N, neoxanthin; Or, the origin.

There was a 3-fold increase of β-carotene in plants expressing *CrtB*, followed by the accumulation of phytofluene and phytoene (Table 2.1). Concerning the xanthophylls, we noted that lutein was increased 1.5-fold while the violaxanthin content decreased in contrast to neoxanthin (Table 2.1). None of these changes was observed in control plants.

	Carotenoids (mg/g fresh weight)						Chlorophylls (mmol/mol chlorophyll)
Plant	Phytoene	Phytofluene	β-carotene	Lutein	Violaxanthin	Neoxanthin	*a* + *b*
Control	0	0	65 ± 4	130 ± 8	48 ± 4	10 ± 2	1100 ± 55
TTU51 CTP *CrtB*	208 ± 10	50 ± 9	194 ± 5	191 ± 14	14 ± 2	45 ± 3	985 ± 36

The values given are the ± SD of the analysis of 3 samples.

Table 2.1. Pigment composition of control and transfected *N. benthamiana* leaves

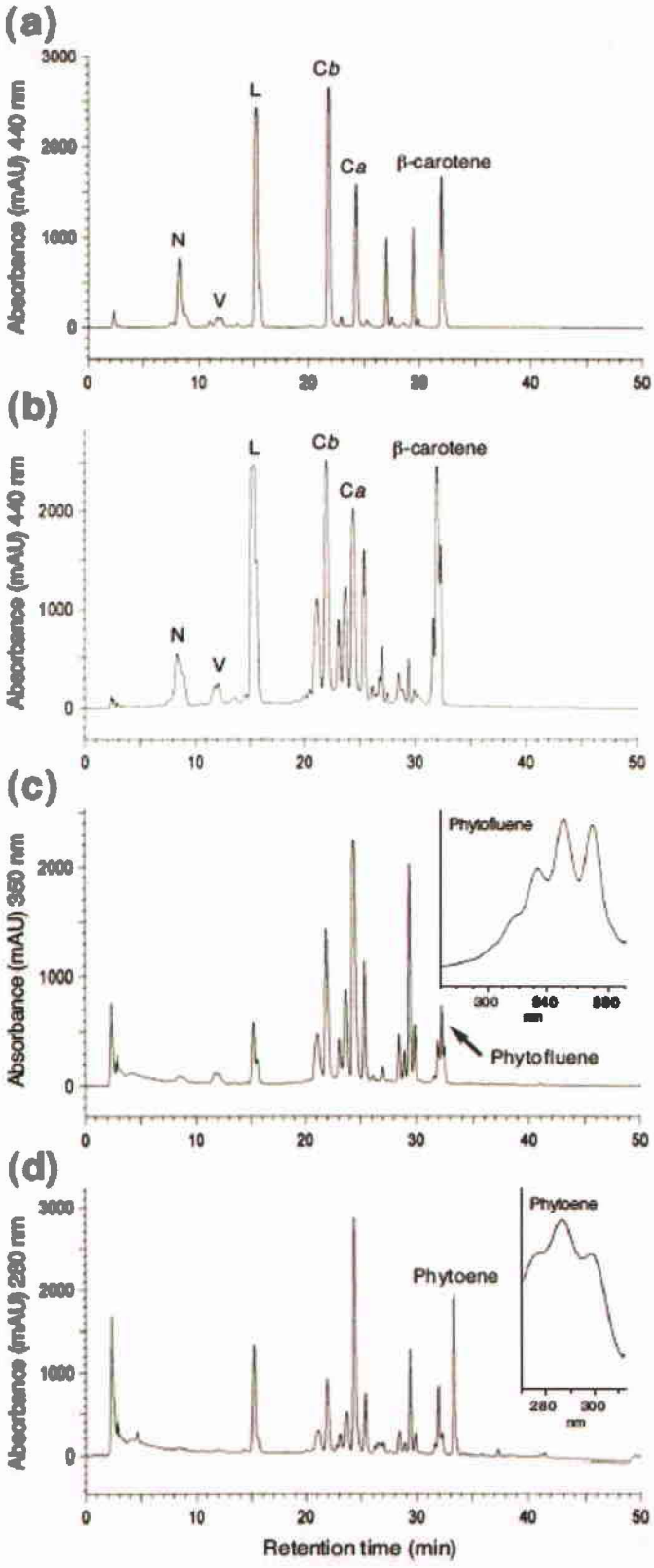
(a)
Absorbance (mAU) 440 nm
N
V
L
Cb
Ca
β-carotene
(b)
Absorbance (mAU) 440 nm
N
V
L
Cb
Ca
β-carotene
(c)
Absorbance (mAU) 350 nm
Phytofluene
300
340
380
nm
Phytofluene
(d)
Absorbance (mAU) 280 nm
Phytoene
Phytoene
280
300
nm
Retention time (min)

Figure 2.5. HPLC profiles of pigment content from control and transfected *N. benthamiana*. (a) Pigments from control plant detected at 440 nm; (b-d) Pigments from TTU51 CTP CrtB plants detected respectively at 440, 350 and 280 nm. Pigment analysis was carried out as described under Experimental procedures. Abbreviations refer to N, neoxanthin; V, violaxanthin; L, lutein; C*a*, chlorophyll *a*; C*b*, chlorophyll *b*. Peaks eluting between 27 to 30 min represent chlorophyll derivatives.

2.3.4 Transcriptional profiling of plants overexpressing phytoene synthase

A 10K potato cDNA microarray system developed at The Institute for Genomic Research (TIGR) was employed to analyze global gene expression in plants overexpressing phytoene synthase derived from *Erwinia herbicola*, and to determine the cause of the phenotypic color change in the new leaves. Solanaceous species share similar genomes with respect to gene content and genome organization, and there is a high degree of nucleotide sequence homology among the members of this family. For example, examination of sequence homology for phytoene synthase and phytoene desaturase between *N. benthamiana* and that of the potato cDNAs spotted on the array revealed an 80-90% identity. In an effort to extract gene expression data for *N. benthamiana*, the heterologous potato system was selected, despite the fact that the two species display diverse adaptive features. Initial studies to determine hybridization conditions utilized *N. benthamiana* (non-infected) as a control and revealed good hybridization for highly conserved genes, and for genes recognized to be involved in viral defense, such as heat shock proteins (Lu et al., 2003). This data was consistent with another study that

demonstrated that tobamoviruses cause an induction of various heat shock genes and genes involved in plant stress and defense responses in *Arabidopsis* (Whitham et al., 2003).

By digesting the TTU51 CTP *CrtB* vector with *Kpn*I and *Stu*I, and ligating the 6163 bp fragment to a 4397 bp fragment from a *Kpn*I-*Stu*I digested viral vector (pBS 740 AT #120) that carries a ribozyme (Turpen et al., 1993), a modified viral vector, TTU51 CTP *CrtB* RZ was developed. The construction of this vector eliminated the need for linearization prior to *in vitro* transcription reactions. Infectious RNA was prepared by *in vitro* transcription using a T7 DNA-dependent RNA polymerase and was used to mechanically inoculate two lower leaves of *N. benthamiana* plants using carborundum. Control plants were transfected with a construct that expresses the green florescent protein (TTOSA1 APE pBAD), in an attempt to subtract out the effects of the viral vector. Microarray hybridizations were performed at ten days post-inoculation (10 dpi).

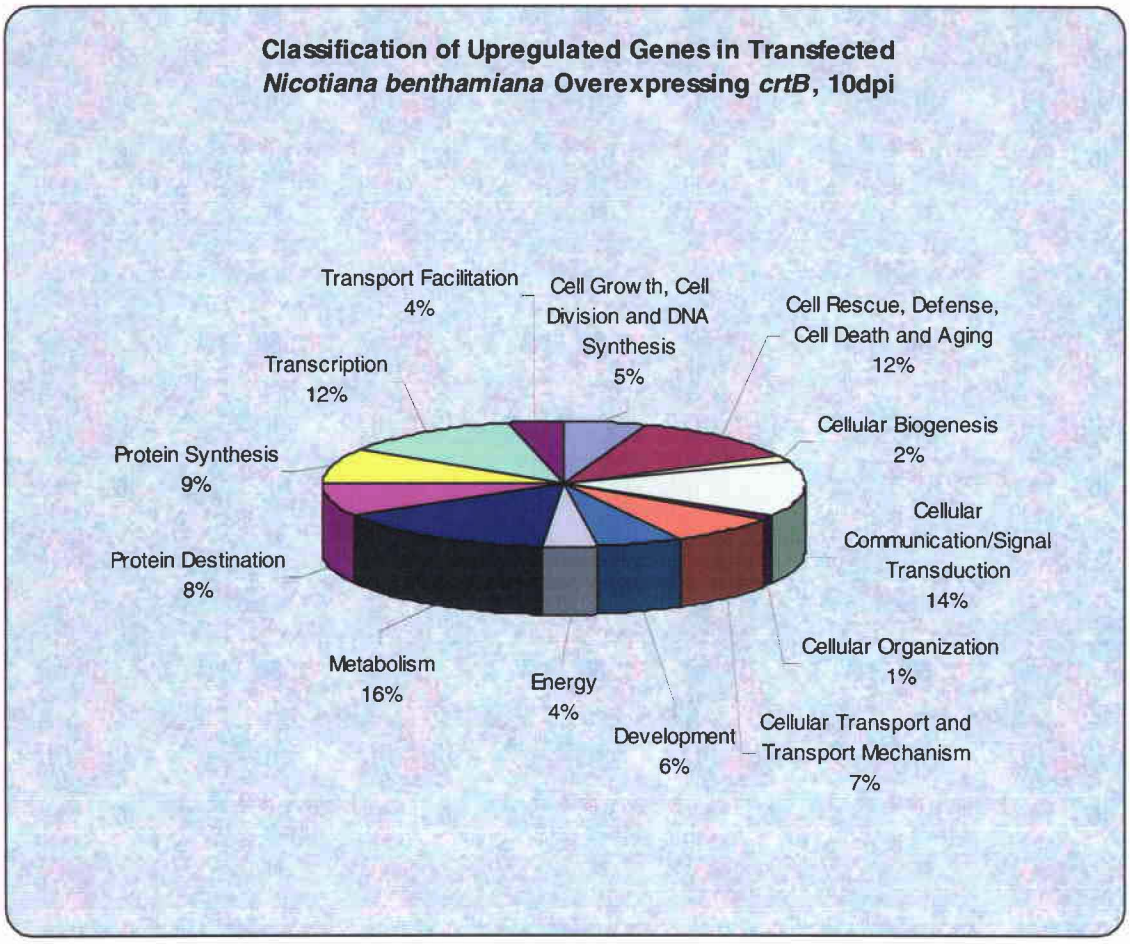

Figure 2.6. Classification of upregulated genes in transfected *N. benthamiana* overexpressing *CrtB*, 10dpi, as compared to GFP transfected plants.

Transcriptional profiling identified 165 annotated genes that were upregulated with a 2-fold or greater expression, and genes were classified by comparing TIGR annotations to the MIPS scheme (Munich Information Center for Protein Sequences). Upregulated genes included a large number of defense genes (12%) that protect against toxins and oxidative stress, including glutamate-cysteine ligase, mitogen-activated protein (MAP) kinase 4, monodehydroascorbate reductase, methionine sulfoxide reductase, glutathione S-transferase T5, and TMV-induced protein I. Other upregulated genes included those

involved in metabolism (16%); cell communication/signal transduction (14%); transcription (12%); transport (11%); protein synthesis (9%); protein destination (8%); and to a lesser extent, genes involved in energy; development; cellular biogenesis; and cellular organization (Figure 2.6). Unexpectedly, there was also a greater than 2-fold change in gene expression in endogenous *N. benthamiana* phytoene synthase (*psy)* and phytoene desaturase (*pds*) genes.

2.3.5 Expression of a mammalian defensin in transfected plants

Defensins are a family of small, cationic antimicrobial peptides that are found in the mammalian immune system (Lehrer, 1991). They contain a core of three disulfide bonds and are rich in arginine residues. Since purified defensins can kill a wide range of gram-positive and gram-negative bacterial, fungal, and enveloped viral pathogens, they may be difficult to produce in recombinant expression systems. Defensins also occur in plants and have been shown to confer acquired resistance to plant pathogens (Kanzaki et al., 2002). We are specifically interested in the production of alpha-defensins because they have anti-HIV activity (Chang *et al.*, 2003;Mackewicz *et al.*, 2003).

Figure 2.7. *N. benthamiana* infected with TTU51A CTP *CrtB* NP1 transcripts.

In our study we fused a synthetic gene encoding rabbit NP1 defensin (Selsted et al., 1985) to the C-terminal end of phytoene synthase and determine whether the expression of the chimeric CrtB NP1 enzyme could be visually monitored in plants. In this experiment, a unique *Eco*RV site was inserted in *CrtB* by PCR mutagenesis directly upstream of the stop codon. A synthetic NP1 sequence was then cloned adjacent to *CrtB*, infectious RNA was made *in vitro*, and directly applied to plants. One to two weeks post inoculation, leaves from plants transfected with TTU51A CTP *CrtB* NP1 developed an orange phenotype (Fig. 2.7). The 39-KDa CrtB NP1 fusion accumulated in systemically infected leaves and was analyzed by immunoblotting, using *E. coli* produced phytoene synthase and an NP1 fusion as standards. Both phytoene synthase and the C-terminal fusion to NP1 were detected in transfected plants using a rabbit anti-CrtB antibody. The CrtB NP1 chimera was approximately 3.5 kDa larger than phytoene synthase (Fig. 2.8a, lanes 2, 3). Recombinant CrtB NP1 from sytemically infected *N. benthamiana* also cross-reacted with a guinea pig anti-NP1 antibody (Fig. 2.8b, lane 3). No detectable

cross-reacting protein was observed in the non-infected *N. benthamiana* control plants (Fig. 2.8b, lane 1) or those that were infected with TTU51 CTP *CrtB* (Fig. 2.8b, lane 2). These results suggest that CrtB NP1 fusion is targeted to the chloroplast in an enzymatically active form.

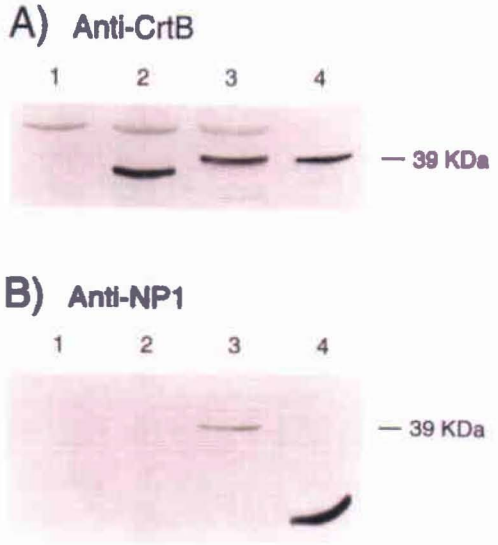

Figure 2.8. Protein analysis of a transfected *N. benthamiana* plant 11 days after inoculation. (a) Western blot analysis (anti-CrtB). Lanes: 1, non-infected *N. benthamiana*; 2, *N. benthamiana* transfected with TTU51 CTP *CrtB*; 3, *N. benthamiana* transfected with TTU51A CTP *CrtB* NP1; 4, *E. coli* expressed CTP *CrtB*. (b) Western blot analysis (anti-NP1). Lanes: 1, non-infected *N. benthamiana*; 2, *N. benthamiana* transfected with TTU51 CTP *CrtB*; 3, *N. benthamiana* transfected with TTU51A CTP *CrtB* NP1; 4, *E. coli* expressed NP1 fusion.

2.4 DISCUSSION

In this study, the *Erwinia herbicola* phytoene synthase gene was placed under the control of the TMV coat protein subgenomic promoter in TTU51, a hybrid tobamoviral vector. Approximately 4 to 6 days after transfection, systemically infected plants developed a bright orange phenotype that can be a useful visible marker to study viral replication and movement. Transfected plants can be analyzed at various times post inoculation because the detection of the phytoene synthase phenotype does not require removal or preparation of the infected tissue. By comparison, histochemical staining for the detection of β-glucuronidase (GUS) activity often requires the excision of the leaf from the plant and the removal of chlorophyll by ethanol or chloral hydrate (Jefferson *et al.*, 1987). Since this procedure damages the sample, additional analysis of the same tissue at various times after inoculation is impossible. The transient expression of CrtB has similar attributes to the viral expression of the green fluorescent protein (GFP) (Baulcombe et al., 1995), (Heinlein et al., 1995), (Casper, 1996). Both are nondestructive and can be directly visualized in whole plants at various times during development. One main difference in the two markers is that the detection of CrtB does not require ultraviolet lights. The CrtB orange phenotype can be easily seen in normal sunlight. This attribute may be beneficial when screening a large number of plants or in outdoor field conditions.

Enzyme assays of isolated chloroplasts revealed that along with the increase in phytoene synthase level there is a concomitant decrease in chlorophyll synthetase activity. Chlorophyll biosynthesis may also decrease due to the channeling, of

geranylgeranyl pyrophosphate (GGPP) toward the synthesis of carotenoids. In a similar vein, it has been shown that the channeling of GGPP to the synthesis of phytoene and lycopene, reduces its availability for the biosynthesis of the plant hormones gibberellins, and induces dwarfism in tomato (Fray et al., 1995). This finding is supported by the increased accumulation of β-carotene giving the orange color along with the accumulation of phytofluene and phytoene and lutein. Our data show that like in seeds, the overexpression of phytoene synthase can lead to massive synthesis of carotenoid in photosynthetic leaves which do not form structures for their disposal as shown in non-green plastids (Camara et al., 1995). Due to their potential inability to constitute molecular rivets for plastid membranes (Gruszecki, 1999), the amount of β-carotene, phytoene and phytofluene probably represents the maximum that could allow the function of the photosynthetic membranes.

Results from the microarray revealed an upregulation of the endogenous phytoene synthase and phytoene desaturase genes at the 10 dpi point. This result may explain the accumulation of phytofluene (Fig. 5c, Table 1). These results were also observed in microarray studies in which plants transfected with antisense phytoene desaturase were compared to wild type plants (data not shown). Since an accumulation of phytoene occurs due to crtB overexpression as well as phytoene desaturase knock-out (Kumagai *et al.*, 1995), this result could suggest that the availability of phytoene may play a key role as a regulator of carotenoid gene expression in leaves as noted in seeds (Lindgren *et al.*, 2003; Shewmaker *et al.*, 1999; Wong *et al.*, 2004).

The transient expression of phytoene synthase can be used in other areas of molecular biology. Since purification tags have helped in the detection and isolation of heterologous proteins, we decided to determine whether phytoene synthase could tolerate C-terminal fusions. In this context it is interesting to note that in fungi, phytoene synthase is fused to lycopene cyclase (Arrach et al., 2001). In this experiment a rabbit antimicrobial peptide (defensin) was selected because it is difficult to produce in other recombinant expression systems, and has potential therapeutic properties as a peptide based antibiotic. The rabbit defensin NP1 contained three dipeptide Arg repeats that may be a substrate for a KEX2-like protease. Since this enzyme is located in the ER/Golgi complex, it may be difficult to secrete NP1 without concomitant cleavage. In our experiment, without the concomitant cleavage, we targeted the defensin to the chloroplast using a phytoene synthase fusion, and visually monitored the expression in plants. One week after inoculation, systemically infected tissue containing a chimeric gene encoding phytoene synthase and NP1 developed an orange phenotype. The 39-KDa CrtB NP1 fusion accumulated in upper non-inoculated leaves and the hybrid protein cross-reacted to anti-phytoene synthase and anti-defensin antibodies. No detectable cross-reacting protein was observed in the non-infected *N. benthamiana* control plants. These results suggest that the phytoene synthase NP1 fusion is targeted to the chloroplast in an enzymatically active form. It is interesting to note that a reciprocal experiment fusing NP1 to the C-terminal end of GFP destroyed the ability of GFP to be fluorescent. Phytoene synthase fusions can be isolated from chloroplasts using anti-CrtB affinity chromatography. The transient, directed expression of CrtB NP1 by a viral vector

suggests that this system may be useful to sequester other biologically active peptides in the plant chloroplasts.

Finally through the use of a viral vector encoding a bacterial phytoene synthase gene we have induced an uncontrolled buildup of carotenoid in chloroplasts, a characteristic feature of chromoplasts. Transcriptional profiling revealed that the expression of endogenous phytoene synthase and phytoene desaturase was triggered under these conditions. This reveals that the step catalyzed by phytoene synthase represents a crucial target for the regulation of carotenoid biosynthesis. Further analysis of this phenomenon is thus expected to provide insight into the mechanisms involved. Our study also revealed that the ability of phytoene synthase to tolerate C-terminal fusion and the phenotypic orange coloration due the accumulation of β-carotene could be developed as an alternative strategy to transplastomic technology.

2.5 EXPERIMENTAL PROCEDURES

2.5.1 Plasmid Constructions

The chloroplast-targeting phytoene synthase expression vector, TTU51 CTP *CrtB* (Figure 1), was constructed in several subcloning steps. First, a unique *Sph*I site was inserted in the start codon for the *Erwinia herbicola* phytoene synthase gene by polymerase chain reaction (PCR) mutagenesis (Saiki et al., 1985) using oligonucleotides *CrtB* M1S 5' CCA AGC TTC TCG AGT GCA GCA TGC AGC AAC CGC CGC TGC TTG AC 3' (upstream) and *CrtB* P300 5' AAG ATC TCT CGA GCT AAA CGG GAC GCT GCC AAA GAC CGG CCG G 3' (downstream). The *CrtB* PCR fragment was subcloned into pBluescript (Stratagene) at the *Eco*RV site, creating plasmid pBS664. A 938 bp *Sph*I, *Xho*I *CrtB* fragment from pBS664 was then subcloned into a vector containing the sequence encoding the *N. tabacum* chloroplast transit peptide (CTP) for the small subunit of ribulose-1,5-bisphosphate carboxylase (RUBISCO) (O'Neal et al., 1987), creating plasmid pBS670. Next, the tobamoviral vector, TTU51, was constructed. A 1020 bp fragment from the tobacco mild green mosaic virus (TMGMV; U5 strain) containing the viral subgenomic promoter, coat protein gene, and the 3' end was isolated by PCR using TMGMV primers 5' GGC TGT GAA ACT CGA AAA GGT TCC GG 3' (upstream) and 5' CGG GGT ACC TGG GCC GCT ACC GGC GGT TAG GGG AGG 3' (downstream), subcloned into the *Hinc*II site of Bluescript KS-, and verified by dideoxynucleotide sequencing (Sanger et al., 1977). This clone contains a naturally occurring duplication of 147 bp that includes the whole upstream pseudoknot domain in the 3' noncoding region. The hybrid viral cDNA consisting of TMV-U1 and TMGMV

was constructed by swapping a 1-Kb *Xho*I-*Kpn*I TMGMV fragment into TTO1 (Kumagai et al., 1995), creating plasmid TTU51. Finally, the 1.1 Kb *Xho*I CTP *CrtB* fragment from pBS670 was subcloned into the *Xho*I of TTU51, creating plasmid TTU51 CTP *CrtB*. As a CTP negative control, a 942 bp *Xho*I fragment containing the *CrtB* gene from pBS664 was subcloned into TTU51, creating plasmid TTU51 *CrtB*.

TTU51A CTP *CrtB* NP1 was designed to express a phytoene synthase defensin fusion in the chloroplasts. First, a unique *Sph*I site was inserted 3 bp upstream of the stop codon for the *CrtB* gene by PCR mutagenesis using oligonucleotides *CrtB* M1S 5' CCA AGC TTC TCG AGT GCA GCA TGC AGC AAC CGC CGC TGC TTG AC 3' (upstream) and *CrtB* His 5' CTA GAT CTC CTA GGT TAG TGA TGG TGA TGG TGA TGG ATA TCA ACG GGA CGC TGC CAA AGA CCC CG 3' (downstream). The 1106 bp *Xho*I, *Eco*RV CTP *CrtB* fragment was then subcloned into a plasmid containing a synthetic rabbit NP1 gene consisting of the following sequence: 5' GAT ATC GAA GGT CGT GTG GTC TGT GCG TGC AGA CGA GCG CTG TGC CTG CCG CGG GAA CGT CGT GCG GGT TTC TGC CGT ATC CGT GGT CGT ATC CAC CCA CTC TGC TGC CGC CGC TAA CCT AGG 3'. The resulting plasmid was called TTO1A CTP *CrtB* NP1. A unique *Avr*II site was inserted upstream of the TMGMV coat subgenomic promoter by PCR mutagenesis using oligonucleotides *Cla*I 5' TAA TCG ATG ATG ATT CGG AGG CTA C 3' (upstream) and NP1 R33AS 5' CCG GTC GAC CTA GGT TAG CGG CGG CAG CAG AGT GGG 3' (downstream). The 1228 bp *Xho*I, *Sal*I CTP *CrtB* NP1 fragment was then subcloned into TTU51, creating plasmid TTU51A CTP *CrtB* NP1.

TTOSA1 APE pBAD was designed to express the green florescent protein (GFP) in the cytoplasm. Using PCR mutagenesis the *Sph*I site in the 126K replicase open reading

frame (ORF) of TTO1A was removed using oligonucleotide 5' CGT CCA GGT TGG GCA TAC AGC AGT GTA CAT ATG C 3' and a unique *Pme*I site was inserted at the 3' end of tomato mosaic virus cDNA (fruit necrosis strain; ToMV-FN) (Valverde, 1991) using oligonucleotide 5' CGG GGT ACC GTT TAA ACT GGG CCC CAA CCG GGG GTT CCG GG 3'. A 1.4 Kb *Xho*I, *Avr*II fragment from TTO1A 103L (Kumagai et al., 2000) containing the rice α-amylase OS103 cDNA was inserted, creating plasmid TTOSA1 APE 103L. A unique *Sph*I site (start codon) and a unique *Avr*II site (adjacent to the stop codon) was inserted in the jellyfish *Aequorea victoria* GFP cDNA by PCR mutagenesis using oligonucleotides GFP M1S 5' TAA GCA TGC TGA AAG GAG AAG AAC TTT TCA CTG GAG TT 3' (upstream) and GFP K238 5' TAC CTA GGA GAT ATC CTT GTA TAG TTC ATC CAT GCC ATG TGT 3' (downstream), subcloned into TTOSA1 APE 103L, creating plasmid TTOSA1 APE pBAD.

2.5.2 In vitro transcriptions, inoculations, and analysis of transfected plants

N. benthamiana plants were inoculated with *in vitro* transcripts of *Kpn*I-digested TTU51 CTP *CrtB*, TTU51A CTP *CrtB* NP1, TTOSA1 APE pBAD, and TTU51 CTP *CrtB* RZ as described (Dawson, 1986).

2.5.3 Immunological detection of CrtB and NP1 from transfected *N. benthamiana*

Total soluble plant protein concentrations were determined (Bradford, 1976) using bovine serum albumin as a standard. The proteins were analyzed on a 0.1% SDS/12.5% polyacrylamide gel (Laemmli, 1970) and transferred by electroblotting for 1 hr to a nitrocellulose membrane (Towbin, 1979). The blotted membranes were incubated for 1 hr with a 2000-fold dilution of anti-CrtB antiserum (phytoene synthase detection) or 2000-fold dilution of anti-NP1 antisera (defensin detection). Using standard protocols, the antisera was raised in rabbits (Robert Sargeant, Ramona, CA) against a synthetic N-terminal phytoene synthase 15-mer peptide MSQPPLLDHATQTM +C conjugated to KLH (Immuno-Dynamic, Inc.) or in guinea pigs (Babco) against a synthetic 33-mer rabbit NP1 peptide conjugated to ovalbumin. The enhanced chemiluminescence horseradish peroxidase-linked, goat anti-rabbit IgG and goat anti-guinea pig IgG assays (Cappel Laboratories) were performed according to the manufacturer's (Amersham) specifications. The blotted membranes were subjected to film exposure times of up to 10 seconds.

2.5.4 Phytoene synthase assay

The chloroplast were prepared as described previously (Camara, 1993). The phytoene synthase assays were carried out in an incubation mixture (0.5 ml final volume) buffered with Tris-HCL, pH 7.6, containing [^{14}C] geranylgeranyl PP (100,000 cpm)(prepared using pepper GGPP synthase expressed in *E. coli*), 1 mM ATP, 5 mM $MnCl_2$, 1 mM

$MgCl_2$, Triton X-100 (20 mg per mg of chloroplast protein) and chloroplast suspension equivalent to 2 mg protein. After 2 h incubation at 30°C, the reaction products were extracted with chloroform methanol (Camara, 1993), subjected to TLC on a silica gel plate developed with benzene/ ethyl acetate (90/10), and autoradiographed.

2.5.5 Chlorophyll synthetase assay

For the chlorophyll synthetase assay, the isolated chloroplasts were lysed by osmotic shock before incubation. The reaction mixture (0.2 ml, final volume) consisting of 50 mM Tris-HCL (pH 7.6) containing [^{14}C] geranylgeranyl PP (100,000 cpm), 5 $MgCl_2$, 1 mM ATP, and ruptured plastid suspension equivalent to 1 mg protein was incubated for 1 hr at 30°C. The reaction products were analyzed as described previously.

2.5.6 Pigment analysis

Total pigments extracts from leaves of non-inoculated and transfected *N. benthamiana*, were separated by thin-layer chromatography on a silica gel plate using hexane/acetone (60/40, v/v) as developing solvent (Kumagai et al., 1998) and by HPLC coupled to diode array detection as described previously (Bouvier et al., 2003) except a linear gradient of acetonitrile:water, 90/10 v/v) to 100% ethylacetate for 45 min was used. Authentic standards were use to calibrate the detector response.

2.5.7 cDNA microarray analysis

At ten days post-inoculation (10 dpi), RNA was isolated separately (Qiagen RNeasy) from two *crtB*-inoculated plants and from two GFP-inoculated plants, and quantified. Fluorescently-labeled cDNA was generated for microarray screening. Total RNA (25 μg) was reverse transcribed (Invitrogen Fluoroscript) and labeled with Amersham cyanine-3 (Cy3) dye for each *crtB*-inoculated plant or with cyanine-5 (Cy5) dye for each of the control plants. Cy3/C5 populations were mixed and hybridized to each of two 10K potato cDNA microarrays (TIGR) using recommended hybridization conditions. A detailed description of TIGR Potato Microarray optimized methods, the array design, and the cDNA description file used for this study (GenePix Array List, GAL file version1) can be found at the following URL:
http://www.tigr.org/tdb/potato/microarray_comp.shtml.
Signal data were obtained by scanning slides in an Affymetrix 286 scanner using Jaguar software, and data analysis was performed using GeneSpring software (Silicon Genetics, Redwood City). Jaguar spot files and spreadsheets of GeneSpring processed data reflecting transcript abundance is made available as supplementary material.

2.6 ACKNOWLEDGEMENTS

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2.8 SUPPLEMENTARY MATERIALS

Microarray data tables for gene expression in *N. benthamiana* infected with TTU51 CTP-*CrtB*-RZ transcripts are provided in Appendix I in Excel format for easy viewing. The following data was submitted as Supplementary Material to *The Plant Journal* that is intended for access through the on-line edition. Normalized gene expression data represent the ratio of Cy3 signals of *crtB*-transfected experimental samples to cy5 signals of GFP-transfected controls.

Raw cy3 and cy5 signal data for each of the two microarrays was combined for import into GeneSpring software for analysis. Gene lists generated from LOWESS normalized values for 2-fold upregulated genes. The "Combined Clusters" file lists the upregulated genes that were clustered using K-means for 5 clusters, (100 iterations) for the standard correlation. The TIGR Master Gene Table (Versions 1 and 2) for the clones on the 10K cDNA potato microarray can be found at the following url: www.tigr.org.

CHAPTER 3. TRANSCRIPTIONAL CHANGES IN CAROTENOID GENES INDUCED BY TOBAMOVIRAL TRANSFECTION

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3.1 ABSTRACT

Manipulation of carotenoid biosynthesis was accomplished by transfecting *Nicotiana benthamiana* with *tobamoviral* vectors that carry genes encoding enzymes in the pathway. Transcriptional profiling was conducted using a heterologous cDNA microarray system and quantitative real-time PCR (QRT-PCR) to determine the impact of transfection on the expression of endogenous phytoene synthase (*psy*) and phytoene desaturase (*pds*), in order to gain insight into regulation of the synthesis of these enzymes. Plants were transfected with tobamoviral vectors carrying a *crtB* (phytoene synthase) cDNA derived from *Erwinia herbicola*, or with viral vectors carrying an antisense construct of a gene encoding phytoene desaturase derived from ripening tomato. Previous reports indicate that both transfections result in an accumulation of colorless phytoene, which may act as a signal in the regulation of the pathway. Microarray analysis shows an elevation of endogenous *psy and pds* mRNAs in the *crtB*-transfected plant experiments, which was validated using QRT-PCR, as well as an accumulation of transcripts from IPP isomerase and β-carotene hydroxylase. At ten days post-inoculation plants transfected with a partial *pds* antisense construct developed photobleaching in newly formed leaves due to cytoplasmic knock-down of phytoene desaturase mRNA. The delayed phenotype is indicative of an early suppression of the host silencing machinery by the *tobamoviral* vector. Microarray analysis revealed high

levels of virally derived *pds* transcript, suggesting that at this time point the virus is escaping RNA silencing. A differential QRT-PCR analysis discerned that although the viral vector *pds* transcript is high, there is a 78-fold decrease in the endogenous *pds* transcript accumulation compared to wild type *N. benthamiana.* Other genes in the pathway did not show significant changes in mRNA levels with the exception of a 5-fold decrease in transcript levels of a putative 9-cis-epoxycarotenoid dioxygenase (NCED), a key regulatory enzyme in abscisic acid biosynthesis. QRT-PCR analysis of norflurazon-treated plants that also accumulate phytoene, showed a 5-fold reduction in levels of *pds* transcripts by QRT-PCR. Overall, these data indicate that manipulation of the carotenoid pathway using *tobamoviral* vectors can impact the accumulation of transcripts both upstream and downstream of targeted enzymes, but that phytoene does not have a direct role in the regulation of the pathway.

3.2 INTRODUCTION

Carotenoids are 40-carbon isoprenoid pigments that contain conjugated double bonds, endowing fruits, flowers and leaves with a range of beautiful colors. Essential components of photosynthetic membranes, they function to protect chlorophylls from photo-oxidation, and to assist in the harvesting of light (Cunningham and Gantt, 1998). In addition, they are precursors of the hormone abscisic acid (ABA), and provide nutritional benefits in fruits as precursors to essential vitamins and as antioxidants (Bramley, 2002).

In eukaryotes, carotenoid enzymes are encoded from nuclear genes. Upon translation in the cytoplasm, enzymes are modified with a transit peptide, and are transported to the chloroplast where the carotenoid biosynthesis pathway proceeds. Genes encoding these enzymes have been cloned from bacteria, algae, fungi and plants, and have been used for metabolic engineering and biotechnological applications in both carotenogenic and non-carotenogenic organisms (Hirschberg, 2001). Such investigations had shed light upon pathway regulation in photosynthetic tissue, which is distinct from those mechanisms operating in fruits and flowers (Bramley, 2002; Thelander, 1986).

Key factors determining the regulation of the pathway have not as yet been identified, although light, stress, and stage of development appear to influence gene induction (Giuliano, *et al.*, 2003; Liu, *et al.*, 2004; Simkin, *et al.*, 2003). Regulation of carotenoid biosynthesis in plant leaves is thought to be tight and to occur at the level of transcription, and has been investigated by creating transgenic plants or by studying

mutants that possess altered carotenoid synthesis and accumulation (Bramley, 2002). In genetically-manipulated plants, accumulated intermediates, including phytoene and lycopene, or the decrease in chlorophyll levels (Giuliano, *et al.*, 1993) (Corona, *et al.*, 1996) have been proposed as possible signals influencing transcriptional rates.

The use of bleaching herbicides that compete with carotenoid desaturation reactions have been employed to study two of the early enzymes in the pathway, phytoene synthase (*psy*) and phytoene desaturase (*pds*). Norflurazon, [NF, (4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)one)] is a chlorosis-inducing herbicide that causes a noncompetitive inhibition of *pds*, leading to an accumulation of phytoene and a subsequent photobleaching of leaves and other organs (Breitenbach, *et al.*, 2001) (Simkin, *et al.*, 2000). Phytoene is a colorless carotenoid that is unable to protect the plants against photo-oxidation of the chlorophylls (Jung et al, 2000).

Simkin *et al* measured the amount of accumulated phytoene in norflurazon-treated *Capsicum annum* plantlets to be 1.6x that of an untreated control. Using reverse-phase HPLC, Kumagai *et al* showed that treatment with norfluorazon causes the level of phytoene in *Nicotiana benthamiana* plants to rise to 74x that of a control plant (Kumagai, *et al.*, 1995). Transgenic *N. tabacum* plants expressing antisense *pds* also accumulate phytoene (Busch, *et al.*, 2002). Levels of *psy* and *pds* transcripts have been measured in these phytoene-accumulating plants to gain insight into regulation of carotenoid enzyme synthesis. Traditionally, levels of *pds* were difficult to assay using Northern blot analysis, although the development of reverse-transcription PCR polymerase chain

reaction (RT-PCR) assays provided a level of sensitivity that enabled these rare transcripts to be detected (Corona, *et al.*, 1996; Giuliano, *et al.*, 1993).

Over the past ten years, published reports using RT-PCR to assay carotenoid enzyme transcripts have been inconsistent. Giuilano *et al* studied both organ-specific and temporal expression of *psy* and *pds* in tomato seedlings and in the phytoene-accumulating *ghost* mutant. They demonstrated that norflurazon treatment of plants causes a 2-fold increase in the level of transcript of *psy* mRNA and a 10-fold increase in *pds* mRNA using reverse-transcription assays (Giuliano, *et al.*, 1993). In contrast to Giuliano's results, no change in transcript levels of *psy* or *pds* was found in norflurazon-treated pepper using comparative RT-PCR (Simkin, *et al.*, 2003), nor was there any change in the levels of zeta-carotene desaturase (*zds*) or plastid terminal oxidase (*ptox*). No significant induction of *pds* gene expression was found in norflurazon-treated *Arabidopsis* plants or in white sectors of the *Arabidopsis immutans* variegation mutant using a competitive RT-PCR method (Wetzel and Rodermel, 1998). However, activation of the *pds* promoter occurs during chemically induced arrest of pigment biosynthesis due to norflurazon treatments (Corona, *et al.*, 1996).

Transfecting plants with viral vectors carrying sense or antisense constructs that have homology to endogenous genes is an alternative method that can provide insight into carotenoid biosynthesis regulation. By mechanically inoculating leaves with infectious viral transcripts, endogenous mRNAs are degraded in the cytoplasm by virus-induced gene silencing (VIGS) causing a decrease in the levels of carotenogenic

enzymes. Within days of inoculation, plants present with phenotypes that are striking in appearance, often with alterations of the natural pigmentation. Transfection technology can be effectively combined with transcriptional profiling methods to gain insight into pathway regulation. Microarray studies have been used extensively to investigate viral transfection in animal systems, but microarray analyses have not been reported for transfected plants. In addition, quantitative real-time PCR (QRT-PCR) enables validation of targets identified in microarray studies, and provides an enhanced level of sensitivity over traditional RT-PCR.

This investigation seeks to further explore regulatory mechanisms by examining the transcriptional changes in carotenoid genes induced by tobamoviral transfection. Previous reports have shown that *N. benthamiana* plants transfected with a tobamoviral vector expressing phytoene synthase have a level of phytoene 11x that of the untransfected control, and those transfected with a tobamoviral vector (TTO1 PDS^{-}) expressing an antisense *pds* have a level 51x that of the untransfected control (Kumagai, *et al.*, 1995). The effect of this accumulation of phytoene in young *N. benthamiana* plants was examined using 10K potato cDNA microarrays developed by The Institute for Genomic Research (TIGR) and QRT-PCR technologies.

3.3 RESULTS

3.3.1 *Nicotiana benthamiana* Plant Transfections

For the transcriptional profiling experiments, *Nicotiana benthamiana* plants were transfected at the 6-8 leaf stage with *in vitro* transcripts of *tobamoviral* vectors that over-express phytoene synthase, TTU51 CTP *crtB* RZ (Kumagai MH, 2004), or cause a cytoplasmic knock-down of phytoene desaturase mRNA ,TTO1 *PDS*$^-$ (Kumagai, *et al.*, 1995) using a rub-inoculation technique. Symptoms of virus infection first appeared at 4 dpi in the *CrtB*-inoculated leaves, with yellow-to-orange color changes continuing to develop in newly formed leaves. Transfection with this vector causes an upregulation of genes in the carotenoid biosynthesis pathway by targeting a bacteria-derived phytoene synthase to the chloroplast.

Transfection with TTO1 *PDS*$^-$ produces the antisense transcript of a partial *pds* cDNA derived from ripening tomato, causing photobleaching at approximately 10 dpi in newly formed leaves (Figure 3.2) and eventually in stems and flowers. This plasmid contains the open reading frames (ORFs) encoding the 126-, 183-, and 30-kDa proteins (126K, 185/54K, and 30K) that are under the control of the SP6 promoter; a *Xho*I fragment of the partial tomato phytoene desaturase cDNA in the negative orientation that is under the control of the TMV-U1 coat protein subgenomic promoter. The subgenomic promoter is located within the minus strand of the 30K ORF. Control plants were transfected with a viral vector, TTOSA1 APE pBAD that expresses green fluorescent protein (GFP), to minimize gene expression changes due to effects of the virus.

3.3.2 Bioinformatics Analysis

Sequence information for the tomato *pds* insert in the TTO1 PDS$^-$ viral vector was derived using BLAST alignments (Figure 3.1). Cloning primers (Kumagai, *et al.*, 1995) and sequence information for *pds* available in NCBI for *Lycopersicon esculentum* (*Le*) mRNA (complete cds, M88683) were aligned to determine the insert sequence and length (647 bp).

(a)

Alignment of LePDSclone__F to GB_PL1:TOMPHYTDES

```
     M88683 Lycopersicon esculentum phytoene
     desaturase(pds) mRNA, complete cds. 6/1995
           Length = 2321

 Score = 44.1 bits (22), Expect = 0.001
 Identities = 22/22 (100%)
 Strand = Plus / Minus


Query: 9    tgtgttcttcagttttctgtca 30
             ||||||||||||||||||||||
Sbjct: 1589 tgtgttcttcagttttctgtca 1568
```

Alignment of LePDSclone__R to GB_PL1:TOMPHYTDES

```
         M88683 Lycopersicon esculentum phytoene
          desaturase (pds) mRNA, complete cds. 6/1995
          Length = 2321

 Score = 36.2 bits (18), Expect = 0.23
 Identities = 18/18 (100%)
 Strand = Plus / Plus


Query: 12  ttgatttctccgaagctt 29
           ||||||||||||||||||
Sbjct: 943 ttgatttctccgaagctt 960
```

(b)

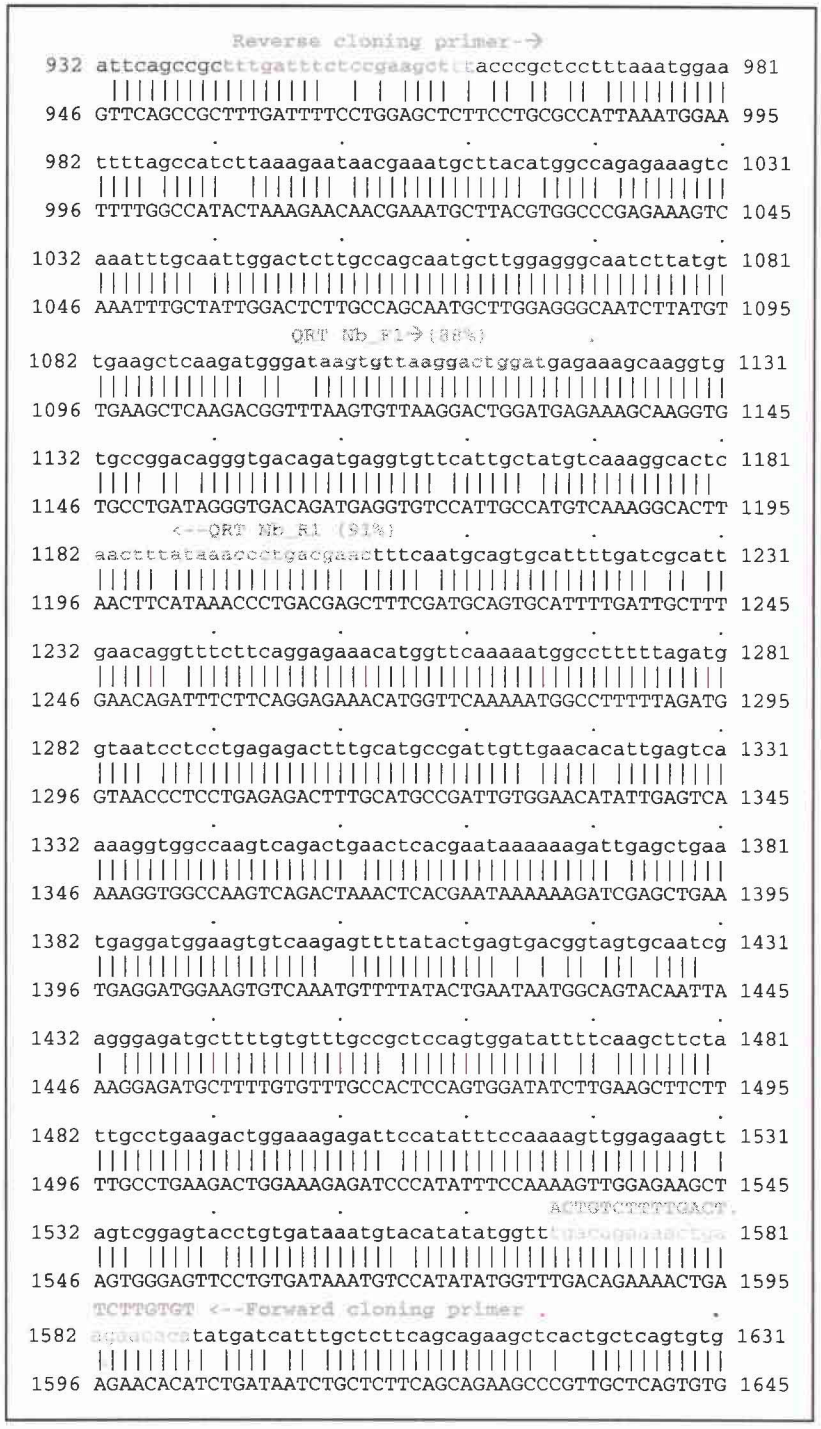

Figure 3.1. Determination of phytoene desaturation (*pds*) insert sequence in TTO1 PDS-viral vector. (a) BLAST Alignments for cloning primers LePDSclone__F and LePDSclone_R against *Lycopersicon esculentum* (*Le*) mRNA, complete cds (M88683). (b) Sequence alignment of *Le pds* viral vector insert to *Nb pds* cDNA, depicting QRT-

PCR primers *Nb*PDS_F1 and *Nb*PDS_R1 (amplicon = 111 bp). Top sequence represents *Le pds* M88683 and bottom sequence represents *Nb pds* I23876 (Sequence 3 from US patent #5539093).

Although *Nicotiana benthamiana* has served as a model organism for virus-induced gene silencing (VIGS) studies, little information about its genetics has been publicly available until recently. cDNAs encoding carotenoid biosynthesis enzymes in this plant that encode *psy* and *pds* are found in published patents (Kumagai MH, 2004; Kumagai, 1999), and in addition, a large number of sequences are now available in the NCBI database. As of January 2004, TIGR Gene Index reported over 6100 unique sequences. Access to sequence information for *N. benthamiana* was critical in understanding the potential for the transcripts of the viral vector inserts to hybridize to the potato cDNAs in the microarray study (Table 3.1).

	Nb*pds* Seq#3	TTO1 PDS-	St*pds* (*Le*)
Nb*pds* Seq#3	100%	92%	88%
TTO1 PDS-	92%	100%	96%
St*pds* (*Le*)	88%	96%	100%

Table 3.1. Comparison of *pds* sequence homology of *Nicotiana benthamiana* and *Lycopersicon esculentum* to *Solanum tuberosum* to determine the possibility of transcript hybridization in microarray experiments.

3.3.3 Microarray Design and Analysis: Changes in Transcript Levels of Carotenoids in *CrtB*- and PDS_{as}-transfected plants

For transcriptional profiling studies, *N. benthamiana* plants were transfected with the tobamoviral vector, TTO1 PDS$^-$, which carries a partial antisense *pds* cDNA from ripening tomato (Kumagai, *et al.*, 1995). Leaves were harvested at 10 dpi for microarray assays of carotenoid gene transcripts (Figure 3.2).

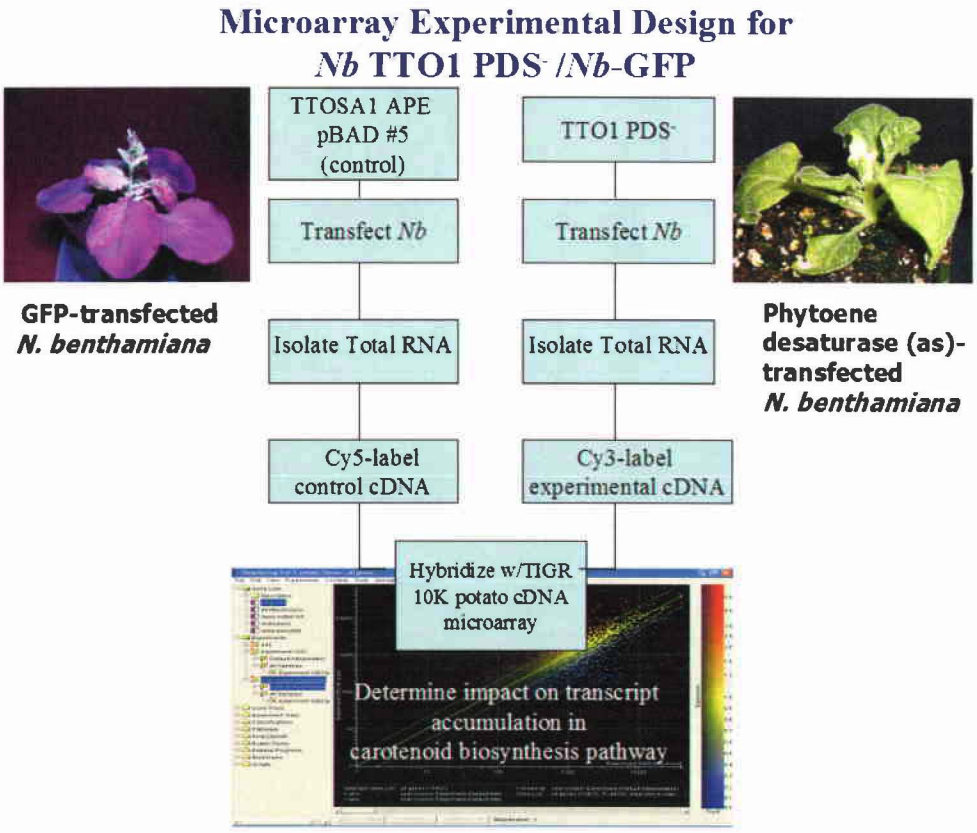

Figure 3.2. Microarray design for analysis of transcript abundance in virus-transfected *N. benthamiana* plants (10 days post-inoculation) using TTOSA1 APE pBAD-transfected plants (expressing GFP) as controls.

Leaf RNA was isolated from two treated and two control plants and first strand cDNA was synthesized using cyanine-3 and cyanine-5 fluorescent dyes. Differentially labeled cDNAs were quantified, pooled, and hybridized to heterologous 10K potato cDNA microarrays purchased through The Institute for Genomic Research (TIGR). Microarray data analysis was using GeneSpring software (Agilent Technologies, formerly Silicon Genetics) revealed unexpectedly high levels of *pds* transcript, an average 160-fold change ($p < 0.0009$) at ten days post-inoculation (dpi) compared to plants transfected with a GFP control.

No significant fold change was detected for *psy* or for other genes in the pathway that could be assayed using this microarray (Figure 3.3). However, a putative 9-cis-epoxycarotenoid dioxygenase (NCED) showed a 5-fold decrease in transcript level compared to control levels. This enzyme catalyzes a key regulated step in the abscisic acid (ABA) biosynthesis pathway in higher plants. In contrast, microarray analysis of leaf carotenogenesis enzymes in the *crtB*-transfected plants at 10 dpi showed increased endogenous transcripts for isopentenyl pyrophosphate (IPP) isomerase (17x), *psy* (3x) *pds* (5x), and β-carotene hydroxylase (12x).

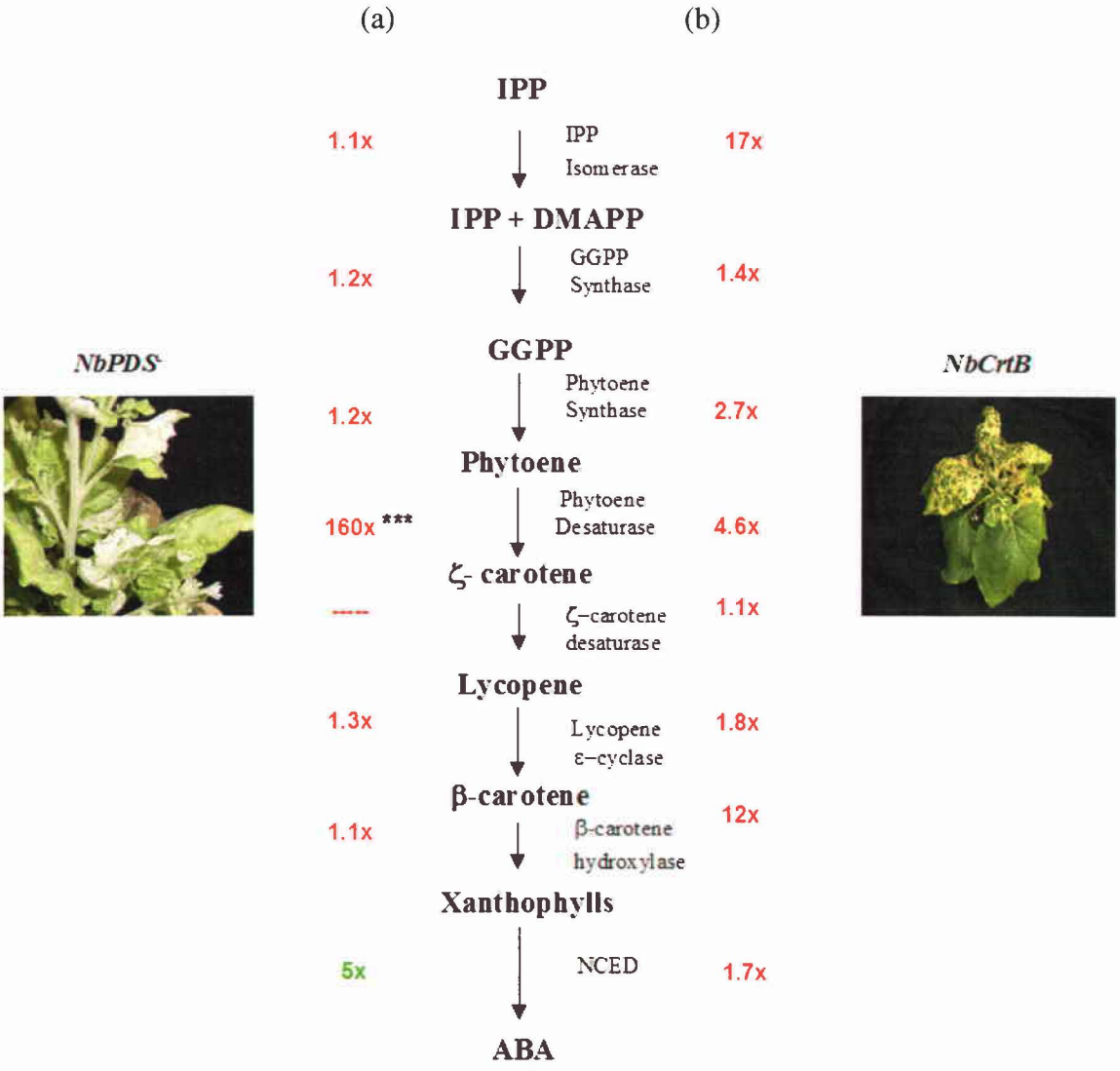

Figure 3.3. Microarray fold change for enzymes in the carotenoid biosynthesis pathway. (a) *PDSas*-transfected *Nb*; and (b) *CrtB*-transfected *Nb*. All values shown in red represent upregulation or no significant change, with the exception of a 5-fold decrease in NCED, a putative 9-cis-epoxycarotenoid dioxygenase, shown in green. (** accumulated *pds* transcript due to viral vector insert).

3.3.4 Quantitative Real-Time PCR Assays

In order to validate the microarray findings of both *CrtB* and *PDS*-transfection experiments, a relative quantitative real-time PCR (QRT-PCR) was performed as an independent assay to examine the abundance of *psy* and *pds* mRNAs. Primers for both target and reference genes were designed using BioRad's Beacon Designer 2.1. cDNAs synthesized from RNA at 10 dpi from both *CrtB*- and GFP-transfected plants were used as template for the QRT reactions. Internal reference genes included the small subunit of rubisco (GenBank #X01722), a large ribosomal protein (RPL37a) (GenBank #BQ515266), or ubiquitin (GenBank #CK294769).

CrtB-transfection of plants produces a bacterial phytoene synthase. We previously reported (Kumagai, *et al.*, 2004)that levels of endogenous *psy* and *pds* are elevated at 10 days post-inoculation (dpi) using microarray analysis. Additional assays using QRT-PCR confirmed that these transcripts accumulate in the leaves of *crtB*-transfected plants. Pfaffl method (Pfaffl, 2001) calculations that take into account efficiencies of amplification for both target and reference genes show a 2-fold accumulation of endogenous *psy* compared to GFP-transfected controls. In *CrtB*-treated plants, efficiency of amplification of the *psy* target was 95.0% (correlation coefficient 1.00). Efficiency of amplification of the rubisco reference gene was 98.6% (correlation coefficient .999). The possibility that levels of endogenous *psy* were elevated due to viral vector contributions was ruled out. The QRT-primers designed to amplify endogenous *psy* do not amplify the *CrtB* viral vector insert in conventional RT-PCR at the melting

temperature used in QRT-PCR presumably because of the low homology between the bacterial *crtB* and the endogenous *pds* (data not shown). QRT-PCR analysis also showed a 13-fold increase in *pds* in *CrtB*-treated plants compared to GFP-transfected control plants. Efficiencies of amplification of the *pds* target and rubisco reference genes were 95.5% (correlation coefficient .999) and 99.2%, respectively. The melt curve analysis showed no primer dimers for either assay.

In order to assay the levels of endogenous *pds* in *PDSas*-transfected *N. benthamiana,* two sets of QRT-PCR primers (Figure 3.4) were designed to distinguish between transcript accumulation due to the viral vector insert (*Nb*PDS_F1 and *Nb*PDS_R1), and that due to the endogenous plant *pds* (*Nb*PDS_F2 and *Nb*PDS_R2).

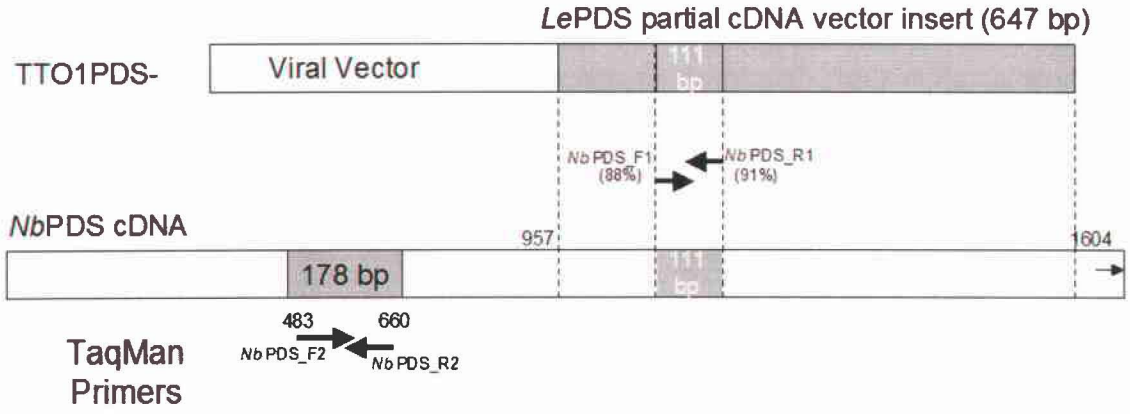

Figure 3.4. TaqMan Primer Design. Regions of *pds* cDNA depicting amplification using primer sets that lie within the viral vector insert (QRT primer Set#1: *Nb*PDS_F1 and *Nb*PDS_R1) and outside the viral vector insert (QRT primer Set#2: *Nb*PDS_F2 and *Nb*PDS_R2). (Not drawn to scale)

The *N. benthamiana pds* sequence (Fitzmaurice WP, 1996) was used to design the "inside" primers (Figure 3.1) and these were then blasted against the *Le pds* mRNA to determine the degree of homology which showed approximately 90%. To assay the levels of endogenous *pds*, primers were designed outside of the region of homology to the tomato-derived *pds* partial cDNA, in order to eliminate contributions from amplification of the viral vector insert in QRT reactions.

In conventional reverse transcription PCR, amplification of *pds* using primer set #1 produced an amplicon of 111 bp for both the viral vector plasmid and for the cDNA template derived from *Nb PDS*-treated plants. Amplification of *pds* using primer set #2 produced an amplicon of 178 bp only for the cDNA template derived from *Nb PDS*-treated plants. Information regarding potential amplification of the viral vector insert (lane 8) was used to differentiate its contributions from those of endogenous transcript amplification in QRT-PCR reactions (Figure 3.5).

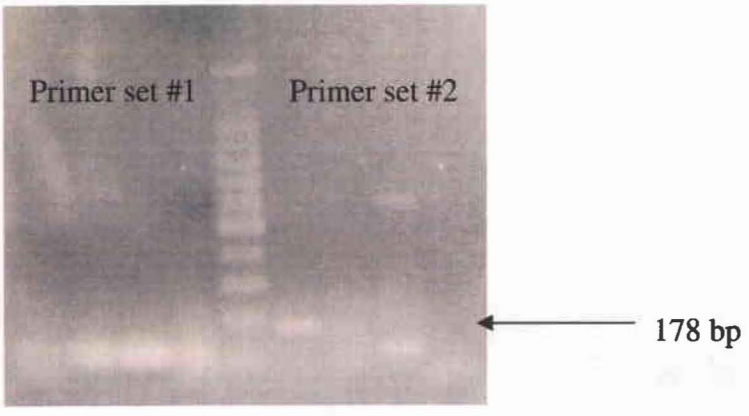

Figure 3.5. RT-PCR reaction depicting amplification of phytoene with primer set #1 (Lanes 1-4) and primer set #2 (Lanes 6-9), 1.5% agarose gel. Templates are *Nb* wild type cDNA in lanes 1 and 6; *Nb PDS*⁻ cDNA in lanes 2 and 7; and Viral vector plasmid TTO1 *PDS*⁻ in lanes 3 and 8. Lanes 4 and 9 shows no template control. Lane 5 is Promega 100 bp ladder.

QRT-PCR was then performed on *PDSas*-transfected *N. benthamiana* plants using primer set# 1, *Nb*PDS_F1 and *Nb*PDS_R1, with an expected amplicon of 111 bp. Assays using GFP-transfected plants as controls showed that *PDSas*-transfected *N. benthamiana* had 282-fold higher levels of *pds* mRNA transcript (data not shown), confirming findings in the microarray data. QRT-PCR assays using untransfected plants as controls also revealed higher levels of *pds* in the *PDSas*-transfected plants, indicated that the viral vector was contributing to high *pds* levels (Figure 3.6a).
To eliminate contributions from the virus, and to obtain additional information about the effects of phytoene accumulation on endogenous *pds* transcript accumulation, a comparison of *PDSas*-transfected, *Nb* wild type, and norflurazon-treated plants was performed using QRT-PCR primer set#2.

N. benthamiana plants were treated with norflurazon (NF) by applying 5 mL to the base of the plant at the 6-8 leaf stage. These plants showed a photobleaching effect beginning at 2 days post application, and RNA was extracted from leaves at this time and prepared for QRT-PCR assays. Relative QRT-PCR showed that compared to wild type, pds_{as}-treated plants present with a 78-fold decrease and NF-treated plants show a 5-fold

decrease in transcript (Figure 3.6b). Products of QRT-PCR were run out on a 1.5% agarose electrophoresis gel to check for the presence of additional bands due to any non-specific binding of the viral insert. No additional bands were apparent (data not shown), and the melt curve analyses indicated a lack of primer dimers.

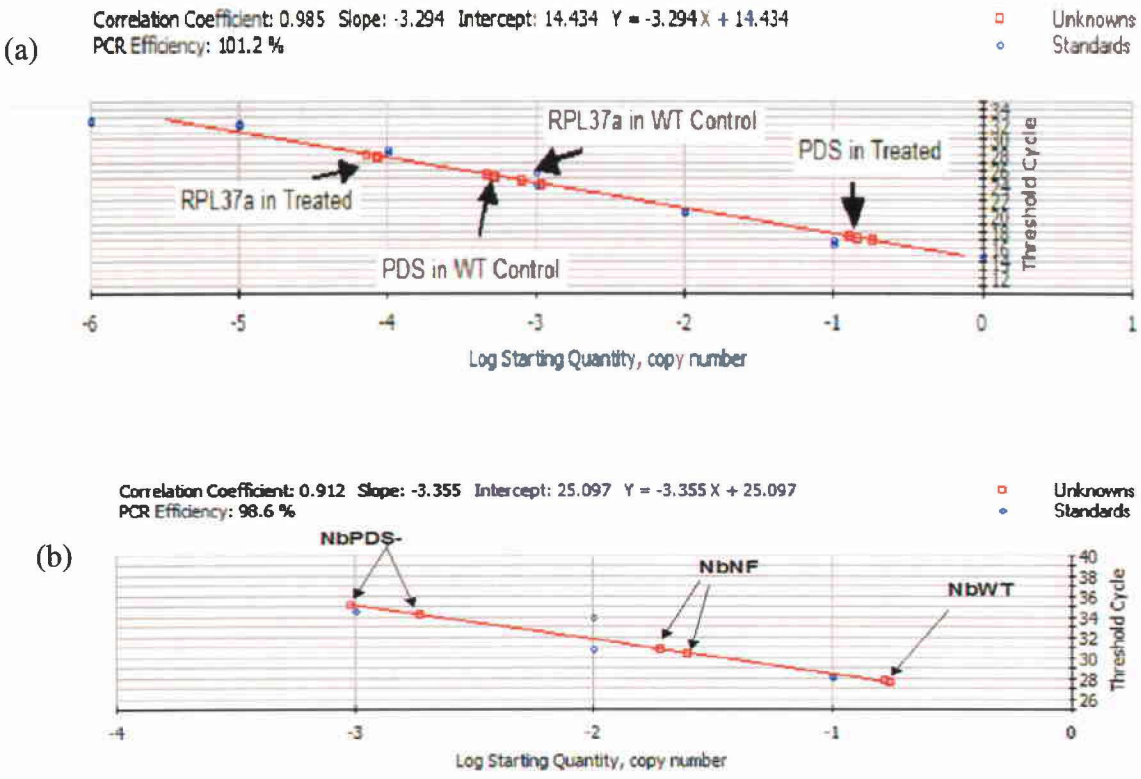

Figure 3.6. BioRad iCycler QRT-PCR graphical analysis of *pds* levels.

(a) Depicting high levels of *pds* due to viral vector insert amplification in transfected plants compared to wild type controls (untransfected), using primer set #1; and

(b) QRT-PCR graphical analysis of *pds* transcript abundance in *pds*-transfected plants, in wild type controls, and in norflurazon-treated plants using QRT primer set#2.

The levels of *pds* transcript accumulation were also assayed in GFP-transfected plants (Table 3.2) to ascertain its use as a control in microarray and QRT-PCR studies. GFP-transfected controls show levels of *pds* mRNA comparable to that in the pds_{as}-transfected plant.

Constructs	Amount normalized *pds* mRNA[a] (AU)[b]	Relative Abundance of *pds* mRNA levels[c] (%)
Wild Type (untransfected)	1.35	100
Norflurazon-treated	0.27	20
Nb TTOSA1 APE pBAD-transfected	0.02	1.7
Nb TTO1 PDS^--transfected	0.01	1.2

[a] Value represents the mean from one experiment. For each sample, QRT-PCR was performed in duplicate. [b] Arbitrary unit. [c] relative to wild type control

Table 3.2. Quantitative real-time PCR determination of relative abundance of endogenous *pds* mRNA in TTO1 PDS_{as}-transfected *Nicotiana benthamiana* plants compared to norflurazon-treated, untransfected wild type and GFP-transfected control plants.

3.4 DISCUSSION

This investigation studies the effects of TTU51 CTP *CrtB* RZ and TTO1 PDS$^-$ transfection on carotenogenic enzymes in young *N. benthamiana* leaves. Previous reports indicate that both transfections accumulate phytoene (Kumagai, *et al.*, 1995), which may influence regulation of carotenoid biosynthesis genes. Overexpression of phytoene synthase with the TTU51 CTP *CrtB* RZ viral vector causes an increase in endogenous transcript accumulation at 10 dpi for enzymes upstream of *psy* (IPP isomerase) and downstream of *psy* (*pds* and β-carotene hydroxylase). Microarray data shows elevation of enzymes leading to the formation of ζ-carotene and β–carotene, yellow and orange-colored pigments, respectively. The approximate 5-fold increase ($p < 0.03$) in expression of *pds* that catalyzes the formation of ζ-carotene and the 12-fold increase ($p < 0.05$) in expression of β-carotene hydroxylase that catalyzes the formation of β–carotene may contribute to the orange-yellow phenotype seen in leaves, stems, flowers, and roots. The use of a heterologous insert derived from *Erwinia herbicola* enables a clear distinction in the interpretation of microarray and QRT-PCR data for impact on endogenous *psy* transcripts, due to low homology of the bacterial sequence to *Nb* and potato. The levels of endogenous *psy* in *CrtB*-transfected plants are also increased at 10 dpi.

All carotenoids are derived from isopentenyl diphosphate (IPP) (Bramley, 2002). Isomerization of IPP to dimethylallyl diphosphate (DMAPP) is catalyzed by IPP isomerase, which shows a 17-fold increased level of transcript ($p < 0.04$) in the *CrtB*-

transfected plants compared to the GFP-transfected controls. It is reasonable to suggest that the increase in IPP isomerase upstream of *psy* leads to a greater concentration of DMAPP, the activated substrate for the formation of geranylgeranylpyrophosphate (GGPP). Two molecules of GGPP condense to form 15-*cis* phytoene in a reaction catalyzed by *psy*. Therefore, overexpression of *CrtB* appears to cause an increase in phytoene because of increased transcript levels of IPP isomerase, which then drives the pathway to proceed at elevated levels. It appears that phytoene accumulation is a by-product rather than the signal for upregulation of this gene in the pathway. A microarray analysis of *CrtB*-transfected plants at additional time points would shed light on the cause of the increase in IPP isomerase transcripts. Overall, these data also indicate a potential use of the TTU51 CTP *CrtB* RZ viral vector for metabolic engineering of leaf carotenogenesis in *N. benthamiana* and other solanaceous plants for targeted increase in pathway intermediates.

In the pds_{as}-transfection studies, the sequence of the viral vector tomato-derived insert shares 92% homology with endogenous *N. benthamiana pds*, which is sufficient for gene silencing of endogenous *pds* transcripts to occur. Analysis of pds_{as}-transfected plants at 10 dpi using a heterologous potato cDNA microarray showed an average of 160-fold increase of *pds* mRNA compared to the GFP-transfected control. These results indicated a possible elevation of transcripts because of viral vector contributions. The cDNAs for microarray hybridization were synthesized using oligo d(T) priming; the viral insert is partial and lacks a poly-(A) tail. However, there is a run of five As in the insert cDNA that may result in the synthesis of cDNA during the reverse-transcription

reactions. The tomato-derived *pds* insert shares 96% homology with the potato cDNA clone sequences on the microarray, whereas the homology of endogenous *N. benthamiana pds* is 88%. cDNAs produced from viral template would cause a spurious elevation in the *pds* fold-change on the microarray.

While there was a possibility that the elevation of endogenous *pds* was real, QRT-PCR was employed to address this question and two sets of quantitative real-time PCR primers were designed using *N. benthamiana* sequence information. The first set was designed within the 647 bp partial cDNA insert sequence present in TTO1 PDS$^-$, with primers sharing 90% homology. Conventional PCR confirmed that the QRT-PCR primer set #1 (*NbPDS*_F1 and *NbPDS*_R1) could amplify the insert in the viral vector. QRT-PCR experiments showed a 370-fold increase over the GFP control plant levels and a 280-fold increase in *pds* transcript accumulation over the untransfected plants, indicating a contribution from the viral vector. Primer set #2 (*NbPDS*_F2 and *NbPDS*_R2) was designed upstream of the region of homology to the endogenous *pds* transcript (Figure 5). QRT-PCR experiments using primer set #2 showed that the endogenous levels of *pds* were significantly decreased (78x) when compared to levels in wild type control.

The cDNA for the real-time PCR experiments was synthesized using BioRad's iScript, which employs both oligo-d(T) and random primers. The use of two QRT-PCR primer sets enabled us to determine that cDNAs were synthesized from the viral vector template causing an elevation in *pds* transcript levels. It appears that viral vector inserts lacking poly-(A)s can also serve as templates for cDNA synthesis using oligo-d(T)

priming, hence the elevated levels observed on the microarray for the pds_{as}-transfection experiments.

It is also important to also consider the issue of posttranscriptional gene silencing. The conventional wisdom has been that viruses are both targets and inducers of PTGS. The phenotype of the pds_{as}-treated plant provides important clues. It is not until 10 dpi that the photobleaching of the leaves is just beginning to progress in the newly forming leaves. This may be related to the stability of the PDS enzyme. At this time point, results of the microarray and QRT-PCR using primer set #1 show that the *tobamoviral* vector has caused a high level of *pds* transcript to accumulate, suggesting that the virus may be capable of evading RNA silencing by the host plant, as well as impeding the degradation of homologous gene transcripts. However, QRT-PCR using primer set #2 shows a reduction in the accumulation of endogenous *pds*. Therefore, while the endogenous transcript is targeted for degradation, the viral genome is not. There is currently a lack of knowledge regarding the amount of the viral-derived RNA that is available in the plant for gene silencing.

Investigations have demonstrated that TMV infection can reverse GFP silencing in leaves of transgenic *N. benthamiana* plants (Voinnet, *et al.*, 1999). In addition, studies of tomato mosaic virus (ToMV) implicate the 130K replicase protein as a suppressor of posttranscriptional gene silencing, potentially blocking the utilization of small interfering RNAs (siRNAs) (Kubota, *et al.*, 2003). TMV may use its replicase protein to suppress

gene silencing in a manner similar to (ToMV), as evidenced by a delay in the phenotype, and an accumulation of viral inserts at the 10 dpi point.

Microarray data show that other genes in the carotenoid biosynthesis pathway are not significantly affected by phytoene accumulation. However, NCED, a putative 9-cis-epoxycarotenoid dioxygenase, showed a 5-fold decrease in transcript level compared to control levels. NCED catalyzes the cleavage of 9-*cis* epoxycarotenoids, a key regulatory step in the abscisic acid (ABA) biosynthesis pathway of higher plants. ABA plays a major role in the adaptation of plants to environmental stress, plant growth and development (Han, *et al.*, 2004), and it is increased in response to attack by a variety of pathogens. The microarray data suggests that the TTO1 PDS^- viral vector may be able to evade host RNA-silencing defenses by downregulating the production of ABA. Recent studies on plant virus suppression mechanisms suggest that in addition to virus-encoded suppressor proteins, the "escape" mechanism used by viruses may be mediated by plant host responses to different types of stress, including heat shock (Taliansky, *et al.*, 2004). Data from additional microarray experiments (not shown) using wild type untransfected plants as controls as well as from published microarray studies of *tobamovirus*-infected plants reveal an upregulation of heat shock proteins, such as Hsp70s (Whitham, *et al.*, 2003).

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Viral Vector Constructs

For the transcriptional profiling experiments, control plants were transfected with TTOSA1 APE pBAD (Kumagai *et al.*, submitted for publication 2005) and experimental plants overexpressing phytoene synthase (*crtB*) were transfected with a modified TTU51 CTP *crtB* (Kumagai *et al.*, submitted for publication 2005). By digesting a TTU51 CTP *crtB* vector with *Kpn*I and *Stu*I, and ligating the 6163 bp fragment to a 4397 bp fragment from a *Kpn*I-*Stu*I digested viral vector 740AT#120 (Kumagai *et al.*, 2002) that carries a ribozyme, a modified viral vector, TMV CTP *crtB*-RZ was developed. The construction of this vector eliminates the need for linearization prior to *in vitro* transcription reactions. The TTO1 PDS$^-$ viral vector (Kumagai, *et al.*, 1995) contains a partial tomato phytoene desaturase cDNA in the antisense orientation that is under the control of the TMV-U1 coat protein subgenomic promoter.

3.5.2 Plant Inoculations and Treatments

N. benthamiana plants were grown from seed and kept under lights at 25C. *In vitro* transcription reactions were performed for TTU51 CTP *crtB* RZ (T7 promoter), and for TTOSA1 APE pBAD and TTO1 PDS$^-$ (SP6 promoter) using Ambion mMessage mMachine *in vitro* transcription kits. At the 6-8 leaf stage of development, two lower leaves were rub-inoculated with *in vitro* transcripts using carborundum. Norflurazon treatments involved application of 5 mL of 10 *u*M norflurazon (Sigma) to the base of *N. benthamiana* plants at the 8-leaf stage.

3.5.3 Labeling and Hybridization for cDNA microarrays

At 10 days post-inoculation, *CrtB*-transfected *N. benthamiana* plant leaf material was ground in liquid nitrogen. Total RNA (25µg) was extracted using Qiagen RNeasy kits, quantified, and differentially labeled with fluorescent cyanine-3 (cy3) or cyanine-5 (cy5) dyes (Amersham, Cat Nos. PA53022, PA55022) using Invitrogen Fluoroscript kit (Cat# L1013-01) for first-strand cDNA synthesis.

For pds_{as}-transfected plants, leaf material at 10 dpi was ground in liquid nitrogen. Total RNA was extracted using TRIzol® (Invitrogen Cat. No. 15596-018), and cleaned using Qiagen RNeasy Clean-Up protocol. Invitrogen SuperScript Direct cDNA Labeling System (Cat# L1015-01) was used for the *PDS* microarray study. Quality and quantity of RNA was checked on a 1% agarose gel, Shimadzu spectrophotometer, or Agilent 2100 Bioanalyzer and cy-dye labeled cDNA was quantified using a Beckmann spectrophotometer. As a control for both microarray studies, RNA from GFP-transfected plants was isolated, quantified, reverse transcribed and labeled with cy5 dye using the same procedures. Equal amounts of labeled treated and control cDNA were mixed and hybridized in replicate to 10K potato cDNA microarrays from TIGR (The Institute for Genomic Research). Hybridization and post-hybridization washes were performed using recommended hybridization conditions found in protocols developed by TIGR. A detailed description of TIGR Potato Microarray optimized methods, the array design, and the cDNA description file used for this study (GenePix Array List, GAL file versions

1and 2) can be found at the following URL:

http://www.tigr.org/tdb/potato/microarray_comp.shtml.

Scanning of the microarrays for the *crtB* experiments was performed using an Affymetrix 286 scanner. Scanning of the microarrays for the *pds* experiments was performed using the BioRad VersArray ChipReader. Raw signal data was combined into text files that were imported into GeneSpring (Agilent, formerly Silicon Genetics) software for background subtraction, normalization by LOWESS and analysis. Gene lists were generated for 2-fold upregulated genes

3.5.4 Quantitative Real-time PCR (QRT-PCR) Primer Design

QRT-PCR primer sets were designed using BioRad's Beacon Designer 2.1. Sense and antisense primers for quantitative real-time PCR for *psy* and *pds* were derived from U.S. patent #5,539,093, sequence #1 and sequence #3, respectively. The forward primer for phytoene synthase is *Nb*Psy1_F (5' GCTGGTACGGTTGGGTTGAT 3'); reverse primer for phytoene synthase is *Nb*Psy1_R (5' GCATGTGCTAATTCATCTTGAGGT 3'), product length = 191 bp, Tm = 86.1°C. *PDS* primer set #1 (Figure 2 and 5) amplifies a region of *N. benthamiana pds* that has homology to the viral vector tomato *pds* insert. The forward primer for phytoene desaturase is *Nb*Pds3_F1 (5' CGGTTTAAGTGTTAAGGACTGGAT 3') and the reverse primer is *Nb*Pds3_R1 (5' AGCTCGTCAGGGTTTATGAAGT 3'), product length = 111 bp, Tm = 85.6°C. Primer set #2 was designed to a region of endogenous *pds* from position 483 to position 660. The forward primer is *Nb*Pds3_F2 (5' TGGGGCATAAGTTAAGGATTCG 3') and

the reverse primer is *Nb*Pds3_R2 (5' TTAGTTGGGCGTGAGGAAGT 3'), product length = 178 bp, Tm = 86.3°C.

Several sets of primers for controls were used: ribulose-1,5-bisphosphate carboxylase (rubisco) small subunit (*Nicotiana sylvestris* GenBank #X01722) forward primer is RubSSU_F (5' ACAAGAAGAAGTACGAGACTCTCT 3') and reverse primer is RubSSU_R (5' CGAACATAGGTAGCTTCCACATG 3'), product length = 204, Tm = 87.1°C; ubiquitin (*N. benthamiana* GenBank #CK294769) forward primer is *Nb*UBI_F (5' CAACATCCAGAAGGAGTCTACC 3') and ubiquitin *Nb*UBI_R (5' GCCAGCGAAAATCAACCTCT 3'), product length = 193 bp, Tm = 85°C; and 60S Ribosomal Protein RPL37a_F (5' AGGTTAGCCAGCATAGCAAGT 3') and 60S Ribosomal ProteinPL37a_R (5' CCGCAATCTTTACATCCCCAAA 3'), product length = 94, Tm = 84.5°C.

3.5.5 cDNA Synthesis and QRT-PCR Assays

RNA that was extracted for microarray studies at 10 dpi was digested with amplification grade DNase I (Invitrogen Cat. No. 18068-015) and used for first strand cDNA synthesis for QRT-PCR assays. cDNA was synthesized using iScript cDNA Synthesis kit (BioRad Catalog #170-8890) for treated and control plants, in which RNA is primed with oligo $dT_{(20)}$ and random primers. A ten-fold dilution series was prepared for standard curves. Reaction mixtures for standards and unknowns were comprised of BioRad SYBR Green Supermix, cDNA template, and QRT-PCR primers at 200 nM concentration, and applied to a 96-well plate. QRT-PCR and melt curve reactions were performed on a BioRad

iCycler using the thermal protocol: Cycle 1: 3 mins at 95°C; Cycle 2: 40 repeats of 10 sec at 95°C, 15 sec at optimum Tm of primers; Cycle 3: 1 min at 95°C; Cycle 4: 1 min at 55°C; Cycle 5: 80 repeat of 10 sec at 55°C, increasing 0.5°C. Efficiencies were determined for target and reference genes, when possible, and Pfaffl's method of calculating fold change ratios was used (Pfaffl, 2001).

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4. TRANSCRIPTIONAL CHANGES IN THE ARF MULTIGENE FAMILY INDUCED BY TOBAMOVIRAL TRANSFECTION

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FOREWORD

ADP-ribosylation factors (ARFs) are members of a multigene family of GTP-binding proteins that are highly conserved at the nucleotide level across the kingdoms. In plants, ARF-1 is known to have a role in membrane trafficking, recruiting coatomer coats, and interacting with phospholipase D in signaling pathways. In a forward genomics screen using recombinant *tobacco mosaic virus* vectors, Kumagai *et al.* demonstrated that an *Arabidopsis thaliana ARF-1* expressed in antisense orientation leads to a severely stunted phenotype in *Nicotiana benthamiana* plants (U. S. Patent #6,426,185).

My contributions are an extension of this work to explore the use of viral vectors in a functional genomic study of a multigene family. My experiments were designed to understand the increasing severity of the dwarf phenotype by examining the transcriptional changes induced in ARF_{as}-transfected *N. benthamiana* plants. Using cDNA microarray and quantitative real-time PCR analyses, a time-course investigation was conducted over a 20-day period, in parallel with a Western blot analysis, to determine the levels of *ARF*-1 mRNA and protein, as well as to examine changes in transcript accumulation of other genes.

4.1 ABSTRACT

ADP-ribosylation factors (ARFs) are members of a multigene family of GTP-binding proteins that are highly conserved at the nucleotide level across the kingdoms. In plants, ARF-1 is known to have a role in membrane trafficking, recruiting coatomer coats, and interacting with phospholipase D in signaling pathways. A forward genomics screen using recombinant *tobacco mosaic virus* vectors revealed that an *Arabidopsis thaliana ARF-1* expressed in antisense orientation leads to a severely stunted phenotype in *Nicotiana benthamiana* plants. To understand changes in gene expression induced by *tobamoviral* transfection, transcriptional profiling was performed using a heterologous cDNA microarray and quantitative real-time PCR. A microarray time-course analysis shows that transcripts of the viral vector *ARF* insert accumulate to high levels in the plant, indicating that the virus is escaping RNA silencing. Over a 20-day period, there is an accumulation of endogenous plant transcripts that have a gene expression pattern that parallels that of the viral vector *ARF* transcript. Detection of ARF proteins by Western analysis shows that ARF protein levels gradually decline and that all ARF family members are knocked down by 20 days post-inoculation. Phenotypically, the plant shows a progression in stunted leaf development and signs of necrosis at 20 dpi, at which point, 383 genes are downregulated. A Welch ANOVA identified 16 genes for further characterization that may play a role in G-protein signaling. This study demonstrates that viral vector technology can be combined with transcriptional profiling for a functional genomic analysis of a multigene family.

4.2 INTRODUCTION

Tobamoviral vectors have been developed for gene silencing studies, in addition to the heterologous expression of proteins. Transfected plants produce phenotypic or biochemical changes that result from endogenous gene silencing or overexpression. In forward genomic screens, construction of cDNA libraries in viral vectors can be used to systematically analyze large numbers of infected plants (Kumagai *et al.,* 2002; Baulcombe, 1999). By sequencing the nucleic acid insert in the cDNA viral vector, phenotypes can be associated with gene sequences. In this investigation, a gene from *Arabidopsis thaliana* encoding an ADP-ribosylation factor (*ARF-1*) in the antisense orientation was identified in a forward genomics screen (Kumagai, 2002).

The *Arabidopsis* genome contains 93 genes that encode small GTP-binding proteins, including RAB, RHO, RAN and ARF GTPases (Vernoud *et al.*, 2003). These proteins function as molecular switches that cycle between "active" and "inactive" states by binding to and hydrolyzing GTP. ARFs are highly conserved at the nucleotide level among mammals (Price *et al.*, 1996) and other eukaryotic organisms (Kahn *et al.*, 1991). In plants, published reports have focused mainly on the role of ARF within the secretory pathway, deciphering its role in intracellular trafficking (Memon, 2004). Studies conducted in *A. thaliana* protoplasts using dominant-negative mutant studies implicate ARF-1, but not ARF-3 in intracellular trafficking (Lee *et al.*, 2002) and in *Nicotiana* protoplasts, dominant-negative mutants demonstrate a role for ARF-1 in the vacuolar-

sorting route (Pimpl *et al.*, 2003). In *Arabidopsis* and tobacco-cultured cells, dominant-negative mutants reveal ARF's role in the maintenance of the Golgi organization (Takeuchi *et al.*, 2002). Expression of ARF-1 in an antisense orientation in *Solanum tuberosum* results in alteration in plant phenotype, an increase in starch accumulation in tubers, and an increase in glucose synthesis in sink organs. Repression of ARF in potato results in 14-3-3 gene activation (Zuk *et al.*, 2003).

In mammals, ADP ribosylation factors function in a number of different roles in addition to its role in the secretion. ARF-1 is known to participate in actin remodeling, pathogenic mechanisms, cytoskeletal organization, and signal transduction (Randazzo *et al.*, 2000). It also functions in cell proliferation and is highly studied for its role in cancer development (Colicelli, 2004; Randazzo *et al.*, 2000; Zuk *et al.*, 2003). In addition to their role in secretion, plant ARFs may also function as targets of signal transduction in the cell periphery, as is reported for mammalian ARFs. In this investigation, microarrays will be used to filter the global gene expression profile to a small subset of genes to conduct further studies related to ARF's role in signal transduction.

The use of microarrays in functional genomics studies (Stuart *et al.*, 2003) is based on the hypothesis that genes traveling in the same pathway will show similar gene expression changes when a system is perturbed. In this investigation, this "guilt by association" approach (Quackenbush, 2003) was used to analyze gene expression changes occurring in plants transfected with antisense *ARF* to identify genes associated

with G-protein signaling. Plants were studied over time using microarray and quantitative real-time PCR technologies to understand the role of the ARF family and to associate phenotype with gene expression changes. In addition, the impact of the viral vector as it replicates and accumulates its *ARF* antisense transcript was evaluated.

4.3 RESULTS

4.3.1 Construction of an *Arabidopsis thaliana* cDNA Library in an RNA Viral Vector

An *Arabidopsis thaliana* CD4-13 cDNA library (*Arabidopsis* Biological Resource Center) with inserts of 0.5-1 kb was digested with *Not*I, and subcloned into the *tobacco mosaic virus* vector, pBS740, under the transcriptional control of a tobamovirus subgenomic promoter. Following transformation, approximately 2000 recombinant plasmid DNAs were isolated from C600 cells from overnight cultures using a BioRobot (Qiagen), and infectious RNAs from 430 independent clones were directly applied to the leaves of *N. benthamiana* plants. One to two weeks after inoculation, transfected plants were visually monitored for changes in growth rates, morphology, and color. Plants that were severely stunted were selected for further analysis.

4.3.2 Forward Genomics Screen Reveals a Gene Encoding a GTP Binding Protein

DNA sequence analysis of one of the clones causing a stunted phenotype contained an

Arabidopsis GTP binding protein open reading frame (ORF) in the antisense orientation (Figure 4.1). A TMV-U1 subgenomic promoter of the viral vector that is located within the minus strand of the 30-kDa ORF controlled the synthesis of the CD4-13 antisense subgenomic RNA.

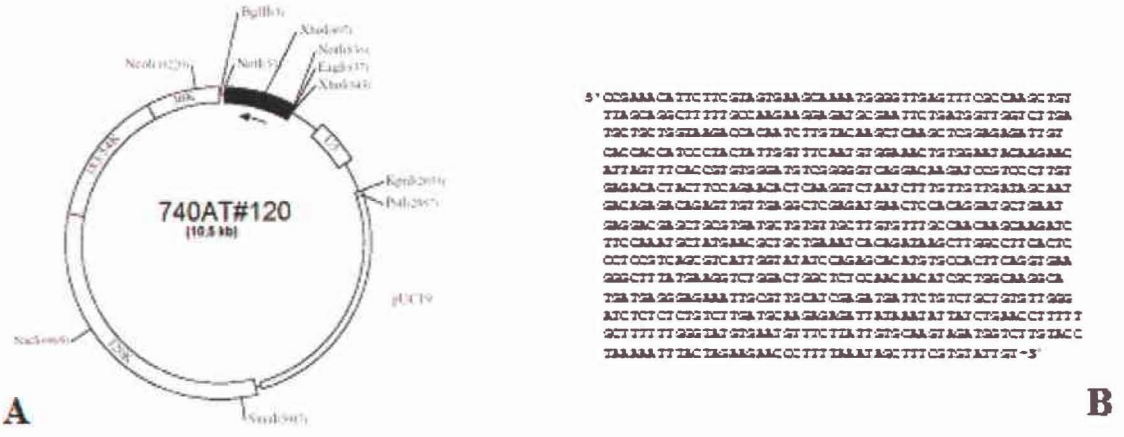

Figure 4.1. ADP Ribosylation Factor-1 Expression Vector. A) The viral vector 740 AT#120 contains the TMV-U1 126-, 183- and 30 kDa ORFs, the TMV U5 coat protein, T7 promoter, part of the pUC19 plasmid, and B) an *Arabidopsis thaliana ARF-1* cDNA in antisense orientation.

The 782 bp *Not*I fragment of 740 AT #120 containing the ADP-ribosylation factor (*ARF*) cDNA was characterized. Nucleotide sequence analysis and amino acid sequence comparisons were performed using DNA Strider, PCGENE and National Center for Biotechnology Information (NCBI) Blast programs. 740 AT #120 contained an open reading frame (ORF) in the antisense orientation that encodes a protein of 181 amino

acids with an apparent molecular weight of 20,579 daltons. The nucleotide sequence from 740 AT #120 exhibits a high degree of homology (81-84% identity and positive) to rice, barley, carrot, corn and *A. thaliana* DNA encoding *ARFs*. The amino acid sequence derived from 740 AT #120 exhibits an even higher degree of homology (96-98% identity and 97-98% positive) to ARFs from rice, carrot, corn and *A. thaliana*. The homology of viral vector *At ARF* to a cloned partial *N. benthamiana ARF* was 85%, and to *N. benthamiana* sequence (Genbank Accession #CN743800) was 84%. The phenotype of the plant suggested that this level of homology was sufficiently high for a virus-induced gene silencing (VIGS) knock-down of the transcript in the cytoplasm.

A heterologous potato cDNA microarray system developed by The Institute for Genomic Research (TIGR) was used to determine the impact of ARF_{as}-transfection on *N. benthamiana.* Because a highly homologous sequence was used to induce a VIGS phenotype, an analysis of the homology of *ARF-1* sequences of *N. benthamiana* and of the *Arabidopsis* viral insert to *Solanum tuberosum* was required (Table 4.1). There were five annotated potato clones, spotted in duplicate, that were identified as targets for *ARF* transcript hybridization. Although it was recognized that there were additional sequences on the microarray to which *ARF*s could potentially hybridize, there was not sufficient annotation to determine this. Limited information regarding *N. benthamiana ARF* mRNA sequences was found in several online databases, including NCBI, TIGR *Nb* Gene Index, and USPTO (published patents). This sequence information enabled an analysis of *N. benthamiana* homology to potato cDNA clones on the microarray.

S. tuberosum ARF clones on cDNA microarray	Viral vector At ARF-1 SeqID20, US Patent #6,426,185 (773 bp)	Nb ARF (391 bp); SeqID27, US Patent # 6,426,185*	Nb ARF (582 bp) (5') EST CN743800 SAL_US006xj22f1.a. [gi:47508797]	Nt ARF (553 bp) SeqID136, Patent #WO03012096
ARF (BQ508962)	442/528 (83%)	332/387 (85%)	449/488 (92%)	278/307 (90%)
ARF (BQ512403)	429/527 (81%)	328/390 (84%)	399/486 (82%)	211/252 (83%)
ARF (BQ519021)	315/376 (83%)	288/301 (95%)	283/328 (86%)	220/263 (83%)
Probable ARF (BQ117540)	43/53 (81%)	NS	NS	NS
ARF-like (ARL) (BQ121152)	NS	NS	NS	NS
740 AT #120 Viral Vector At ARF-1 (SeqID20)	100%	332/390 (85%)	412/487 (84%)	222/265 (83%)

Table 4.1. Identities from *ARF* BLAST Alignments. Homology of *At ARF* and *Nicotiana ARF* sequence to clones on the TIGR potato cDNA microarray. (*Same as SeqID14, US Patent # 6,700,040); "NS" indicates no significant homology by NCBI Blast (bl2seq) search. *At* (*Arabidopsis thaliana*); *Nb* (*Nicotiana benthamiana*); *Nt* (*Nicotiana tabacum*).

Over 80% homology over several hundred bases was found between the *Arabidopsis* and *Nicotiana ARF* sequences to three of the clones on the potato microarray (BQ508962, BQ512403, and BQ519021), suggesting that cross-hybridization could occur

on the microarray. Previous studies have estimated that cross-hybridization can occur if related genes have greater than 70-80% sequence identity in *Arabidopsis* (Girke *et al.*, 2000) and about 80% identity in maize (McGonigle *et al.*, 2000).

4.3.3 Microarray Time Course Analysis

A time-course analysis was conducted at 4-day intervals over a 20-day period to investigate gene expression changes due to the effects of antisense *ARF-1*. Phenotypic appearance of the plant showed a progression of curled and stunted leaf development, and necrosis was apparent by 20 dpi (Figure 4.2). Leaves were harvested beginning at 4 dpi, and RNA was extracted for cDNA synthesis for both *ARF*-treated and GFP-treated plants.

Figure 4.2. Phenotype of *ARF*-1_{as}-transfected *N. benthamiana plants*. Clockwise, 4dpi, 8dpi, 12 dpi, 16 dpi, and 20 dpi (center).

Differentially labeled cDNA from treated and control plants was mixed and hybridized to replicate cDNA potato micorarrays from each time point. Microarrays were scanned using a BioRad VersArray ChipReader, and raw signal data for treated and control samples was imported into GeneSpring for analysis. Background signals were subtracted and normalization was performed using LOWESS (locally weighted scatterplot smoothing). Genes were filtered for expression and confidence, and lists were generated for genes exhibiting over 2-fold expression, p-value < 0.05. An analysis of all time points of a 2-fold or greater transcript accumulation revealed a short list of "upregulated" genes (Table 4.2). A multiple testing correction (Benjamini and Hochberg false discovery rate) was used minimize significant genes due to chance.

GENBANK ACCESSION #	TIGR ANNOTATION
BQ519023	F5I14.2 gene product (Arabidopsis thaliana)
BQ515350	ATP synthase beta subunit (Lycopersicon esculentum)
BQ508962	ADP Ribosylation factor (Oryza sativa japonica cultivar group)
BQ506989	Probable shaggy-like protein-kinase dzeta [imported] (Arabidopsis thaliana)
BQ511644	*similar to* UP|Q8X085 (Q8X085) Related to pre-mRNA splicing SRp75 (Predicted protein), *partial (6%)* EST619059
BQ514939	ATP synthase beta subunit (*Primula gaubaeana*)
BQ519021	ADP-ribosylation factor [imported], pepper
BQ512403	ADP-ribosylation factor [imported], rice

Table 4.2. Upregulated genes in ARF_{as}-treated plants. Potato clones showing 2-fold or greater hybridization of *N. benthamiana* transcript for all time points, t-test p-value < 0.05. The cross-gene error model was active. The Benjamini and Hochberg MTC is a false discovery rate type of error control.

Three clones on the potato array (BQ508962, BQ519021, and BQ512403) with ARF annotations showed high signal intensity levels over time compared to GFP-transfected controls. Transcripts from transfected *N. benthamiana* hybridized to these clones and were progressively and significantly elevated until 20 dpi, at which point accumulation dropped (Table 4.3). It appeared that viral vector transcripts were hybridizing to the ARF clones on the microarray.

Potato cDNA Clone	**Normalized Ratio (p-value)**				
	4dpi	**8 dpi**	**12 dpi**	**16 dpi**	**20 dpi**
ARF (BQ508962), homology to rice	15 (0.17)	**16 (0.01)**	**63 (0.0005)**	**310 (0.05)**	48 (0.29)
ARF (BQ512403), homology to rice	10 (0.23)	**13 (0.002)**	128 (0.07)	**92 (0.05)**	5 (.71)
ARF (BQ519021), homology to pepper	11 (.23)	**13 (0.03)**	19 (0.17)	39 (0.07)	7 (0.64)

Table 4.3. Normalized ratios of *ARF* transcripts in *ARFas*-transfected plants compared to GFP-transfected plants. Signal intensities were normalized using LOWESS (GeneSpring).

ARF (BQ508962) was selected for further analysis, and a condition tree of "like ARF" was generated (Figure 4.3) to determine plant genes that paralleled the accumulation of virus-derived transcripts.

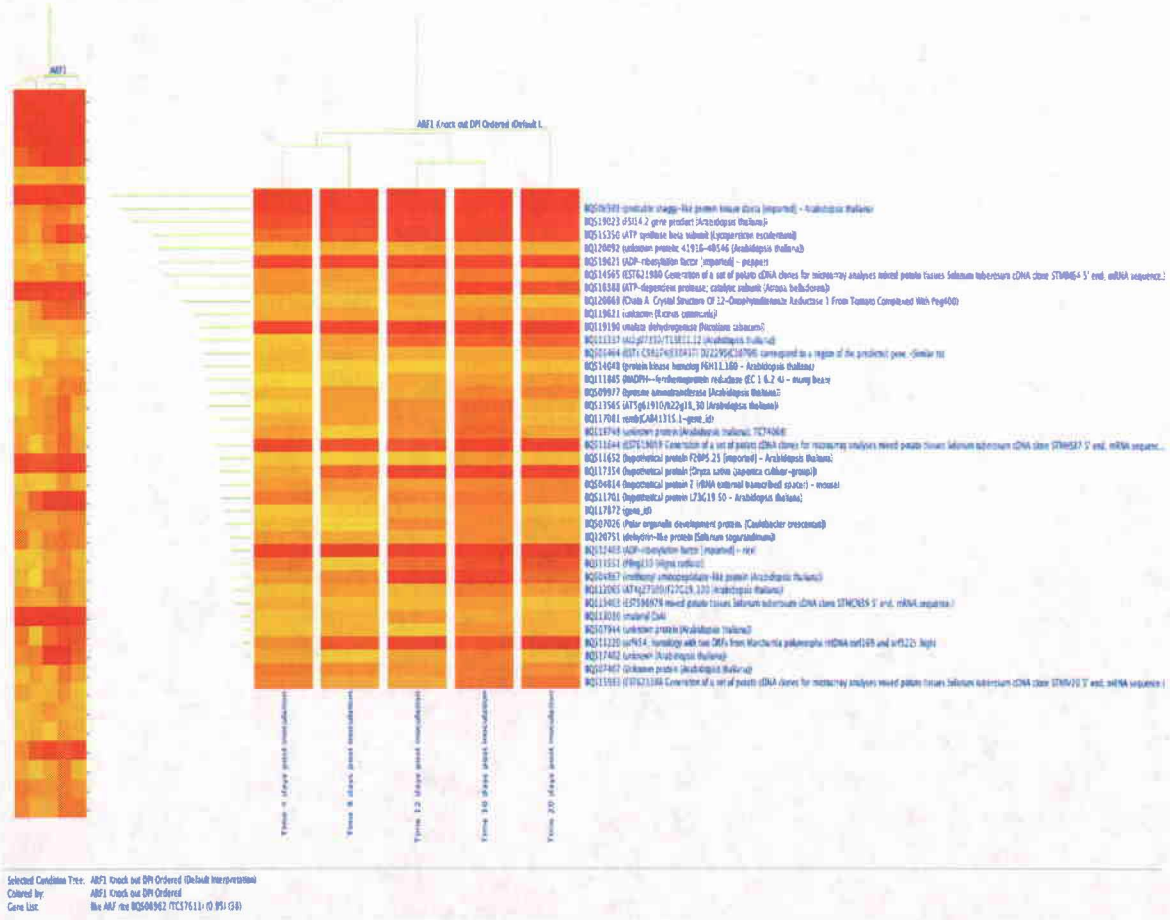

Figure 4.3. Condition tree of "Like ARF".

Genes with an expression pattern similar to ARF over the 20-day period were clustered using a similarity measure for standard correlation. Table 4.4 shows a list generated by a K-means clustering of "Like ARF (BQ508962)" based on the log of ratio mode (weight 1.0), for five clusters.

set1	
BQ111357	At2g07350/T13E11.12 {Arabidopsis thaliana}
BQ111885	NADPH--ferrihemoprotein reductase (EC 1.6.2.4) - mung bean
BQ113030	malonyl CoA
BQ505464	ESTs C99174(E10437) D22295(C10709) correspond to a region of the predicted gene.~Similar to
BQ509977	tyrosine aminotransferase {Arabidopsis thaliana}
BQ511220	orf454; homology with two ORFs from Marchantia polymorpha mtDNA (orf169 and orf322) high
set2	
BQ112065	AT4g27500/F27G19_100 {Arabidopsis thaliana}
BQ113403	EST598979 mixed potato tissues Solanum tuberosum cDNA clone STMCN39 5' end, mRNA sequence.
BQ120092	unknown protein; 41916-40546 {Arabidopsis thaliana}
BQ120669	Chain A Crystal Structure Of 12-Oxophytodienoate Reductase 1 From Tomato Complexed With Peg400
BQ506989	probable shaggy-like protein kinase dzeta [imported] - Arabidopsis thaliana
BQ507944	unknown protein {Arabidopsis thaliana}
BQ514565	EST621980 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIM64 5' end, mRNA sequence.
BQ519021	ADP-ribosylation factor [imported] - pepper
BQ519023	F5I14.2 gene product {Arabidopsis thaliana}
set3	
BQ117081	emb|CAB41315.1~gene_id
BQ117354	hypothetical protein {Oryza sativa (japonica cultivar-group)}
BQ117872	gene_id
BQ504867	methionyl aminopeptidase-like protein {Arabidopsis thaliana}
BQ507026	Polar organelle development protein. {Caulobacter crescentus}
BQ512403	ADP-ribosylation factor [imported] - rice
BQ513565	AT5g61910/k22g18_30 {Arabidopsis thaliana}
BQ515350	ATP synthase beta subunit {Lycopersicon esculentum}
set4	
BQ119190	malate dehydrogenase {Nicotiana tabacum}
BQ119621	unknown {Ricinus communis}
BQ120751	dehydrin-like protein {Solanum sogarandinum}
BQ504814	hypothetical protein 2 (rRNA external transcribed spacer) - mouse
BQ507407	Unknown protein {Arabidopsis thaliana}
BQ511644	EST619059 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMHS87 5' end, mRNA sequence.
BQ511701	hypothetical protein L73G19.50 - Arabidopsis thaliana
BQ514048	protein kinase homolog F6H11.160 - Arabidopsis thaliana
BQ515983	EST623398 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIV20 5' end, mRNA sequence.
set5	
BQ119748	unknown protein {Arabidopsis thaliana}; TC74068
BQ508962	ADP-ribosylation factor {Oryza sativa (japonica cultivar-group)}
BQ511551	PBng110 {Vigna radiata}
BQ511652	hypothetical protein F20P5.25 [imported] - Arabidopsis thaliana
BQ516388	ATP-dependent protease; catalytic subunit {Atropa belladonna}
BQ517402	unknown {Arabidopsis thaliana}

Table 4.4. Gene list based on K-means clustering of "Like ARF (BQ508962)"

An analysis of downregulated genes was also conducted. At 20 dpi, downregulated genes cluster into 5 distinct sets (Figure 4.4). Between 16 and 20 dpi, endogenous plant gene transcript levels drop sharply in all sets, paralleling the progressively stunted phenotype seen in the plant.

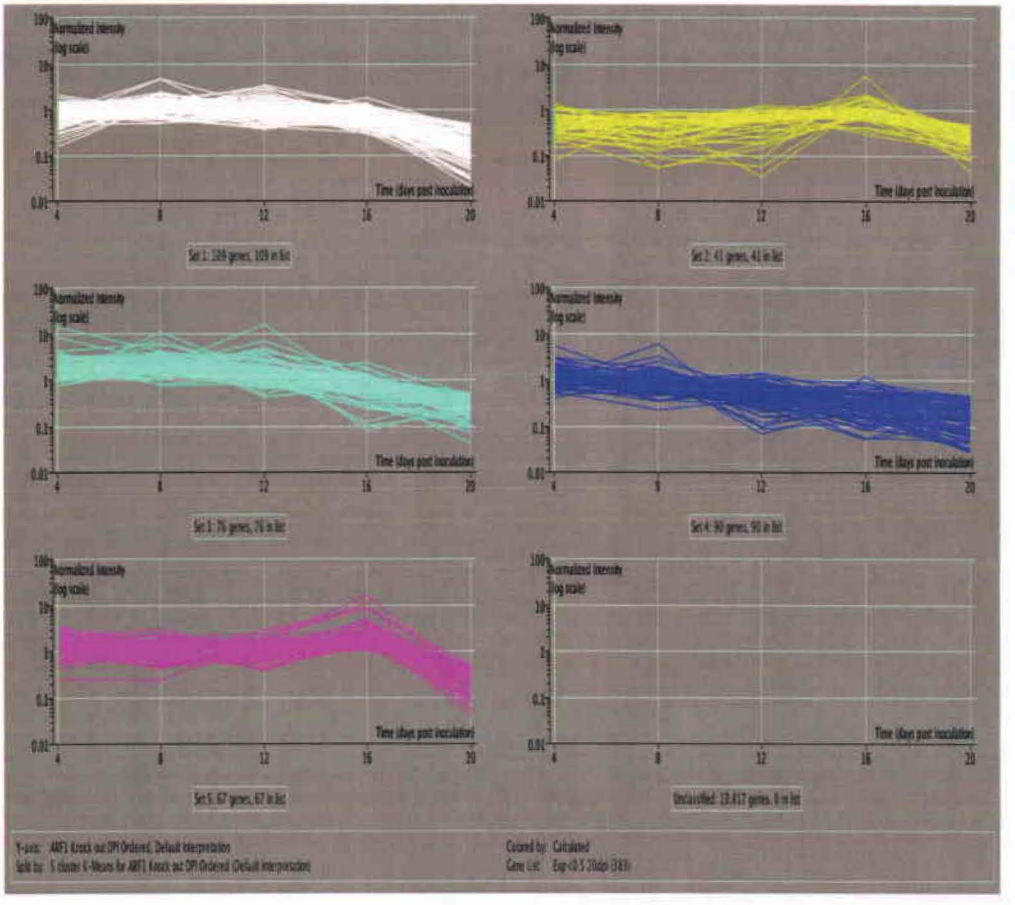

Figure 4.4. Downregulated genes, 20 dpi. Genes downregulated by *ARF*as treatment cluster into 5 distinct groups.

Set 5 of the cluster was selected for further analysis. A Welch analysis of variance (ANOVA), a parametric test, in which variances are not assumed equal, was performed to

determine genes at 20 dpi that have statistically significant differences from other time points (Table 4.5). This restriction tested 67 genes, with about 3 genes expected to pass the restriction by chance, using a p-value cutoff of 0.05, and yielded a list of 16 genes that are targeted for further characterization.

BQ114084	1.06E-04	BURP domain-containing protein {Bruguiera gymnorrhiza}
BQ113521	0.00158048	50S ribosomal protein L29 (Boehm and Bonifacino, 2001)
BQ113359	0.0057062	Chlorophyll A-B binding protein 13 chloroplast precursor (LHCII type III CAB-13). [Tomato]
BQ120697	0.00942126	Ribulose bisphosphate carboxylase/oxygenase activase chloroplast precursor (RuBisCO activase) (RA).
BQ113095	0.01147712	H-Protein precursor {Flaveria pringlei}
BQ120783	0.01346456	unknown {Arabidopsis thaliana}; TC65789
BQ115111	0.02458749	acyl CoA reductase-like protein - Arabidopsis thaliana
BQ515352	0.02706783	photosystem II protein X precursor - Arabidopsis thaliana
BQ509988	0.02769142	superoxide dismutase (EC 1.15.1.1) (Fe) - curled-leaved tobacco
BQ115182	0.0306387	unknown protein {Arabidopsis thaliana}
BQ113829	0.03450371	unknown protein {Arabidopsis thaliana}
BQ119924	0.03467785	EST605500 mixed potato tissues Solanum tuberosum cDNA clone STMEN55 5' end, mRNA sequence.
BQ114077	0.03814301	Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) (NAD(P)H-dependent glycerol-3-phosphate
BQ515383	0.04117716	putative 60S RIBOSOMAL PROTEIN L36 {Oryza sativa (japonica cultivar-group)}
BQ118924	0.0430134	oligouridylate binding protein {Nicotiana plumbaginifolia}; TC58199
BQ513474	0.04482397	EST620889 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIF38 5' end, mRNA sequence.

Table 4.5. Welch ANOVA of Downregulated Cluster, 20 dpi, Set 5

4.3.4 Quantitative Real-Time PCR Assays

ARF1$_{as}$-transfection of plants produces an antisense *ADP-Ribosylation Factor* 1 that was predicted to cause a knock-down of endogenous transcript in the cytoplasm, based on the stunted appearance of the plant. However, microarray analysis shows that levels of *ARF*s are elevated over a twenty-day post-inoculation period compared to the GFP-transfected control plants. It was recognized that hybridization of the viral vector may have contributed to elevated levels in the microarray studies. To determine whether *ARF*-1 endogenous transcripts were being degraded by VIGS, quantitative real-time PCR (QRT-PCR) primers were designed such that they would not amplify the viral vector insert, using sequence information from the potato BQ508962 ARF rice ORF. The forward primer had 100% alignment with Nb *ARF* CN743800 and *Nt ARF*, and the reverse primer showed 95% alignment with *Nt ARF*. The QRT-PCR primers did not show significant homology by NCBI BLAST alignment to the 740AT #120 viral vector *ARF* insert. A 1.5% agarose gel electrophoresis of reverse transcription-PCR (RT-PCR) amplified products demonstrates that the QRT-PCR primers amplify the *ARF*$_{as}$-treated plant cDNA, but not the viral vector cDNA plasmid (Figure 5, lanes 6 and 7). To test the quality of the treated and control cDNA, primers that amplify ubiquitin were used, producing a 200 bp amplicon (Figure 4.5, lanes 1-4).

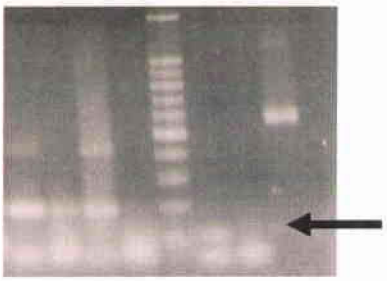

Figure 4.5. 1.5% Agarose Gel Electrophoresis Gel of Reverse Transcription PCR reaction products. Performance of QRT-PCR target and reference primers in *ARF*-1_{as}-transfected *Nb* plants at Tm of 58C. Lanes 1-4, QRT ubiquitin reference primers as follows: Lane 1) Nb-ARF_{as}-transfected cDNA; Lane 2) Nb GFP-transfected cDNA; Lane 3) Nb WT cDNA; Lane 4) No template control (water). Lanes 6-9, QRT ARF1 primers as follows: Lane 6) *Nb ARF*-1_{as}-transfected; Lane 7) Viral vector 740 AT #120; Lane 8) Viral vector 740 AT #120 amplified with cloning primers, ARF1M1S and ARF1A180A (amplicon = 540 bp); Lane 9) No template control (water). Lane 5 shows Promega 100 bp ladder.

A relative quantitative real-time PCR (QRT-PCR) was then performed at the Tm optimized in the RT reaction to examine the abundance of *ARF*-1 mRNAs in transfected plant leaves. cDNAs synthesized from RNA at 12 dpi from both *ARFas*- and GFP-transfected plants were used as template for standards and unknowns in the QRT reactions. Pfaffl method calculations (Pfaffl, 2001) that take into account efficiencies of amplification for both target and reference genes show a reduction of endogenous *ARF* compared to that of GFP-transfected controls. In ARF_{as}-treated plants, efficiency of amplification of the target was 97.9% (correlation coefficient 0.998, slope –3.374). Efficiency of amplification of the reference gene could not be obtained. Calculations

using delta-delta Ct showed that transcript levels of endogenous *ARF* were 2.4x lower that level of ARF in control samples (Figure 4.6a). In a second QRT assay, efficiency of amplification of the target in ARF_{as}-treated plants was 98.5% (correlation coefficient 0.986, slope –3.358). Efficiency of amplification of the reference gene was 81% (correlation coefficient .992, slope –3.881). Calculations using the Pfaffl method showed that transcript levels of endogenous *ARF* were again, 2.4x lower that level of ARF in control samples (Figure 4.6b). Melt curve analyses show *ARF* amplification products melted at predicted Tm.

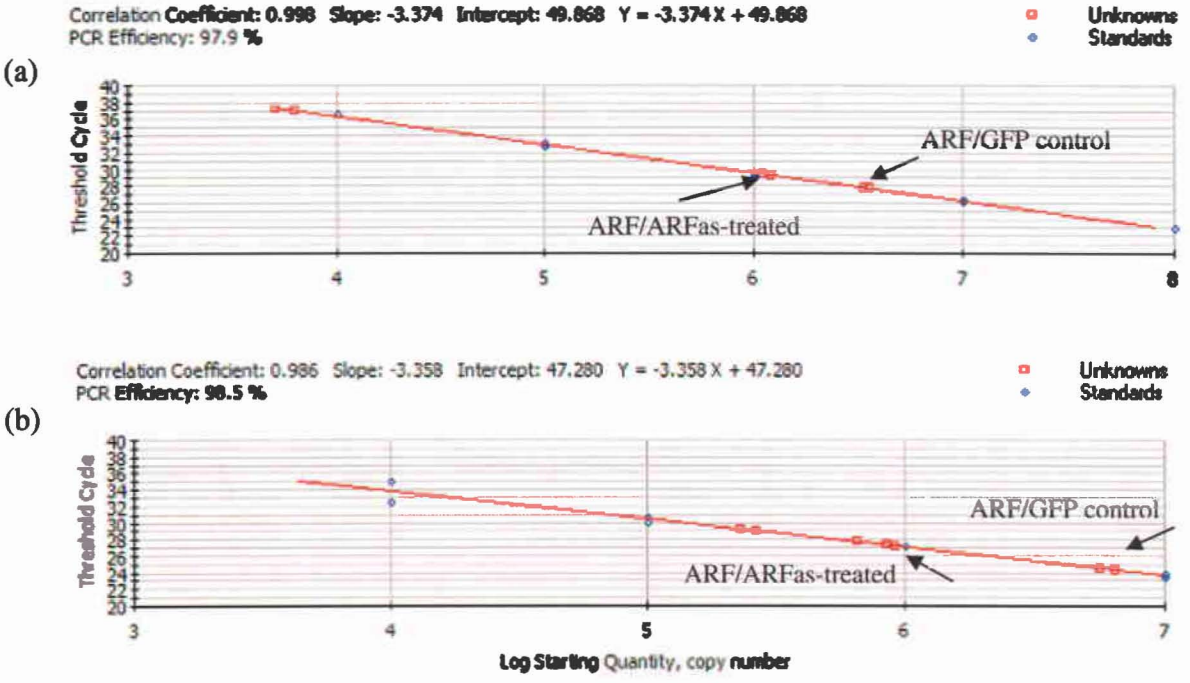

Figure 4.6. QRT-PCR graphical analysis of *ARF* transcript accumulation at 12 dpi, compared to GFP-transfected controls.

4.3.5 Western Blot Analysis

A new viral vector, TTOSA1 ARF1+ was constructed to overexpress *ARF*. TTOSA1 ARF+-transfected plants and uninfected wild type *N. benthamiana* that express basal levels of *ARF*-1 were selected as controls for a Western blot analysis. Reverse-transcription assays were performed to verify transcript accumulation in the TTOSA1 ARF+-transfected plants (Figure 4.7). In addition, an *Arabidopsis thaliana* ARF-1 T-DNA insertion mutant (Salk _136703) was also assayed to determine the ability of the antibody to detect other members of the ARF family in a plant species.

Figure 4.7. 1% Agarose Gel Electrophoresis of Reverse-Transcription PCR products shows amplification of *ARF*-1 in overexpression (left), uninfected (central), and antisense-transfected (right) *N. benthamiana* plants using ARF1M1S and ARF1A180A primers. Lane 1) NEB 1 kb ladder; Lanes 2 and 3) *ARF*-1+ transfected plant cDNA (7 dpi); Lane 4) *Nb* uninfected WT cDNA; Lane 5) *ARF*-1+ transfected plant cDNA (35 dpi); Lane 6) *ARF*-1_{as} -transfected plant cDNA (8 dpi).

Crude protein extracts from each time point were assayed for the presence of ARF protein (Figure 4.8). The primary antibody used for the protein blots was a mouse

monoclonal that detects ARF1, ARF3, ARF5, and ARF6, and ARF4 to a lesser degree. This antibody is known to be effective in other species, but had not been tested in plants.

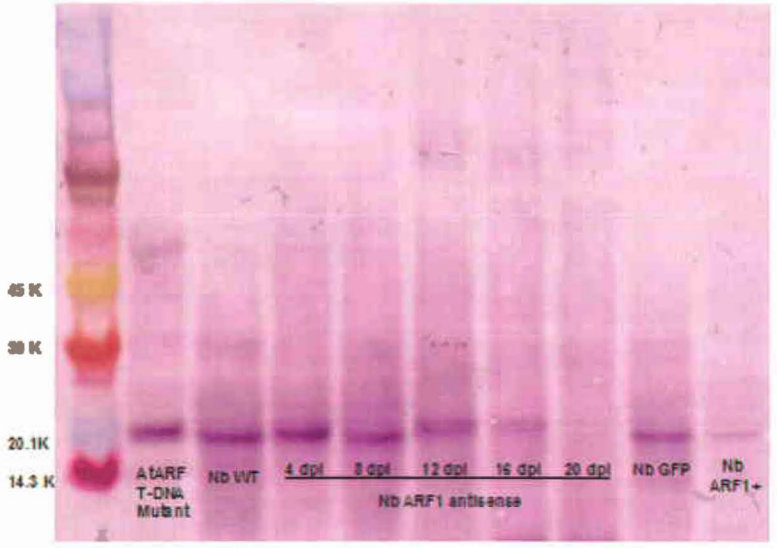

Figure 4.8. Western analysis of ARF1 leaf proteins (crude extract). Approximately 30 µg protein loaded per well. Lane 1. Molecular weight ladder (Amersham Biosciences RPN756PS Rainbow Marker) ; Lane 2. *Arabidopsis thaliana* ARF1 T-DNA Insertion Mutant (Salk _136703); Lane 3. *N. benthamiana* uninfected wild type control; Lanes 4-8. *N. benthamiana* transfected with *A. thaliana* antisense ADP-Ribosylation Factor 1, 4 dpi, 8 dpi, 12 dpi, 16 dpi, 20 dpi; Lane 9. *N. benthamiana* transfected with GFP, 16 dpi; Lane 10. Nb ARF1+ sense construct. (12.5% Polyacrylamide Gel Electrophoresis. Affinity Bioreagents ARF-1 mouse monoclonal primary antibody. Alkaline phosphatase secondary antibody.)

4.4 DISCUSSION

N. benthamiana plants develop a severely stunted phenotype of the newly developing leaves when transfected with a *tobamoviral* vector carrying an antisense insert of a partial cDNA of *ADP Ribosylation factor*-1 (*ARF*-1). This suggests that an *Arabidopsis* antisense transcript with homology to an endogenous target can hinder the expression of a gene in *N. benthamiana.* Transcriptional profiling was conducted in an attempt to correlate plant phenotype and gene function.

Information on the genetics of *N. benthamiana* is limited; however, USPTO, NCBI, and TIGR databases were useful sources of information for *ARF* gene sequences. Oligonucleotide-based GeneChips™ and cDNA micorarrays are not commercially available for *N. benthamiana.* Instead, a heterologous potato cDNA microarray system from TIGR was employed, enabling a large amount of gene expression data to be extracted. Hybridization information provided insight into the *N. benthamiana* genome. By cross-referencing gene sequences of potato in the TIGR database to which treated plants hybridized, QRT-PCR primers were developed that amplify *ARF* transcripts in *N. benthamiana.* Therefore, target validation of the microarray data using QRT-PCR is facilitated. Amplification of transcripts in this plant also enables gene-specific information to be acquired in subsequent cloning and sequencing experiments.

At 4, 8, 12, 16, and 20 days post-inoculation, RNA was extracted from transfected *N. benthamiana* plants for cDNA synthesis and hybridized to the potato microarrays. The cDNA microarray data showed that over time, ADP-ribosylation factors show the highest accumulation of transcript. While RNA was primed with oligo-d(T) to avoid synthesis of cDNA from the viral vector, microarray data suggested that the highly elevated levels of *ARF* transcript were due to viral vector contributions. It was also possible that other members in the ARF family that share homology to *ARF*-1 were hybridizing to the arrays.

To acquire additional information, QRT-PCR reactions were performed. Conventional reverse-transcription PCR (RT-PCR) determined that the QRT-PCR primers do not cause amplification of the viral vector *At ARF* insert. QRT-PCR results indicated that at 12 dpi, accumulation of endogenous *ARF*-1 transcript was 2.4-fold less than that of the GFP-transfected control plants. These results suggest that levels of *ARF-1* transcript are due to amplification of endogenous ARF-1, rather than from viral vector contributions. However, the possibility remains that *Nb* transcripts from other members of the ARF family are amplified. An additional consideration is that *tobamoviral* vectors designed to cause VIGS phenotypes may actually cause suppression of homology–dependent gene silencing due to their viral replicase protein. Additional QRT-PCR experiments at earlier time points would provide information about the progression of *ARF*-1 transcript degradation.

Although there is probably no homology-dependent VIGS occurring in the GFP-transfected plant, RNA silencing of the viral genome may occur. In the ARF_{as}-transfected plant, both VIGS and RNA silencing of the viral genome should be operating. It was expected that genes involved in homology-dependent silencing would show changes in expression levels throughout the time course. A virus-transfected plant was selected as a control in order to eliminate or at least minimize the effects of genes related to RNA silencing against the virus.

The phenotypes of both plants indicate high expression of the insert, and therefore viability of the virus. In addition, the transcript data from the microarray indicates a high level of accumulation of virus-derived *ARF* transcripts over time. Is there an impact of this transcript accumulation on endogenous gene expression? The microarray data would suggest so. Over time on the microarray, there is an increase in the accumulation of endogenous plant transcripts (Table 4) that have gene expression patterns that parallel the viral vector *ARF* transcript accumulation. A question arises as to whether changes in transcript levels reflect an induction by the virus in its attempt to escape silencing, or whether they are changing because of VIGS of endogenous *ARF*.

Western blot assays showed that over time there was a steady decline in levels of *ARF* protein. Available documentation on the use of the primary antibodies used in these protein experiments indicated that the ARF monoclonal antibody had not been tested in plant species. It was expected that protein levels in the *ARF*as-transfected plants would be knocked down. Therefore, uninfected wild type *N. benthamiana* plants were used as

controls to show that ARF proteins can be detected in plant species. In addition, *Arabidopsis* ARF-1 T-DNA insertion mutants were used to show that other ARF protein members could also be detected. In the *Nb*-transfection time course, the Western blot analysis showed that ARF proteins were detectable but steadily declining over a 16-day period, and that they were not detectable at 20 dpi. It is not clear whether the ARF-1 protein is produced over the course of the first 12 days, although by this point the transcript level had dropped over 2-fold. It is likely that the primary antibody also detected ARF-3, ARF-4, ARF-5, and ARF-6. It is clear, however, that the 740 AT #120 viral vector can cause a complete knock-down of the ARF protein family by 20 dpi. It appears that transfection with the new viral vector construct, TTOSA1 ARF1+ may also cause a knock-down of ARF protein, rather than the high levels expected due to overexpression. The phenotype appears as stunted as the antisense-transfection plants at 8 dpi, and Western analysis indicate that protein levels at 16 dpi are close to those at 12-16 dpi of the ARF_{as}-transfected plants.

Because no ARF proteins are detectable at the 20-day point, a further analysis to understand transcriptional changes between 16 and 20 dpi is warranted. There are 383 genes that are downregulated due to the *ARF*as-treatment at this time point that cluster into 5 distinct groups (Figure 4.5). Sixteen genes from set 5 (Table 4.5) showing statistically significant differences from other time points were identified for further characterization to shed light on the role of the ARF family in *N. benthamiana*.

In conclusion, a *tobamoviral* transfection using a highly homologous *ARF* derived from *Arabidopsis* can effectively knock down production of an entire family of proteins in *N. benthamiana*. The effects of this knock-down on gene expression provides insight into the role that this family plays in the plant, and perhaps in other species, as well. In human studies where it is often difficult to study genes and gene families, plants could serve as a model for understanding gene and protein expression. When it desirable to knock down an entire family of proteins, studies using viral transfection have the advantage over those involving transgenic plants that require multiple genes to be transformed and often result in a lethal phenotype.

In the case of ARF, human and plant sequences have high homology at the nucleotide level. Ultraconserved elements in the human genome have been characterized, with orthologs present in rat, mouse, chicken and dog genomes, and many of the identified segments are also significantly conserved in fish (Bejerano *et al.*, 2004). These represent a class of genetic elements whose function and evolutionary origins have not yet been determined, although they appear to be involved in the regulation of transcription and development. Perhaps such regulatory elements have orthologs in plants. Plant transfections with human or other animal *ARF* sequences could provide insight into roles in these organisms as well.

4.5 EXPERIMENTAL PROCEDURES

4.5.1 Plasmid Constructions

An *Arabidopsis thaliana* cDNA library was constructed in a *tobamoviral* vector. Four *A. thaliana* cDNA libraries were obtained from the *Arabidopsis* Biological Resource Center (ABRC) and size-fractionated with inserts of 0.5-1 kb (CD4-13), 1-2 kb (CD4-14), 2-3 kb (CD4-15), and 3-6 kb (CD4-16). The *Arabidopsis thaliana CD4-13* cDNA library was digested with *Not*I. DNA fragments between 500 and 1000 bp were isolated by trough elution and subcloned into the *Not*I site of the tobacco mosaic virus vector, pBS740, placed under the transcriptional control of a tobamovirus subgenomic promoter. *E. coli* C600 competent cells were transformed with the pBS740 AT library and colonies containing *Arabidopsis* cDNA sequences were selected on LB Amp 50 ug/ml. Recombinant C600 cells were automatically picked using a Flexys robot (Genomic Solutions) and then transferred to a 96 well flat bottom block containing terrific broth (TB) Amp 50 ug/ml.

Digestion with *Not*I in most cases liberated the entire *A. thaliana* cDNA insert because the original library was assembled with *NotI* adapters. *Not*I is an 8-base cutter that infrequently cleaves plant DNA. In order to insert the *Not*I fragments into a transcription plasmid, the pBS735 transcription plasmid was digested with *Pac*I/*Xho*I and ligated to an adapter DNA sequence created from the oligonucleotides 5'-TCGAGCGGCCGCAT-3' and 5'-GCGGCCGC-3'. The resulting plasmid pBS740 (Figure 4.1) contains a unique *Not*I restriction site for bi-directional insertion of *Not*I fragments from the CD4-13

library. Recovered colonies were prepared from these for plasmid minipreps with a Qiagen BioRobot 9600™. The plasmid DNA preparations were performed on the BioRobot9600™ in a 96-well format and yielded transcription quality DNA. An *Arabidopsis* cDNA library was ligated into the plasmid and analyzed by agarose gel electrophoresis to identify clones with inserts. Clones with inserts are transcribed in vitro and inoculated onto *N. benthamiana.*

An *ARF-1* viral vector construct in the sense orientation was built using site-directed mutagenesis to amplify the 740 AT #120 insert. Forward Primer ARF1M1S (5' CACC GTCGACTGCAGCATG GGG TTG AGT TTC GCC AAG CTG TTT AGC 3') and reverse primer ARF1A180A (5'TCCCTAGGATCATGCCTTGCCAGCG ATGTTGTTGGAGAGCC 3') were used to re-orient the insert, which was then cloned into a linearized TTOSA1 APE pBAD viral vector with *Sal*I and *Avr*II restriction enzyme sites, creating TTOSA1 ARF-1+ . The nucleotide sequencing of 740 AT #120 was carried out by dideoxy termination using double stranded templates (Sanger *et al.*, 1977).

4.5.2 Plant Treatments for Transcriptional Profiling and Protein Analyses

N. benthamiana plants were grown from seed and kept under lights at 25C. *In vitro* transcription reactions were performed for 740 AT #120 (T7 promoter), and for TTOSA1 APE pBAD and TTOSA1 ARF-1+ (SP6 promoter) using Ambion mMessage mMachine *in vitro* transcription kits. At the 4-6 leaf stage of development, two lower leaves were rub-inoculated with *in vitro* transcripts using carborundum. At 4, 8, 12, 16, and 20 days post-inoculation time points, *ARFas-* and GFP-transfected plant leaves displaying a

phenotype were harvested for RNA and protein extractions. Leaves from plants transfected with TTOSA *ARF*-1+ construct were harvested at 7 dpi for extraction of crude protein.

Seeds obtained from ABRC for ARF-1 T-DNA Insertion (Salk_136703) were surface sterilized, germinated on water agar, and transferred to 4-inch pots containing potting soil. Leaves were harvested and RNA extracted using Qiagen RNeasy kits. Reverse-transcription PCR was performed using the following primers, to confirm the presence of the insertion: Left genomic primer (5' GGAATTTTTGGAGCCTCAAGATTGT 3'), Right genomic primer (5' TTCCGCAGAATCATCAACCATT 3'), and Left border primer of pROK2 (5' GCGTGGACCGCTTGCTGCAACT 3'). Plants were confirmed for the presence of the insertion by analyzing RT-PCR products on a 1% agarose gel electrophoresis (data not shown).

4.5.3 Labeling and Hybridization for cDNA microarrays

Total RNA was extracted using TRIzol® (Invitrogen Cat. No. 15596-018), and cleaned using Qiagen RNeasy Clean-Up protocol. Invitrogen SuperScript Direct cDNA Labeling System (Cat# L1015-01) was used to differentially label with fluorescent cyanine-3 (cy3) or cyanine-5 (cy5) dyes (Amersham, Cat Nos. PA53022. Quality and quantity of RNA was checked on a 1% agarose gel, and on a Shimadzu spectrophotometer. Cy-dye labeled cDNA was quantified using a Beckmann spectrophotometer. As a control for both microarray studies, RNA from GFP-transfected plants was isolated, quantified,

reverse transcribed and labeled with cy5 dye using the same procedures. Equal amounts of labeled treated and control cDNA were mixed and hybridized in replicate to 10K potato cDNA microarrays from TIGR (The Institute for Genomic Research). Hybridization and post-hybridization washes were performed using recommended hybridization conditions found in protocols developed by TIGR. A detailed description of TIGR Potato Microarray optimized methods, the array design, and the cDNA description file used for this study (GenePix Array List, GAL file versions 1and 2) can be found at the following URL: http://www.tigr.org/tdb/potato/microarray_comp.shtml.

4.5.4 Microarray Scanning and Analysis

Scanning of the microarrays for the experiments was performed using the BioRad VersArray ChipReader. Raw signal data was combined into text files that were imported into GeneSpring (Agilent, formerly Silicon Genetics) software for background subtraction, normalization by LOWESS and analysis. Gene lists were generated for 2-fold upregulated and downregulated genes.

K-means clustering of gene list "Like ARF (BQ508962)" was based on the following interpretation: ARF1 knock-down (default interpretation), Log of Ratio mode (weight 1.0). The parameters used were: number of clusters, 5; number of iterations, 100. The similarity measure was standard correlation. Downregulated genes were also clustered using K-means, with 5 clusters, 100 iterations, and a standard correlation similarity measure. A Welch ANOVA (parametric test, variances not assumed equal) was

performed on genes from set 5 of the downregulated clusters with statistically significant differences among the following groups based on values of 'Time': 12 days post inoculation, 16 days post inoculation, 20 days post inoculation, 8 days post inoculation, 4 days post inoculation; p-value cutoff 0.05, no multiple testing correction. This restriction tested 67 genes, with about 3.35 genes expected to pass the restriction by chance.

4.5.5 Quantitative Real-time PCR

QRT-PCR primers for both target and reference genes were designed using BioRad's Beacon Designer 2.1. Sense and antisense primers for quantitative real-time PCR for ARF-1 were derived from potato clone BQ508962 (ADP-Ribosylation Factor, rice). The forward primer for *ARF-1* is ARF1-1_F (GCCGAAATAACTGATAAGCTTGGA); reverse primer is *ARF*1_R (CCAATCAAGACCCTCATAAAGTCC), product length = 108 bp, product Tm = 86.3°C.

Primers for reference genes were aldehyde oxidase (BQ112643) and *Nb* phytoene synthase. Forward primer is 5' TTCGTCCAAAACATCTGGTGAAC 3' (23 bp), Tm=58.4°C, GC%=43.5; and reverse primer is 5'ACTGGTAATATTGCAGGGACATCT 3' (24 bp), Tm=58.5°C, GC%=41.7, product length=162, Tm=88.2°C. The forward primer for phytoene synthase is *Nb*Psy1_F 5' GCTGGTACGGTTGGGTTGAT 3'; reverse primer for phytoene synthase is *Nb*Psy1_R 5' GCATGTGCTAATTCATCTTGAGGT 3', product length = 191 bp, Tm = 86.1°C. Ubiquitin (*N. benthamiana*) forward primer is *Nb*UBI_F

5'CAACATCCAGAAGGAGTCTACC3' and ubiquitin *Nb*UBI_R

5'GCCAGCGAAAATCAACCTCT3', product length = 193 bp, Tm = 85°C.

Total RNA was digested with amplification grade DNase I (Invitrogen Cat. No. 18068-015) for first strand cDNA synthesis for QRT-PCR assays. cDNA was synthesized using iScript cDNA Synthesis kit (BioRad Cat. No. 170-8890) for treated and control plants, in which RNA is primed with oligo $dT_{(20)}$ and random primers. A ten-fold dilution series was prepared for standard curves. Reaction mixtures for standards and unknowns were comprised of BioRad SYBR Green Supermix (Cat. No. 170-8880), cDNA template, and QRT-PCR primers at 200 nM concentration, and applied to a 96-well plate. QRT-PCR and melt curve reactions were performed on a BioRad iCycler using the thermal protocol: Cycle 1: 3 mins at 95°C; Cycle 2: 40 repeats of 10 sec at 95°C, 15 sec at optimum Tm of primers; Cycle 3: 1 min at 95°C; Cycle 4: 1 min at 55°C; Cycle 5: 80 repeat of 10 sec at 55°C, increasing 0.5C. When efficiencies could be determined for target and reference genes, Pfaffl's method of calculating fold change ratios was used (Pfaffl, 2001 #64); otherwise, fold change was calculated using $2^{(\Delta\Delta Ct)}$.

4.5.6 Protein Isolation and Western Blot

Proteins were isolated from symptomatic leaves at each point in the time course for *ARF-1*-transfected plants using an extraction buffer (10 mM Tris.bis.propane (pH 6.0), 5 mM $CaCl_2$, and 10 mM β-mercaptoethanol). As a control, proteins were isolated from uninfected wild type plants and from TTOSA1 *ARF*+ -transfected plants. Proteins were also extracted from leaves of *Arabidopsis* T-DNA insertion mutant plants (Salk_136703)

and used to determine ability of ARF-1 primary antibody to detect other members of the ARF family of proteins. Leaf material was massed and homogenized in liquid nitrogen using a mortar and pestle. Protein extraction buffer was added to the pulverized leaf powder in a 1:1 ratio. The mixture was centrifuged for 3 minutes at 14,000 rpm at 4°C. The supernatant was transferred to a new tube, and then centrifuged for 3 minutes at 14,000 rpm at 4°C.

Crude protein extracts were quantified using Bradford reagent (Sigma B6916), and electrophoresed on a NuPAGE 4-12% Bis-Tris gel in 1X MES SDS running buffer (500 mL) for about 2 hr. The proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech) in 1X Tris-Glycine + 20% methanol, 1L. After transfer, the membrane was rinsed 2x with TTBS (Tween Tris-Buffer Saline, Tween-20 1:2000 TBS) before incubating in 5% BSA overnight at 4°C to block non-specific binding. The membrane was washed 5x with TTBS (5 min per wash). After washing, the membrane was incubated for 3 hours with the appropriate primary antibody, ARF-1 mouse monoclonal (Affinity Bioreagents, Cat. No. MA3-060) that was diluted in TTBS containing 1% BSA. The membrane was then washed 3X with TTBS (5 min per wash). It was incubated with secondary antibody (goat antimouse) diluted in TTBS (1:5000) containing 1% BSA for 1 hour at room temperature. It was washed 3x with TTBS, incubated in the appropriate substrate for visualization, and detected by an alkaline phosphatase (AP) conjugate substrate kit.

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CHAPTER 5. CONCLUSIONS

Specific issues in the development of a transcriptional profiling system were addressed in order to study the utility of plant viral vector technology in metabolic engineering and functional genomic applications. Reflecting upon my work in its entirety, I have synthesized evidence from the data and have drawn conclusions regarding the use of heterologous hybridizations, the choice of controls and the impact of the virus and the viral insert on transcript accumulation.

Initial studies of TTU51/CTP-*CrtB*-RZ-transfected *Nicotiana benthamiana* using wild type non-infected controls produced reliable signal data using heterologous cDNA microarrays for the hybridization of the related solanaceous species, *Nicotiana benthamiana* and *Solanum tuberosum*. Signal information for thousands of genes was obtained with the 10K potato microarrays. A particularly favorable finding was that transfected plants showed an increase in heat shock proteins compared to controls. This was consistent with published data that *tobamovirus*-infection in *Arabidopsis thaliana* upregulates heat shock genes as a defense response.

Table A3 in Appendix I depicts data for subsequent hybridizations that used GFP-transfected plants as controls. Microarray conditions were adjusted to optimize hybridization temperature, and to reduce the background signal, such that it could be subtracted out prior to analysis. Microarray #753 was a single sample; #677 and #681 were hybridized together; and slides #9733 - #9737 and #5146 were also hybridized together. When analyzed together, 112 genes show an upregulation in seven of nine

biological replicate hybridizations, demonstrating a high level of precision. In addition, independent experiments using quantitative real-time PCR (QRT-PCR) validated microarray findings that phytoene synthase and phytoene desaturase transcript levels are elevated in TTU51/CTP-*CrtB*-RZ-transfected *Nicotiana benthamiana* compared to GFP-transfected controls. This supports the use of the TIGR 10K potato microarray as a reliable tool for the extraction of *N. benthamiana* gene expression data in transfected plants.

As discussed in Chapter 1, a virus-infected plant expressing green fluorescent protein (GFP) was selected as a control in an attempt to eliminate or minimize changes in gene expression that occur because of virus infection, and to understand the specific impact of the *crtB*, *pds* and *ARF1* insert sequences in the treatment groups. Microarray analyses showed that heat shock genes were no longer upregulated when these controls were employed.

An assumption was made that GFP expression does not interfere with endogenous gene expression, although a microarray analysis of a GFP-transfected plant using a wild type non-infected control plant was not performed. However, the data in this research show that the use of GFP-transfected plants as controls is not ideal, at least for investigations of the carotenoid pathway. Evidence from quantitative real-time PCR experiments (Chapter 3, Table 3.2) indicates that GFP production impacts on the transcript accumulation of phytoene desaturase (*pds*), showing a highly reduced level compared to that of uninfected *N. benthamiana*. Phenotypically, GFP-transfected plants

show a fairly normal growth and development pattern, but in natural light the sections of leaves that are expressing GFP actually appear lighter in pigmentation compared to sectors not expressing GFP. This may be indicative of a change in carotenoid levels. In addition, HPLC data show that there is a 30% reduction in the total measured carotenoids (nmol/g) of a GFP-transfected *N. benthamiana* plant compared to that of an uninfected plant (Appendix II).

It is unclear why GFP-transfected controls show levels of *pds* mRNA comparable to that in the pds_{as}-transfected plants. Carotenoids absorb in the 460-550 nm range, protecting chlorophylls from photobleaching. Wild type GFP absorbs light at wavelengths of 395 nm and 475 nm, and red-shifted GFP mutants absorb at 490 nm. If expression of the GFP protein aids in protecting chlorophylls from photo-oxidation, this may impact the transcription of genes encoding carotenoid biosynthesis enzymes.

What are the implications then for the microarray data? The white phenotype of a pds_{as}-transfected plant is apparently due to a drop in carotenoid levels that leads to a subsequent photobleaching of leaves and other organs. So it would be expected that enzymes downstream in the pathway would be affected. Microarray data in the pds_{as}-transfection study show that transcript levels did not significantly decrease for these enzymes. However, the data probably do not accurately reflect the actual levels of transcripts of the other enzymes, because of the masking effects of the GFP control. Additional quantitative real-time PCR assays using uninfected plant controls would shed light on the actual levels of carotenoid transcripts in the pathway.

If GFP-transfected plants were to be used as controls in future studies, I would recommend that microarray analyses be performed to better understand the effects of GFP expression on the investigated pathway, by hybridizing GFP-transfected plant cDNA to wild type non-infected plant cDNA. Alternatively, if wild type non-infected plants are selected as controls, additional information about VIGS could be explored, particularly if it is correlated with the timing of the appearance of the phenotype, as well as a profile of protein abundance.

The length of the viral vector insert also confounded results. If the goal is to query the expression of the endogenous plant gene that has a homologous counterpart in the viral vector, conclusions cannot be drawn from microarray data alone. Both *pds* and *ARF* antisense transfections resulted in high levels of the endogenous plant gene on the microarray. Further analyses using conventional reverse-transcription PCR (RT-PCR) and QRT-PCR assays were necessary to distinguish between endogenous plant gene and viral vector sequence contributions. In the experiments outlined in this dissertation, the viral vectors had very long inserts (600-800 bp), requiring a careful analysis of sequence homology among the various species, and limiting the possibilities for primer design. Vector sequences unexpectedly served as templates when synthesizing cDNAs using oligo $d(T)_{20}$ priming leading to 1) hybridization on the cDNA microarray, and 2) amplification in the real-time PCR reactions.

One alternative that would possibly prevent the hybridization of viral vector insert sequences on cDNA microarrays would be to construct the viral vectors with shorter inserted sequences. Very short direct inverted-repeat (IR) viral vector inserts {Lacomme, 2003 #68} leading to desired or even more effective phenotypes would not serve as effective templates for cDNA synthesis because of the folded nature of the IR. A shorter sequence would also open up more options for the design of the QRT-PCR primers to query the endogenous gene.

Another possibility would be to use an oligo-based array. More affordable options now exist for microarray studies. Combi-Matrix (http://www.combimatrix.com/) creates oligonucleotide custom arrays (902 or 12K) by synthesizing 35-40 bp sequences based on GenBank accession numbers on a 5x22mm semiconductor using an electrochemical detritylation process. The custom arrays are user-defined, so that viral sequences can be used as controls. Fragmentation of labeled cDNA is performed prior to hybridization. If insert sequence cDNA is synthesized from viral template it would potentially hybridize to the oligos on the array. However, positive signals from the virus controls would serve as a red flag, indicating the need for additional assays.

With respect to the plant's ability to launch an RNA-mediated defense against the virus, phenotypic observations and data from microarray and real-time PCR experiments show that the virus escapes degradation. Levels of viral transcript are highly elevated in both pds_{as} and ARF_{as} transfections, indicating virus viability. Phenotypes are not only

delayed, but they are prolonged. *Tobamoviruses* use the replicase protein to suppress RNA silencing. Engineered TMV vectors with inserts may cause a delay in the expression of the phenotype by suppressing the homology-dependent silencing of the endogenous mRNA, as well as preventing targeting and degradation of its own genome. An analysis of short interfering RNAs (siRNAs) would shed additional light on the progression of VIGS in the plant. Overall, it appears that there is an advantage in using these vectors in functional genomics studies since the virus wins out over the plant in the RNA silencing race at least for several weeks following transfection.

I would also like to make a few comments about the terminology used in microarray and other gene expression assays. I utilized the terms "upregulation" and "downregulation" conservatively because it suggests that regulation at the transcriptional level is affected. Perhaps this is true, but the issue of transcript stability must also be considered. Microarray and QRT-PCR are more correctly indicating transcript accumulation at a given point in time, and levels of accumulation do not necessarily equate to a change in regulatory elements. I also opted for the term "knock down" rather than the term "knock-out" to describe the impact of viral vectors carrying antisense sequences. "Knock-out" is a term that is used to describe a null mutation in a gene that is introduced into a genome by homologous recombination causing replacement of the normal allele. The phenotype of the plants in the ARF_{as} transfections is progressively stunted, but protein and transcript levels remain high until the 20 dpi assays, at which point they show a complete knock down.

Overall, I would recommend the use of transcriptional profiling of plants transfected with *tobamoviral* vectors, for both metabolic engineering studies and for functional genomic investigations. If the goal is to correlate phenotype with gene function, I would incorporate additional experimental parameters, such as time or environmental conditions. Performing a "snapshot" study at one time point produces a very large amount of information for microarray studies. It is not possible to draw conclusions regarding gene function, as higher order statistical tests cannot be performed. Finally, I would also recommend that the study design include biological replication, and that metabolic profiling and Western analyses be performed in order to acquire protein data in tandem with the transcriptional profiling. In this way, a more comprehensive picture of the impact of transfection can be acquired.

APPENDIX I

Microarray data tables with normalized gene expression ratios are provided in this Appendix. Normalized gene expression data represent the ratio of Cy3 signals of transfected experimental samples to cy5 signals of GFP-transfected controls in *N. benthamiana*. Ratios were generated by combining raw cy3 and cy5 signal data for each microarray experiment and importing the file into GeneSpring software for analysis. Gene lists generated from LOWESS normalized values for 2-fold upregulated genes or downregulated genes. The TIGR Master Gene Table (Versions 1 and 2) for the clones on the 10K cDNA potato microarray can be found at the following url: www.tigr.org. The tables are as follows:

A1. Normalized Upregulated Gene Expression Ratios (*CrtB*RZ/GFP) in "Classification of Upregulated Genes" (Figure 2.6) 166

A2. Normalized Upregulated Gene Expression Ratios (*CrtB*RZ/GFP) for Selected Genes in Carotenoid Biosynthesis Pathway 182

A3. Normalized Upregulated Gene Expression Ratios (*CrtB*RZ/GFP), 2-fold or greater occurring in 7 of 9 Replicates 183

A4. Normalized Upregulated Gene Expression Ratios (*PDS*/GFP), 2-fold or greater 194

A5. Normalized Upregulated Gene Expression Ratios (*ARF*/GFP), 2-fold or greater, at 4, 8, 12, 16 and 20 dpi 205

A6. Normalized Downregulated Gene Expression Ratios (*ARF*/GFP) in Set 5, "Downregulated Genes, 20 dpi" (Figure 4.4) 218

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ513194	Cell division cycle protein 48 homolog. (Bell pepper) (Capsicum annuum)	4.527 (4.436 to 4.618)	0.0443	13.21 (13.13 to 13.29)	0.00373	Cell Growth, Cell Division and DNA Synthesis
BQ507451	contains similarity to the auxin-independent growth promoter~gene_id	3.391 (2.417 to 4.364)	0.555	27.19 (26.92 to 27.46)	0.0216	Cell Growth, Cell Division and DNA Synthesis
BQ514390	DNA-damage inducible protein DDI1-like (Arabidopsis thaliana)	2.763 (2.149 to 3.378)	0.528	12.32 (11.24 to 13.40)	0.0166	Cell Growth, Cell Division and DNA Synthesis
BQ118979	Induced stolen tip protein TUB8 (Fragment). (Potato) (Solanum tuberosum)	2.600 (2.518 to 2.682)	0.0289	2.879 (2.809 to 2.95)	0.0167	Cell Growth, Cell Division and DNA Synthesis
BQ118419	MAR-binding protein (Nicotiana tabacum)	4.039 (1.83 to 6.247)	0.333	24.79 (18.08 to 31.50)	0.0134	Cell Growth, Cell Division and DNA Synthesis
BQ121688	photomorphogenesis repressor protein-like (Arabidopsis thaliana)	2.416 (2.140 to 2.693)	0.424	79.51 (42.97 to 116.1)	0.00975	Cell Growth, Cell Division and DNA Synthesis
BQ515065	probable 24-sterol C-methyltransferase (EC 2.1.1.41) - common tobacco	2.236 (0.748 to 3.724)	0.532	10.30 (10.02 to 10.59)	0.0212	Cell Growth, Cell Division and DNA Synthesis
BQ113671	tonneau 1a (Arabidopsis thaliana)	3.713 (3.657 to 3.770)	0.127	4.122 (3.939 to 4.305)	0.0183	Cell Growth, Cell Division and DNA Synthesis
BQ514038	translation elongation factor eEF1Balpha (clone 2) (validated) - Arabidopsis thaliana	3.312 (2.755 to 3.868)	0.0554	3.763 (3.184 to 4.343)	0.0155	Cell Growth, Cell Division and DNA Synthesis
BQ114714	ubiquitin-protein ligase 2 (Arabidopsis thaliana)	10.07 (9.869 to 10.28)	0.00912	3.56 (3.318 to 3.802)	0.0126	Cell Growth, Cell Division and DNA Synthesis; Protein Destination
BQ512152	At1g66240 T6J19_6 (Arabidopsis thaliana)	2.669 (2.537 to 2.802)	0.189	5.152 (5.048 to 5.255)	0.0173	Cell Rescue, Defense, Cell Death and Aging

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ508437	At1g79380 T8K14_20 (Arabidopsis thaliana)	2.167 (2.123 to 2.211)	0.219	6.527 (6.42 to 6.634)	0.0154	Cell Rescue, Defense, Cell Death and Aging
BQ113636	Glutamate--cysteine ligase chloroplast precursor (EC 6.3.2.2) (Gamma-glutamylcysteine synthetase)	4.545 (4.127 to 4.962)	0.0427	4.141 (3.983 to 4.298)	0.0119	Cell Rescue, Defense, Cell Death and Aging
BQ115048	glutathione synthase (EC 6.3.2.3) 2 - tomato; TC54503	5.209 (3.695 to 6.722)	0.114	17.29 (13.07 to 21.50)	0.0194	Cell Rescue, Defense, Cell Death and Aging
BQ515006	glutathione transferase (EC 2.5.1.18) class-phi - Commerson s wild potato	6.447 (3.41 to 9.484)	0.352	26.68 (20.29 to 33.07)	0.00959	Cell Rescue, Defense, Cell Death and Aging
BQ513598	hypothetical protein F18B13.2 (imported) - Arabidopsis thaliana	10.09 (9.912 to 10.26)	0.0522	20.38 (20.23 to 20.53)	0.00264	Cell Rescue, Defense, Cell Death and Aging
BQ516445	mitochondrial formate dehydrogenase precursor (Solanum tuberosum)	5.966 (2.380 to 9.551)	0.192	8.823 (7.925 to 9.720)	0.00987	Cell Rescue, Defense, Cell Death and Aging
BQ120295	Mitogen-activated protein kinase homolog 4 (EC 2.7.1.-) (MAP kinase 4) (AtMPK4). (Mouse-ear cress); TC50308	4.877 (3.268 to 6.486)	0.338	50.23 (44.01 to 56.45)	0.00674	Cell Rescue, Defense, Cell Death and Aging
BQ121623	monodehydroascorbate reductase (Brassica juncea); TC49833	2.303 (0.472 to 4.133)	0.626	11.70 (11.31 to 12.1)	0.00971	Cell Rescue, Defense, Cell Death and Aging
BQ121207	monodehydroascorbate reductase (NADH) (EC 1.6.5.4) cytosolic - tomato	2.243 (1.333 to 3.153)	0.581	9.509 (9.175 to 9.842)	0.0102	Cell Rescue, Defense, Cell Death and Aging
BQ516892	Peptide methionine sulfoxide reductase (EC 1.8.4.6) (Protein- methionine-S-oxide reductase)	4.651 (4.374 to 4.927)	0.0865	13.59 (13.07 to 14.11)	0.0163	Cell Rescue, Defense, Cell Death and Aging

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ508930	probable DnaJ protein (imported) - Arabidopsis thaliana	6.387 (4.700 to 8.073)	0.428	13.07 (12.08 to 14.06)	0.0064	Cell Rescue, Defense, Cell Death and Aging
BQ518442	Probable glutathione S-transferase (EC 2.5.1.18) (Auxin-induced protein PGNT1 PCNT110).	5.845 (4.269 to 7.422)	0.0593	11.07 (8.499 to 13.65)	0.00601	Cell Rescue, Defense, Cell Death and Aging
BQ120348	probable thioredoxin At2g35010 (imported) - Arabidopsis thaliana	5.417 (3.237 to 7.597)	0.427	16.36 (15.02 to 17.69)	0.0193	Cell Rescue, Defense, Cell Death and Aging
BQ517745	putative calcineurin B-like protein (Oryza sativa)	3.665 (3.518 to 3.812)	0.309	11.59 (10.06 to 13.12)	0.00353	Cell Rescue, Defense, Cell Death and Aging
BQ514137	putative glutathione S-transferase T5 (Lycopersicon esculentum)	5.694 (3.672 to 7.716)	0.179	7.09 (7.023 to 7.156)	0.00267	Cell Rescue, Defense, Cell Death and Aging
BQ507009	putative lysophospholipase (Arabidopsis thaliana)	3.541 (2.946 to 4.135)	0.237	9.675 (8.52 to 10.83)	0.0209	Cell Rescue, Defense, Cell Death and Aging
BQ510641	putative resistance protein (Lycopersicon esculentum)	16.89 (16.65 to 17.12)	0.000611	52.82 (52.23 to 53.4)	0.000316	Cell Rescue, Defense, Cell Death and Aging
BQ507505	TMV-induced protein I (Capsicum annuum)	27.36 (26.18 to 28.54)	0.00137	69.12 (67.48 to 70.75)	0.00118	Cell Rescue, Defense, Cell Death and Aging
BQ117125	heme oxygenase 1 (Lycopersicon esculentum)	3.807 (2.112 to 5.502)	0.325	7.662 (6.629 to 8.695)	0.0123	Cellular Biogenesis
BQ119649	polygalacturonase (EC 3.2.1.15) 1 beta chain precursor - tomato	38.15 (37.85 to 38.45)	0.000349	34.27 (32.35 to 36.19)	0.000556	Cellular Biogenesis
BQ112806	probable chaperonin-containing TCP-1 complex gamma chain F9D12.18 - Arabidopsis thaliana	3.086 (3.077 to 3.096)	0.323	14.74 (13.19 to 16.28)	0.00691	Cellular Biogenesis

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ118416	At2g03890 T18C20.9 (Arabidopsis thaliana)	3.549 (3.448 to 3.650)	0.11	15.33 (14.98 to 15.68)	0.00343	Cellular Communication/Signal Transduction
BQ516912	AT3g01490 F4P13_4 (Arabidopsis thaliana)	2.151 (1.72 to 2.582)	0.632	12.06 (11.63 to 12.49)	0.013	Cellular Communication/Signal Transduction
BQ511382	AT3g16910 K14A17_3 (Arabidopsis thaliana)	2.044 (1.973 to 2.116)	0.182	5.332 (4.472 to 6.193)	0.0129	Cellular Communication/Signal Transduction
BQ115193	CBL-interacting protein kinase 6 (Arabidopsis thaliana)	2.978 (2.450 to 3.505)	0.149	28.66 (27.70 to 29.63)	0.00561	Cellular Communication/Signal Transduction
BQ112245	GDP dissociation inhibitor - common tobacco	2.252 (1.896 to 2.607)	0.0932	15.96 (14.89 to 17.02)	0.00525	Cellular Communication/Signal Transduction
BQ118661	GTP-binding protein - garden pea	2.198 (2.094 to 2.303)	0.143	28.68 (28.43 to 28.92)	0.00268	Cellular Communication/Signal Transduction
BQ515193	GTP-binding protein F23K16.150 - Arabidopsis thaliana; TC43752	4.528 (4.085 to 4.970)	0.0395	10.11 (9.979 to 10.24)	0.0119	Cellular Communication/Signal Transduction
BQ510930	GTP-binding protein-like (Arabidopsis thaliana)	3.926 (3.206 to 4.646)	0.0656	15.9 (15.47 to 16.32)	0.00207	Cellular Communication/Signal Transduction
BQ112592	hypothetical protein F20D21.27 (imported) - Arabidopsis thaliana	2.335 (2.015 to 2.656)	0.196	25.26 (21.94 to 28.59)	0.00313	Cellular Communication/Signal Transduction
BQ118224	protein kinase-like protein (Arabidopsis thaliana)	4.285 (3.947 to 4.624)	0.0933	46.19 (40.66 to 51.73)	0.00864	Cellular Communication/Signal Transduction
BQ114148	protein phosphatase 2C (PP2C) (Fagus sylvatica)	10.92 (8.396 to 13.44)	0.0957	13.18 (12.56 to 13.81)	0.00625	Cellular Communication/Signal Transduction

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ511591	protein T27G7.10 (imported) - Arabidopsis thaliana	2.004 (1.751 to 2.256)	0.536	9.287 (9.208 to 9.366)	0.0109	Cellular Communication/Signal Transduction
BQ514070	putative GTP-binding protein; 106556-109264 (Arabidopsis thaliana)	2.197 (2.120 to 2.275)	0.0555	12.65 (12.40 to 12.89)	0.00085	Cellular Communication/Signal Transduction
BQ513539	Ser Thr specific protein phosphatase 2A B regulatory subunit beta isoform	2.086 (0.195 to 3.976)	0.719	10.83 (10.63 to 11.02)	0.0182	Cellular Communication/Signal Transduction
BQ510123	Ser Thr specific protein phosphatase 2A B regulatory subunit beta isoform	2.054 (2.035 to 2.073)	0.67	22.69 (19.72 to 25.66)	0.00832	Cellular Communication/Signal Transduction
BQ113170	Serine threonine protein phosphatase PP1 isozyme 1 (EC 3.1.3.16). (Common tobacco)	4.618 (4.134 to 5.101)	0.015	10.61 (8.467 to 12.74)	0.00619	Cellular Communication/Signal Transduction
BQ508186	AT4g01050 F2N1_31 (Arabidopsis thaliana)	2.216 (2.167 to 2.266)	0.0701	5.369 (5.118 to 5.621)	0.0114	Cellular Organization
BQ516782	protein epsilon subunit of mitochondrial F1-ATPase (imported) - Arabidopsis thaliana	2.904 (2.584 to 3.225)	0.3	12.38 (12.27 to 12.5)	0.0139	Cellular Organization
BQ508320	Nuclear transport factor 2 (NTF-2). (Mouse-ear cress) (Arabidopsis thaliana)	5.334 (5.054 to 5.615)	0.0338	12.29 (11.08 to 13.49)	0.00548	Cellular Transport and Transport Mechanism
BQ121865	vesicle transport v-SNARE (vesicle soluble NSF attachment protein receptor) protein	5.795 (5.787 to 5.804)	0.00263	17.91 (17.84 to 17.97)	0.000634	Cellular Transport and Transport Mechanism
BQ507309	alanyl-tRNA synthetase (Arabidopsis thaliana)	9.491 (9.488 to 9.493)	0.00536	10.2 (9.469 to 10.93)	0.00521	Cellular Transport and Transport Mechanisms
BQ510962	chloroplast outer envelope protein OEP86 precursor - garden pea	2.941 (2.672 to 3.210)	0.19	22.76 (20.21 to 25.32)	0.00433	Cellular Transport and Transport Mechanisms

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ512157	coatomer protein complex beta prime; beta -COP protein (Arabidopsis thaliana)	2.675 (2.585 to 2.765)	0.0219	2.817 (2.637 to 2.998)	0.0201	Cellular Transport and Transport Mechanisms
BQ518306	GTP-binding protein Rab7b - common tobacco; TC49504	2.654 (2.105 to 3.204)	0.565	7.911 (7.500 to 8.322)	0.0205	Cellular Transport and Transport Mechanisms
BQ506270	Probable 28 kDa Golgi SNARE protein (Golgi SNAP receptor complex member 1). (Mouse-ear cress)	2.704 (1.682 to 3.727)	0.256	13.89 (12.93 to 14.84)	0.00677	Cellular Transport and Transport Mechanisms
BQ120994	putative vacuolar proton ATPase subunit E (Lycopersicon esculentum)	5.908 (5.733 to 6.084)	0.00366	2.734 (2.647 to 2.822)	0.0206	Cellular Transport and Transport Mechanisms
BQ113310	SRP receptor homolog FtsY precursor chloroplast (validated) - Arabidopsis thaliana	7.409 (6.313 to 8.506)	0.00469	8.564 (8.438 to 8.689)	0.00143	Cellular Transport and Transport Mechanisms
BQ113941	SRP receptor homolog FtsY precursor chloroplast (validated) - Arabidopsis thaliana	8.017 (7.432 to 8.603)	0.00224	6.342 (6.279 to 6.406)	0.00221	Cellular Transport and Transport Mechanisms
BQ505638	vacuolar sorting protein 35 homolog - Arabidopsis thaliana	9.215 (3.857 to 14.57)	0.0395	10.79 (6.592 to 14.99)	0.0192	Cellular Transport and Transport Mechanisms
BQ117979	vesicle transport v-SNARE (vesicle soluble NSF attachment protein receptor) protein	9.782 (9.633 to 9.931)	0.00115	6.672 (6.643 to 6.702)	0.00209	Cellular Transport and Transport Mechanisms
BQ516467	AIM1 protein - Arabidopsis thaliana; TC53381	5.701 (3.519 to 7.884)	0.165	13.62 (9.33 to 17.91)	0.0149	Development
BQ111846	AT3g02110 F1C9_10 (Arabidopsis thaliana)	32.08 (31.99 to 32.17)	0.000368	3.692 (3.657 to 3.727)	0.00771	Development
BQ117338	auxin response factor 6 (ARF6) (imported) - Arabidopsis thaliana	11.86 (9.465 to 14.26)	0.0181	16.75 (15.36 to 18.14)	0.00767	Development

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ511502	auxin-induced protein IAA8 - tomato (fragment)	8.3 (7.339 to 9.260)	0.141	7.932 (5.900 to 9.964)	0.0193	Development
BQ121263	B12D protein (Ipomoea batatas)	3.058 (1.385 to 4.731)	0.361	12.09 (10.53 to 13.65)	0.005	Development
BQ119389	Cell elongation protein diminuto. (Garden pea) (Pisum sativum); TC55756	6.576 (5.851 to 7.301)	0.0188	6.715 (5.752 to 7.679)	0.0137	Development
BQ512285	homeobox 2 protein (Lycopersicon esculentum)	4.567 (2.656 to 6.478)	0.433	20.40 (19.84 to 20.97)	0.00827	Development
BQ516656	HUA enhancer 2 (Arabidopsis thaliana)	3.168 (2.475 to 3.862)	0.289	19.96 (19.06 to 20.86)	0.00391	Development
BQ115588	LEUNIG (Arabidopsis thaliana)	2.504 (1.826 to 3.183)	0.262	9.633 (7.752 to 11.51)	0.011	Development
BQ119668	brassinosteroid-insensitive protein BRI1 - Arabidopsis thaliana	3.672 (3.522 to 3.823)	0.241	7.527 (6.279 to 8.775)	0.0177	Development; Cellular Communication/Signal Transduction
BQ515554	ATP synthase alpha chain (EC 3.6.3.14). (Common tobacco) (Nicotiana tabacum)	2.322 (2.051 to 2.594)	0.0526	20.39 (18.87 to 21.92)	0.00252	Energy
BQ515387	cytochrome P450 (Arabidopsis thaliana)	4.513 (4.013 to 5.013)	0.0847	8.302 (8.038 to 8.565)	0.00737	Energy
BQ114047	electron transfer flavoprotein beta-subunit-like (Arabidopsis thaliana)	5.402 (5.050 to 5.755)	0.378	10.01 (9.888 to 10.13)	0.00816	Energy
BQ518982	NIFS-like protein CpNifsp precursor (Arabidopsis thaliana)	15.51 (14.55 to 16.47)	0.0103	12.13 (11.94 to 12.33)	0.00644	Energy

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ121095	probable cytochrome P450 F13O11.25 (imported) - Arabidopsis thaliana	3.653 (3.602 to 3.704)	0.0654	6.312 (6.153 to 6.472)	0.0198	Energy
BQ115950	peroxisomal multifunctional protein (Oryza sativa)	2.734 (2.150 to 3.318)	0.685	10.09 (8.179 to 12.00)	0.0159	Energy; Development;
BQ113843	Proteasome subunit beta type 1 (EC 3.4.25.1) (20S proteasome alpha subunit F)	8.428 (8.306 to 8.549)	0.0247	34.51 (31.8 to 37.22)	0.00355	Meabolism
BQ512336	3-dehydroquinate dehydratase (EC 4.2.1.10) shikimate 5-dehydrogenase (EC 1.1.1.25) - tomato	6.84 (6.055 to 7.625)	0.0163	4.113 (3.832 to 4.393)	0.0103	Metabolism
BQ514003	3-oxoacyl-(acyl-carrier-protein) synthase (Capsicum chinense)	2.01 (1.791 to 2.228)	0.753	54.64 (50.37 to 58.92)	0.0109	Metabolism
BQ121840	acetyl-CoA synthetase-like protein (Arabidopsis thaliana)	2.308 (2.239 to 2.376)	0.144	12.18 (12.11 to 12.25)	0.00278	Metabolism
BQ119634	ATP-dependent Clp protease regulatory subunit CLPX (Arabidopsis thaliana)	2.473 (1.213 to 3.733)	0.629	13.13 (12.44 to 13.83)	0.00614	Metabolism
BQ116302	Auxin-induced protein PCNT115. (Common tobacco) (Nicotiana tabacum)	27.77 (24.37 to 31.16)	0.0115	3.054 (3.026 to 3.082)	0.0138	Metabolism
BQ517075	beta Galactosidase-like protein - Arabidopsis thaliana	3.747 (3.120 to 4.373)	0.373	7.849 (6.431 to 9.267)	0.0172	Metabolism
BQ112288	cold-induced glucosyl transferase (Solanum sogarandinum)	43.67 (43.38 to 43.96)	0.0013	125.1 (124.5 to 125.6)	0.000219	Metabolism

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ118537	CTP synthase (Arabidopsis thaliana)	3.584 (3.204 to 3.963)	0.171	18.03 (14.62 to 21.43)	0.00897	Metabolism
BQ518130	Cysteine desulfurase mitochondrial precursor (EC 4.4.1.-). (Mouse-ear cress) (Arabidopsis thaliana)	6.420 (5.612 to 7.229)	0.0148	11.88 (11.21 to 12.54)	0.00388	Metabolism
BQ118862	Cysteine desulfurase mitochondrial precursor (EC 4.4.1.-). (Mouse-ear cress) (Arabidopsis thaliana); TC42609	2.976 (2.656 to 3.297)	0.0422	18.10 (17.78 to 18.42)	0.00241	Metabolism
BQ517292	cytochrome P450 (EC 1.14.-.-) 81B1c - Jerusalem artichoke; TC54452	2.723 (2.591 to 2.856)	0.375	12.63 (11.33 to 13.94)	0.0113	Metabolism
BQ113430	FRO2-like protein; NADPH oxidase-like (Arabidopsis thaliana)	3.534 (3.494 to 3.575)	0.00863	2.811 (2.794 to 2.829)	0.0183	Metabolism
BQ510567	glucan 1 3-beta-glucosidase (EC 3.2.1.58) (imported) - common tobacco	3.496 (3.288 to 3.705)	0.05	5.667 (5.078 to 6.255)	0.0147	Metabolism
BQ116391	GMP synthase 61700-64653 (imported) - Arabidopsis thaliana; TC53227	3.102 (2.513 to 3.691)	0.596	17.71 (17.36 to 18.06)	0.0141	Metabolism
BQ512271	probable glucosyl transferase (imported) - Arabidopsis thaliana	12.97 (10.57 to 15.37)	0.0334	137.1 (134.4 to 139.9)	0.00184	Metabolism
BQ508885	probable glucosyl transferase (imported) - Arabidopsis thaliana	6.106 (3.723 to 8.49)	0.289	15.17 (14.84 to 15.51)	0.014	Metabolism
BQ120828	Probable glutathione S-transferase (EC 2.5.1.18) (Auxin-induced protein PGNT35 PCNT111).	3.632 (0.424 to 6.840)	0.352	4.435 (4.395 to 4.476)	0.00472	Metabolism

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ506914	probable phosphoglycerate dehydrogenase (EC 1.1.1.95) - Arabidopsis thaliana	3.015 (2.441 to 3.588)	0.111	12.74 (9.702 to 15.78)	0.00958	Metabolism
BQ117212	probable polygalacturonase (EC 3.2.1.15) 1 - tomato	8.951 (8.903 to 8.998)	0.00127	24.31 (24.31 to 24.31)	0.000485	Metabolism
BQ113221	putative S1 protein (Arabidopsis thaliana); TC54400	12.34 (11.98 to 12.7)	0.0155	6.997 (6.932 to 7.062)	0.00241	Metabolism
BQ117370	S-adenosyl-L-methionine	3.614 (3.31 to 3.918)	0.0212	25.63 (22.87 to 28.38)	0.00291	Metabolism
BQ508377	Zeatin O-xylosyltransferase (EC 2.4.1.204) (Zeatin O-beta-D-xylosyltransferase).	10.28 (8.783 to 11.77)	0.0559	15.39 (15.31 to 15.48)	0.0041	Metabolism
BQ507676	RAD23 protein (Lycopersicon esculentum)	5.861 (5.623 to 6.100)	0.0491	14.04 (12.12 to 15.96)	0.00854	Metabolism; Cell Growth, Cell Division and DNA Synthesis
BQ120185	3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 (EC 1.1.1.34) (HMG-CoA reductase 2) (HMG2.2).	3 (2.788 to 3.211)	0.448	25.49 (23.16 to 27.82)	0.0126	Metabolism; Cell Rescue, Defense, Cell Death and Aging
BQ115026	cytochrome P450 (Arabidopsis thaliana)	9.365 (8.553 to 10.18)	0.0383	12.62 (10.34 to 14.9)	0.00408	Metabolism; Cell Rescue, Defense, Cell Death and Aging
BQ509879	probable cullin protein - tomato	2.485 (1.727 to 3.243)	0.475	4.852 (4.613 to 5.092)	0.0214	Metabolism; Cell Rescue, Defense, Cell Death and Aging
BQ113646	xanthine dehydrogenase homolog T11I11.130 - Arabidopsis thaliana	2.419 (0.539 to 4.299)	0.578	44.88 (37.28 to 52.49)	0.00866	Metabolism; Cell Rescue, Defense, Cell Death and Aging

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ512781	26S proteasome regulatory particle non-ATPase subunit5 (Oryza sativa)	2.083 (2.043 to 2.123)	0.185	16.74 (16.41 to 17.07)	0.00151	Protein Destination
BQ116223	At1g12410 F5O11_7 (Arabidopsis thaliana)	2.471 (0.884 to 4.057)	0.448	9.542 (8.926 to 10.16)	0.00593	Protein Destination
BQ512511	At1g64230 F22C12_17 (Arabidopsis thaliana)	5.672 (5.491 to 5.853)	0.0798	4.917 (4.409 to 5.425)	0.0162	Protein Destination
BQ111622	Regulator of nonsense transcripts 1 homolog. (Mouse-ear cress) (Arabidopsis thaliana)	2.911 (2.576 to 3.245)	0.284	10.11 (10.08 to 10.15)	0.00816	Protein Destination
BQ119542	signal recognition particle 54K protein - tomato (cv. Rentita)	3.468 (3.448 to 3.488)	0.425	7.482 (7.078 to 7.886)	0.0213	Protein Destination
BQ511894	syntaxin related protein AtVam3p (Arabidopsis thaliana)	3.165 (2.852 to 3.479)	0.282	6.482 (5.702 to 7.261)	0.0132	Protein Destination
BQ114231	transport protein particle component Bet3p-like protein (Arabidopsis thaliana)	3.652 (3.452 to 3.851)	0.192	5.013 (4.237 to 5.789)	0.0184	Protein Destination
BQ519244	ubiquinol--cytochrome-c reductase (EC 1.10.2.2) 11K protein - potato	3.189 (2.679 to 3.699)	0.0902	13.06 (13.03 to 13.09)	0.00434	Protein Destination
BQ112099	ubiquitin activating enzyme 2 (Arabidopsis thaliana)	4.466 (2.446 to 6.486)	0.134	17.31 (15.22 to 19.40)	0.00237	Protein Destination
BQ513173	ubiquitin-conjugating enzyme-like protein (Arabidopsis thaliana)	2.077 (1.631 to 2.524)	0.502	5.448 (4.924 to 5.972)	0.0218	Protein Destination
BQ512785	zinc metalloprotease (insulinase family) (Arabidopsis thaliana)	2.694 (2.361 to 3.028)	0.059	2.783 (2.696 to 2.871)	0.0194	Protein Destination

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ515965	40S ribosomal protein S11. (Soybean) (Glycine max)	6.070 (5.217 to 6.923)	0.0252	31.52 (19.93 to 43.12)	0.00701	Protein Synthesis
BQ510858	40S ribosomal protein S6. (Garden asparagus) (Asparagus officinalis)	7.882 (5.158 to 10.61)	0.032	10.41 (9.728 to 11.09)	0.00471	Protein Synthesis
BQ512246	60S ribosomal protein L35 (Euphorbia esula)	3.709 (3.676 to 3.743)	0.0346	10.22 (10.2 to 10.24)	0.00442	Protein Synthesis
BQ513262	At1g07830 F24B9_7 (Arabidopsis thaliana)	6.519 (5.690 to 7.347)	0.0525	5.851 (5.567 to 6.134)	0.0149	Protein synthesis
BQ121217	eukaryotic initiation factor 3H1 subunit (Arabidopsis thaliana)	4.636 (3.874 to 5.399)	0.474	30.55 (30.31 to 30.79)	0.00932	Protein Synthesis
BQ515507	Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta)	10.21 (10.07 to 10.34)	0.0131	5.972 (5.826 to 6.117)	0.00578	Protein synthesis
BQ116593	hypothetical protein AAF19747.1 (imported) - Arabidopsis thaliana	14.56 (11.61 to 17.50)	0.119	10.23 (7.928 to 12.53)	0.0149	Protein Synthesis
BQ514275	N2 N2-dimethylguanosine tRNA methyltransferases-like protein - Arabidopsis thaliana	2.884 (2.76 to 3.009)	0.114	3.494 (3.485 to 3.502)	0.0208	Protein Synthesis
BQ513623	plastid ribosomal protein S6 precursor (Spinacia oleracea)	2.873 (2.654 to 3.092)	0.11	15.84 (14.96 to 16.73)	0.00311	Protein Synthesis
BQ514749	Protein translation factor SUI1 homolog. (Japanese willow) (Salix bakko)	5.764 (5.73 to 5.798)	0.00267	9.547 (9.484 to 9.609)	0.0012	Protein Synthesis
BQ115876	putative cysteinyl-tRNA synthetase (Arabidopsis thaliana)	3.281 (2.856 to 3.705)	0.274	12.79 (12.50 to 13.08)	0.0106	Protein Synthesis

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ507646	putative RNA-binding protein (Arabidopsis thaliana)	2.333 (2.167 to 2.5)	0.397	17 (13.45 to 20.54)	0.00666	Protein Synthesis
BQ518386	rna-binding protein (Schizosaccharomyces pombe)	2.209 (0.968 to 3.451)	0.832	13.24 (8.101 to 18.38)	0.015	Protein Synthesis
BQ506043	RPS6-like protein (Arabidopsis thaliana)	2.068 (1.786 to 2.351)	0.403	18.83 (17.83 to 19.83)	0.00405	Protein Synthesis
BQ112260	AT3g14110 MAG2_6 (Arabidopsis thaliana)	6.732 (6.673 to 6.79)	0.011	4.562 (4.528 to 4.597)	0.00631	Signal Transduction
BQ510794	contains similarity to guanylate binding protein~gene_id	9.95 (9.867 to 10.03)	0.0011	6.165 (5.765 to 6.565)	0.00324	Signal Transduction
BQ114948	contains similarity to kinase~gene_id	4.232 (3.645 to 4.82)	0.119	3.848 (3.73 to 3.967)	0.012	Signal Transduction
BQ507290	cyclophilin-40 (Arabidopsis thaliana)	5.255 (2.2 to 8.309)	0.204	32.39 (28.88 to 35.89)	0.0047	Signal Transduction
BQ515951	cyclophilin-40 (Arabidopsis thaliana)	4.922 (3.994 to 5.849)	0.468	22.99 (15.89 to 30.08)	0.0102	Signal Transduction
BQ114069	FKBP12 interacting protein (FIP37) - Arabidopsis thaliana	4.115 (3.606 to 4.624)	0.188	28.17 (26.62 to 29.73)	0.0102	Signal Transduction
BQ120931	phosphoprotein phosphatase (EC 3.1.3.16) catalytic beta chain - alfalfa	2.469 (1.849 to 3.09)	0.274	8.91 (7.803 to 10.02)	0.0158	Signal Transduction
BQ518562	At2g21320 F3K23.8 (Arabidopsis thaliana)	3.351 (1.396 to 5.307)	0.134	3.887 (3.519 to 4.255)	0.0156	Transcription

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ516498	At2g21320 F3K23.8 (Arabidopsis thaliana)	7.280 (1.509 to 13.05)	0.19	52.87 (48.12 to 57.61)	0.00506	Transcription
BQ514543	bZIP DNA-binding protein HBF-1 - soybean	2.522 (2.148 to 2.896)	0.286	14.26 (12.53 to 15.99)	0.00581	Transcription
BQ112534	H1flk (Arabidopsis thaliana)	4.637 (4.227 to 5.046)	0.00682	3.908 (3.822 to 3.994)	0.00681	Transcription
BQ512011	hypothetical protein AT4g07410 (imported) - Arabidopsis thaliana; TC52272	3.748 (3.207 to 4.29)	0.0848	12.81 (10.98 to 14.63)	0.0133	Transcription
BQ509021	hypothetical protein M4E13.100 - Arabidopsis thaliana	2.320 (1.789 to 2.852)	0.111	11.78 (8.632 to 14.93)	0.00989	Transcription
BQ507050	hypothetical protein T10K17.240 - Arabidopsis thaliana	2.091 (2.086 to 2.096)	0.267	9.323 (8.489 to 10.16)	0.0177	Transcription
BQ510191	polypyrimidine tract-binding RNA transport protein-like (Arabidopsis thaliana)	11.62 (10.87 to 12.38)	0.0556	9.736 (9.551 to 9.922)	0.00932	Transcription
BQ518440	probable DNA-binding protein - garden pea	3.395 (2.996 to 3.794)	0.359	16.41 (12.86 to 19.96)	0.0129	Transcription
BQ509651	probable zinc finger protein (imported) Arabidopsis thaliana	7.734 (6.750 to 8.718)	0.331	20.25 (19.53 to 20.98)	0.0054	Transcription
BQ117380	Putative splicing factor activator protein (Arabidopsis thaliana)	4.941 (4.329 to 5.553)	0.432	21.92 (17.8 to 26.05)	0.02	Transcription
BQ510333	putative zinc finger protein (Arabidopsis thaliana)	2.504 (1.519 to 3.489)	0.262	8.738 (7.790 to 9.687)	0.00531	Transcription

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ505018	spliceosomal-like protein - Arabidopsis thaliana	13.12 (1.967 to 24.27)	0.0599	48.36 (35.26 to 61.46)	0.00489	Transcription
BQ510182	splicing factor-like protein (Arabidopsis thaliana)	3.475 (3.377 to 3.573)	0.347	13.61 (12.11 to 15.11)	0.0157	Transcription
BQ113472	splicing factor-like protein (Arabidopsis thaliana)	2.474 (1.933 to 3.014)	0.253	17.89 (14.73 to 21.05)	0.00677	Transcription
BQ117367	Transcription initiation factor IIB (TFIIB). (Soybean) (Glycine max)	3.863 (3.345 to 4.380)	0.0928	6.595 (6.075 to 7.114)	0.0106	Transcription
BQ117445	U1 small nuclear ribonucleoprotein 70 kDa (U1 SNRNP 70 kDa) (snRNP70) (U1-70K). (Mouse-ear cress)	3.028 (1.936 to 4.119)	0.194	8.468 (6.432 to 10.50)	0.0179	Transcription
BQ118578	U1 small nuclear ribonucleoprotein 70 kDa (U1 SNRNP 70 kDa) (snRNP70) (U1-70K). (Mouse-ear cress)	3.151 (3.095 to 3.207)	0.0934	9.916 (9.315 to 10.52)	0.00925	Transcription
BQ116100	U3 snoRNP-associated-like protein (imported) - Arabidopsis thaliana; TC41546	3.744 (2.805 to 4.683)	0.0339	24.65 (21.73 to 27.56)	0.0013	Transcription
BQ513985	WRKY transcription factor 22 (Arabidopsis thaliana)	10.47 (8.683 to 12.25)	0.0228	29.91 (27.24 to 32.58)	0.0118	Transcription
BQ119928	ribosomal protein L27-like - Arabidopsis thaliana	4.407 (4.015 to 4.8)	0.0775	12.53 (9.720 to 15.34)	0.0163	Translation
BQ515130	CLC-Nt2 protein (Nicotiana tabacum)	2.559 (2.239 to 2.879)	0.14	6.718 (6.231 to 7.206)	0.00936	Transport Facilitation

A1. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Classification of Upregulated Genes (Reference Figure 2.6)

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value	MIPS Classification
BQ117855	cytochrome c oxidase subunit 6b-1 (Oryza sativa)	5.767 (5.428 to 6.105)	0.301	65.46 (56.37 to 74.55)	0.00421	Transport Facilitation
BQ120630	glutathione-conjugate transporter AtMRP4 (imported) - Arabidopsis thaliana	5.613 (5.464 to 5.762)	0.363	36.71 (36.27 to 37.14)	0.0187	Transport Facilitation
BQ115067	nodulin-like protein (imported) - Arabidopsis thaliana	5.299 (5.271 to 5.327)	0.166	27.32 (27.15 to 27.5)	0.00492	Transport Facilitation
BQ510156	permease 1 (Arabidopsis thaliana)	8.702 (6.666 to 10.74)	0.0148	12.04 (10.78 to 13.3)	0.00744	Transport Facilitation
BQ508139	probable nitrate transporter (imported) - Arabidopsis thaliana	5.790 (4.357 to 7.223)	0.0977	46.12 (41.56 to 50.67)	0.00917	Transport Facilitation

A2. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Upregulated Selected Genes in Carotenoid Biosynthesis Pathway

GenBank ID#	TIGR Gene Annotation 0677 and 0681 10 dpi	Sample crtB0677 normalized	Sample crtB0677 t-test p-value	Sample crtB0681 normalized	Sample crtB0681 t-test p-value
BQ509967	beta-carotene hydroxylase (Lycopersicon esculentum)	6.031 (5.401 to 6.661)	0.0355	2.141 (2.036 to 2.247)	0.123
BQ116393	beta-carotene hydroxylase (Lycopersicon esculentum)	4.338 (3.823 to 4.852)	0.279	5.488 (5.266 to 5.71)	0.395
BQ112964	isopentenyl diphosphate isomerase 2 (Nicotiana tabacum); TC53575	4.124 (4.036 to 4.213)	0.0143	3.754 (2.377 to 5.131)	0.201
BQ516016	Phytoene dehydrogenase chloroplast precursor (EC 1.14.99-.-) (Phytoene desaturase). (Tomato)	4.607 (4.381 to 4.832)	0.0126	2.632 (2.614 to 2.649)	0.0532
BQ115584	Phytoene synthase 2 chloroplast precursor (EC 2.5.1.-) (Fragment). (Tomato)	2.607 (1.593 to 3.62)	0.536	2.135 (1.998 to 2.272)	0.388

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ112704	40S ribosomal protein SA (p40). (Carrot) (Daucus carota)	3.082 (2.851 to 3.314)	0.761 (0.753 to 0.768)	0.891 (0.886 to 0.895)	18.43 (16.38 to 20.47)	17.17 (15.19 to 19.15)	3.043 (2.738 to 3.347)	27.39 (22.96 to 31.81)	5.874 (3.288 to 8.461)	2.453 (2.415 to 2.491)
BQ116332	50S ribosomal protein L12 chloroplast precursor (CL12). (Wood tobacco) (Nicotiana sylvestris)	33.06 (31.2 to 34.93)	0.612 (0.563 to 0.661)	1.1 (1.092 to 1.108)	12.44 (10.89 to 13.99)	9.013 (7.684 to 10.34)	10.37 (9.54 to 11.2)	9.493 (1.447 to 17.54)	4.433 (3.87 to 4.996)	3.239 (3.138 to 3.341)
BQ116168	50S ribosomal protein L12 chloroplast precursor (CL12). (Wood tobacco) (Nicotiana sylvestris)	8.642 (7.657 to 9.627)	40.83 (34.19 to 47.48)	0.963 (0.951 to 0.975)	13.46 (12.71 to 14.2)	6.95 (6.637 to 7.263)	2.046 (1.878 to 2.214)	1.038 (1.025 to 1.051)	22.15 (21 to 23.3)	2.706 (2.179 to 3.233)
BQ119646	50S ribosomal protein L21 chloroplast precursor (CL21) (CS-L7). (Spinach) (Spinacia oleracea)	7.991 (7.303 to 8.68)	3.393 (3.365 to 3.42)	2.156 (2.148 to 2.163)	2.323 (2.054 to 2.591)	21.77 (19.89 to 23.64)	8.558 (8.209 to 8.907)	0.803 (0.68 to 0.926)	23.6 (20.4 to 26.8)	3.616 (3.361 to 3.871)
BQ112187	50S ribosomal protein L24 chloroplast precursor (CL24). (Common tobacco) (Nicotiana tabacum)	11.02 (10.49 to 11.56)	2.185 (2.183 to 2.187)	0.982 (0.978 to 0.987)	1.436 (1.423 to 1.449)	8.414 (6.641 to 10.19)	2.862 (2.81 to 2.914)	9.456 (8.658 to 10.25)	12.33 (11.34 to 13.32)	2.369 (2.241 to 2.496)
BQ116353	60S ribosomal protein L35 (Euphorbia esula)	2.497 (2.325 to 2.67)	1.41 (1.407 to 1.413)	1.552 (1.548 to 1.555)	8.627 (6.962 to 10.29)	12.54 (10.16 to 14.91)	14.77 (13.41 to 16.13)	24.58 (21.73 to 27.43)	23.86 (22.35 to 25.36)	2.141 (2.039 to 2.244)
BQ112991	60S ribosomal protein L6 (YL16-like). (Common ice plant) (Mesembryanthemum crystallinum)	2.03 (1.79 to 2.269)	0.644 (0.536 to 0.752)	0.969 (0.968 to 0.971)	33.62 (23.45 to 43.8)	10.32 (9.131 to 11.51)	4.653 (3.54 to 5.765)	43.85 (32.2 to 55.5)	25.08 (22.6 to 27.55)	4.898 (4.596 to 5.201)
BQ117113	60S ribosomal protein L7. (Mouse-ear cress) (Arabidopsis thaliana)	2.575 (2.378 to 2.772)	0.518 (0.49 to 0.545)	0.96 (0.957 to 0.962)	2.751 (2.217 to 3.285)	27.03 (25.16 to 28.9)	5.077 (4.919 to 5.235)	2.673 (2.576 to 2.769)	45.72 (35.95 to 55.5)	2.399 (1.989 to 2.81)
BQ115641	60S ribosomal protein L7. (Mouse-ear cress) (Arabidopsis thaliana)	8.11 (6.325 to 9.896)	4.963 (4.861 to 5.064)	0.691 (0.686 to 0.695)	27.76 (27.44 to 28.08)	15.1 (14.22 to 15.98)	1.395 (1.37 to 1.42)	3.271 (2.809 to 3.734)	2.5 (2.134 to 2.866)	2.504 (2.125 to 2.884)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ112351	60S ribosomal protein L7. (Mouse-ear cress) (Arabidopsis thaliana)	2.907 (2.552 to 3.261)	0.774 (0.656 to 0.893)	1.041 (0.833 to 1.248)	6.022 (5.427 to 6.616)	7.28 (4.539 to 10.02)	6.718 (4.822 to 8.613)	16.38 (0.55 to 32.2)	22.27 (18.4 to 26.15)	3.095 (2.821 to 3.369)
BQ508897	70 kDa peptidylprolyl isomerase (EC 5.2.1.8) (Peptidylprolyl cis-trans isomerase) (Cyclophilin)	3.32 (3.282 to 3.358)	13.37 (12.41 to 14.33)	1.479 (1.474 to 1.484)	30.36 (29.3 to 31.42)	25.65 (22.8 to 28.5)	8.002 (7.379 to 8.625)	31.83 (28 to 35.65)	18.37 (16.15 to 20.6)	1.896 (1.568 to 2.225)
BQ115922	70 kDa peptidylprolyl isomerase (EC 5.2.1.8) (Peptidylprolyl cis-trans isomerase) (Cyclophilin); TC53461	3.359 (2.789 to 3.93)	5.07 (5.051 to 5.09)	1.105 (1.094 to 1.116)	18.93 (16.52 to 21.34)	1.644 (1.191 to 2.097)	15.5 (15.07 to 15.92)	40.15 (38.8 to 41.5)	5.93 (3.67 to 8.19)	2.328 (2.231 to 2.424)
BQ120891	A_IG002N01.18 gene product (Arabidopsis thaliana)	7.616 (5.09 to 10.14)	0.813 (0.751 to 0.875)	0.742 (0.735 to 0.749)	12.3 (9.91 to 14.7)	17.35 (15.75 to 18.94)	3.492 (2.716 to 4.269)	3.104 (0.217 to 5.991)	15.06 (12.94 to 17.18)	2.848 (2.829 to 2.866)
BQ117718	alanine aminotransferase (Arabidopsis thaliana); TC49691	2.447 (2.119 to 2.775)	0.671 (0.417 to 0.924)	1.252 (1.212 to 1.293)	3.838 (3.651 to 4.026)	19.88 (15.35 to 24.4)	20.95 (19.55 to 22.35)	3.37 (2.966 to 3.774)	3.705	2.282 (2.096 to 2.468)
BQ506157	alpha-tubulin (Nicotiana tabacum)	3.136 (0.32 to 5.952)	13.45 (8.468 to 18.44)	10.61 (1.84 to 19.37)	24.7 (20.4 to 29)	15.28 (12.19 to 18.38)	3.801 (3.602 to 4.001)	24.37 (20.1 to 28.65)	1.469 (1.449 to 1.49)	1.128 (1.11 to 1.146)
BQ513537	AT3g01490 F4P13_4 (Arabidopsis thaliana)	2.459 (2.279 to 2.64)	2.151 (1.72 to 2.582)	12.06 (11.63 to 12.49)	8.483 (6.987 to 9.979)	32.43 (29.79 to 35.07)	0.598 (0.585 to 0.611)	2.859 (2.771 to 2.947)	3.803 (2.831 to 4.776)	1.202 (0.967 to 1.437)
BQ111846	AT3g02110 F1C9_10 (Arabidopsis thaliana)	15.06 (13.54 to 16.59)	32.08 (31.99 to 32.17)	3.692 (3.657 to 3.727)	0.84 (0.704 to 0.977)	1.638 (1.625 to 1.652)	3.449 (3.367 to 3.532)	2.78 (2.614 to 2.947)	4.665 (4.382 to 4.947)	3.661 (3.439 to 3.884)
BQ511382	AT3g16910 K14A17_3 (Arabidopsis thaliana)	4.037 (3.283 to 4.791)	2.044 (1.973 to 2.116)	5.332 (4.472 to 6.193)	0.53 (0.0163 to 1.044)	5.189 (5.147 to 5.231)	6.75 (6.2 to 7.3)	10.72 (9.8 to 11.65)	7.2 (6.55 to 7.85)	1.436 (1.129 to 1.744)
BQ511426	AT3g45260 F18N11_20 (Arabidopsis thaliana)	6.611 (4.552 to 8.67)	0.713 (0.666 to 0.761)	2.945 (2.915 to 2.975)	7.99 (7.493 to 8.487)	3.883 (3.708 to 4.058)	5.257 (4.922 to 5.592)	13.83 (12.69 to 14.97)	5.873 (5.322 to 6.425)	1.406 (1.367 to 1.445)
BQ505231	AT3g52990 F8J2_160 (Arabidopsis thaliana)	4.988 (4.852 to 5.124)	0.611 (0.599 to 0.623)	1.484 (1.458 to 1.511)	7.095 (6.738 to 7.451)	9.321 (8.267 to 10.38)	30.07 (27.09 to 33.05)	21.51 (18.2 to 24.82)	36.05 (27.7 to 44.4)	2.931 (2.305 to 3.558)
BQ512875	AT3g53110 T4D2_40 (Arabidopsis thaliana)	1.953 (1.883 to 2.024)	0.567 (0.554 to 0.58)	2.129 (2.019 to 2.239)	18.6 (16.84 to 20.35)	32.94 (32.25 to 33.62)	36.63 (32.35 to 40.9)	4.834 (4.21 to 5.458)	2.264 (1.327 to 3.2)	2.462 (2.087 to 2.836)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ120122	AT4g02530 T10P11_17 (Arabidopsis thaliana)	3.811 (1.717 to 5.904)	2.538 (2.267 to 2.809)	1.136 (0.86 to 1.412)	10.19 (9.982 to 10.39)	9.716 (9.026 to 10.41)	12.35 (10.31 to 14.39)	18.46 (18.19 to 18.73)	5.373 (2.984 to 7.762)	2.038 (2.013 to 2.062)
BQ112434	AT5g14910 F2G14_30 (Arabidopsis thaliana)	2.634 (1.508 to 3.761)	0.842 (0.0814 to 1.603)	1.773 (0.412 to 3.135)	3.861 (3.198 to 4.524)	4.991 (3.935 to 6.047)	5.598 (5.076 to 6.119)	7.551 (5.565 to 9.538)	7.024 (4.771 to 9.278)	2.239 (1.828 to 2.65)
BQ114689	AT5g28840 F7P1_20 (Arabidopsis thaliana)	5.336 (3.781 to 6.891)	2.565 (2.564 to 2.565)	0.292 (0.0299 to 0.554)	5.295 (5.031 to 5.559)	50.25 (42.53 to 57.97)	7.083 (6.815 to 7.35)	6.683 (6.514 to 6.853)	6.763 (5.924 to 7.601)	1.637 (0.916 to 2.358)
BQ117338	auxin response factor 6 (ARF6) (imported) - Arabidopsis thaliana	5.638 (3.996 to 7.28)	11.86 (9.465 to 14.26)	16.75 (15.36 to 18.14)	1.367 (1.289 to 1.445)	31.57 (29.65 to 33.5)	31.25 (24.8 to 37.7)	5.825 (4.558 to 7.092)	3.146 (2.736 to 3.555)	2.099 (1.884 to 2.315)
BQ517075	beta Galactosidase-like protein - Arabidopsis thaliana	2.388 (1.168 to 3.607)	3.747 (3.12 to 4.373)	7.849 (6.431 to 9.267)	10.12 (7.898 to 12.35)	1.81 (1.311 to 2.309)	2.827 (2.82 to 2.834)	4.21 (3.895 to 4.524)	5.587 (5.475 to 5.699)	1.81 (1.486 to 2.134)
BQ116393	beta-carotene hydroxylase (Lycopersicon esculentum)	0.121 (0.0975 to 0.145)	4.338 (3.823 to 4.852)	5.488 (5.266 to 5.71)	17.91 (16.71 to 19.11)	4.788 (4.206 to 5.369)	12.5 (10.65 to 14.35)	4.131 (3.923 to 4.338)	1.521 (1.514 to 1.527)	9.072 (0.0611 to 18.08)
BQ114643	beta-mannosidase enzyme (Lycopersicon esculentum)	4.618 (3.923 to 5.314)	3.017 (2.192 to 3.841)	3.483 (2.483 to 4.484)	25.77 (21.95 to 29.6)	19.32 (17.4 to 21.25)	12.04 (11.84 to 12.25)	3.009 (2.524 to 3.494)	9.825 (9.3 to 10.35)	1.874
BQ511755	biotin carboxyl carrier protein subunit precursor (Glycine max)	3.332 (2.564 to 4.1)	8.293 (7.512 to 9.073)	2.78 (2.757 to 2.803)	1.022 (0.725 to 1.318)	5.2 (4.984 to 5.417)	54.6 (40.4 to 68.8)	1.428 (1.159 to 1.698)	2.28 (1.838 to 2.722)	2.625 (2.142 to 3.109)
BQ120158	Brassinosteroid-regulated protein BRU1. (Soybean) (Glycine max)	2.658 (2.464 to 2.851)	0.441 (0.419 to 0.463)	1.241 (1.235 to 1.248)	4.302 (3.319 to 5.285)	3.723 (2.583 to 4.863)	2.284 (2.181 to 2.386)	2.881 (0.291 to 5.471)	5.618 (5.531 to 5.705)	2.402 (2.395 to 2.41)
BQ119360	Cell division protein ftsH homolog chloroplast precursor (EC 3.4.24.-) (Fragment). (Bell pepper); TC41599	3.224 (2.947 to 3.502)	2.827 (2.635 to 3.019)	2.775 (0.01 to 5.549)	2.822 (2.254 to 3.39)	5.274 (1.788 to 8.761)	1.12 (1.085 to 1.154)	4.795 (3.958 to 5.631)	3.429 (3.269 to 3.589)	2.43 (2.019 to 2.84)
BQ119389	Cell elongation protein diminuto. (Garden pea) (Pisum sativum); TC55756	4.233 (3.607 to 4.86)	6.576 (5.851 to 7.301)	6.715 (5.752 to 7.679)	24.85 (24.2 to 25.5)	5.294 (5.179 to 5.41)	0.914 (0.0335 to 1.795)	2.454 (2.313 to 2.596)	18.73 (17.55 to 19.9)	3.329 (2.966 to 3.693)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ119676	chaperonin 21 precursor (Lycopersicon esculentum)	2.271 (1.842 to 2.699)	0.943 (0.907 to 0.979)	1.056 (1.056 to 1.057)	2.355 (2.178 to 2.532)	3.197 (3.166 to 3.229)	7.705 (6.683 to 8.727)	3.2 (0.851 to 5.55)	21.69 (19.65 to 23.74)	6.203 (5.987 to 6.418)
BQ112312	Chlorophyll A-B binding protein 7 chloroplast precursor (LHCI type II CAB 7). (Tomato)	6.238 (5.129 to 7.347)	0.59 (0.569 to 0.61)	0.911 (0.856 to 0.966)	13.53 (12.95 to 14.12)	3.188 (3.071 to 3.305)	2.085 (1.705 to 2.465)	4.187 (3.448 to 4.926)	3.982 (3.074 to 4.889)	2.521 (2.225 to 2.818)
BQ117504	chloroplast 50S ribosomal protein L15 (Arabidopsis thaliana)	12.61 (10.54 to 14.68)	2.64 (2.552 to 2.727)	1.1 (1.091 to 1.109)	39.59 (36.76 to 42.43)	35.35 (33.66 to 37.04)	15.01 (14.57 to 15.45)	31.37 (29.32 to 33.41)	2.864 (2.825 to 2.903)	3.429 (3.28 to 3.578)
BQ117403	Chloroplast 50S ribosomal protein L16. (Common tobacco) (Nicotiana tabacum)	8.015 (7.639 to 8.391)	2.364 (2.314 to 2.415)	1.462 (1.433 to 1.491)	13.64 (9.366 to 17.91)	70.34 (69.18 to 71.5)	8.813 (8.711 to 8.915)	4.506 (4.309 to 4.702)	5.365 (4.109 to 6.622)	2.661 (2.545 to 2.776)
BQ515209	contains similarity to phytocyanin early nodulin-like protein~gene_id	3.222 (3.021 to 3.423)	0.252 (0.143 to 0.362)	0.468 (0.317 to 0.619)	10.9 (10.05 to 11.76)	11.73 (11.2 to 12.25)	15.67 (14.95 to 16.39)	20.22 (16.52 to 23.92)	3.187 (3.06 to 3.313)	2.344 (1.899 to 2.79)
BQ519227	CycD3;2 (Lycopersicon esculentum)	1.028 (0.771 to 1.285)	2.366 (1.886 to 2.846)	2.23 (1.656 to 2.804)	2.9 (1.3 to 4.5)	7.825 (0.85 to 14.8)	2.4 (1.35 to 3.45)	5.075 (2.05 to 8.1)	7.525 (0.65 to 14.4)	4.033 (0.0819 to 7.983)
BQ509702	cyclin-dependent protein kinase p34cdc2 (Lycopersicon esculentum)	1.866 (1.064 to 2.668)	2.383 (2.114 to 2.651)	2.332 (2.069 to 2.595)	2.042 (1.891 to 2.193)	1.481 (1.27 to 1.692)	14.13 (11.85 to 16.4)	2.066 (2.019 to 2.114)	5.85 (4 to 7.7)	2.067 (1.94 to 2.195)
BQ505800	diminuto (Arabidopsis thaliana)	3.579 (0.314 to 6.844)	1.387 (0.0217 to 2.752)	1.268 (0.091 to 2.446)	12.35 (10.38 to 14.31)	3.603 (2.99 to 4.216)	20.7 (18.87 to 22.53)	17.99 (15.56 to 20.43)	2.398 (2.193 to 2.602)	2.836 (2.805 to 2.867)
BQ117876	ESTs AU069293(C53946) AU077613(E20660) correspond to a region of the predicted gene.; Similar to	3.005 (2.723 to 3.287)	0.963 (0.603 to 1.323)	2.292 (2.235 to 2.349)	1.36 (0.826 to 1.894)	13.64 (13.43 to 13.85)	11.23 (11 to 11.45)	2.023 (1.956 to 2.091)	3.03 (2.832 to 3.229)	4.702 (4.084 to 5.319)
BQ116416	Eukaryotic translation initiation factor 5 (eIF-5). (Kidney bean French bean) (Phaseolus vulgaris)	4.207 (3.599 to 4.816)	1.079 (1.04 to 1.118)	1.339 (1.33 to 1.348)	14.09 (13.76 to 14.42)	6.644 (5.527 to 7.762)	3.129 (3.042 to 3.215)	3.189 (3.153 to 3.225)	19.65 (13.2 to 26.1)	3.291 (3.239 to 3.343)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ514908	F1N19.10 (Arabidopsis thaliana)	2.022 (1.9 to 2.143)	11.92 (10.03 to 13.82)	12.54 (12.01 to 13.06)	6.775 (0.75 to 12.8)	14.83 (2.4 to 27.25)	2.074 (1.847 to 2.3)	2.69 (2.292 to 3.088)	11.26 (10.48 to 12.04)	2.071 (2.068 to 2.074)
BQ112396	ferredoxin--nitrite reductase (EC 1.7.7.1) - common tobacco (fragment); TC52992	4.319 (4.231 to 4.408)	0.37 (0.334 to 0.405)	0.842 (0.824 to 0.861)	11.95 (9 to 14.9)	11.88 (11.53 to 12.22)	11.92 (11.8 to 12.05)	2.292 (2.226 to 2.358)	12.75 (12.2 to 13.3)	2.805 (2.391 to 3.219)
BQ118303	GDP dissociation inhibitor - common tobacco	1 (0.956 to 1.044)	7.571 (7.516 to 7.627)	1.857 (1.824 to 1.891)	2.968 (2.888 to 3.047)	7.859 (7.288 to 8.431)	3.436 (2.86 to 4.011)	26.85 (25.27 to 28.43)	6.845 (6.788 to 6.903)	9.98 (8.355 to 11.61)
BQ114627	*homologue to* PIR|A05035|A05035 translation initiation factor IF-1 homolog - common tobacco chloroplast (fragment) {Nicotiana tabacum;} , *partial (60%)*	3.175 (3.174 to 3.177)	1.211 (1.2 to 1.223)	0.996 (0.993 to 0.998)	3.463 (2.961 to 3.965)	7.104 (6.081 to 8.128)	27.98 (24.73 to 31.23)	3.861 (3.222 to 4.499)	28.76 (28.14 to 29.39)	2.679 (1.71 to 3.647)
BQ505226	*homologue to* UP|Q7X9K1 (Q7X9K1) Ribosomal Pr 117 (Fragment), *partial (27%)*	2.438 (1.845 to 3.032)	6.079 (2.4 to 9.759)	5.418 (3.732 to 7.104)	2.459 (2.379 to 2.539)	12.62 (11 to 14.25)	0.745 (0.557 to 0.933)	2.063 (1.405 to 2.721)	3.246 (1.947 to 4.544)	1.211 (0.718 to 1.705)
BQ118868	hydroxyproline-rich glycoprotein homolog (Arabidopsis thaliana)	0.819 (0.78 to 0.859)	2.653 (1.513 to 3.794)	3.402 (3.006 to 3.798)	11.75 (10.84 to 12.66)	28.29 (26.31 to 30.28)	3.365 (3.349 to 3.38)	3.38 (3.188 to 3.571)	15.47 (14.56 to 16.39)	2.633 (2.376 to 2.889)
BQ511013	hypothetical protein AAF27112.1 (imported) - Arabidopsis thaliana	2.141 (1.723 to 2.558)	1.211 (1.061 to 1.361)	2.612 (2.364 to 2.86)	30.58 (29.93 to 31.23)	37.73 (35.5 to 39.95)	4.189 (3.5 to 4.878)	6.329 (4.915 to 7.742)	5.31 (4.878 to 5.743)	3.064 (2.833 to 3.294)
BQ515625	hypothetical protein AAF80647.1 (imported) - Arabidopsis thaliana	0.703 (0.352 to 1.054)	7.451 (0.328 to 14.57)	2.502 (2.471 to 2.533)	3.426 (2.229 to 4.623)	15.1 (9.129 to 21.07)	3.061 (1.98 to 4.142)	36.95 (33.25 to 40.65)	3.152 (3.05 to 3.254)	1.658 (1.388 to 1.929)
BQ111389	hypothetical protein AT4g22120 - Arabidopsis thaliana	2.254 (1.61 to 2.899)	1.769 (1.669 to 1.869)	28.91 (13.71 to 44.12)	8.183 (7.778 to 8.588)	3.48 (0.7 to 6.261)	25.25 (12.35 to 38.15)	3.903 (3.496 to 4.309)	1.292 (0.887 to 1.696)	2.258 (1.913 to 2.603)
BQ120353	hypothetical protein M7J2.60 - Arabidopsis thaliana	2.192 (1.166 to 3.218)	1.866 (1.793 to 1.939)	0.567 (0.443 to 0.692)	7.25 (6.756 to 7.745)	4.243 (3.543 to 4.943)	7.161 (5.187 to 9.135)	3.245 (2.765 to 3.726)	4.232 (1.929 to 6.536)	2.531 (2.381 to 2.681)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ514005	hypothetical protein T18N14.110 - Arabidopsis thaliana	2.15 (0.908 to 3.392)	3.249 (3.024 to 3.474)	2.083 (1.834 to 2.331)	1.206 (0.955 to 1.457)	9.996 (8.249 to 11.74)	5.521 (5.036 to 6.006)	8.08 (6.131 to 10.03)	13.73 (11.5 to 15.95)	1.933 (0.415 to 3.451)
BQ506826	hypothetical protein~similar to Arabidopsis thaliana hypothetical protein F20O9.120 (Oryza sativa)	3.68 (3.306 to 4.053)	2.093 (1.719 to 2.468)	10.07 (8.118 to 12.03)	4.275 (2.75 to 5.8)	2.655 (1.549 to 3.761)	3.925 (3.45 to 4.4)	2.425 (2.25 to 2.6)	1.726 (0.966 to 2.485)	0.999 (0.58 to 1.419)
BQ113795	L3 Ribosomal protein (Medicago sativa subsp. x varia)	2.024 (1.855 to 2.193)	0.701 (0.678 to 0.724)	1.042 (1.04 to 1.044)	2.816 (2.381 to 3.251)	7.246 (6.88 to 7.612)	2.496 (2.418 to 2.573)	27.73 (25.15 to 30.3)	14.67 (13.95 to 15.39)	8.724 (7.692 to 9.755)
BQ119650	low density lipoprotein B-like protein (Arabidopsis thaliana)	4.44 (4.194 to 4.685)	1.589 (0.93 to 2.248)	4.434 (4.13 to 4.737)	28.23 (24.62 to 31.84)	11.96 (11.2 to 12.72)	11.55 (10.84 to 12.27)	4.651 (3.135 to 6.166)	30.33 (29.35 to 31.3)	2.691 (2.578 to 2.804)
BQ507033	magnesium transporter protein (Arabidopsis	2.249 (2.209 to 2.289)	2.284 (2.048 to 2.52)	1.416 (1.309 to 1.524)	2.195 (1.788 to 2.602)	2.702 (2.172 to 3.231)	3.745 (0.123 to 7.368)	4.2 (0.3 to 8.1)	9.6 (8.5 to 10.7)	1.729 (1.568 to 1.891)
BQ115799	methionine synthase (Solanum tuberosum)	2.756 (2.67 to 2.842)	0.606 (0.475 to 0.737)	1.015 (1.013 to 1.018)	3.01 (2.942 to 3.078)	6.555 (6.275 to 6.834)	13.06 (12.54 to 13.59)	2.091 (1.971 to 2.211)	19.62 (19.6 to 19.65)	4.356 (4.326 to 4.385)
BQ114043	mRNA-binding protein precursor (imported) - tomato (fragment)	2.692 (2.139 to 3.246)	0.718 (0.708 to 0.728)	2.959 (2.944 to 2.975)	18.94 (17.07 to 20.8)	6.01 (5.839 to 6.182)	25.88 (24.75 to 27)	1.844 (1.661 to 2.027)	18.81 (16.45 to 21.17)	2.811 (2.676 to 2.946)
BQ111668	mRNA-binding protein precursor (imported) - tomato (fragment); TC53732	8.96 (8.142 to 9.777)	0.568 (0.535 to 0.601)	6.123 (5.467 to 6.78)	5.686 (4.991 to 6.382)	12.09 (11.34 to 12.84)	21.45 (19.9 to 23)	2.144 (1.824 to 2.464)	18.04 (15.39 to 20.69)	3.831 (3.586 to 4.077)
BQ116314	NADPH-protochlorophyllide oxidoreductase (Vigna	13.86 (12.62 to 15.1)	0.343 (0.342 to 0.343)	0.408 (0.378 to 0.439)	13.73 (10.15 to 17.3)	25.93 (24.1 to 27.75)	10.65 (9.4 to 11.9)	14.07 (13.38 to 14.76)	10.65 (9.9 to 11.4)	2.812 (2.77 to 2.854)
BQ113765	nascent polypeptide associated complex alpha chain (Pinus taeda)	2.327 (1.566 to 3.089)	0.85 (0.752 to 0.948)	0.9 (0.899 to 0.901)	12.74 (12.71 to 12.76)	48.31 (47.63 to 49)	7.95 (7.149 to 8.752)	51 (43.6 to 58.4)	34.5 (33.4 to 35.6)	2.892 (2.016 to 3.767)
BQ514682	No annotation	2.041 (1.973 to 2.11)	3.003 (2.803 to 3.204)	5.393 (5.342 to 5.444)	5.978 (4.795 to 7.161)	12.24 (11.68 to 12.79)	23.02 (22.4 to 23.65)	8.563 (8.15 to 8.976)	15.07 (14.9 to 15.25)	0.683 (0.152 to 1.214)
BQ517778	No annotation	0.142 (0.0194 to 0.265)	4.983 (3.114 to 6.853)	1.948 (1.238 to 2.657)	18.07 (15.07 to 21.07)	27.19 (25.66 to 28.71)	7.173 (6.024 to 8.322)	28.91 (22.29 to 35.52)	28.15 (26.9 to 29.4)	3.791 (3.432 to 4.151)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ116524	oligouridylate binding protein (Nicotiana plumbaginifolia); TC53672	2.456 (0.665 to 4.248)	4.906 (2.809 to 7.003)	12.32 (8.387 to 16.25)	2.878 (2.87 to 2.885)	20.2 (16 to 24.4)	3.759 (3.547 to 3.972)	21.02 (20.5 to 21.55)	2.547 (1.837 to 3.257)	1.659 (1.5 to 1.817)
BQ504945	Ornithine carbamoyltransferase chloroplast precursor (EC 2.1.3.3) (OTCase); TC41428	2.085 (2.064 to 2.106)	2.148 (1.991 to 2.306)	3.547 (0.169 to 6.926)	1.834 (0.494 to 3.174)	5.423 (5.319 to 5.527)	4.722 (3.076 to 6.369)	3.477 (2.29 to 4.664)	0.895 (0.526 to 1.263)	4.656 (0.225 to 9.088)
BQ516892	Peptide methionine sulfoxide reductase (EC 1.8.4.6) (Protein- methionine-S-oxide reductase)	2.643 (0.733 to 4.553)	4.651 (4.374 to 4.927)	13.59 (13.07 to 14.11)	7.44 (6.734 to 8.147)	8.731 (7.444 to 10.02)	15.88 (13.08 to 18.68)	9.514 (9.272 to 9.756)	2.335 (2.075 to 2.594)	1.151 (0.961 to 1.341)
BQ120178	peroxiredoxin (Phaseolus vulgaris)	2.136	0.815 (0.767 to 0.863)	0.943 (0.94 to 0.947)	14.9 (13.61 to 16.19)	12.9 (12.07 to 13.72)	8.005 (7.764 to 8.245)	21.89	10.24 (10.19 to 10.29)	5.829 (5.459 to 6.2)
BQ112405	PEX14 (Arabidopsis thaliana)	0.931 (0.715 to 1.148)	1.307 (0.741 to 1.872)	3.865 (3.402 to 4.328)	26.13 (25.86 to 26.41)	13.25 (13.14 to 13.37)	12.23 (9.1 to 15.35)	21.55 (19.4 to 23.7)	6.675 (1.5 to 11.85)	2.58 (2.309 to 2.852)
BQ117614	phosphate transport protein G7 mitochondrial - soybean	2.824 (2.778 to 2.87)	21.52 (20.39 to 22.66)	0.736 (0.664 to 0.809)	35.55 (32.9 to 38.2)	2.189 (2.005 to 2.374)	16.36 (13.07 to 19.66)	4.326 (0.236 to 8.416)	14.73 (13.35 to 16.1)	2.591 (2.519 to 2.664)
BQ116188	Phosphoglycerate kinase cytosolic (EC 2.7.2.3). (Common tobacco) (Nicotiana tabacum)	6.041 (5.382 to 6.699)	1.882 (1.812 to 1.952)	2.485 (2.481 to 2.489)	22.4 (21.96 to 22.83)	2.282 (2.186 to 2.379)	2.603 (2.499 to 2.707)	1.126 (1.116 to 1.136)	17.52 (17.45 to 17.59)	2.901 (2.778 to 3.025)
BQ116142	Phosphoglycerate kinase cytosolic (EC 2.7.2.3). (Common tobacco) (Nicotiana tabacum)	2.011 (1.887 to 2.136)	1.435 (1.41 to 1.46)	2.005 (1.981 to 2.028)	2.54 (2.499 to 2.581)	13.68 (11.19 to 16.17)	15.18 (13.79 to 16.56)	18.43 (14.72 to 22.14)	4.484 (4.42 to 4.548)	3.14 (3.044 to 3.236)
BQ507192	Plastidic ATP ADP-transporter. (Potato) (Solanum tuberosum)	1.722 (1.64 to 1.804)	1.993 (1.814 to 2.172)	10.8 (10.79 to 10.81)	13.46 (8.882 to 18.03)	2.388 (2.361 to 2.416)	44.02 (40.65 to 47.4)	3.36 (2.953 to 3.766)	16.3 (15.56 to 17.04)	4.945 (4.23 to 5.66)
BQ510570	polypyrimidine tract-binding RNA transport protein-like (Arabidopsis thaliana)	2.331 (2.328 to 2.334)	1.661 (1.131 to 2.192)	6.947 (6.531 to 7.362)	3.071 (3.016 to 3.126)	3.883 (3.456 to 4.311)	2.315 (0.975 to 3.656)	2.661 (2.318 to 3.005)	2.213 (1.774 to 2.652)	2.144 (1.966 to 2.322)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ116110	probable aminotransferase 101422-99564 (imported) - Arabidopsis thaliana	10.92 (9.792 to 12.05)	4.007 (3.495 to 4.519)	2.683 (2.325 to 3.04)	3.701 (1.788 to 5.614)	0.896 (0.795 to 0.997)	12.15 (11.2 to 13.1)	2.17 (1.989 to 2.351)	2.026 (1.428 to 2.624)	1.627 (1.44 to 1.813)
BQ518442	Probable glutathione S-transferase (EC 2.5.1.18) (Auxin-induced protein PGNT1 PCNT110).	3.534 (2.615 to 4.453)	5.845 (4.269 to 7.422)	11.07 (8.499 to 13.65)	39.2 (33.35 to 45.05)	22.94 (21.05 to 24.83)	17.35 (17.14 to 17.56)	4.302 (3.731 to 4.874)	1.714 (1.49 to 1.939)	1.4 (1.379 to 1.422)
BQ114493	probable ribosomal protein L34 (imported) - Arabidopsis thaliana; TC45114	9.296 (7.536 to 11.06)	0.338 (0.328 to 0.348)	1.128 (0.878 to 1.378)	3.441 (1.66 to 5.223)	4.184 (3.375 to 4.992)	11.61 (11.31 to 11.91)	11.29 (5.98 to 16.6)	29.56 (29.42 to 29.69)	10.39 (5.648 to 15.13)
BQ119527	Proteasome subunit alpha type 4 (EC 3.4.25.1) (20S proteasome alpha subunit C)	1.881 (1.841 to 1.922)	5.222 (5.202 to 5.242)	2.291 (2.27 to 2.312)	0.93 (0.758 to 1.103)	2.187 (1.949 to 2.425)	53.55 (49.2 to 57.9)	13.01 (9.612 to 16.42)	2.111 (1.963 to 2.259)	2.624 (2.55 to 2.697)
BQ117181	protein kinase-like protein (Arabidopsis thaliana)	4.027 (3.473 to 4.58)	1.901 (1.101 to 2.7)	3.753 (3.451 to 4.056)	2.105 (2.084 to 2.125)	28.87 (21.15 to 36.6)	5.018 (0.809 to 9.227)	3.283 (3.093 to 3.474)	6.65 (6.25 to 7.05)	0.948 (0.781 to 1.114)
BQ512694	putative aquaporin TIP3 (Vitis berlandieri x Vitis rupestris)	0.285 (0.138 to 0.432)	5.446 (2.292 to 8.6)	6.348 (5.82 to 6.877)	8.262 (6.361 to 10.16)	1.66 (1.466 to 1.853)	42.57 (33.9 to 51.25)	2.258 (2.069 to 2.448)	2.443 (1.978 to 2.908)	2.028 (1.598 to 2.457)
BQ117343	putative heat-shock protein (Arabidopsis thaliana)	1.314 (0.734 to 1.894)	3.629 (3.54 to 3.718)	2.086 (1.958 to 2.214)	7.539 (6.683 to 8.394)	39.28 (38.58 to 39.98)	6.464 (5.864 to 7.063)	3.988 (3.906 to 4.07)	3.787 (2.607 to 4.967)	1.495 (1.249 to 1.742)
BQ511792	putative protein (Arabidopsis thaliana)	1.607 (1.546 to 1.669)	8.356 (7.537 to 9.174)	3.467 (3.131 to 3.804)	11.03 (9.95 to 12.12)	21.17 (19.95 to 22.4)	1.27 (1.254 to 1.285)	4.01 (2.883 to 5.137)	2.159 (0.887 to 3.431)	2.672 (2.191 to 3.154)
BQ510641	putative resistance protein (Lycopersicon esculentum)	12.16 (11.92 to 12.41)	16.89 (16.65 to 17.12)	52.82 (52.23 to 53.4)	1.82 (1.514 to 2.125)	8.221 (8.016 to 8.426)	5.079 (4.74 to 5.417)	31.37 (29.35 to 33.4)	4.608 (4.343 to 4.872)	2.94 (2.401 to 3.478)
BQ507672	putative ribosomal protein L10 (Arabidopsis thaliana)	5.434 (4.964 to 5.903)	0.585 (0.548 to 0.621)	1.028 (1.009 to 1.048)	2.829 (2.755 to 2.902)	52 (48.75 to 55.25)	3.956 (3.734 to 4.178)	3.085 (2.925 to 3.244)	26.57 (21.55 to 31.6)	3.433 (2.66 to 4.207)
BQ116132	ribosomal protein L10 chloroplast - common	2.181 (2.018 to 2.344)	0.92 (0.896 to 0.945)	1.962 (1.96 to 1.965)	9.197 (8.447 to 9.947)	4.761 (4.699 to 4.823)	3.545 (3.361 to 3.729)	32.1 (31.73 to 32.48)	6.21 (5.795 to 6.626)	3.212 (2.994 to 3.43)
BQ121684	ribosomal protein L13 (Arabidopsis thaliana)	9.027 (8.863 to 9.191)	2.414 (2.243 to 2.586)	1.331 (1.315 to 1.348)	2.379 (2.041 to 2.718)	2.683 (2.306 to 3.059)	1.207 (0.726 to 1.689)	5.064 (4.757 to 5.37)	2.781 (2.685 to 2.878)	3.169 (2.658 to 3.679)
BQ117085	ribosomal protein L33 (Castanea sativa)	2.4 (2.203 to 2.598)	0.58 (0.579 to 0.581)	0.814 (0.81 to 0.819)	5.694 (5.065 to 6.324)	4.354 (4.331 to 4.376)	8.86 (5.695 to 12.03)	2.826 (2.794 to 2.858)	18.35 (17.45 to 19.25)	2.681 (2.649 to 2.713)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ505879	ribosomal protein S28 (Prunus persica)	1.019 (0.13 to 1.908)	3.393 (0.103 to 6.683)	1.461 (0.106 to 2.816)	9.714 (8.715 to 10.71)	8.361	15.48 (13.31 to 17.65)	2.832 (2.411 to 3.253)	20.92 (13.4 to 28.45)	3.257 (2.986 to 3.529)
BQ119087	ribosomal protein S5 (Spinacia oleracea)	2.812 (2.378 to 3.245)	0.933 (0.922 to 0.944)	0.888 (0.88 to 0.895)	6.844 (6.709 to 6.979)	3.227 (3.118 to 3.335)	2.067 (2.004 to 2.131)	3.664 (3.537 to 3.791)	6.375 (5.865 to 6.885)	2.95 (2.081 to 3.82)
BQ111660	rna binding protein-like - Arabidopsis thaliana; TC45014	3.756 (3.413 to 4.1)	0.826 (0.824 to 0.827)	4.322 (4.2 to 4.445)	2.595 (1.562 to 3.629)	7.125 (5.55 to 8.7)	3.75 (2.05 to 5.45)	0.487 (0.451 to 0.523)	3.5 (2.8 to 4.2)	2.127 (1.381 to 2.873)
BQ117361	RNA helicase (Vigna radiata)	6.16 (4.337 to 7.982)	1.42 (1.26 to 1.579)	1.624 (1.338 to 1.91)	17.36 (14.85 to 19.87)	28.87 (18.78 to 38.97)	15.54 (12.78 to 18.31)	3.695 (1.809 to 5.581)	24.36 (23.95 to 24.77)	3.492 (3.43 to 3.554)
BQ120064	S-adenosylmethionine decarboxylase; SAMDC (Solanum tuberosum)	0.342 (0.217 to 0.467)	2.793 (2.292 to 3.294)	4.782 (4.112 to 5.452)	31.75 (29.15 to 34.35)	2.411 (0.163 to 4.659)	5.361 (5.056 to 5.667)	9.832 (7.039 to 12.62)	2.708 (2.484 to 2.933)	1.781 (1.516 to 2.046)
BQ511409	*similar to* UP|Q7QH57 (Q7QH57) AgCP9225 (Fragment), *partial (3%)*	9.266 (8.997 to 9.536)	35.09 (34.74 to 35.45)	28.58 (28.17 to 29)	2.182 (2.115 to 2.248)	0.91 (0.87 to 0.95)	36.93 (32.55 to 41.3)	0.993 (0.949 to 1.037)	3.374 (3.06 to 3.687)	2.204 (2.006 to 2.401)
BQ114539	*similar to* UP|Q93VK5 (Q93VK5) At1g31800/68069_m00159, *partial (15%)*	9.72 (9.24 to 10.2)	4.018 (1.938 to 6.098)	18.84 (17.24 to 20.44)	8.286 (6.988 to 9.584)	1.186 (0.996 to 1.377)	7.175 (3.25 to 11.1)	6.975 (0.95 to 13)	6.275 (5.3 to 7.25)	1.261 (1.048 to 1.473)
BQ111885	*similar to* UP|Q9AU08 (Q9AU08) NADPH-cytochrome P450 oxydoreductase isoform 1, *partial (23%)*	6.079 (5.94 to 6.218)	9.747 (9.704 to 9.791)	3.163 (3.156 to 3.171)	23.62 (23.51 to 23.72)	4.758 (4.469 to 5.046)	4.415 (3.711 to 5.118)	3.125 (3.072 to 3.178)	3.157 (3.094 to 3.219)	2.87 (2.844 to 2.895)
BQ511601	*similar to* UP|Q9LN01 (Q9LN01) T6D22.15, *partial (4%)*	7.708 (7.447 to 7.97)	3.316 (3.306 to 3.327)	81.56 (73.69 to 89.44)	11.83 (11.8 to 11.85)	3.226 (2.865 to 3.587)	15.2 (13.25 to 17.15)	8.75 (5 to 12.5)	1.586 (1.463 to 1.708)	1.321 (0.801 to 1.841)
BQ505018	spliceosomal-like protein - Arabidopsis thaliana	0.089 (0.0242 to 0.154)	13.12 (1.967 to 24.27)	48.36 (35.26 to 61.46)	4.735 (4.183 to 5.286)	1.005 (0.704 to 1.305)	4.359 (3.894 to 4.824)	7.301 (7.247 to 7.354)	2.584 (2.364 to 2.804)	2.118 (1.9 to 2.337)
BQ112317	sulfur (Nicotiana tabacum)	6.004 (5.763 to 6.246)	1.169 (1.12 to 1.217)	1.202 (1.196 to 1.209)	25.2 (19.07 to 31.33)	18.78 (14.42 to 23.14)	23.83 (19.65 to 28)	2.967 (1.858 to 4.076)	20.19 (17.92 to 22.46)	2.773 (2.398 to 3.148)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ120129	thylakoid lumenal 17.4 kD protein chloroplast precursor (P17.4) (Arabidopsis thaliana)	3.051 (2.574 to 3.528)	2.108 (2.049 to 2.167)	3.104 (2.372 to 3.837)	3.764 (3.596 to 3.931)	2.079 (0.402 to 3.756)	16.27 (15.45 to 17.1)	23.87 (21.05 to 26.7)	0.733 (0.714 to 0.752)	1.754 (1.564 to 1.943)
BQ120993	thylakoid lumenal 17.4 kD protein chloroplast precursor (P17.4) (Arabidopsis thaliana)	3.185 (2.542 to 3.827)	3.236 (2.328 to 4.144)	1.718 (1.668 to 1.767)	2.27 (2.085 to 2.456)	2.14 (0.471 to 3.808)	13.13 (3.5 to 22.75)	11.99 (10.27 to 13.72)	3.04 (2.951 to 3.129)	1.426 (1.387 to 1.465)
BQ117445	U1 small nuclear ribonucleoprotein 70 kDa (U1 SNRNP 70 kDa) (snRNP70) (U1-70K).	2.983 (2.015 to 3.952)	3.028 (1.936 to 4.119)	8.468 (6.432 to 10.5)	2.437 (2.366 to 2.507)	3.653 (3.322 to 3.984)	3.336 (2.473 to 4.199)	14.05 (13.95 to 14.15)	3.558 (3.049 to 4.066)	2.068 (1.701 to 2.436)
BQ517894	ubiquitin carrier protein 4 (Glycine max)	1.08 (1.073 to 1.087)	5.383 (1.764 to 9.002)	8.824 (6.333 to 11.31)	18.69 (16.93 to 20.45)	13.85 (12.9 to 14.79)	7.813 (3.317 to 12.31)	12.74 (12.54 to 12.95)	3.116 (2.649 to 3.583)	2.061 (1.892 to 2.23)
BQ113088	unknown protein (Arabidopsis thaliana)	2.963 (2.663 to 3.263)	6.299 (6.254 to 6.344)	9.932 (9.87 to 9.993)	6.908 (4.341 to 9.476)	1.927 (1.734 to 2.12)	2.066 (1.625 to 2.506)	12.31 (9.817 to 14.81)	2.77 (2.22 to 3.319)	1.622 (1.295 to 1.949)
BQ116425	unknown protein (Arabidopsis thaliana)	3.015 (2.992 to 3.038)	3.547 (3.114 to 3.98)	8.944 (7.462 to 10.43)	8.201 (7.664 to 8.738)	11.34 (10.59 to 12.08)	19.16 (12.41 to 25.91)	46.62 (45.05 to 48.2)	5.343 (4.366 to 6.32)	3.579 (3.129 to 4.029)
BQ513123	Unknown protein (Arabidopsis thaliana)	1.294 (1.246 to 1.343)	4.293 (2.85 to 5.736)	21.35 (12.35 to 30.36)	7.744 (7.159 to 8.328)	7.58 (7.543 to 7.616)	5.442 (0.186 to 10.7)	7.359 (5.866 to 8.851)	2.033 (1.928 to 2.139)	0.841 (0.759 to 0.923)
BQ112219	Unknown protein (Arabidopsis thaliana)	4.518 (3.585 to 5.45)	2.048 (1.95 to 2.147)	0.82 (0.188 to 1.452)	7.8 (4.6 to 11)	9.266 (7.625 to 10.91)	11.98 (10.25 to 13.7)	12.15 (10.34 to 13.97)	3.657 (2.665 to 4.649)	4.305 (3.788 to 4.822)
BQ506690	unnamed protein product (Glycine max)	3.011 (2.665 to 3.357)	2.037 (1.608 to 2.467)	0.862 (0.819 to 0.905)	15.98 (15.24 to 16.72)	7.579 (6.916 to 8.243)	6.743 (3.821 to 9.664)	14.77 (12.25 to 17.28)	17.8 (15.55 to 20.05)	0.973 (0.936 to 1.01)
BQ517311	UP|Q9SVZ1 (Q9SVZ1) Protein kinase-like protein, *partial (5%)*	5.868 (4.673 to 7.064)	4.196 (3.377 to 5.015)	2.984 (2.904 to 3.064)	25.89 (19.57 to 32.22)	3.198 (2.949 to 3.447)	3.454 (2.005 to 4.903)	12.12 (11.73 to 12.51)	1.169 (1.164 to 1.175)	2.394 (2.175 to 2.613)
BQ515181	*weakly similar to* UP|AAR92349 (AAR92349) At1g32690, *partial (35%)*	2.621 (2.448 to 2.795)	1.054 (1.029 to 1.08)	2.422 (2.244 to 2.6)	5.401 (4.656 to 6.146)	29.87 (24.25 to 35.49)	5.753 (5.26 to 6.247)	4.26 (2.212 to 6.307)	3.455 (2.837 to 4.073)	1.379 (1.325 to 1.434)

A3. Microarray Experiment: TTU51 CTP *CrtB*-RZ/TTOSA1 APE pBAD (GFP)
Normalized Gene Expression Ratios, 2-fold or Greater occurring in 7 of 9 Replicates

Normalized Gene Expression (2-fold or greater) at 10 days post inoculation for 7 of 9 replicates		Nb TTU51 CTP-CrtB-RZ vs. Nb TTOSA1 APE pBAD (GFP)								
GenBank ID#	TIGR Annotation	Microarray BarCode #								
		753	677	681	9733	9734	9735	9736	9737	5146
BQ508637	*weakly similar to* UP|Q94A38 (Q94A38) AT5g46250/MPL12_3, *partial (45%)*	0.398 (0.377 to 0.418)	4.348 (2.915 to 5.781)	4.962 (4.96 to 4.965)	12.35 (11.25 to 13.45)	2.465 (2.143 to 2.787)	24 (22.7 to 25.3)	0.894 (0.683 to 1.105)	12.87 (9.65 to 16.1)	2.03 (1.724 to 2.335)
BQ112914	Xylose isomerase (EC 5.3.1.5). (Mouse-ear cress) (Arabidopsis thaliana); TC54670	2.333 (2.164 to 2.502)	0.778 (0.743 to 0.812)	1.631 (1.601 to 1.661)	23.5 (20.35 to 26.65)	19.3 (14 to 24.6)	4.601 (3.316 to 5.886)	21.75 (19.3 to 24.2)	3.884 (3.229 to 4.538)	2.422 (2.226 to 2.618)
BQ111387	Zeta-carotene desaturase chloroplast precursor (EC 1.14.99.30) (Carotene 7 8-desaturase). (Tomato); TC50983	2.321 (2.232 to 2.41)	1.216 (0.124 to 2.309)	0.939 (0.307 to 1.571)	24.77 (20.38 to 29.16)	2.081 (1.912 to 2.251)	5.914 (2.888 to 8.939)	3.206 (2.681 to 3.731)	16.98 (15.8 to 18.17)	2.081 (1.73 to 2.431)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
Normalized Upregulated Gene Expression Ratios, 2-fold or Greater

GenBamk ID#	TIGR Annotation	File Name GSImportPD SGFP9689.txt normalized	File Name GSImportPD SGFP9689.tx t control	File Name GSImportPD SGFP9689.txt raw	File Name GSImportPD SGFP9690.tx t normalized	File Name GSImportPDS GFP9690.txt control	File Name GSImportPD SGFP9690.txt raw
BQ516126	10 kDa chaperonin (Protein CPN10) (Protein GROES). [Rape] {Brassica napus}	2.351 (1.692 to 3.009)	18.14 (17.61 to 18.68)	42.65 (30.7 to 54.6)	2.39 (0.99 to 3.79)	10 (9.944 to 10.06)	23.9 (9.9 to 37.9)
BQ113219	2-oxoglutarate-dependent dioxygenase {Solanum chacoense}; TC57619	3.905 (2.28 to 5.53)	10 (7.223 to 12.78)	39.05 (22.8 to 55.3)	67.32 (1.41 to 133.2)	10 (9.617 to 10.38)	673.2 (14.1 to 1,332.3)
BQ113767	3-hydroxyisobutyryl-coenzyme A hydrolase-like protein {Arabidopsis thaliana}	6.275 (0.75 to 11.8)	10 (8.734 to 11.27)	62.75 (7.5 to 118)	23.59 (0.53 to 46.66)	10 (9.823 to 10.18)	236 (5.3 to 466.6)
BQ119694	60S ribosomal protein L18. [Mouse-ear cress] {Arabidopsis thaliana}	14.32 (1.811 to 26.84)	10.27 (8.389 to 12.16)	147.2 (18.6 to 275.7)	4.729 (1.471 to 7.988)	14.96 (13.01 to 16.91)	70.75 (22 to 119.5)
BQ111892	Actin 97. [Potato] {Solanum tuberosum}	3.79 (1.76 to 5.82)	10 (7.589 to 12.41)	37.9 (17.6 to 58.2)	3.548 (2.317 to 4.778)	33.36 (7.869 to 58.85)	118.4 (77.3 to 159.4)
BQ117076	Adenosylhomocysteinase (EC 3.3.1.1) (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase)	8.21 (0.0125 to 16.41)	191.9 (23.15 to 360.6)	1,575.3 (2.4 to 3,148.2)	2.782 (1.64 to 3.925)	18.29 (6.237 to 30.35)	50.9 (30 to 71.8)
BQ111834	apospory-associated protein C {Arabidopsis thaliana}; TC71404	2.561 (1.152 to 3.97)	10.5 (7.685 to 13.32)	26.9 (12.1 to 41.7)	2.24 (1.76 to 2.72)	10 (9.946 to 10.05)	22.4 (17.6 to 27.2)
BQ513849	arabinogalactan-protein precursor {Nicotiana alata}	3.495 (1.66 to 5.33)	10 (6.31 to 13.69)	34.95 (16.6 to 53.3)	2.101 (2.028 to 2.173)	10.35 (4.59 to 16.12)	21.75 (21 to 22.5)
BQ113729	aspartate kinase (EC 2.7.2.4) / homoserine dehydrogenase (EC 1.1.1.3) precursor -	17.32 (1.49 to 33.14)	10 (9.71 to 10.29)	173.1 (14.9 to 331.4)	10.72 (1.41 to 20.03)	10 (9.156 to 10.84)	107.2 (14.1 to 200.3)
BQ507250	At1g08920/F7G19_20 {Arabidopsis thaliana}	2.11 (1.91 to 2.31)	10 (7.589 to 12.41)	21.1 (19.1 to 23.1)	46.11 (9.38 to 82.84)	10 (8.716 to 11.28)	461.1 (93.8 to 828.4)
BQ121075	At1g09020/F7G19_11 {Arabidopsis thaliana}	3.499 (1.04 to 5.957)	20.87 (6.471 to 35.26)	73 (21.7 to 124.3)	460.7 (5.703 to 915.6)	28.12 (24.24 to 32)	12,955.25 (160.4 to 25,750.1)
BQ120901	At1g54320/F20D21_50 {Arabidopsis thaliana}	29.57 (0.727 to 58.42)	13.89 (7.928 to 19.86)	410.8 (10.1 to 811.6)	2.331 (0.63 to 4.032)	14.76 (10.7 to 18.82)	34.4 (9.3 to 59.5)
BQ505137	AT3g16950/K14A17_7 {Arabidopsis thaliana}; TC68185	13.74 (0.0342 to 27.45)	371.1 (11.49 to 730.8)	5,099.8 (12.7 to 10,186.9)	2.317 (0.866 to 3.767)	15.82 (11.85 to 19.79)	36.65 (13.7 to 59.6)
BQ507093	AT3g54650/T5N23_10 {Arabidopsis thaliana}; TC61286	2.354 (1.704 to 3.004)	14.08 (11.99 to 16.18)	33.15 (24 to 42.3)	5.538 (1.046 to 10.03)	10.13 (9.54 to 10.72)	56.1 (10.6 to 101.6)
BQ118264	AT4g17300/dl4685w {Arabidopsis thaliana}	46.58 (0.618 to 92.54)	17.14 (9.162 to 25.12)	798.4 (10.6 to 1,586.2)	3.76 (0.409 to 7.11)	19.3 (7.778 to 30.82)	72.55 (7.9 to 137.2)
BQ507569	AT4g35230/F23E12_210 {Arabidopsis thaliana}	13.22 (0.01 to 26.43)	215.2 (11.69 to 418.6)	2,843.5 (0.4 to 5,686.7)	2.385 (0.798 to 3.972)	10.9 (10.42 to 11.38)	26 (8.7 to 43.3)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
Normalized Upregulated Gene Expression Ratios, 2-fold or Greater

GenBamk ID#	TIGR Annotation	File Name GSImportPDSGFP9689.txt normalized	File Name GSImportPDSGFP9689.txt control	File Name GSImportPDSGFP9689.txt raw	File Name GSImportPDSGFP9690.txt normalized	File Name GSImportPDSGFP9690.txt control	File Name GSImportPDSGFP9690.txt raw
BQ113588	ATP phosphoribosyl transferase {Arabidopsis thaliana}; TC70793	23.61 (0.0423 to 47.19)	333.3 (9.396 to 657.3)	7,871.3 (14.1 to 15,728.4)	2.31 (2.01 to 2.61)	10 (8.481 to 11.52)	23.1 (20.1 to 26.1)
BQ117738	auxin response factor 1 [imported] - Arabidopsis thaliana	2.945 (1.76 to 4.13)	10 (8.716 to 11.28)	29.45 (17.6 to 41.3)	4.97 (2.49 to 7.45)	10 (6.176 to 13.82)	49.7 (24.9 to 74.5)
BQ505921	Chain A Semi-Reduced Inhibitor-Bound Cyclic Nucleotide Phosphodiesterase From Arabidopsis Thaliana	2.341 (0.651 to 4.031)	16.45 (10.43 to 22.47)	38.5 (10.7 to 66.3)	3.615 (0.42 to 6.81)	10 (8.573 to 11.43)	36.15 (4.2 to 68.1)
BQ516315	Chlorophyll A-B binding protein 1B chloroplast precursor (LHCII type I CAB-1B) (LHCP). [Tomato]	3.669 (0.0885 to 7.249)	496 (243.9 to 748.2)	1,819.8 (43.9 to 3,595.6)	2.321 (1.64 to 3.001)	90.89 (36.11 to 145.7)	210.9 (149.1 to 272.8)
BQ121945	class IV endochitinase {Vitis vinifera}	3.862 (1.328 to 6.396)	15.59 (9.209 to 21.97)	60.2 (20.7 to 99.7)	16.79 (0.78 to 32.8)	10 (9.792 to 10.21)	167.9 (7.8 to 328)
BQ120916	conserved hypothetical protein {Neurospora crassa}	152 (4.104 to 300)	10.84 (10.01 to 11.68)	1,648.5 (44.5 to 3,252.5)	77.41 (0.555 to 154.3)	34.94 (28.42 to 41.47)	2,705.2 (19.4 to 5,391)
BQ514465	contains ESTs D47173(S12339) AU096192(S12386)~unknown protein	3.59 (0.85 to 6.33)	10 (8.77 to 11.23)	35.9 (8.5 to 63.3)	6.775 (4.19 to 9.36)	10 (9.2 to 10.8)	67.75 (41.9 to 93.6)
BQ121975	contains similarity to transcription factor~gene_id	2.175 (1.67 to 2.68)	10 (6.513 to 13.49)	21.75 (16.7 to 26.8)	2.369 (0.278 to 4.461)	14.77 (7.859 to 21.68)	35 (4.1 to 65.9)
BQ518783	contains similarity to unknown protein~dbj|BAA91048.1~gene_id	27.68 (0.75 to 54.61)	10 (5.738 to 14.26)	276.8 (7.5 to 546.1)	2.935 (0.873 to 4.997)	11.57 (11 to 12.14)	33.95 (10.1 to 57.8)
BQ115975	cyclin B-type - common tobacco	8.788 (0.609 to 16.97)	10.68 (6.459 to 14.9)	93.85 (6.5 to 181.2)	2.403 (1.116 to 3.69)	18.37 (8.661 to 28.09)	44.15 (20.5 to 67.8)
BQ517650	Cytochrome P450 71D10 (EC 1.14.-.-). [Soybean] {Glycine max}	4.055 (1.42 to 6.69)	10 (7.968 to 12.03)	40.55 (14.2 to 66.9)	2.565 (1.81 to 3.32)	10 (8.502 to 11.5)	25.65 (18.1 to 33.2)
BQ114712	deacetylvindoline 4-O-acetyltransferase {Catharanthus roseus}	2.687 (0.871 to 4.503)	12.17 (11.82 to 12.53)	32.7 (10.6 to 54.8)	6.837 (1.035 to 12.64)	10.24 (9.806 to 10.68)	70.05 (10.6 to 129.5)
BQ519018	dimethylaniline monooxygenase (N-oxide-forming)-like protein {Arabidopsis thaliana}	3.293 (1.223 to 5.364)	10.8 (7.355 to 14.24)	35.55 (13.2 to 57.9)	35.53 (0.965 to 70.09)	14.19 (6.469 to 21.91)	504.2 (13.7 to 994.6)
BQ513618	dirigent protein {Forsythia x intermedia}	2.27 (2.13 to 2.41)	10 (5.913 to 14.09)	22.7 (21.3 to 24.1)	13.27 (10.75 to 15.78)	10 (8.725 to 11.28)	132.6 (107.5 to 157.8)
BQ111672	DNA repair protein-like {Arabidopsis thaliana}	134.1 (0.873 to 267.3)	12.71 (9.255 to 16.16)	1,704.15 (11.1 to 3,397.2)	16.19 (0.515 to 31.86)	12.24 (8.663 to 15.82)	198.1 (6.3 to 389.9)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
Normalized Upregulated Gene Expression Ratios, 2-fold or Greater

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BQ513513	Elicitor inducible gene product Nt-SubE80 {Nicotiana tabacum}	2.826 (1.805 to 3.846)	10.64 (9.779 to 11.49)	30.05 (19.2 to 40.9)	2.183 (0.01 to 4.374)	279.8 (89.35 to 470.3)	610.8 (-2.4 to 1,224.1)
BQ119210	enoyl-[acyl-carrier-protein] reductase (NADH) (EC 1.3.1.9) 2 precursor - common tobacco; TC61049	12.32 (0.688 to 23.94)	37.07 (27.3 to 46.83)	456.5 (25.5 to 887.6)	62.13 (0.831 to 123.4)	43.31 (36.63 to 49.99)	2,691 (36 to 5,346)
BQ111782	EST597358 mixed potato tissues Solanum tuberosum cDNA clone STMCD06 5' end, mRNA sequence.	3.565 (0.81 to 6.32)	10 (7.013 to 12.99)	35.65 (8.1 to 63.2)	3.07 (1.23 to 4.91)	10 (7.601 to 12.4)	30.7 (12.3 to 49.1)
BQ112222	EST597798 mixed potato tissues Solanum tuberosum cDNA clone STMCF87 5' end, mRNA sequence.	3.45 (1.84 to 5.06)	10 (7.721 to 12.28)	34.5 (18.4 to 50.6)	6.48 (0.79 to 12.17)	10 (9.574 to 10.43)	64.8 (7.9 to 121.7)
BQ114549	EST600125 mixed potato tissues Solanum tuberosum cDNA clone STMCU77 5' end, mRNA sequence.	4.736 (1.325 to 8.147)	10.57 (9.214 to 11.92)	50.05 (14 to 86.1)	2.47 (2.03 to 2.91)	10 (7.584 to 12.42)	24.7 (20.3 to 29.1)
BQ117388	EST602964 mixed potato tissues Solanum tuberosum cDNA clone STMDS35 5' end, mRNA sequence.	3.427 (0.01 to 6.846)	1,651.927 (7.173 to 3,296.681)	5,661.55 (13.8 to 11,309.3)	19.19 (0.96 to 37.42)	10 (9.127 to 10.87)	191.9 (9.6 to 374.2)
BQ119120	EST604696 mixed potato tissues Solanum tuberosum cDNA clone STMEH39 5' end, mRNA sequence.	2.429 (1.603 to 3.254)	10.73 (8.607 to 12.85)	26.05 (17.2 to 34.9)	3.67 (0.27 to 7.07)	10 (9.411 to 10.59)	36.7 (2.7 to 70.7)
BQ506824	EST614239 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMGM65 5' end, mRNA sequence.	3.575 (0.203 to 6.948)	12.84 (8.702 to 16.97)	45.9 (2.6 to 89.2)	19.58 (8.85 to 30.32)	10 (7.556 to 12.44)	195.8 (88.5 to 303.2)
BQ511645	EST619060 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMHS88 5' end, mRNA sequence.	12.23 (1.429 to 23.02)	16.17 (7.961 to 24.37)	197.6 (23.1 to 372.2)	2.24 (0.59 to 3.89)	10 (7.863 to 12.14)	22.4 (5.9 to 38.9)
BQ512462	EST619877 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMHY54 5' end, mRNA sequence.	2.169 (0.944 to 3.394)	12.61 (8.084 to 17.13)	27.35 (11.9 to 42.8)	5.765 (1.53 to 10)	10 (9.637 to 10.36)	57.65 (15.3 to 100)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
Normalized Upregulated Gene Expression Ratios, 2-fold or Greater

GenBamk ID#	TIGR Annotation	File Name GSImportPD SGFP9689.txt normalized	File Name GSImportPD SGFP9689.tx t control	File Name GSImportPD SGFP9689.txt raw	File Name GSImportPD SGFP9690.tx t normalized	File Name GSImportPDS GFP9690.txt control	File Name GSImportPD SGFP9690.txt raw
BQ513543	EST620958 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIF81 5' end, mRNA sequence.	10.29 (1.9 to 18.67)	10.79 (8.75 to 12.82)	110.9 (20.5 to 201.4)	45.67 (0.106 to 91.23)	35.86 (33.78 to 37.94)	1,637.75 (3.8 to 3,271.7)
BQ517506	EST624921 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMJE13 5' end, mRNA sequence.	6.67 (1.3 to 12.04)	10 (7.572 to 12.43)	66.7 (13 to 120.4)	6.695 (4.23 to 9.16)	10 (7.807 to 12.19)	66.95 (42.3 to 91.6)
BQ517525	EST624940 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMJE29 5' end, mRNA sequence.	345.8 (0.614 to 690.9)	14.66 (9.023 to 20.3)	5,069.75 (9 to 10,130.5)	2.065 (1.02 to 3.111)	11.38 (6.38 to 16.38)	23.5 (11.6 to 35.4)
BQ517913	EST625328 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMJG84 5' end, mRNA sequence.	2.491 (1.684 to 3.299)	10.16 (9.697 to 10.61)	25.3 (17.1 to 33.5)	7.49 (1.81 to 13.17)	10 (7.695 to 12.31)	74.9 (18.1 to 131.7)
BQ518186	EST625601 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMJI60 5' end, mRNA sequence.	4.208 (0.63 to 7.785)	11.26 (7.619 to 14.91)	47.4 (7.1 to 87.7)	349.2 (0.772 to 697.5)	13.72 (10.71 to 16.74)	4,791.75 (10.6 to 9,572.9)
BQ518992	EST626407 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMJN29 5' end, mRNA sequence.	2.159 (0.0495 to 4.268)	1,277.959 (15.88 to 2,540.04)	2,758.85 (63.3 to 5,454.4)	2.605 (1.475 to 3.734)	17.49 (17.16 to 17.81)	45.55 (25.8 to 65.3)
BQ116482	Eukaryotic translation initiation factor 5 (eIF-5). [Kidney bean French bean] {Phaseolus vulgaris}	3.07 (1.273 to 4.867)	16.42 (10.57 to 22.26)	50.4 (20.9 to 79.9)	2.569 (0.486 to 4.651)	15.63 (11.61 to 19.65)	40.15 (7.6 to 72.7)
BQ513020	Expressed protein {Arabidopsis thaliana}	2.37 (0.58 to 4.16)	10 (9.867 to 10.13)	23.7 (5.8 to 41.6)	14.88 (1.66 to 28.1)	10 (6.058 to 13.94)	148.8 (16.6 to 281)
BQ113979	extensin homolog - potato (fragment); TC66021	2.165 (1.242 to 3.088)	17.39 (15.62 to 19.16)	37.65 (21.6 to 53.7)	4.345 (0.88 to 7.81)	10 (9.632 to 10.37)	43.45 (8.8 to 78.1)
BQ511506	F1N19.26 {Arabidopsis thaliana}	17.9 (0.69 to 35.11)	10 (9.969 to 10.03)	179 (6.9 to 351.1)	7.336 (0.635 to 14.04)	27.87 (16.23 to 39.52)	204.4 (17.7 to 391.2)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
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GenBamk ID#	TIGR Annotation	File Name GSImportPDSGFP9689.txt normalized	File Name GSImportPDSGFP9689.txt control	File Name GSImportPDSGFP9689.txt raw	File Name GSImportPDSGFP9690.txt normalized	File Name GSImportPDSGFP9690.txt control	File Name GSImportPDSGFP9690.txt raw
BQ518078	F20B17.13 {Arabidopsis thaliana}	2.149 (0.718 to 3.581)	11.56 (6.26 to 16.86)	24.85 (8.3 to 41.4)	19.04 (0.01 to 38.76)	10 (9.2 to 10.8)	190.4 (-6.7 to 387.6)
BQ120711	ferredoxin--nitrite reductase (EC 1.7.7.1) - common tobacco (fragment); TC65206	8.334 (0.0402 to 16.63)	472.9 (12.21 to 933.6)	3,941.3 (19 to 7,863.6)	3.365 (1.27 to 5.46)	10 (8.313 to 11.69)	33.65 (12.7 to 54.6)
BQ509136	galactonolactone dehydrogenase (EC 1.3.2.3) - broccoli	6.55 (0.89 to 12.21)	10 (9.693 to 10.31)	65.5 (8.9 to 122.1)	6.102 (1.428 to 10.78)	18.07 (10.88 to 25.26)	110.2 (25.8 to 194.7)
BQ506746	gamma tubulin {Nicotiana tabacum}; TC66286	19.67 (1.86 to 37.48)	11.4 (9.471 to 13.33)	224.2 (21.2 to 427.3)	12.01 (0.575 to 23.45)	26.25 (21.52 to 30.98)	315.3 (15.1 to 615.5)
BQ514335	gene_id	2.033 (0.835 to 3.23)	12.69 (7.726 to 17.66)	25.8 (10.6 to 41)	2.962 (1.575 to 4.349)	13.59 (10.6 to 16.58)	40.25 (21.4 to 59.1)
BQ120127	gene_id	2.245 (0.151 to 4.339)	36.39 (7.551 to 65.24)	81.7 (5.5 to 157.9)	65.98 (0.288 to 131.7)	27.74 (12.54 to 42.95)	1,830.5 (8 to 3,653)
BQ114150	gene_id	2.84 (0.72 to 4.96)	10 (8.461 to 11.54)	28.4 (7.2 to 49.6)	3.017 (2.291 to 3.743)	433.7 (27.13 to 840.3)	1,308.4 (993.6 to 1,623.2)
BQ111871	gene_id	4.965 (1.11 to 8.82)	10 (9.828 to 10.17)	49.65 (11.1 to 88.2)	8.555 (0.89 to 16.22)	10 (6.357 to 13.64)	85.55 (8.9 to 162.2)
BQ118343	Heat shock cognate protein 80. [Tomato] {Lycopersicon esculentum}	2.606 (2.163 to 3.05)	14.1 (13.59 to 14.6)	36.75 (30.5 to 43)	2.21 (1.25 to 3.17)	10 (9.413 to 10.59)	22.1 (12.5 to 31.7)
BQ511515	Heat shock protein 83. [Violet Japanese morning glory] {Pharbitis nil}	5.251 (1.687 to 8.814)	17.85 (14.08 to 21.61)	93.7 (30.1 to 157.3)	4.489 (3.863 to 5.116)	22.75 (21.99 to 23.52)	102.2 (87.9 to 116.4)
BQ517923	highly similar to rice zinc finger protein {Arabidopsis thaliana}	3.205 (2.05 to 4.36)	10 (7.679 to 12.32)	32.05 (20.5 to 43.6)	60.54 (3.56 to 117.5)	10 (8.112 to 11.89)	605.4 (35.6 to 1,175.2)
BQ516869	histone H3 (variant H3R-21) - rice	3.528 (0.868 to 6.187)	33.86 (25.42 to 42.31)	119.5 (29.4 to 209.5)	4.569 (2.104 to 7.035)	48.15 (33.07 to 63.23)	220 (101.3 to 338.7)
BQ514906	histone H4 - tomato	2.393 (1.387 to 3.399)	33.74 (17.95 to 49.54)	80.75 (46.8 to 114.7)	6.107 (4.773 to 7.44)	18.56 (15.37 to 21.76)	113.3 (88.6 to 138.1)
BQ516317	hypothetical 122 kd avirulence protein in avrbs3 region {Xanthomonas campestris}	3.155 (1.39 to 4.92)	10 (7.877 to 12.12)	31.55 (13.9 to 49.2)	9.535 (0.47 to 18.6)	10 (9.195 to 10.8)	95.35 (4.7 to 186)
BQ511967	hypothetical protein {Arabidopsis thaliana}	2.135 (1.324 to 2.946)	12.76 (12.52 to 13.01)	27.25 (16.9 to 37.6)	2.537 (0.952 to 4.122)	11.13 (9.376 to 12.89)	28.25 (10.6 to 45.9)
BQ113769	hypothetical protein {Citrus x paradisi}	96.48 (0.695 to 192.3)	10.22 (9.335 to 11.1)	985.7 (7.1 to 1,964.3)	2.69 (1.689 to 3.691)	10.89 (6.239 to 15.55)	29.3 (18.4 to 40.2)

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BQ507338	hypothetical protein {Oryza sativa (japonica cultivar-group)}	2.043 (1.582 to 2.504)	11.06 (10.73 to 11.39)	22.6 (17.5 to 27.7)	566.3 (0.542 to 1,132.081)	19.94 (16.41 to 23.47)	11,292.9 (10.8 to 22,575)
BQ515450	hypothetical protein {Plasmodium falciparum 3D7}	50.34 (1.19 to 99.48)	10 (7.236 to 12.76)	503.3 (11.9 to 994.8)	6.45 (0.79 to 12.11)	10 (7.299 to 12.7)	64.5 (7.9 to 121.1)
BQ121087	hypothetical protein AAF34300.1 [imported] - Arabidopsis thaliana	4.86 (1.15 to 8.57)	10 (9.359 to 10.64)	48.6 (11.5 to 85.7)	37.64 (0.67 to 74.62)	10 (6.492 to 13.51)	376.5 (6.7 to 746.2)
BQ119497	hypothetical protein AAF98563.1 [imported] - Arabidopsis thaliana	11.75 (0.91 to 22.59)	10 (9.566 to 10.43)	117.5 (9.1 to 225.9)	2.52 (0.76 to 4.28)	10 (8.644 to 11.36)	25.2 (7.6 to 42.8)
BQ509662	hypothetical protein AAG21619.1 [imported] - Arabidopsis thaliana	2.223 (1.917 to 2.529)	11.27 (9.917 to 12.62)	25.05 (21.6 to 28.5)	108.4 (0.16 to 216.6)	105.4 (20.73 to 190)	11,419.1 (16.9 to 22,821.3)
BQ518394	hypothetical protein At2g14170 [imported] - Arabidopsis thaliana	133.4 (1.162 to 265.7)	13.77 (13.42 to 14.13)	1,837.85 (16 to 3,659.7)	2.167 (1.039 to 3.295)	19.06 (10.73 to 27.38)	41.3 (19.8 to 62.8)
BQ517259	hypothetical protein F22K18.50 - Arabidopsis thaliana	5.95 (1.43 to 10.47)	10 (9.889 to 10.11)	59.5 (14.3 to 104.7)	28.59 (0.836 to 56.34)	16.38 (14.49 to 18.27)	468.3 (13.7 to 922.9)
BQ514213	hypothetical protein F25P22.8 [imported] - Arabidopsis thaliana; TC63890	547.4 (0.553 to 1,094.296)	11.22 (9.814 to 12.62)	6,139.9 (6.2 to 12,273.6)	23.61 (1.09 to 46.13)	10 (7.331 to 12.67)	236.1 (10.9 to 461.3)
BQ119042	hypothetical protein F33A8.3 - Caenorhabditis elegans	2.654 (0.268 to 5.041)	50.41 (10.17 to 90.65)	133.8 (13.5 to 254.1)	3.031 (1.334 to 4.729)	26.84 (9.968 to 43.7)	81.35 (35.8 to 126.9)
BQ116570	hypothetical protein F3L17.50 - Arabidopsis thaliana; TC60825	359.4 (0.472 to 718.4)	32.38 (26.88 to 37.89)	11,639.6 (15.3 to 23,263.9)	26.73 (0.179 to 53.29)	48.64 (48.21 to 49.07)	1,300.3 (8.7 to 2,591.9)
BQ112551	Hypothetical protein R03E9.3 {Caenorhabditis elegans}; TC65748	6.044 (0.145 to 11.94)	44.16 (7.027 to 81.29)	266.9 (6.4 to 527.4)	47.82 (0.49 to 95.16)	15.7 (14.87 to 16.53)	750.9 (7.7 to 1,494.1)
BQ509654	hypothetical protein T13J8.10 - Arabidopsis thaliana	2.387 (0.719 to 4.056)	14.33 (13.98 to 14.67)	34.2 (10.3 to 58.1)	20.49 (1.84 to 39.14)	12.23 (11.33 to 13.13)	250.6 (22.5 to 478.6)
BQ507687	hypothetical protein T19K4.120 - Arabidopsis thaliana	14.47 (0.45 to 28.49)	10 (8.68 to 11.32)	144.7 (4.5 to 284.9)	5.71 (1.29 to 10.13)	10 (4.975 to 15.02)	57.1 (12.9 to 101.3)
BQ510660	hypothetical protein T29A15.230 - Arabidopsis thaliana	2.651 (0.61 to 4.692)	15.24 (15.08 to 15.4)	40.4 (9.3 to 71.5)	67.88 (0.0489 to 135.7)	165.7 (16.89 to 314.6)	11,249 (8.1 to 22,489.9)
BQ508839	L-galactose dehydrogenase {Arabidopsis thaliana}; TC61846	29.8 (0.26 to 59.34)	10 (9.926 to 10.07)	298 (2.6 to 593.4)	21.27 (1.69 to 40.84)	10 (9.502 to 10.5)	212.7 (16.9 to 408.4)
BQ509617	Machado-Joseph disease MJD1a-like protein - Arabidopsis thaliana	2.351 (1.821 to 2.881)	13.02 (10.53 to 15.5)	30.6 (23.7 to 37.5)	4.067 (2.126 to 6.008)	11.05 (9.554 to 12.55)	44.95 (23.5 to 66.4)

A4. Microarray Experiment: TTO1 *PDS*/TTOSA1 APE pBAD (GFP)
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BQ506042	major intrinsic protein 2 {Solanum tuberosum}	3.272 (0.554 to 5.99)	20.23 (18.01 to 22.46)	66.2 (11.2 to 121.2)	5.139 (2.111 to 8.167)	10.9 (10.76 to 11.03)	56 (23 to 89)
BQ117274	malate dehydrogenase {Nicotiana tabacum}	26.75 (0.41 to 53.1)	10 (9.875 to 10.12)	267.5 (4.1 to 531)	2.845 (1.199 to 4.491)	14.76 (11.88 to 17.64)	42 (17.7 to 66.3)
BQ117127	NTGP4 {Nicotiana tabacum}	2.332 (2.007 to 2.657)	12.31 (8.604 to 16.01)	28.7 (24.7 to 32.7)	2.35 (0.74 to 3.96)	10 (8.015 to 11.98)	23.5 (7.4 to 39.6)
BQ114465	OSJNBa0033H08.5 {Oryza sativa}	69.54 (0.153 to 138.9)	110.6 (11.97 to 209.2)	7,690.5 (16.9 to 15,364.1)	67.47 (0.298 to 134.6)	32.16 (11.96 to 52.37)	2,170 (9.6 to 4,330.4)
BQ119808	phloem calmodulin-like-domain protein kinase PCPK1 {Cucurbita maxima}	5.675 (1.43 to 9.92)	10 (9.347 to 10.65)	56.75 (14.3 to 99.2)	2.475 (2.38 to 2.57)	10 (9.84 to 10.16)	24.75 (23.8 to 25.7)
BQ117933	phosphomevalonate kinase {Hevea brasiliensis}	3.255 (1.44 to 5.07)	10 (8.08 to 11.92)	32.55 (14.4 to 50.7)	54.74 (0.67 to 108.8)	10 (8.092 to 11.91)	547.4 (6.7 to 1,088.2)
BQ516016	Phytoene dehydrogenase chloroplast precursor (EC 1.14.99.-) (Phytoene desaturase). [Tomato]	171.1 (119.2 to 223)	10 (7.192 to 12.81)	1,711 (1,192.2 to 2,229.8)	151.7 (150.8 to 152.7)	10.78 (9.882 to 11.68)	1,635.85 (1,625.7 to 1,646)
BQ516950	plastidic cysteine synthase 1 {Solanum tuberosum}	3.185 (1.39 to 4.979)	51.64 (19.84 to 83.44)	164.4 (71.8 to 257.1)	3.261 (1.285 to 5.237)	39.76 (23.2 to 56.32)	129.6 (51.1 to 208.2)
BQ117973	PLIC-2 {Mus musculus}	9.16 (0.53 to 17.79)	10 (7.752 to 12.25)	91.6 (5.3 to 177.9)	2.15 (1.06 to 3.24)	10 (8.452 to 11.55)	21.5 (10.6 to 32.4)
BQ513893	probable acyl-CoA synthetase [imported] - Arabidopsis thaliana	16.96 (1.17 to 32.75)	10 (7.3 to 12.7)	169.6 (11.7 to 327.5)	3.042 (2.379 to 3.705)	12.06 (11.36 to 12.77)	36.7 (28.7 to 44.7)
BQ515205	probable acyl-CoA synthetase [imported] - Arabidopsis thaliana	3.301 (0.704 to 5.897)	17.19 (3.811 to 30.58)	56.75 (12.1 to 101.4)	5.3 (2.14 to 8.46)	10 (9.745 to 10.25)	53 (21.4 to 84.6)
BQ507959	probable ankyrin [imported] - Arabidopsis thaliana	3.109 (1.01 to 5.208)	11.48 (8.405 to 14.56)	35.7 (11.6 to 59.8)	3.115 (2.42 to 3.81)	10 (9.68 to 10.32)	31.15 (24.2 to 38.1)
BQ505857	probable dna repair/transcription protein - fission yeast (Schizosaccharomyces pombe)	87.88 (1.29 to 174.5)	10 (8.895 to 11.11)	878.8 (12.9 to 1,744.6)	2.433 (0.72 to 4.147)	12.64 (11.65 to 13.62)	30.75 (9.1 to 52.4)
BQ519088	probable flavonol 3-O-glucosyltransferase [imported] - Arabidopsis thaliana	2.441 (0.691 to 4.19)	14.32 (13.13 to 15.51)	34.95 (9.9 to 60)	256.7 (0.762 to 512.6)	24.42 (21.24 to 27.61)	6,269.3 (18.6 to 12,520)
BQ112205	probable glucosyltransferase [imported] - Arabidopsis thaliana; TC69112	7.38 (0.81 to 13.95)	10 (9.451 to 10.55)	73.8 (8.1 to 139.5)	110.9 (0.897 to 220.9)	10.03 (6.3 to 13.76)	1,112.3 (9 to 2,215.6)
BQ510874	probable replication factor F16M19.6 [imported] - Arabidopsis thaliana	9.668 (0.555 to 18.78)	23.23 (22.92 to 23.54)	224.6 (12.9 to 436.3)	48.83 (43.82 to 53.84)	38.7 (29.94 to 47.47)	1,889.8 (1,695.9 to 2,083.7)

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BQ507586	probable RING zinc finger protein T23K23.8 [imported] - Arabidopsis thaliana	5.466 (0.674 to 10.26)	21.22 (12.16 to 30.29)	116 (14.3 to 217.7)	126.1 (1.033 to 251.1)	23.13 (22.52 to 23.74)	2,916.15 (23.9 to 5,808.4)
BQ518325	probable sugar transporter [imported] - Arabidopsis thaliana	2.568 (0.729 to 4.408)	11.39 (11.03 to 11.75)	29.25 (8.3 to 50.2)	61.17 (6.775 to 115.6)	12.25 (9.869 to 14.63)	749.4 (83 to 1,415.8)
BQ511358	probable WRKY-type DNA binding protein [imported] - Arabidopsis thaliana	12.72 (1.011 to 24.43)	11.18 (8.559 to 13.79)	142.2 (11.3 to 273)	3.899 (1.362 to 6.436)	12.34 (7.585 to 17.09)	48.1 (16.8 to 79.4)
BQ112305	protein F14D16.25 [imported] - Arabidopsis thaliana	5.675 (0.63 to 10.72)	10 (4.562 to 15.44)	56.75 (6.3 to 107.2)	2.995 (0.52 to 5.47)	10 (9.032 to 10.97)	29.95 (5.2 to 54.7)
BQ517122	protein kinase Ck2 regulatory subunit 2 {Nicotiana tabacum}	4.222 (0.692 to 7.751)	10.84 (10.62 to 11.06)	45.75 (7.5 to 84)	2.55 (0.97 to 4.13)	10 (8.345 to 11.65)	25.5 (9.7 to 41.3)
BQ509754	Putative activator-like transposable element {Oryza sativa (japonica cultivar-group)}; TC57651	11.84 (4.66 to 19.02)	10 (5.024 to 14.98)	118.4 (46.6 to 190.2)	6.008 (4.463 to 7.552)	25.25 (14.02 to 36.48)	151.7 (112.7 to 190.7)
BQ511484	putative AMP-binding protein {Arabidopsis thaliana}	2.345 (2.21 to 2.48)	10 (5.481 to 14.52)	23.45 (22.1 to 24.8)	670.8 (1.016 to 1,340.504)	17.02 (10 to 24.04)	11,415.95 (17.3 to 22,814.6)
BQ506561	putative formin binding protein {Oryza sativa (japonica cultivar-group)}	2.835 (1.32 to 4.35)	10 (8.143 to 11.86)	28.35 (13.2 to 43.5)	2.78 (1.36 to 4.2)	10 (8.547 to 11.45)	27.8 (13.6 to 42)
BQ511591	putative GTP-binding protein; 106556-109264 {Arabidopsis thaliana}	3.342 (1.628 to 5.056)	12.16 (10.59 to 13.74)	40.65 (19.8 to 61.5)	11.85 (9.08 to 14.61)	12.24 (9.984 to 14.49)	144.9 (111.1 to 178.8)
BQ120822	putative HesB-like protein {Arabidopsis thaliana}	2.072 (0.793 to 3.352)	12.11 (6.434 to 17.79)	25.1 (9.6 to 40.6)	2.458 (0.652 to 4.265)	10.43 (5.537 to 15.33)	25.65 (6.8 to 44.5)
BQ513588	putative kinesin light chain gene {Oryza sativa (japonica cultivar-group)}	12.8 (0.0242 to 25.57)	218.7 (15.99 to 421.3)	2,798.7 (5.3 to 5,592)	3.136 (1.074 to 5.198)	12.95 (11.64 to 14.25)	40.6 (13.9 to 67.3)
BQ517520	putative merR-family transcriptional regulator {Streptomyces coelicolor A3(2)}	8.96 (1.61 to 16.31)	10 (8.765 to 11.23)	89.6 (16.1 to 163.1)	8.16 (1.55 to 14.77)	10 (9.237 to 10.76)	81.6 (15.5 to 147.7)
BQ118922	putative mitogen-activated protein kinase MAPK {Prunus armeniaca}; TC68334	10.77 (0.331 to 21.21)	30.79 (16.4 to 45.17)	331.6 (10.2 to 653.1)	5.019 (0.823 to 9.215)	11.55 (9.872 to 13.22)	57.95 (9.5 to 106.4)
BQ111972	putative phosphatidate phosphohydrolase {Arabidopsis thaliana}; TC60158	2.295 (2.05 to 2.54)	10 (8.756 to 11.24)	22.95 (20.5 to 25.4)	2.845 (1.35 to 4.34)	10 (8.555 to 11.45)	28.45 (13.5 to 43.4)
BQ511260	putative polyprotein (aspartic proteinase reverse transcriptase ribonuclease H)	5.48 (1.94 to 9.02)	10 (8.02 to 11.98)	54.8 (19.4 to 90.2)	4.373 (1.302 to 7.445)	25.12 (19.46 to 30.77)	109.8 (32.7 to 187)
BQ510095	putative protein {Arabidopsis thaliana}; TC66219	2.132 (1.025 to 3.239)	12.1 (9.656 to 14.55)	25.8 (12.4 to 39.2)	2.502 (0.837 to 4.167)	14.93 (12.44 to 17.42)	37.35 (12.5 to 62.2)

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BQ510641	putative resistance protein {Lycopersicon esculentum}	4.998 (1.302 to 8.694)	12.59 (9.009 to 16.18)	62.95 (16.4 to 109.5)	7.942 (0.4 to 15.48)	34.53 (7.849 to 61.2)	274.2 (13.8 to 534.6)
BQ115797	putative sugar transporter {Oryza sativa}	8.915 (4.16 to 13.67)	10 (8.445 to 11.55)	89.15 (41.6 to 136.7)	11.62 (0.01 to 23.22)	975.6 (7.522 to 1,943.732)	11,333.099 (7.3 to 22,658.898)
BQ512566	putative vesicle transport protein {Arabidopsis thaliana}	5.55 (0.89 to 10.21)	10 (8.239 to 11.76)	55.5 (8.9 to 102.1)	5.89 (1.17 to 10.61)	10 (9.121 to 10.88)	58.9 (11.7 to 106.1)
BQ113397	putative WD-40 repeat protein MSI2 {Arabidopsis thaliana}	3.866 (0.804 to 6.928)	11.2 (9.456 to 12.95)	43.3 (9 to 77.6)	3.534 (1.074 to 5.993)	10.43 (10.07 to 10.79)	36.85 (11.2 to 62.5)
BQ117403	ribosomal protein L16 {Atropa belladonna}	2.836 (0.247 to 5.425)	51.48 (39.44 to 63.52)	146 (12.7 to 279.3)	6.319 (0.177 to 12.46)	62.65 (46.08 to 79.22)	395.9 (11.1 to 780.6)
BQ113965	S-adenosylmethionine synthetase 3 (EC 2.5.1.6) (Methionine adenosyltransferase 3)	46.1 (0.173 to 92.02)	17.32 (5.875 to 28.76)	798.2 (3 to 1,593.4)	4.893 (4.732 to 5.053)	19.31 (17.86 to 20.77)	94.5 (91.4 to 97.6)
BQ514457	Similar to putative transcription factor (AF062890) {Oryza sativa (japonica cultivar-group)}	8.124 (0.804 to 15.44)	26.26 (16.33 to 36.18)	213.3 (21.1 to 405.5)	2.01 (1.814 to 2.206)	12.24 (12.03 to 12.45)	24.6 (22.2 to 27)
BQ112020	sulfur {Nicotiana tabacum}	2.355 (1.96 to 2.75)	10 (8.728 to 11.27)	23.55 (19.6 to 27.5)	13.79 (0.323 to 27.25)	78.05 (64.57 to 91.52)	1,076.15 (25.2 to 2,127.1)
BQ513463	T32G6.22/T32G6.22 {Arabidopsis thaliana}	2.114 (1.345 to 2.882)	10.41 (6.894 to 13.92)	22 (14 to 30)	5.009 (3.748 to 6.271)	10.03 (9.663 to 10.4)	50.25 (37.6 to 62.9)
BQ509737	T7N9.23 {Arabidopsis thaliana}	4.6 (1.45 to 7.75)	10 (7.114 to 12.89)	46 (14.5 to 77.5)	10.34 (0.55 to 20.14)	10 (8.654 to 11.35)	103.4 (5.5 to 201.4)
BQ111911	tetratricoredoxin {Nicotiana tabacum}	4.403 (1.046 to 7.76)	10.32 (5.189 to 15.45)	45.45 (10.8 to 80.1)	2.44 (1.19 to 3.69)	10 (7.545 to 12.45)	24.4 (11.9 to 36.9)
BQ515762	TMV response-related gene product {Nicotiana tabacum}	2.285 (1.524 to 3.047)	10.44 (9.977 to 10.9)	23.85 (15.9 to 31.8)	4.15 (1 to 7.3)	10 (8.76 to 11.24)	41.5 (10 to 73)
BQ120917	tospovirus resistance protein B {Lycopersicon esculentum}	6.301 (0.169 to 12.43)	37.22 (10.37 to 64.08)	234.5 (6.3 to 462.8)	2.095 (0.539 to 3.651)	98.23 (17.32 to 179.1)	205.8 (52.9 to 358.7)
BQ121776	Transcribed sequence with weak similarity to protein ref:NP_568521.1 (A.thaliana) putative protein [Arabidopsis thaliana]	2.869 (1.144 to 4.593)	11.54 (9.992 to 13.09)	33.1 (13.2 to 53)	9.885 (0.69 to 19.08)	10 (9.157 to 10.84)	98.85 (6.9 to 190.8)
BQ505323	Transcribed sequences	8.285 (0.95 to 15.62)	10 (8.188 to 11.81)	82.85 (9.5 to 156.2)	2.69 (0.61 to 4.77)	10 (7.857 to 12.14)	26.9 (6.1 to 47.7)

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BQ517108	Transcribed sequences	9.046 (0.389 to 17.7)	16.97 (3.59 to 30.35)	153.5 (6.6 to 300.4)	2.385 (0.958 to 3.812)	10.34 (6.287 to 14.39)	24.65 (9.9 to 39.4)
BQ117244	UDP-glucose 6-dehydrogenase (EC 1.1.1.22) (UDP-Glc dehydrogenase) (UDP-GlcDH) (UDPGDH). [Soybean]	5.093 (0.988 to 9.197)	15.99 (7.427 to 24.56)	81.45 (15.8 to 147.1)	3.428 (2.918 to 3.938)	11.96 (9.778 to 14.15)	41 (34.9 to 47.1)
BQ512283	unknown {Arabidopsis thaliana}	1,036.9 (0.562 to 2,073.246)	11.75 (10.31 to 13.19)	12,182.75 (6.6 to 24,358.9)	2.025 (1.25 to 2.8)	10 (6.008 to 13.99)	20.25 (12.5 to 28)
BQ509651	unknown {Arabidopsis thaliana}	2.407 (1.724 to 3.09)	11.42 (10 to 12.84)	27.5 (19.7 to 35.3)	2.7 (1.88 to 3.52)	10 (9.786 to 10.21)	27 (18.8 to 35.2)
BQ112625	unknown {Arabidopsis thaliana}	5.935 (1.17 to 10.7)	10 (8.727 to 11.27)	59.35 (11.7 to 107)	4.301 (0.0651 to 8.537)	124.3 (18.21 to 230.5)	534.8 (8.1 to 1,061.5)
BQ115451	unknown protein [imported] - Arabidopsis thaliana	4.177 (1.648 to 6.706)	12.2 (11.73 to 12.66)	50.95 (20.1 to 81.8)	184.7 (53.35 to 316.1)	18.04 (14.79 to 21.3)	3,332.95 (962.5 to 5,703.4)
BQ508798	unknown protein {Arabidopsis thaliana}	107.4 (1.09 to 213.8)	10 (9.956 to 10.04)	1,074.45 (10.9 to 2,138)	52.63 (0.29 to 105)	10 (9.709 to 10.29)	526.3 (2.9 to 1,049.7)
BQ519217	unknown protein {Arabidopsis thaliana}	2.195 (2.13 to 2.26)	10 (9.121 to 10.88)	21.95 (21.3 to 22.6)	2.329 (1.263 to 3.395)	11.16 (9.249 to 13.08)	26 (14.1 to 37.9)
BQ508609	unknown protein {Arabidopsis thaliana}	2.223 (1.598 to 2.848)	15.77 (8.691 to 22.84)	35.05 (25.2 to 44.9)	25.81 (23.36 to 28.26)	71.07 (13.36 to 128.8)	1,834.3 (1,660.4 to 2,008.3)
BQ511156	unknown protein {Arabidopsis thaliana}	2.242 (2.168 to 2.317)	17.48 (16.54 to 18.42)	39.2 (37.9 to 40.5)	95.14 (0.529 to 189.7)	15.89 (12.53 to 19.25)	1,511.8 (8.4 to 3,015.2)
BQ505377	Unknown protein {Arabidopsis thaliana}	2.935 (1.04 to 4.83)	10 (7.192 to 12.81)	29.35 (10.4 to 48.3)	2.223 (0.75 to 3.696)	11.07 (10.11 to 12.02)	24.6 (8.3 to 40.9)
BQ116573	Unknown protein {Arabidopsis thaliana}	2.953 (0.716 to 5.189)	12.57	37.1 (9 to 65.2)	2.293 (1.432 to 3.155)	12.36 (6.839 to 17.89)	28.35 (17.7 to 39)
BQ511947	Unknown protein {Arabidopsis thaliana}	3.571 (1.335 to 5.806)	11.38 (11.27 to 11.5)	40.65 (15.2 to 66.1)	15.69 (1.66 to 29.71)	10 (9.736 to 10.26)	156.9 (16.6 to 297.1)
BQ116951	Unknown protein {Arabidopsis thaliana}	3.892 (3.367 to 4.418)	13.51 (9.942 to 17.09)	52.6 (45.5 to 59.7)	2.336 (0.727 to 3.946)	13.08 (9.963 to 16.19)	30.55 (9.5 to 51.6)
BQ517193	unknown protein {Arabidopsis thaliana}	38.16 (0.29 to 76.04)	40.74 (17.72 to 63.77)	1,555 (11.8 to 3,098.2)	16.78 (0.377 to 33.18)	12.2 (11 to 13.39)	204.6 (4.6 to 404.7)
BQ509801	unknown protein {Arabidopsis thaliana}	6.59 (0.0516 to 13.13)	215.3 (4.198 to 426.3)	1,418.5 (11.1 to 2,825.8)	2.455 (0.82 to 4.09)	10 (9.786 to 10.21)	24.55 (8.2 to 40.9)

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BQ513098	unknown protein {Arabidopsis thaliana}	604.8 (1.092 to 1,208.507)	14.65 (12.97 to 16.33)	8,860.45 (16 to 17,704.9)	6.94 (2.03 to 11.85)	10 (9.461 to 10.54)	69.4 (20.3 to 118.5)
BQ121803	Unknown protein {Arabidopsis thaliana}	63.02 (0.914 to 125.1)	14.33 (8.58 to 20.09)	903.4 (13.1 to 1,793.8)	9.305 (6.62 to 11.99)	10 (8.716 to 11.28)	93.05 (66.2 to 119.9)
BQ112843	vitellogenin {Anolis pulchellus}	61.86 (6.06 to 117.6)	10 (9.931 to 10.07)	618.5 (60.6 to 1,176.5)	2.194 (0.854 to 3.534)	11.12 (10.85 to 11.39)	24.4 (9.5 to 39.3)
BQ113896	Wound-induced aspartate proteinase CDI inhibitor precursor. [Potato] {Solanum tuberosum}	2.538 (0.656 to 4.421)	10.52 (9.877 to 11.16)	26.7 (6.9 to 46.5)	2.335 (1.57 to 3.1)	10 (8.981 to 11.02)	23.35 (15.7 to 31)
BQ515839	WRKY transcription factor 50 {Arabidopsis thaliana}	2.295 (1.715 to 2.874)	11.13 (10.24 to 12.03)	25.55 (19.1 to 32)	27.05 (1.49 to 52.6)	10 (6.592 to 13.41)	270.5 (14.9 to 526)
BQ515883	wsv319 {shrimp white spot syndrome virus}	2.044 (1.179 to 2.909)	11.96 (9.036 to 14.89)	24.45 (14.1 to 34.8)	2.052 (1.574 to 2.53)	10.35 (9.394 to 11.32)	21.25 (16.3 to 26.2)

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 4 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ505231	AT3g52990/F8J2_160 {Arabidopsis thaliana}	2.926 (2.121 to 3.518)	0.0272	1.268 (0.289 to 1.991)	0.602	1.109 (0.81 to 1.256)	0.762	1.056 (0.176 to 3.537)	0.948	1.084 (0.666 to 1.818)	0.895
BQ515350	ATP synthase beta subunit {Lycopersicon esculentum}	2.588 (1.758 to 3.428)	0.0135	3.339 (3.21 to 3.544)	0.0028	10.62 (8.387 to 12.7)	0.00021	13.62 (10.98 to 16.94)	0.00182	3.779 (1.615 to 8.122)	0.283
BQ111722	chlorophyll a/b-binding protein type I precursor - tomato; TC57676	2.415 (2.023 to 2.82)	0.0214	3.114 (0.478 to 6.879)	0.246	1.752 (1.22 to 2.83)	0.146	1.136 (0.734 to 1.444)	0.709	0.289 (0.104 to 0.594)	0.26
BQ116285	DNA segment KIST 6~data source	4.655 (2.62 to 6.281)	0.0239	0.835 (0.441 to 1.253)	0.736	1.284 (0.958 to 1.524)	0.494	2.364 (1.283 to 5.86)	0.195	16.93 (1.034 to 414.7)	0.446
BQ505499	EST612914 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMGE48 5' end, mRNA sequence.	3.568 (2.926 to 4.088)	0.0276	1.192 (0.491 to 1.709)	0.665	1.21 (0.773 to 1.792)	0.619	1.671 (0.865 to 2.104)	0.268	2.797 (1.693 to 4.858)	0.141
BQ518663	Hypothetical 65.0 kDa protein ycf82 (ORF519). [Euglenophycean alga] {Astasia longa}	3.435 (2.771 to 4.502)	0.0354	0.891 (0.616 to 1.561)	0.775	1.01 (0.812 to 1.407)	0.979	1.32 (0.01 to 4.036)	0.651	0.797 (0.36 to 1.33)	0.756
BQ509850	hypothetical protein {Arabidopsis thaliana}	8.154 (6.399 to 11.39)	0.0133	0.674 (0.35 to 1.102)	0.476	0.917 (0.536 to 1.4)	0.845	1.991 (0.777 to 4.606)	0.448	1.772 (0.857 to 3.457)	0.464
BQ508413	hypothetical protein T15C9.70 - Arabidopsis thaliana; TC67087	3.981 (2.408 to 6.093)	0.0261	1.395 (0.516 to 2.011)	0.457	1.725 (0.805 to 5.116)	0.529	0.759 (0.384 to 1.112)	0.677	1.257 (1.028 to 1.704)	0.698
BQ115464	mRNA-binding protein precursor [imported] - tomato (fragment)	3.271 (2.617 to 3.604)	0.0121	2.065 (1.337 to 2.652)	0.0656	1.266 (0.494 to 1.7)	0.541	0.981 (0.662 to 1.502)	0.956	0.309 (0.251 to 0.417)	0.29
BQ121659	nucleoside triphosphatase putative {Arabidopsis thaliana}	2.574 (2.082 to 3.308)	0.0371	1.351 (0.7 to 2.506)	0.595	0.65 (0.216 to 1.236)	0.533	0.988 (0.457 to 1.542)	0.979	0.937 (0.503 to 1.74)	0.924

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 4 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ114946	peptidylprolyl isomerase (EC 5.2.1.8) ROF1 - Arabidopsis thaliana	2.275 (2.136 to 2.408)	0.041	1.15 (0.939 to 1.604)	0.623	1.415 (1.019 to 1.93)	0.343	1.867 (0.688 to 3.761)	0.251	1.835 (1.148 to 2.195)	0.229
BQ121461	probable alanine aminotransferase F5A18.24 - Arabidopsis thaliana	2.15 (1.601 to 2.626)	0.0541	1.126 (0.906 to 1.469)	0.594	0.94 (0.821 to 1.222)	0.863	0.858 (0.288 to 1.991)	0.782	0.637 (0.374 to 1.073)	0.565
BQ112325	probable alanine--glyoxylate transaminase (EC 2.6.1.44) [imported] - Arabidopsis thaliana	2.876 (1.582 to 3.746)	0.037	1.441 (0.461 to 2.43)	0.425	0.676 (0.01 to 1.589)	0.647	1.964 (0.738 to 3.893)	0.243	0.482 (0.268 to 0.633)	0.421
BQ116628	putative mitogen-activated protein kinase {Arabidopsis thaliana}; TC66313	2.846 (2.151 to 3.769)	0.0214	0.87 (0.37 to 1.489)	0.742	0.554 (0.463 to 0.757)	0.329	0.788 (0.15 to 1.673)	0.654	0.806 (0.665 to 0.956)	0.755
BQ111587	ribosomal protein L1 [imported] - spinach	2.967 (2.353 to 3.602)	0.0234	0.867 (0.466 to 1.313)	0.671	0.562 (0.213 to 0.902)	0.337	0.97 (0.557 to 1.256)	0.935	0.497 (0.2 to 1.353)	0.445
BQ116332	ribosomal protein L12-1a {Nicotiana tabacum}	4.697 (3.827 to 6.081)	0.0165	3.139 (2.146 to 5.437)	0.0467	0.595 (0.0783 to 1.887)	0.654	1.217 (0.739 to 1.613)	0.687	1.062 (0.611 to 1.391)	0.926
BQ517273	serine/threonine protein kinase-like protein {Arabidopsis thaliana}	2.963 (2.333 to 4.452)	0.0352	1.672 (0.688 to 2.698)	0.228	0.912 (0.682 to 1.26)	0.809	0.53 (0.01 to 1.994)	0.466	0.56 (0.261 to 1.178)	0.515
BQ509988	superoxide dismutase (EC 1.15.1.1) (Fe) - curled-leaved tobacco	2.301 (2.076 to 2.66)	0.0331	1.992 (0.677 to 3.342)	0.156	1.255 (0.594 to 3.062)	0.792	1.959 (1.064 to 4.428)	0.203	0.384 (0.316 to 0.523)	0.318
BQ508853	Transcribed sequence with weak similarity to protein pir:T10205 (A.thaliana) T10205 hypothetical protein F25G13.120 - Arabidopsis thaliana	6.175 (2.042 to 7.976)	0.0218	1.022 (0.374 to 1.801)	0.965	0.474 (0.111 to 1.044)	0.376	0.997 (0.235 to 2.686)	0.996	0.919 (0.756 to 0.999)	0.902

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 4 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ515412	Transcribed sequences	2.479 (2.14 to 2.881)	0.0255	1.417 (0.721 to 1.904)	0.324	2.773 (1.163 to 4.26)	0.0387	5.227 (1.818 to 11.14)	0.147	0.349 (0.0402 to 2.748)	0.674
BQ519208	unknown {Arabidopsis thaliana}	4 (1.13 to 5.938)	0.0384	0.936 (0.554 to 1.215)	0.848	0.741 (0.538 to 0.913)	0.517	0.908 (0.483 to 1.325)	0.83	1.123 (0.967 to 1.23)	0.853
BQ115852	unknown protein {Arabidopsis thaliana}; TC57794	3.214 (2.374 to 4.309)	0.0198	1.286 (1.071 to 1.616)	0.389	1.404 (1.048 to 1.835)	0.368	1.539 (0.714 to 2.125)	0.347	0.692 (0.426 to 0.802)	0.631

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 8 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ112187	50S ribosomal protein L24 chloroplast precursor (CL24). [Common tobacco] {Nicotiana tabacum}	2.665 (1.592 to 4.846)	0.106	3.304 (1.974 to 4.314)	0.0238	2.86 (0.01 to 8.71)	0.191	0.558 (0.179 to 1.045)	0.37	0.417 (0.229 to 0.746)	0.337
BQ515145	actin-like protein {Arabidopsis thaliana}	1.134 (0.519 to 2.131)	0.77	3.153 (1.205 to 4.028)	0.0427	3.558 (0.397 to 5.73)	0.0691	1.916 (0.01 to 11.75)	0.425	0.505 (0.227 to 0.999)	0.405
BQ512403	ADP-ribosylation factor [imported] - rice	10.21 (3.832 to 25.64)	0.232	13.48 (9.091 to 21.38)	0.0023	127.9 (52.63 to 264.3)	0.067	91.83 (30.49 to 339.7)	0.0545	4.904 (0.0278 to 156.1)	0.711
BQ117369	aspartate carbamoyltransferase {Solanum tuberosum}	0.299 (0.013 to 5.327)	0.669	5.232 (3.263 to 7.978)	0.0206	2.284 (0.844 to 6.138)	0.147	0.724 (0.0764 to 1.368)	0.622	6.346 (1.221 to 23.15)	0.338
BQ112434	AT5g14910/F2G14_30 {Arabidopsis thaliana}	1.956 (0.861 to 4.988)	0.492	2.247 (1.627 to 2.917)	0.0181	0.937 (0.747 to 1.223)	0.864	0.885 (0.632 to 1.336)	0.745	0.673 (0.472 to 1.045)	0.595
BQ515350	ATP synthase beta subunit {Lycopersicon esculentum}	2.588 (1.758 to 3.428)	0.0135	3.339 (3.21 to 3.544)	0.0028	10.62 (8.387 to 12.7)	0.0002	13.62 (10.98 to 16.94)	0.00182	3.779 (1.615 to 8.122)	0.283
BQ514939	ATP synthase beta subunit {Primula gaubaeana}	2.283 (1.396 to 3.2)	0.135	2.963 (1.895 to 3.934)	0.034	46.83 (0.01 to 334.2)	0.0705	29.28 (0.01 to 220)	0.192	3.91 (0.578 to 23.1)	0.542
BQ112460	ATP synthase C chain (EC 3.6.3.14) (Lipid-binding protein) (Subunit III).; TC65117	1.728 (1.513 to 1.973)	0.0939	2.231 (1.378 to 3.147)	0.0484	6.591 (5.219 to 7.709)	0.0021	13.84 (5.711 to 23.32)	0.0353	0.709 (0.0205 to 14.9)	0.903
BQ112730	chlorophyll a/b-binding protein (cab-11) - tomato	1.487 (1.213 to 1.888)	0.198	2.609 (2.336 to 2.997)	0.003	1.674 (1.383 to 2.156)	0.117	0.505 (0.06 to 1.315)	0.311	0.086 (0.042 to 0.118)	0.151
BQ113365	chlorophyll a/b-binding protein (cab-11) - tomato	1.808 (1.132 to 3.202)	0.265	3.86 (3.358 to 4.487)	0.0009	0.859 (0.429 to 1.597)	0.777	0.24 (0.0674 to 0.44)	0.156	0.159 (0.126 to 0.255)	0.188
BQ115145	Chlorophyll A-B binding protein 1B chloroplast precursor (LHCII type I CAB-1B) (LHCP). [Tomato]	1.361 (0.908 to 2.059)	0.532	3.068 (2.239 to 4.036)	0.0201	1.808 (1.299 to 2.412)	0.0995	0.906 (0.379 to 1.63)	0.863	0.235 (0.0972 to 0.545)	0.228
BQ113442	Chlorophyll A-B binding protein 4 chloroplast precursor (LHCII type I CAB-4) (LHCP). [Tomato]	1.074 (0.691 to 1.716)	0.847	3.836 (2.374 to 5.245)	0.02	1.764 (1.028 to 3.345)	0.223	0.371 (0.01 to 0.596)	0.203	0.0995 (0.0687 to 0.123)	0.159

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 8 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ112935	Chlorophyll A-B binding protein 7 chloroplast precursor (LHCI type II CAB-7). [Tomato]	0.903 (0.581 to 1.142)	0.792	6.01 (3.96 to 7.703)	0.0106	0.0867 (0.01 to 0.805)	0.396	0.123 (0.0504 to 0.194)	0.13	0.223 (0.122 to 0.338)	0.222
BQ516559	Chlorophyll A-B binding protein CP24 10A chloroplast precursor (CAB- 10A) (LHCP). [Tomato]	0.949 (0.738 to 1.539)	0.88	2.34 (2.008 to 2.945)	0.0166	1.03 (0.445 to 1.848)	0.951	1.101 (0.0901 to 13.09)	0.972	0.112 (0.0727 to 0.14)	0.166
BQ519227	CycD3;2 {Lycopersicon esculentum}	2.932 (1.309 to 5.596)	0.0827	2.364 (1.566 to 2.862)	0.0498	1.342 (0.932 to 2.253)	0.388	0.488 (0.122 to 1.021)	0.411	0.769 (0.5 to 0.955)	0.723
BQ111934	cysteine proteinase inhibitor - potato; TC65971	2.091 (1.369 to 3.166)	0.0898	3.029 (2.217 to 3.491)	0.0039	0.869 (0.353 to 1.595)	0.781	2.232 (0.01 to 7.55)	0.209	0.0397 (0.01 to 0.174)	0.11
BQ112981	cytochrome p450 {Arabidopsis thaliana}	1.201 (0.735 to 2.769)	0.735	2.534 (1.814 to 3.429)	0.048	0.969 (0.619 to 1.946)	0.946	1.341 (0.01 to 5.434)	0.688	0.756 (0.391 to 1.07)	0.722
BQ119679	Enolase (EC 4.2.1.11) (2-phosphoglycerate dehydratase) (2 phospho-D- glycerate hydro-lyase).	1.482 (0.707 to 2.212)	0.376	2.283 (1.814 to 3.025)	0.0387	1.258 (0.895 to 1.711)	0.504	0.785 (0.436 to 2.029)	0.69	0.973 (0.75 to 1.147)	0.964
BQ113225	EST598801 mixed potato tissues Solanum tuberosum cDNA clone STMCM16 5' end, mRNA sequence.	5.997 (0.442 to 31.93)	0.364	2.556 (1.875 to 3.018)	0.0215	4.651 (1.659 to 12.3)	0.277	0.861 (0.01 to 6.443)	0.919	3.379 (1.446 to 12.24)	0.239
BQ511788	EST619203 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMHT88 5' end, mRNA sequence.	1.461 (0.108 to 2.733)	0.595	5.628 (2.736 to 8.063)	0.0175	2.39 (1.191 to 5.419)	0.0831	1.127 (0.537 to 2.543)	0.84	0.4 (0.129 to 0.576)	0.315
BQ513478	EST620893 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIF40 5' end, mRNA sequence.	1.283 (0.505 to 2.619)	0.588	2.819 (1.983 to 3.864)	0.0365	2.858 (1.129 to 5.967)	0.346	1.083 (0.539 to 2.103)	0.834	0.247 (0.0779 to 0.666)	0.235
BQ505804	Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta)	2.332 (1.431 to 3.736)	0.123	1.997 (1.719 to 2.44)	0.0369	0.493 (0.249 to 1.067)	0.345	1.412 (1.177 to 1.698)	0.335	0.784 (0.45 to 1.393)	0.754

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 8 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ118260	Fe-superoxide dismutase precursor {Medicago sativa}	1.27 (0.322 to 2.985)	0.687	2.023 (1.673 to 2.506)	0.0452	2.85 (1.808 to 3.905)	0.051	1.181 (1.094 to 1.245)	0.729	0.979 (0.783 to 1.279)	0.97
BQ516600	gene_id	1.414 (0.617 to 2.827)	0.559	2.725 (1.642 to 3.963)	0.037	3.78 (2.864 to 4.26)	0.008	1.743 (0.28 to 10.37)	0.799	0.106 (0.0576 to 0.159)	0.163
BQ510594	Heat shock cognate protein 80. [Tomato] {Lycopersicon esculentum}	1.631 (1.162 to 1.957)	0.142	2.209 (1.676 to 2.665)	0.0442	1.18 (0.322 to 2.168)	0.784	1.341 (0.01 to 5.085)	0.835	1.124 (0.311 to 3.485)	0.924
BQ113137	hypothetical protein {Oenothera elata subsp. hookeri}; TC58083	8.271 (2.268 to 35.09)	0.334	2.438 (1.57 to 2.828)	0.0305	1.253 (0.974 to 1.777)	0.43	0.522 (0.316 to 0.761)	0.211	0.0394 (0.01 to 0.175)	0.11
BQ514870	hypothetical protein At2g43500 [imported] - Arabidopsis thaliana	0.987 (0.491 to 1.477)	0.978	2.932 (1.748 to 4.86)	0.0268	10.66 (3.008 to 14.63)	0.0138	5.085 (3.32 to 7.721)	0.0381	0.922 (0.119 to 7.87)	0.97
BQ508800	Hypothetical protein F27E11.3a {Caenorhabditis elegans}; TC61599	1.787 (1.251 to 2.742)	0.124	2.135 (1.727 to 2.822)	0.0343	2.951 (0.01 to 5.887)	0.142	11.65 (0.971 to 41.09)	0.273	0.875 (0.144 to 1.404)	0.815
BQ117149	homologue to UP|Q8LSZ3 (Q8LSZ3) NADPH:protochlorophyllide oxidoreductase	1.297 (0.334 to 2.486)	0.557	2.318 (1.861 to 2.607)	0.0251	0.707 (0.01 to 1.191)	0.541	1.493 (0.866 to 2.482)	0.318	0.507 (0.24 to 0.904)	0.429
BQ117281	Oxygen-evolving enhancer protein 2 chloroplast precursor (OEE2)	1.009 (0.73 to 1.384)	0.977	2.665 (1.938 to 4.066)	0.034	1.457 (1.004 to 1.963)	0.301	1.198 (0.976 to 1.421)	undefined	0.107 (0.0787 to 0.159)	0.163
BQ513774	periaxin-like protein - Arabidopsis thaliana	1.484 (0.598 to 2.655)	0.574	2.158 (1.272 to 2.718)	0.0392	1.146 (0.318 to 4.276)	0.868	0.39 (0.0174 to 0.805)	0.261	0.223 (0.148 to 0.375)	0.22
BQ113573	Photosystem I reaction center subunit XI chloroplast precursor (PSI-L) (PSI subunit V).	1.441 (1.197 to 2.09)	0.244	3.204 (1.965 to 4.928)	0.0303	0.785 (0.385 to 1.252)	0.607	0.24 (0.01 to 0.568)	0.197	0.138 (0.0946 to 0.202)	0.181
BQ511753	predicted protein {Methanosarcina acetivorans str. C2A} [Methanosarcina acetivorans C2A]	1.34 (0.0153 to 2.495)	0.636	2.327 (1.807 to 2.79)	0.0503	1.199 (0.437 to 2.542)	0.763	1.371 (1.233 to 1.564)	0.376	0.841 (0.475 to 1.451)	0.813
BQ117328	Probable 26S proteasome non-ATPase regulatory subunit 3 (26S proteasome subunit S3)	4.059 (0.01 to 9.425)	0.0695	3.034 (2.582 to 3.507)	0.009	5.477 (2.84 to 6.615)	0.0129	14.98 (0.01 to 173.1)	0.325	1.582 (0.118 to 13.09)	0.849

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 8 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ506989	probable shaggy-like protein kinase dzeta [imported] - Arabidopsis thaliana	11.55 (4.474 to 33.25)	0.232	12.84 (9.648 to 18.34)	0.0076	24.05 (16.5 to 34.67)	0.0035	116.7 (26.4 to 557.3)	0.183	17.34 (0.343 to 173.3)	0.415
BQ117401	ribosomal protein L16 {Atropa belladonna}	2.936 (1.903 to 4.062)	0.126	2.561 (1.712 to 3.145)	0.0219	8.291 (6.359 to 9.997)	0.0016	7.623 (3.194 to 11.8)	0.00792	1.743 (0.151 to 11.79)	0.806
BQ512920	T12C24.27 {Arabidopsis thaliana}	1.225 (0.243 to 1.853)	0.646	3.992 (3.063 to 5.357)	0.0135	1.768 (0.537 to 3.729)	0.266	0.787 (0.533 to 1.374)	0.549	0.178 (0.0923 to 0.472)	0.2
BQ516229	Transcribed sequence with moderate similarity to protein ref:NP_192772.1 (A.thaliana) chlorophyll a/b-binding protein - like [Arabidopsis thaliana]	0.871 (0.386 to 1.794)	0.785	4.68 (3.729 to 6.273)	0.0054	0.366 (0.0912 to 1.447)	0.355	0.772 (0.639 to 0.905)	undefined	0.269 (0.154 to 0.427)	0.255
BQ512803	Transcribed sequence with moderate similarity to protein ref:NP_565717.1 (A.thaliana) putative cysteinyl-tRNA synthetase [Arabidopsis thaliana]	0.732 (0.555 to 0.978)	0.55	2.448 (1.881 to 3.341)	0.0344	0.57 (0.01 to 1.005)	0.418	3.11 (0.904 to 8.985)	0.295	1.463 (0.8 to 2.526)	0.522
BQ115289	Transcribed sequence with weak similarity to protein ref:NP_177714.1 (A.thaliana) unknown protein [Arabidopsis thaliana]	1.228 (0.911 to 1.766)	0.537	2.372 (1.714 to 2.988)	0.0324	0.885 (0.42 to 1.298)	0.778	0.484 (0.272 to 1.386)	0.322	1.029 (0.72 to 1.285)	0.966
BQ509009	Transcribed sequences	4.249 (0.556 to 23.08)	0.512	4.635 (2.315 to 6.361)	0.0217	10.48 (2.159 to 45.64)	0.278	1.225 (0.01 to 3.176)	0.837	7.966 (1.013 to 50.3)	0.438
BQ516620	Type I (26 kD) CP29 polypeptide {Lycopersicon esculentum}; TC57676	1.287 (0.608 to 3.176)	0.698	2.764 (1.636 to 3.437)	0.0306	1.145 (0.611 to 1.52)	0.695	0.901 (0.01 to 1.787)	0.85	0.222 (0.0553 to 0.612)	0.22
BQ112776	unknown protein {Arabidopsis thaliana}; TC60335	0.897 (0.208 to 2.888)	0.876	3.513 (2.794 to 4.448)	0.0212	2.597 (0.01 to 10.8)	0.232	2.468 (0.828 to 5.35)	0.338	0.938 (0.616 to 1.467)	0.926

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 12 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ114260	60S ribosomal protein L13a-4. [Mouse-ear cress] {Arabidopsis thaliana}	2.413 (0.502 to 14.25)	0.617	1.59 (0.928 to 2.602)	0.317	2.148 (1.695 to 2.404)	0.0559	0.729 (0.448 to 1.515)	0.524	1.387 (0.7 to 4.077)	0.652
BQ508962	ADP-ribosylation factor {Oryza sativa (japonica cultivar-group)}	14.66 (5.922 to 37.26)	0.171	15.64 (9.706 to 20.21)	0.0098	62.73 (47.34 to 86.17)	0.0005	301.2 (168.6 to 534)	0.0534	47.84 (6.561 to 343.7)	0.285
BQ512525	apocytochrome b6 {Oryza sativa (japonica cultivar-group)}	1.084 (0.527 to 1.928)	0.845	1.358 (0.862 to 1.912)	0.306	3.001 (2.534 to 4.298)	0.0069	3.291 (1.874 to 5.444)	0.0256	0.12 (0.01 to 1.521)	0.51
BQ515350	ATP synthase beta subunit {Lycopersicon esculentum}	2.588 (1.758 to 3.428)	0.0135	3.339 (3.21 to 3.544)	0.0028	10.62 (8.387 to 12.7)	0.0002	13.62 (10.98 to 16.94)	0.0018	3.779 (1.615 to 8.122)	0.283
BQ112460	ATP synthase C chain (EC 3.6.3.14) (Lipid-binding protein) (Subunit III).; TC65117	1.728 (1.513 to 1.973)	0.0939	2.231 (1.378 to 3.147)	0.0484	6.591 (5.219 to 7.709)	0.0021	13.84 (5.711 to 23.32)	0.0353	0.709 (0.0205 to 14.9)	0.903
BQ515554	ATPase alpha subunit {Atropa belladonna}	1.533 (1.21 to 1.882)	0.197	1.232 (0.866 to 1.722)	0.586	4.49 (2.371 to 5.765)	0.0094	11.49 (9.311 to 13.58)	0.0092	0.656 (0.0429 to 7.832)	0.884
BQ112557	beta-glucosidase homolog F8K4.3 - Arabidopsis thaliana	1.308 (0.298 to 4.69)	0.756	2.591 (0.17 to 4.155)	0.118	14.26 (9.685 to 21.89)	0.006	14.4 (0.681 to 35.18)	0.0676	2.594 (0.25 to 28.81)	0.701
BQ114952	copper homeostasis factor [imported] - Arabidopsis thaliana	0.816 (0.376 to 1.895)	0.809	0.387 (0.243 to 0.674)	0.155	2.655 (2.037 to 3.337)	0.0132	1.187 (0.825 to 1.792)	0.565	0.619 (0.38 to 0.972)	0.465
BQ512294	copper homeostasis factor [imported] - Arabidopsis thaliana	0.518 (0.254 to 0.956)	0.308	0.781 (0.454 to 1.382)	0.668	3.32 (2.271 to 3.984)	0.0185	1.235 (0.368 to 1.955)	0.596	0.399 (0.286 to 0.503)	0.32
BQ112071	dnaJ protein homolog - potato	1.821 (0.272 to 2.997)	0.254	0.514 (0.35 to 0.95)	0.213	2.128 (1.63 to 2.69)	0.0543	12.52 (0.378 to 202)	0.431	1.025 (0.386 to 2.265)	0.974

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 12 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ507584	EST614987 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMGR20 5' end, mRNA sequence.	3.294 (0.781 to 15.98)	0.441	1.196 (0.569 to 2.749)	0.783	2.521 (2.303 to 2.846)	0.0216	4.251 (1.171 to 16.17)	0.376	34.71 (3.589 to 291.5)	0.337
BQ516600	gene_id	1.414 (0.617 to 2.827)	0.559	2.725 (1.642 to 3.963)	0.037	3.78 (2.864 to 4.26)	0.008	1.743 (0.28 to 10.37)	0.799	0.106 (0.0576 to 0.159)	0.163
BQ514870	hypothetical protein At2g43500 [imported] - Arabidopsis thaliana	0.987 (0.491 to 1.477)	0.978	2.932 (1.748 to 4.86)	0.0268	10.66 (3.008 to 14.63)	0.0138	5.085 (3.32 to 7.721)	0.0381	0.922 (0.119 to 7.87)	0.97
BQ505670	hypothetical protein F28P22.5 [imported] - Arabidopsis thaliana	0.822 (0.232 to 2.537)	0.828	1.911 (0.895 to 2.296)	0.108	2.463 (1.953 to 2.996)	0.0149	0.456 (0.0243 to 1.351)	0.535	2.308 (0.853 to 10.32)	0.448
BQ113751	Photosystem I reaction center subunit VIII (PSI-I). [Common tobacco] {Nicotiana tabacum}	0.931 (0.712 to 1.419)	0.846	2.082 (0.971 to 3.949)	0.126	2.867 (2.218 to 4.359)	0.0177	4.606 (0.868 to 20.82)	0.488	0.595 (0.102 to 2.937)	0.782
BQ117328	Probable 26S proteasome non-ATPase regulatory subunit 3 (26S proteasome subunit S3)	4.059 (0.01 to 9.425)	0.0695	3.034 (2.582 to 3.507)	0.009	5.477 (2.84 to 6.615)	0.0129	14.98 (0.01 to 173.1)	0.325	1.582 (0.118 to 13.09)	0.849
BQ515072	probable ABC transporter [imported] - Arabidopsis thaliana	0.73 (0.43 to 1.029)	undefined	1.56 (1.06 to 2.482)	0.37	2.455 (2.041 to 2.989)	0.0293	2.134 (1.376 to 4.058)	0.115	0.621 (0.375 to 1.171)	0.559
BQ113207	Probable protease inhibitor P322 precursor. [Potato] {Solanum tuberosum}	3.442 (0.586 to 10.63)	0.105	0.788 (0.27 to 1.14)	0.583	6.534 (4.062 to 9.175)	0.0022	0.547 (0.01 to 31.75)	0.902	2.175 (0.288 to 20.83)	0.743
BQ506989	probable shaggy-like protein kinase dzeta [imported] - Arabidopsis thaliana	11.55 (4.474 to 33.25)	0.232	12.84 (9.648 to 18.34)	0.0076	24.05 (16.5 to 34.67)	0.0035	116.7 (26.4 to 557.3)	0.183	17.34 (0.343 to 173.3)	0.415
BQ117401	ribosomal protein L16 {Atropa belladonna}	2.936 (1.903 to 4.062)	0.126	2.561 (1.712 to 3.145)	0.0219	8.291 (6.359 to 9.997)	0.0016	7.623 (3.194 to 11.8)	0.0079	1.743 (0.151 to 11.79)	0.806

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 12 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ117403	ribosomal protein L16 {Atropa belladonna}	2.7 (0.347 to 5.154)	0.119	2.381 (0.479 to 6.6)	0.129	15.25 (11.37 to 22.75)	0.0032	13.49 (1.925 to 31.27)	0.0247	1.173 (0.0928 to 9.724)	0.948
BQ114627	translation initiation factor IF-1 homolog - common tobacco chloroplast (fragment)	1.907 (0.797 to 3.566)	0.386	1.297 (1.107 to 1.469)	0.243	4.625 (3.937 to 5.944)	0.0124	3.7 (3.168 to 4.198)	0.0007	0.795 (0.0879 to 6.681)	0.92
BQ519261	unknown {Arabidopsis thaliana}	1 (0.407 to 2.715)	0.999	1.669 (1.1 to 2.323)	0.12	2.56 (1.954 to 3.503)	0.0186	0.413 (0.0926 to 0.874)	0.314	0.571 (0.453 to 0.722)	0.456
BQ505483	unknown protein {Arabidopsis thaliana}	1.679 (0.841 to 2.379)	0.241	3.083 (0.632 to 24.42)	0.551	2.436 (1.918 to 3.004)	0.06	0.718 (0.273 to 1.31)	0.623	1.611 (0.967 to 2.172)	0.468

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 16 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ515350	ATP synthase beta subunit {Lycopersicon esculentum}	2.588 (1.758 to 3.428)	0.0135	3.339 (3.21 to 3.544)	0.0028	10.62 (8.387 to 12.7)	0.00021	13.62 (10.98 to 16.94)	0.0018	3.779 (1.615 to 8.122)	0.283
BQ515554	ATPase alpha subunit {Atropa belladonna}	1.533 (1.21 to 1.882)	0.197	1.232 (0.866 to 1.722)	0.586	4.49 (2.371 to 5.765)	0.00936	11.49 (9.311 to 13.58)	0.0092	0.656 (0.0429 to 7.832)	0.884
BQ511025	chitinase-like protein 1 {Arabidopsis thaliana}; TC65808	1.58 (0.485 to 2.693)	0.336	1.732 (0.918 to 3.33)	0.21	1.649 (0.605 to 4.289)	0.301	3.17 (2.258 to 3.867)	0.0053	0.204 (0.0228 to 1.365)	0.496
BQ512208	Cytochrome P450 71B5 (EC 1.14.-.-). [Mouse-ear cress] {Arabidopsis thaliana}	1.886 (0.66 to 4.583)	0.535	0.831 (0.522 to 1.228)	0.643	1.526 (1.116 to 2.382)	0.248	12.74 (8.4 to 18.72)	0.012	1.485 (0.996 to 2.825)	0.519
BQ514087	dehydration-induced protein ERD15 {Lycopersicon esculentum}; TC66339	2.251 (0.378 to 9.88)	0.444	1.139 (0.536 to 1.75)	0.696	1.613 (1.393 to 1.914)	0.193	2.834 (2.472 to 3.268)	0.0344	0.555 (0.189 to 1.83)	0.57
BQ116074	Eukaryotic translation initiation factor 3 subunit 7 (eIF-3 zeta) (eIF3d) (p66). [Mouse-ear cress]	1.528 (0.515 to 3.331)	0.518	1.722 (0.983 to 2.269)	0.132	0.856 (0.37 to 1.203)	0.74	5.117 (3.597 to 7.694)	0.0238	0.882 (0.425 to 1.417)	0.857
BQ519023	F5I14.2 gene product {Arabidopsis thaliana}	3.814 (1.276 to 8.664)	0.311	9.067 (3.511 to 15.37)	0.01	10.52 (2.222 to 21.5)	0.0163	26.26 (17.35 to 37.85)	0.0004	9.828 (0.922 to 80.76)	0.45
BQ113636	Glutamate--cysteine ligase chloroplast precursor (EC 6.3.2.2) (Gamma-glutamylcysteine synthetase)	4.035 (2.04 to 6.592)	0.134	0.795 (0.319 to 2.36)	0.797	2.194 (1.179 to 5.584)	0.262	3.637 (3.044 to 4.39)	0.0265	0.711 (0.339 to 1.055)	0.669
BQ117868	glycine hydroxymethyltransferase (EC 2.1.2.1) - Arabidopsis thaliana; TC57765	1.472 (0.359 to 6.028)	0.665	0.868 (0.41 to 2.033)	0.856	0.734 (0.189 to 2.249)	0.751	4.578 (3.939 to 5.081)	0.0237	1.166 (0.757 to 1.36)	0.804
BQ510509	putative DNA damage repair protein {Oryza sativa (japonica cultivar-group)}	0.482 (0.171 to 1.244)	0.481	1.139 (0.748 to 1.688)	0.642	1.116 (0.973 to 1.311)	0.748	3.163 (2.382 to 4.823)	0.0303	1.004 (0.86 to 1.079)	0.995
BQ514005	putative protein {Vitis riparia}	0.704 (0.01 to 1.726)	0.71	1.07 (0.849 to 1.406)	0.825	1.077 (0.93 to 1.424)	0.845	11.58 (9.318 to 15.85)	0.0048	2.225 (1.449 to 4.008)	0.243

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD
Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 16 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ116177	S-adenosylmethionine decarboxylase proenzyme (EC 4.1.1.50) (AdoMetDC) (SamDC)	1.155 (0.403 to 3.359)	0.903	0.71 (0.477 to 0.902)	0.356	0.75 (0.418 to 1.368)	0.606	12.58 (10.01 to 16.11)	0.0039	1.059 (0.234 to 4.873)	0.973
BQ114627	translation initiation factor IF-1 homolog - common tobacco chloroplast (fragment)	1.907 (0.797 to 3.566)	0.386	1.297 (1.107 to 1.469)	0.243	4.625 (3.937 to 5.944)	0.0124	3.7 (3.168 to 4.198)	0.0007	0.795 (0.0879 to 6.681)	0.92
BQ512292	unknown protein {Arabidopsis thaliana}	0.583 (0.322 to 1.099)	0.437	1.178 (0.521 to 1.709)	0.73	1.097 (0.635 to 2.026)	0.84	4.838 (3.46 to 5.649)	0.0193	1.1 (0.947 to 1.169)	0.884

A5. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD Normalized Gene Expression 2-fold or greater at 4, 8, 12, 16 and 20 dpi

Normalized Gene Expression (2-fold or greater, p-value ≤ 0.05) at 20 days post inoculation compared to other time points		4 days post inoculation		8 days post inoculation		12 days post inoculation		16 days post inoculation		20 days post inoculation	
GenBank ID#	TIGR Annotation	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value	Normalized Gene Expression	t-test p-value
BQ517497	hypothetical protein L5515.04 {Leishmania major}	1.129 (0.259 to 3.391)	0.896	1.242 (1.024 to 1.445)	0.471	0.87 (0.554 to 1.489)	0.738	1.662 (0.646 to 3.797)	0.468	3.194 (2.114 to 3.946)	0.0324
BQ517330	B2 protein. [Carrot] {Daucus carota}	0.541 (0.0289 to 1.481)	0.539	0.72 (0.601 to 0.811)	0.393	0.949 (0.307 to 1.827)	0.909	1.482 (0.114 to 3.018)	0.458	3.725 (2.63 to 4.744)	0.0369

A6. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD Downregulated Normalized Gene Expression 2-fold or lower, Cluster Set 5, 20 dpi

GenBank ID#	TIGR Gene Annotation	Normalized Gene Expression 16 dpi	t-test p value	Normalized Gene Expression 20 dpi	t-test p-value
BQ113521	50S ribosomal protein L29 {Arabidopsis thaliana}	1.23 (0.423 to 1.961)	0.697	0.282 (0.243 to 0.3)	0.251
BQ111936	60S ribosomal protein L12. [Apricot] {Prunus armeniaca}	2.893 (0.347 to 15.64)	0.629	0.277 (0.186 to 0.47)	0.254
BQ115111	acyl CoA reductase-like protein - Arabidopsis thaliana	2.548 (1.561 to 4.996)	0.161	0.159 (0.128 to 0.199)	0.19
BQ516739	AT5g58960/k19m22_160 {Arabidopsis thaliana}	1.536 (1.128 to 2.097)	0.241	0.398 (0.345 to 0.493)	0.327
BQ117156	AT5g64840/MXK3_6 {Arabidopsis thaliana}	1.675 (1.049 to 3.003)	0.287	0.45 (0.304 to 0.607)	0.378
BQ119414	bactinecin 11 {Ovis aries}; TC66043	8.26 (3.61 to 17.21)	0.178	0.484 (0.257 to 0.849)	0.419
BQ507216	beta-fructofuranosidase {Cichorium intybus}; TC69144	1.311 (0.83 to 2.382)	0.52	0.0811 (0.01 to 0.814)	0.355
BQ511755	Biotin carboxyl carrier protein of acetyl-CoA carboxylase chloroplast precursor (BCCP). [Soybean]	1.436 (0.844 to 2.17)	0.385	0.278 (0.116 to 1.024)	0.265
BQ114084	BURP domain-containing protein {Bruguiera gymnorrhiza}	1.358 (0.45 to 5.496)	0.82	0.274 (0.218 to 0.326)	0.248
BQ113195	Catalase isozyme 2 (EC 1.11.1.6). [Tomato] {Lycopersicon esculentum}	2.045 (0.01 to 8.978)	0.631	0.208 (0.121 to 0.293)	0.212
BQ505882	CDH1-D {Gallus gallus}	1.809 (0.427 to 6.643)	0.597	0.199 (0.0324 to 0.833)	0.229
BQ113359	Chlorophyll A-B binding protein 13 chloroplast precursor (LHCII type III CAB-13). [Tomato]	1.255 (0.242 to 6.488)	0.883	0.0957 (0.0784 to 0.131)	0.157
BQ112723	Chlorophyll A-B binding protein 1B chloroplast precursor (LHCII type I CAB-1B) (LHCP). [Tomato]; TC66080	1.51 (0.0246 to 6.571)	0.652	0.105 (0.0226 to 0.593)	0.178
BQ111934	cysteine proteinase inhibitor - potato; TC65971	2.232 (0.01 to 7.55)	0.209	0.0397 (0.01 to 0.174)	0.11
BQ114680	endo-xyloglucan transferase-like protein {Arabidopsis thaliana}	2.314 (1.077 to 3.687)	0.174	0.441 (0.265 to 0.537)	0.395
BQ112759	EST598335 mixed potato tissues Solanum tuberosum cDNA clone STMCJ28 5' end, mRNA sequence.	1.571 (0.211 to 2.733)	0.416	0.177 (0.122 to 0.236)	0.196
BQ119924	EST605500 mixed potato tissues Solanum tuberosum cDNA clone STMEN55 5' end, mRNA sequence.	2.035 (1.296 to 2.494)	0.0952	0.475 (0.317 to 0.652)	0.42
BQ120219	EST605795 mixed potato tissues Solanum tuberosum cDNA clone STMEP43 5' end, mRNA sequence.	3.245 (1.89 to 6.698)	0.0883	0.26 (0.01 to 0.823)	0.291
BQ512765	EST620180 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIA48 5' end, mRNA sequence.	4.325 (1.078 to 23.19)	0.409	0.423 (0.208 to 0.681)	0.359
BQ513474	EST620889 Generation of a set of potato cDNA clones for microarray analyses mixed potato tissues Solanum tuberosum cDNA clone STMIF38 5' end, mRNA sequence.	2.214 (0.733 to 6.575)	0.577	0.149 (0.108 to 0.231)	0.184
BQ120711	ferredoxin--nitrite reductase (EC 1.7.7.1) - common tobacco (fragment); TC65206	1.449 (0.736 to 2.15)	0.416	0.263 (0.128 to 0.539)	0.243
BQ514721	ferritin {Nicotiana tabacum}	3.794 (0.906 to 23.33)	0.508	0.434 (0.243 to 0.822)	0.35
BQ510850	Fructose-1 6-bisphosphatase cytosolic (EC 3.1.3.11); TC58300	1.527 (0.842 to 2.376)	0.367	0.419 (0.288 to 0.537)	0.378

A6. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD Downregulated Normalized Gene Expression 2-fold or lower, Cluster Set 5, 20 dpi

GenBank ID#	TIGR Gene Annotation	Normalized Gene Expression 16 dpi	t-test p value	Normalized Gene Expression 20 dpi	t-test p-value
BQ113039	fructose-1 6-bisphosphatase precursor {Solanum tuberosum}	1.442 (0.204 to 5.846)	0.753	0.307 (0.192 to 0.399)	0.269
BQ114077	Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) (NAD(P)H- dependent glycerol-3-phosphate	15.63 (3.647 to 105.8)	0.289	0.263 (0.105 to 0.622)	0.245
BQ113095	H-Protein precursor {Flaveria pringlei}	2.413 (0.0378 to 20.52)	0.665	0.134 (0.102 to 0.174)	0.176
BQ511606	hypothetical protein {Arabidopsis thaliana}	2.024 (1.159 to 2.771)	0.141	0.225 (0.0645 to 0.713)	0.265
BQ113422	hypothetical protein AAF98579.1 [imported] - Arabidopsis thaliana; TC66766	1.373 (0.969 to 2.062)	0.386	0.272 (0.189 to 0.407)	0.241
BQ112662	hypothetical protein F8J2.180 - Arabidopsis thaliana; TC58551	1.486 (0.768 to 2.381)	0.347	0.411 (0.0784 to 0.667)	0.378
BQ505418	importin alpha 2 {Capsicum annuum}	2.4 (0.512 to 3.703)	0.142	0.0903 (0.01 to 0.836)	0.399
BQ514469	Ire1 homolog-2 {Arabidopsis thaliana}	1.817 (0.732 to 3.535)	0.272	0.47 (0.318 to 0.747)	0.417
BQ517971	laccase (EC 1.10.3.2) - common tobacco (fragment)	1.295 (0.357 to 3.913)	0.766	0.267 (0.0854 to 0.351)	0.245
BQ114043	mRNA-binding protein precursor [imported] - tomato (fragment)	3.017 (0.97 to 6.045)	0.0985	0.413 (0.12 to 0.574)	0.349
BQ118924	oligouridylate binding protein {Nicotiana plumbaginifolia}; TC58199	1.835 (0.286 to 3.139)	0.242	0.255 (0.0944 to 0.707)	0.243
BQ516737	Photosystem II 10 kDa polypeptide chloroplast precursor	2.513 (0.188 to 18.04)	0.712	0.223 (0.0639 to 0.642)	0.248
BQ512815	Photosystem II P680 chlorophyll A apoprotein (CP-47 protein). [Common tobacco] {Nicotiana tabacum}	1.89 (1.276 to 3.59)	0.123	0.0746 (0.01 to 0.573)	0.329
BQ114609	photosystem II protein D1 precursor - soybean chloroplast	2.34 (0.812 to 5.595)	0.48	0.0504 (0.01 to 0.269)	0.167
BQ515352	photosystem II protein X precursor - Arabidopsis thaliana	2.049 (0.383 to 9.66)	0.712	0.207 (0.161 to 0.247)	0.213
BQ119584	plasma membrane intrinsic protein PIP2 {Solanum chacoense}	1.837 (1.038 to 3.194)	0.276	0.413 (0.275 to 0.474)	0.355
BQ120118	probable alanine aminotransferase F5A18.24 - Arabidopsis thaliana	1.757 (0.0785 to 4.363)	0.287	0.159 (0.0944 to 0.225)	0.188
BQ112325	probable alanine--glyoxylate transaminase (EC 2.6.1.44) [imported] - Arabidopsis thaliana	1.964 (0.738 to 3.893)	0.243	0.482 (0.268 to 0.633)	0.421
BQ518440	probable AT-hook DNA-binding protein [imported] - Arabidopsis thaliana	8.43 (0.938 to 122.9)	0.483	0.37 (0.0679 to 0.659)	0.302
BQ113272	probable chaperonin 60 beta chain precursor chloroplast (clone potbchap1) - potato	2.124 (0.611 to 3.564)	0.206	0.33 (0.219 to 0.516)	0.301
BQ119371	Probable vacuolar ATP synthase subunit H (EC 3.6.3.14) (V-ATPase H subunit)	1.492 (0.624 to 3.135)	0.417	0.316 (0.0388 to 0.745)	0.274
BQ510618	Probable WRKY transcription factor 7 (WRKY DNA-binding protein 7). [Mouse-ear cress]	2.211 (1.765 to 3.008)	0.0701	0.456 (0.236 to 0.927)	0.403
BQ118527	protein F5O11.2 [imported] - Arabidopsis thaliana	1.332 (0.807 to 2.304)	0.632	0.0895 (0.012 to 0.677)	0.295
BQ117672	protein T6D22.2 [imported] - Arabidopsis thaliana	1.352 (0.129 to 3.567)	0.682	0.478 (0.31 to 0.648)	0.404

A6. Microarray Experiment: 740AT#120 (antisense ARF-1)/TTOSA1 APE pBAD Downregulated Normalized Gene Expression 2-fold or lower, Cluster Set 5, 20 dpi

GenBank ID#	TIGR Gene Annotation	Normalized Gene Expression 16 dpi	t-test p value	Normalized Gene Expression 20 dpi	t-test p-value
BQ111607	putative 40S ribosomal protein S12 {Oryza sativa (japonica cultivar-group)}	2.432 (1.505 to 4.533)	0.117	0.327 (0.0905 to 0.737)	0.323
BQ515383	putative 60S RIBOSOMAL PROTEIN L36 {Oryza sativa (japonica cultivar-group)}	1.715 (0.629 to 4.814)	0.661	0.327 (0.148 to 0.99)	0.29
BQ507212	putative histidyl tRNA synthetase {Arabidopsis thaliana}	5.004 (3.277 to 9.044)	0.0849	0.0971 (0.01 to 0.952)	0.433
BQ112211	putative protein {Arabidopsis thaliana}	1.846 (0.681 to 2.819)	0.213	0.339 (0.149 to 0.834)	0.327
BQ113949	ribosomal protein L11-like {Nicotiana tabacum}	1.173 (0.741 to 1.411)	0.666	0.49 (0.338 to 0.611)	0.416
BQ120697	Ribulose bisphosphate carboxylase/oxygenase activase chloroplast precursor (RuBisCO activase) (RA).	1.938 (0.733 to 2.78)	0.369	0.11 (0.0712 to 0.129)	0.166
BQ120761	SKIP5-like protein {Lycopersicon esculentum}	1.59 (1.135 to 2.055)	0.323	0.158 (0.0204 to 0.906)	0.394
BQ509988	superoxide dismutase (EC 1.15.1.1) (Fe) - curled-leaved tobacco	1.959 (1.064 to 4.428)	0.203	0.384 (0.316 to 0.523)	0.318
BQ121904	tomato fruit ripening specific URF {Lycopersicon esculentum}; TC64124	0.996 (0.138 to 1.8)	0.993	0.344 (0.193 to 0.473)	0.282
BQ119177	transcription regulator SWI1 - yeast (Saccharomyces cerevisiae)	1.201 (0.01 to 3.861)	0.73	0.237 (0.183 to 0.279)	0.232
BQ516774	ubiquitin / ribosomal protein CEP52 - wood tobacco	3.039 (0.504 to 14.5)	0.561	0.425 (0.0635 to 1.37)	0.367
BQ120783	unknown {Arabidopsis thaliana}; TC65789	1.695 (0.522 to 2.387)	0.277	0.159 (0.108 to 0.208)	0.19
BQ516804	unknown {Arabidopsis thaliana}; TC66030	10.33 (0.573 to 175.4)	0.539	0.0566 (0.0313 to 0.0905)	0.134
BQ511175	unknown {Zea mays}	1.715 (0.951 to 2.99)	0.278	0.487 (0.46 to 0.549)	0.422
BQ111999	unknown protein {Arabidopsis thaliana}	3.61 (2.052 to 5.168)	undefined	0.229 (0.0567 to 0.796)	0.241
BQ113829	unknown protein {Arabidopsis thaliana}	1.649 (1.246 to 2.164)	0.174	0.317 (0.283 to 0.364)	0.3
BQ115182	unknown protein {Arabidopsis thaliana}	1.387 (0.66 to 3.629)	0.536	0.282 (0.164 to 0.452)	0.254
BQ518392	unknown protein {Arabidopsis thaliana}	1.658 (0.0158 to 5.548)	0.665	0.295 (0.102 to 0.761)	0.283
BQ519173	unknown protein {Arabidopsis thaliana}	2.586 (0.177 to 15.22)	0.649	0.308 (0.249 to 0.437)	0.278
BQ113938	Uroporphyrinogen decarboxylase chloroplast precursor (EC 4.1.1.37) (UPD). [Common tobacco]	1.94 (0.392 to 4.484)	0.275	0.382 (0.292 to 0.516)	0.343

APPENDIX II

HPLC data are provided in this appendix for *Nicotiana benthamiana* plants transfected with various constructs, as compared to wild type controls. HPLC data show a reduction in the total measured carotenoids (nmol/g) of a GFP-transfected *N. benthamiana* plant compared to that of non-infected plants. An atypical result shows the accumulation of zeaxanthin in *crtB*-transfected plants at 12 and 14-days post-inoculation.

TMV Vector	Neoxanthin	Violaxanthin	Anther-axanthin	Lutein	Zeaxanthin	chlb	chla	be-car	bb-car	Total chlorophylls	Total Carotenoids	Chlorophyll to Carotenoid Ratio
	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	nmol/g	
crtB 4 dpi	58.4	59.2	0.32	108.6	0	456.5	1383.6	4.7	97.3	1840.1	328.6	5.6
crtB 6 dpi	38.6	46.3	3.7	110.2	0	251.9	729.7	4.3	91.7	981.6	294.9	3.3
crtB 8 dpi	24.7	30.4	3	72.8	0	168.6	484.2	9.3	45.9	652.8	186.2	3.5
crtB10 dpi	11.8	17.5	0.94	57	0	73.8	181.3	8.1	61.3	255.2	156.8	1.6
crtB12 dpi	11.9	18.7	2.2	32.6	0.9	73.4	224.7	4.6	24.5	298	95.4	3.1
crtB14 dpi	19.1	18.4	2	62.1	0.3	104	287.6	7.15	41.1	391.6	150.2	2.6
pds a/s 7 dpi	58.4	62.8	2.4	99.1	0	337.5	1045.7	5.16	19.1	1383.2	246.8	5.6
ADP ribosylation factor-1 a/s	34.6	37	1.6	64.4	0	211.8	692.7	12.1	55	904.4	204.7	4.4
GFP over-expression	55	60.2	2	96	0	346.7	987.9	15.6	69.2	1334.5	298.1	4.5
Wild Type 'A'	51.6	68.2	0.78	121.1	0	429.6	1347.3	27.2	162	1776.9	430.9	4.1
Wild Type 'B'	47.4	53.6	14.2	87.4	0	312.2	972.8	18.2	99.9	1285	320.8	4

crtB phytoene synthase; pds phytoene desaturase; GFP green fluorescent protein; chlb chlorophyll b; chla chlorophyll a; be-car beta-epsilon carotene; bb-car beta-beta carotene