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Characterization of the Pathway Leading to the Synthesis of Salicylic Acid in Plants

Resisting Pathogen Infection

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biological Sciences

by

Alexander Eddo

August 2008

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Keywords: salicylic acid, systemic acquired resistance, shikimate pathway, tobacco
mosaic virus

ABSTRACT

Characterization of the Pathway Leading to the Synthesis of Salicylic Acid in Plants

Resisting Pathogen Infection

by

Alexander Eddo

Salicylic acid is a plant hormone that accumulates with plant-pathogen interaction. This accumulation corresponds to the plant being resistant to infection and without it the plant is susceptible. In this study, primers of genes involved in the normal synthesis of SA were used in RT-PCR to compare gene expression levels in susceptible and resistant plants challenged with tobacco mosaic virus. Because SA synthesis shares chorismate as a common substrate with the synthesis of aromatic amino acids, HPLC was used to determine whether the increase in SA could be attributed to a decrease in amino acid levels. The results suggest that genes of the shikimate pathway are up-regulated in both plant lines but much more quickly in the resistant plant, making differential gene expression a possible cause of SA accumulation. Additionally, results showed a more pronounced decrease in amino acid levels in resistant plants compared to susceptible plants.

DEDICATION

To my dear cousin Dr. Everson Hull who advised me to pursue a master's degree in the interim of trying to get into medical school and who also played a key role in assuring my acceptance to Howard University at the beginning of my tertiary education.
One love couz!

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CHAPTER 1

INTRODUCTION

In 1845 a strange disease struck the potato fields of Ireland and within a period of 5 years about one million people had died not just from starvation but also infections (Edwards and Williams 1956). This period was later termed the Great Irish Famine and it wholly signifies how important it is that plants are able to withstand pathogen infections. Outbreaks such as the potato blight that caused the Irish famine can completely destroy food crops if the plants are not resistant. Although findings are still far from complete, when it comes to disease resistance in plants, great progress has been made at the molecular level to elucidate the various mechanisms involved. It has been shown that the plant hormone salicylic acid (SA) mediates plant defense against pathogens accumulating in both infected and uninfected leaves in response to pathogen attack (Dempsey et al. 1999 and references therein). Plants need SA for the expression of *pathogenesis-related (PR)* gene and the synthesis of defensive compounds associated with both local and systemic acquired resistance (LAR and SAR) (Shah 2003 and references therein).

Salicylic acid has a simple structure composed of a carboxyl and hydroxyl side group attached to a benzene ring (Figure 1). It is a colorless phenol widely used in organic synthesis and has a chemical formula of $C_6H_4(OH)CO_2H$ (Hayat and Ahmad 2007). Also known as 2-hydroxybenzoic acid, salicylic acid is a key ingredient in many skin-care products. It is used for treating acne, psoriasis, calluses, corns, and warts. Salicylic acid causes epidermal cells to slough off more readily, preventing pores from clogging up, and allowing room for new cells to grow (Hayat and Ahmad 2007).

Because of its effect on skin cells, salicylic acid is also used in several shampoos used to treat dandruff (Hayat and Ahmad 2007).

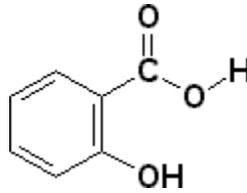


Figure 1 Molecular Structure of Salicylic Acid

Salicylic acid was named after *Salix* (willow) plant where it was first discovered as a major component in the extracts from its bark. Willow bark extracts had been used as a natural anti-inflammatory drug from the ancient time to the 18th century (Rainsford 1984; Weissman 1991). In 1897 the world's first synthetic drug was produced by Bayer Company as an anti-inflammatory agent. The main ingredient in this drug, which is famously known as aspirin, is acetylsalicylic acid (Weissman 1991). Since then, aspirin became one of the most popular drugs and has been widely used by humans for over 100 years. However, very little was known about the role of SA in plants. R. F. White (1979) was the first plant biologist who linked salicylates as disease resistance-inducing chemicals. He and his colleagues injected aspirin into tobacco leaves and showed that it enhanced the resistance to subsequent infection by tobacco mosaic virus (TMV) (White 1979; Antoniw and White 1980). That set the course for subsequent work in this field.

Tobacco Mosaic Virus

The plant disease caused by tobacco mosaic virus is found all over the world. It infects more than 150 types of herbaceous, dicotyledonous plants including many vegetables, flowers, and weeds (Pfleger and Zeyen 1991). Tobacco mosaic virus

infection contributes to serious losses of several crops including tomatoes, peppers, and many ornamentals (Pfleger and Zeyen 1991). It is quite easy to confuse virus-infected plants with plants affected by herbicide or air pollution damage, mineral deficiencies, and other plant diseases. To positively identify TMV infected plants, the service of a plant pathologist is often required with the use of an electron microscope (Pfleger and Zeyen 1991).

Plants such as petunia, snapdragon, tomato, pepper, delphinium, and to a lesser extent muskmelon, cucumber, squash, spinach, ground cherry, phlox, plantain, and jimson weed are common host of tobacco mosaic virus (Pfleger and Zeyen 1991). TMV has a single stranded plus-sense RNA that is packed inside a rigid coat of protein (CP) (Allan et al. 2001). Generally TMV is restricted to plants that are grown in seedbeds and transplanted or plants that are handled frequently though it may infect many other types of plants.

Plant-Pathogen Interaction

As opposed to fungi and bacteria, viruses do not produce spores or other structures capable of penetrating plant parts. Viruses lack an active method of entering plant cells and therefore rely upon mechanically caused wounds, vegetative propagation of plants, or being carried on the mouth parts of chewing insects (Pfleger and Zeyen 1991). Debris of infected plants that remain in the soil are the most common source of virus inoculums as well as certain infected tobacco products such as cigars and tobacco pipes that contaminate workers' hands (Pfleger and Zeyen 1991). Once the virus enters the host, it uses the machinery and metabolism of the host cell to produce multiple copies

of itself (Figure 2). Viruses do not cause disease by consuming or killing cells; instead they take over the metabolic cell processes, resulting in abnormal cell functioning (Pfleger and Zeyen 1991).

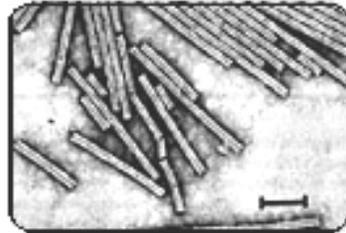


Figure 2 Electron Micrograph of Virus Particles of Tobacco Mosaic Virus from Infected Tomato. The bar represents 200 nanometers or 0.000008 inches. (Adapted from Pfleger and Zeyen 1991)

Common responses of plants to viruses have been divided into the two major categories of cellular stress and developmental defects though they are not necessarily exclusive (Whitham et al. 2006 and references therein). Comparing gene expression in *Arabidopsis thaliana* and *Nicotiana benthamiana* leads to the conclusion that the characteristic changes in gene expression caused by virus infection resembles stress and defense responses. The stress-like responses are characterized by induction of heat shock proteins (HSP) and defense-like responses by induction of *pathogenesis-related* (PR) genes such as *PR-1*, *chitinase* and *β -1-3-glucanase* (Whitham et al. 2006 and references therein).

Plants that either fail to accumulate SA (*nahG* transgenic) or do not produce SA (*sid2* mutant) are susceptible to pathogen infection (Delaney et al. 1994). The *nahG* transgenic plant over-expresses a bacterial salicylate hydroxylase enzyme that quickly metabolizes the SA into catechol (an inactive form of SA), making SA unavailable (Delaney et al. 1994). The *sid2* mutant plant lacks the isochorismate synthase (ICS)

protein required to make SA (Figure 3), which has been knocked out by a T-DNA insertion (Wildermuth et al. 2001).

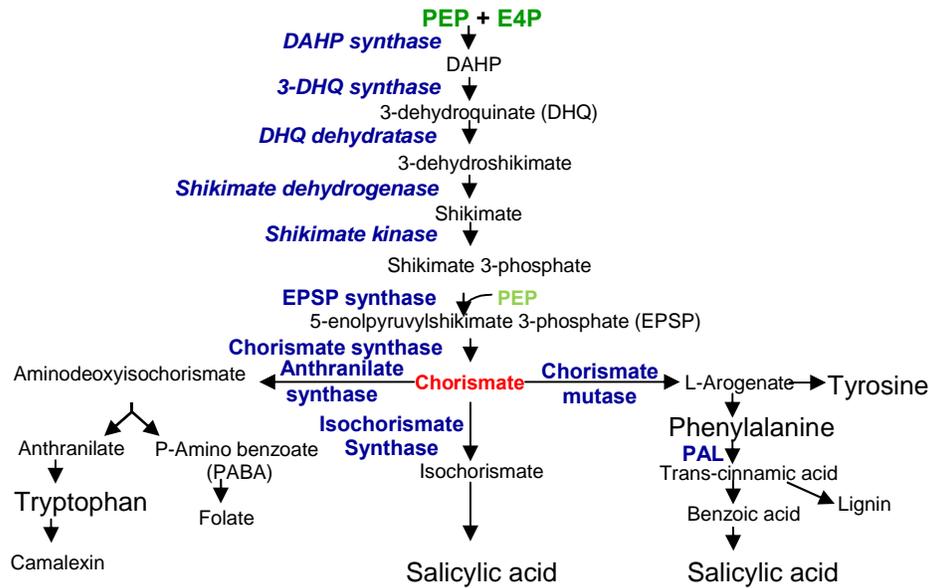


Figure 3 A Schematic Representation of the SA-biosynthetic Pathway. PEP, Phosphoenolpyruvate; E4P, erythrose4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; PAL, phenylalanine ammonia lyase

Mutant *Arabidopsis thaliana* plants such as *dnd1* (*defense, no death 1*) and *mpk4* (*mitogen activated protein kinase 4*) that accumulate high levels of SA are resistant (Yu et al. 1998; Petersen et al. 2000), and exogenously applying SA induces the production of pathogenesis related (PR) defense proteins (Uknes et al. 1996). SA made in plastids is hypothesized to be converted by salicylic acid methyl transferase (SAMT) to methyl salicylate (MeSA) which, being lipid mobile, moves out of the plastid to the cytoplasm (Kumar and Klessig 2003). In the cytoplasm, MeSA is converted back to SA by the methyl salicylate esterase activity of salicylic acid-binding protein 2 (SABP2) (Kumar

and Klessig 2003; Forouhar et al. 2005). The SA released by SABP2 is then responsible for eliciting a broad spectrum resistance response (Kumar and Klessig 2003) (Figure 4).

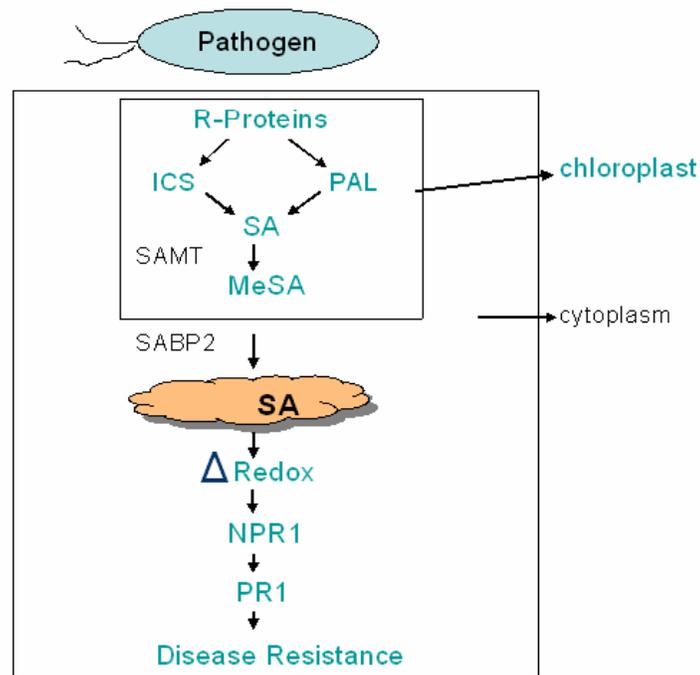


Figure 4 Pathogen Induced Defense Pathway in a Plant Cell. R-proteins, resistance proteins; ICS, isochorismate synthase; SA, salicylic acid; SAMT, salicylic acid methyl transferase; MeSA, methyl salicylic acid; SABP2, salicylic acid binding protein 2; Redox, change in red ox status of cytoplasm; NPR1, non-expressor of pathogenesis-related genes; PR1, pathogenesis related; PAL, phenylalanine ammonia lyase

Surface Resistance-Proteins

Cell surface resistance (R)-proteins are encoded by resistance genes. When a pathogen attacks a plant, the pathogen introduces avirulence (*avr*) proteins into the cell that the R-proteins either recognize or fail to recognize. If a plant recognizes a pathogen, it leads to resistance and the interaction is called an incompatible interaction. In such a case the pathogen is termed avirulent. On the other hand, absence of specific recognition

allows pathogen growth and the plant becomes susceptible. This is known as compatible interaction and the pathogen is termed virulent. However, even in the absence of specific recognition, the plant defense system is functional to a certain level (basal defense) limiting the severity of the disease (Nimchuk et al. 2003). Various works have addressed how R-proteins recognize pathogenic *avr*-proteins and how R-protein activity is regulated during this process (Trotochaud et al. 1999; Wang et al. 2001).

The mechanism used to recognize pathogenic *avr*-proteins, though genetically as simple as a ligand-receptor interaction, may require additional host proteins (Nimchuk et al. 2003). At the same time, studies done on the interaction between tomato and the leaf mold *Cladosporium fulvum* showed that tomato *Cf* proteins are without an obvious signaling domain, suggesting that activation of defense response is mediated through interactions with other partners (Rivas and Thomas 2005). Furthermore, it is suggested that R-protein activity might be regulated by mechanisms such as alternative gene splicing and intermolecular protein interactions involving the N-terminal domain (Nimchuk et al. 2003).

Alternative gene splicing, whereby segments of genes are excised, leads to splice variants of the original genes. Although R-gene variants have not been shown to be functional, they are thought to perhaps participate in R-gene regulation (Nimchuk et al. 2003). It has been shown that molecules of one R-protein domain might interact with molecules of another R-protein via mechanisms such as hydrogen bonding (Hwang et al. 2000). These intermolecular interactions have been suggested to regulate R-Protein activation (Nimchuk et al. 2003)

Studies of R-proteins showed the presence of distinct domains such as the nucleotide binding site (NBS) and leucine rich repeat (LRR), each of which seems to have specific functions (Nimchuk et al. 2003). Domain swapping experiments and mutational analysis of the Arabidopsis NBS-LRR resistance gene *RPS2* showed that specificity of recognition is governed mainly by the LRR domain, while mutations on the NBS site generally eliminate R-protein function (Ellis et al. 2000; Tao et al. 2003). However, studies have also shown that a direct interaction between *avr* and R-proteins may not exist, rather proteins targeted by pathogen virulence factors are the ones associated with R-proteins (Dangl and Jones 2001). This is termed the “Guard Hypothesis.” (Dangl and Jones 2001)

There are two different types of NBS-LRR R-proteins that have an N-terminal coiled coil domain (CC) or a Toll-IL1-receptor like (TIR) domain (Meyers et al. 1999). It is speculated, based on animal models of these domains, that they are protein-protein interaction domains that may interact with signaling partner proteins (Nimchuk et al. 2003). Initial immuno-precipitation studies on R-proteins (*RPS2* and *RPM1*) of Arabidopsis suggested that several proteins can interact with NBS-LRR proteins (Leister and Katagiri 2000). Recent studies suggest that R-proteins require cytosolic heat shock protein 90 (HSP90) to help them function (Shirasu and Schulze-Lefert 2003). Cytosolic HSP90s are protein co-chaperons that are evolutionarily conserved, and they regulate the function and assembly of a wide array of signaling proteins known as client proteins (Pratt and Toft 2003).

Local and Systemic Acquired Resistance

Once plant-pathogen interaction is instigated, the plant's initial response is through a local hypersensitive response that slowly develops into systemic acquired resistance. Hypersensitive response (HR) is mounted to deal with direct pathogen-infected sites in order to restrict the growth and spread of the pathogen to other plant organs. It is characterized by rapid cell death in the region surrounding infection somewhat analogous to the innate immune system found in animals (Allan et al. 2001). However, even prior to HR it has been shown in tobacco plants that there is an initial (phase I) oxidative burst within 4-8 minutes of plant-pathogen interaction (Allan et al. 2001). This burst that generates reactive oxygen species is independent of the tobacco resistance *N* gene and is not involved in expression of defense related genes (Allan et al. 2001). It is thought to be important as a priming mechanism for a later phase II oxidative burst involved in HR (Baker and Orlandi 1995). There is early perception of the virus by the attacked plant even before infection. This perception is through the extra-cellular interaction of plant cell receptors and specific viral coat proteins that occurs in both resistant and susceptible plants (Allan et al. 2001).

In phase I of the HR, the activation of R proteins triggers an ion flux that involves an efflux of hydroxide and potassium outside the cells and an influx of calcium and hydrogen ions into the cell (Orlandi et al. 1992). The cells involved in phase II of the HR generate an oxidative burst by producing reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals, and nitrous oxide (Orlandi et al. 1992). These compounds affect cellular membrane function by inducing lipid peroxidation to cause lipid damage (Baker et al. 1993). Changing ion components in the cell and

breaking down cellular components in the presence of ROS result in the death of affected cells and the formation of local lesions. The deposition of lignin and callose, as well as the production of hydroxyproline-rich glycoproteins, is also triggered by ROS. This deposition serves to reinforce the walls of the cells surrounding the infection site to create a barrier against viral spread (Baker et al. 1993).

The systemic acquired resistance (SAR) response that follows HR can be induced by a number of different pathogens, not just those that cause necrotic lesions, and the resistance observed thereafter is a broad spectrum resistance. During SAR, a number of *pathogenesis-related* genes are induced and SAR activation requires accumulation of endogenous salicylic acid (Ryals et al. 1996). The production of SA therefore proves very crucial for the plant's ability to mount such resistance although SA is not the mobile signal responsible for the SAR response itself (Pallas et al. 1996). SAR requires that a signal moves through the plant phloem from the infected tissue to the systemic tissue (leaves above the primary infected leaves) (Jenns and Kue 1979). Initially, salicylic acid was thought to be this mobile signal because it induces defense responses when applied to plants, is found in phloem exudates of infected leaves, and is required in systemic tissue for SAR (Dempsey et al. 1999 and references therein). However, tobacco grafting studies showed that infected, SA-deficient rootstocks could trigger SAR in wild-type scions. Such results imply that SA is not a mobile SAR signal (Vernooij et al. 1994; Pallas et al. 1996).

Through detailed graft analysis involving silenced-SABP2 tobacco plants, it was recently discovered that methyl salicylate is the phloem-mobile signal for SAR (Park et al. 2007). SAR was blocked when SA methyl transferase (SAMT), which converts SA to

MeSA, was silenced in primary infected tobacco leaves. Moreso, MeSA treatment of lower leaves induced SAR in upper treated leaves which suggests that MeSA is the SAR signal involved in systemic acquired resistance in pathogen-infected tobacco plants (Park et al. 2007).

Two Pathways of Salicylic Acid Production

There are two routes that have been shown to lead to SA production and one of them, involving the phenylalanine ammonia-lyase (PAL) enzyme, has been suggested to have only a minor role in plant systemic defense (Metraux 2002 and references therein). The other route involves SA produced by isochorismate synthase (ICS) (an enzyme localized in plant plastids) from chorismate, the end product of the shikimate pathway (Wildermuth et al. 2001). There was systematic induction of the *ICS1* gene in *Arabidopsis* plants inoculated with an avirulent strain of the *Pseudomonas syringae*, resulting in increased SA accumulation and SAR development in systemic leaves. The expression of *ICS1* and the accumulation of SA occurred at a similar time for these pathogen treatments. The induction also correlated with expression of the *pathogenesis-related (PR-1)* gene, a molecular marker of SAR (Wildermuth et al. 2001). It has been suggested that the SA made by this route is required for SAR while SA made via the PAL enzyme might contribute to basal SA levels present in uninfected plants (Metraux 2002 and references therein).

The Shikimate Pathway

Both routes that produce salicylic acid use the shikimate pathway. Chorismate, the substrate for ICS and a precursor to phenylalanine used by PAL, is made through this pathway (Figure 5). In a sequence of seven metabolic steps, phosphoenolpyruvate (PEP) from glycolysis and erythrose 4-phosphate (E4P) from the pentose phosphate pathway are converted to chorismate, the precursor of the aromatic amino acids and several secondary metabolites (Herrmann and Weaver 1999). The shikimate pathway is found in microorganisms and plants but it has not been found in animals. All enzymes of this pathway have been purified from prokaryotic and eukaryotic plant sources and their respective genes have been characterized from several organisms (Herrmann and Weaver 1999). In higher plants the proteins of the shikimate pathway have amino terminal signal sequences for plastid import, suggesting that the primary location for chorismate synthesis is the plastid (Herrmann and Weaver 1999). Additionally, it is suggested that regulation of the pathway in plants might occur mostly at the genetic level because no physiological feedback inhibitor has been identified (Herrmann and Weaver 1999).

Any of the seven enzymes in the shikimate pathway would be of interest. It is unknown which one, if any, is targeted by the initial signal coming from plant-pathogen interaction. This signal leads to increased level of SA production and accumulation. Additionally, side genes coding for enzymes, anthranilate synthase, and chorismate mutase will also be of interest. These enzymes share chorismate as a substrate for the synthesis of aromatic amino acids and chorismate is also used for direct synthesis of SA.

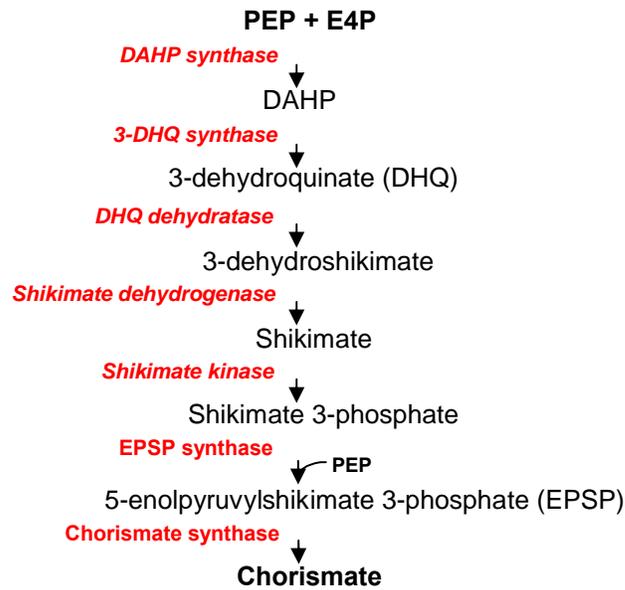


Figure 5 The Shikimate pathway Showing a Sequence of Seven Metabolic Steps Converting Phosphoenolpyruvate and Erythrose 4-phosphate into Chorismate

Isochorismate synthase, another enzyme of particular interest, converts chorismate to isochorismate which is, in turn, converted to SA. There is still some uncertainty as to whether isochorismate synthase and or the PAL enzyme is responsible for the SA accumulation that leads to systemic acquired resistance. Verbene et al. (2000) reported that the transformation of tobacco with bacterial *ICS* and *IPL* genes fused to a strong plant promoter caused overproduction of SA and constitutive expression of defense genes in the transgenic plants.

Mode of Action of Salicylic Acid

Once SA accumulates in the plant chloroplast, it must then travel to the cytoplasm where it is postulated to begin its role in the plant resistant pathway (Herrmann and

Weaver 1999). SA is converted to MeSA, which being lipid mobile, crosses the chloroplast membrane to enter the cytoplasm (Kumar and Klessig 2003). In cytoplasm, MeSA is converted back to SA by the methyl salicylate esterase activity of SABP2. This SA triggers a change in redox potential of the cytoplasm that then activates NPR1 (non-expressor of pathogenesis-related protein 1), a key regulator of defense response in plants. Normally, NPR1 is present as a multimeric protein in the cytoplasm (Mou et al. 2003) that must be converted to its monomeric constituents to enter the nucleus (Kinkema et al. 2000). In the nucleus, monomeric NPR1 interact with members of the TGA family of bZIP transcription factors to activate expression of defense genes such as *PR-1* (Zhang et al. 1999; Zhou et al. 2000; Fan and Dong 2002; Despres et al. 2003; Johnson et al. 2003).

Pathogenesis-Related genes are of several different gene families encode defense proteins that reduce plant susceptibility to pathogens through their antimicrobial properties (Van Loon and Van Strein 1999). Without the initial production and accumulation of SA, plants are unable to express these defense genes to mount broad spectrum SAR (Gaffney et al. 1993; Delaney et al. 1994) and hence become susceptible to pathogenic attacks.

Specific Aim of This Study

Salicylic acid is not only involved in plant defense, but it is also needed for various physiological processes like stomatal closure, flower induction, and heat production (Raskin et al. 1989; Raskin 1992; Chaerle et al. 1999). Now, much is known about the signaling role of SA once it accumulates and how it even participates in a feedback loop

mechanism to fine-tune the resistance process (Shah 2003 and references therein). However, very little is known about the initial signal that causes the accumulation in the first place. This encompasses the main objective of this research to try to determine what molecules might be receiving the signal from the plant-pathogen interaction to cause the increase in accumulation of salicylic acid. Work in this area would contribute to the current understanding of plant defense mechanisms as this could be a point of interest when it comes to creating more resistant plants. It is possible to sensitize the molecules that might be targeted by plant-pathogen interaction under normal plant conditions. As a result, when the plant actually becomes infected, the sensitization can cause a much quicker accumulation of SA leading to enhanced disease resistance.

Two hypotheses were formulated to address the question of which molecules might be responsible for causing SA accumulation. The first of the hypotheses is that the genes coding for enzymes of the shikimate pathway might be up-regulated to allow increased production of SA. Secondly, the conversion of chorismate to metabolites (aromatic amino acids) other than SA might be limited so that a higher amount of chorismate is available for conversion into SA. To address the first hypothesis, the expression of five genes of both the pre- and postchorismate pathway was compared between resistant and susceptible plants infected with tobacco mosaic virus. To test the second hypothesis the levels of aromatic amino acids in both susceptible and resistant plants infected with TMV were analyzed.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

Seeds of resistant tobacco plant line (*Nicotiana tabaccum* c.v. Xanthi NN) containing the 'N' resistance gene and susceptible tobacco plant line (*Nicotiana tabaccum* c.v. Xanthi nn) without the 'N' resistance gene were obtained from Dr. Dharendra Kumar's collection and sown in 3" x 3" square plastic flats and allowed to germinate in Promix (A mixture of Canadian sphagnum peat moss, extra perlite, and doomitic & calcitic limestone). They were grown at a temperature of 22°C with 14-16h light in a growth chamber (Conviron). Ten to fourteen-day old seedlings were individually transferred to 3" x 3" square plastic flats. The seedlings were about ½ an inch tall. Four weeks later, about 4 inches tall, the plants were transferred to larger pots (8" x 5 ½"). Fertilizer (nitrogen, phosphorus, and potassium; 21:5:20) was diluted and applied to the plants about 3 days after they were transferred to the large pots. Two weeks later, the plants were ready for inoculation with tobacco mosaic virus.

Reagents (Chemicals) and Instruments

Materials for RNA isolation and RT-PCR including M-MLV reverse transcriptase, rRNasin, and RQ1 RNase-Free DNase were obtained from Invitrogen (Carlsbad, CA) and Promega (Madison, WI), respectively. Tri-reagent for RNA isolation was obtained from Sigma-Aldrich (St. Louis, MO). Isopropanol, chloroform, formaldehyde, and formamide were available from Fisher Scientific (Madison, WI). For HPLC analysis, pure tyrosine

(T8566), tryptophan (T8941), and phenylalanine (P5482) as well as iso-disc N-4-4 nylon filters (4mm x 0.45µm) were ordered from Sigma-Aldrich (St. Louis, MO).

Ethanol (75%), ethidium bromide (EtBr, 10mg/ml), DNA and RNA loading buffers, DEPC (diethylpyrocarbonate)-treated water, formaldehyde agarose (FA) gel, electrophoresis buffer (10X and 1X MOPS buffer), phosphate buffer (50mM), and gel staining solutions (TAE +EtBr) were prepared as described in Appendix B.

Gene-specific primers were designed using sequences obtained from a public database (NCBI) and synthesized by Sigma-Genosys (Woodlands, TX). An eppendorf gradient PCR Mastercycler (Eppendorf Scientific Inc.) was used for PCR reactions, FastPrep® -24 (MP Biomedicals) for tissue homogenizing, and Varian Pro Star 210-HPLC (Varian Inc.) was used for amino acid analysis. Sorvoll® Biofuge Pico (Kendro Laboratory Products) was used for centrifugation. The Epi Chemi II Darkroom photo imager (UVP Bioimaging Systems) was used to record gel images.

Purified tobacco mosaic virus (18.5mg/ml stock solution) obtained from Dr. Kumar's collection was diluted to a concentration of 1.4µg/ml for plant inoculations.

Infecting Plants with Tobacco Mosaic Virus

Two plants from each line (resistant and susceptible) were selected, one for infection with TMV and the other for mock infection with 50mM phosphate buffer pH 7. Diluted (1.4µg/ml) TMV in 50mM phosphate buffer pH 7 was used for inoculation. A square piece of 4 layers of cheesecloth was cut and washed with distilled water before using for virus inoculations. Two to three leaves (lower position of the rosette) on the selected plants were marked on the edge with a non-toxic marker for identification and

they were dusted with an abrasive (carborundum 320 grit, Fisher Scientific). The abrasive served to help the virus penetrate the leaf tissue. For mock inoculations, plant leaves were gently rubbed with the cheesecloth that was dipped only in phosphate buffer. For TMV inoculations, leaves were rubbed with cheesecloth dipped in diluted TMV solution. Following inoculations, plants were maintained at 22-24°C with 16hrs of light.

Leaf samples were collected from the 2 plant lines at 6 time points (0h, 1h, 2h, 4h, 6h and 12h) by punching out 3 discs with a cork borer (#7) and transferred to 2ml tubes. The zero hour samples were collected immediately prior to inoculations to serve as un-inoculated control.

Total RNA Extraction

Prior to RNA extraction, all glassware was cleaned with detergent and autoclaved. Electrophoresis tanks were cleaned with detergent, rinsed with deionized water, and dried to maintain an RNase-free environment. Water and aqueous solutions were treated with 0.1% DEPC and autoclaved (Sambrook et al. 1989) before use.

Leaf discs were homogenized by using ¼ inch ceramic bead and 250µl of Tri-reagent in a Fast prep-24 for 20 seconds as described by the manufacturer. Once homogenizing was completed, additional 250µl of Tri-reagent was added to the samples, mixed and incubated at room temperature for 5 minutes. To the homogenate, 100µl chloroform was added and mixed vigorously by hand for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh 1.5ml tube. To the aqueous phase 250µl isopropanol was added, mixed well, and incubated at room temperature for 10

minutes. Another round of centrifugation was done for 10 minutes and the pellet was retained. The pellet was rinsed with 0.5ml cold ethanol (75%) made with DEPC-treated water, vortexed, and centrifuged at 8900 rpm for 5 minutes at 4°C. The pellet was air-dried for 10 minutes and resuspended in 25µl DEPC-treated water. To ensure re-suspension, sample tubes were placed in an Eppendorf Mixmate at 1600rpm for 15 minutes. To each of the samples, 2µl DNase, 5µl DNase buffer, and 18µl DEPC water were added and mixed. The samples were incubated at 37°C for 20 minutes in an incubator. DNase treated samples were extracted with 125µl Tri-reagent and 25µl chloroform. RNA was precipitated with 62.5µl isopropanol. After centrifugation, each pellet was resuspended in 50µl of DEPC-treated water.

The concentration of the purified RNA was determined by measuring its optical density (OD) at 260nm using a spectrophotometer. To measure OD, 1.6µl of RNA was diluted to 400µl with DEPC-treated water and was transferred to a cuvette. The amount of RNA in the samples was quantified using the following formula:

$$1 \text{ OD}_{260} = 40\mu\text{g/ml} (0.04\mu\text{g}/\mu\text{l})$$

The quality of the RNA was then analyzed by separating 5µg of RNA on a 1.5% formaldehyde agarose (FA) gel using 1X MOPS as running buffer. The volume of RNA solution that contained 5µg of RNA was determined and noted in Table 1.

Primer Design

For PCR reactions it is desirable to have primers with a melting temperature (TM) between 50°C and 65°C. Designed primers were 17-22 bases long with a base composition of 50%-60% (G+C). The nucleotide sequences of 5 genes of interest; *DAHP*

synthase, chorismate mutase, isochorismate synthase, chorismate synthase, and shikimate kinase, (Appendix C) were obtained from the public database National Center for Biotechnology Information (NCBI). From these sequences, forward and reverse primers (Table 2) were designed to amplify the length of sequences highlighted in appendix C.

Table 1 Quantitation of Extracted RNA

	Sample	OD260nm	RNA $\mu\text{g}/\mu\text{l}$	$\mu\text{l}/1\mu\text{g}$	$\mu\text{l}/5\mu\text{g}$
1	TXN 0hpi	0.055	0.550	1.818	9.091
2	TXN 1hpi	0.058	0.580	1.724	8.621
3	TXN 2hpi	0.062	0.620	1.613	8.065
4	TXN 4hpi	0.051	0.510	1.961	9.804
5	TXN 6hpi	0.054	0.540	1.852	9.259
6	TXN 12hpi	0.042	0.420	2.381	11.905
7	TXS 0hpi	0.065	0.650	1.538	7.692
8	TXS 1hpi	0.085	0.850	1.176	5.882
9	TXS 2hpi	0.133	1.330	0.752	3.759
10	TXS 4hpi	0.066	0.660	1.515	7.576
11	TXS 6hpi	0.193	1.930	0.518	2.591
12	TXS 12hpi	0.040	0.400	2.500	12.500

TXN – TMV infected resistant plant line (*N.t.* Xanthi NN)
 TXS – TMV infected susceptible plant line (*N.t.* Xanthi nn)
 OD – optical density (Absorbance)
 hpi – hours post inoculation

Primers were custom synthesized by Sigma-Genosys (Woodlands, TX). The lyophilized primers were resuspended in DEPC-treated water at a concentration of 100pmol and stored at -20°C until ready for use. Prior to expression profiling, primers were tested with cDNA from leaves of 8 week-old tobacco plants to determine their optimal amplification conditions. Linear range of amplification for each gene was determined by stopping the reaction every 2 cycles between 30-40 cycles and analyzing the samples by agarose gel electrophoresis. The samples in the gel was stained with ethidium bromide and

visualized by transillumination. The number of cycles which showed peak of amplification efficiency was used for future amplifications.

Table 2 Primer Sequences of Genes Studied

GENE	READING FRAME	SEQUENCE	T _m (°C)
DAHP Synthase	FORWARD	GGC TCA ATT TCA GGT ACC	62.2
	REVERSE	GTT AGA GTC GGT AAG TAA	56.9
Shikimate Kinase	FORWARD	CTC ACC TAC CTC TCT CTC A	56.0
	REVERSE	AAG GCT CTG CGA AAC TCT	59.3
Chorismate Synthase	FORWARD	ATC TTC CAA TCT TCA TATA	49.4
	REVERSE	TCC TAG GTG TGG TAA TTC	51.9
Chorismate Mutase	FORWARD	CTT CAA TCT AAG GTT GGT AG	53.0
	REVERSE	CTG AAT ATC ACA GGA AGC AG	57.4
IC Synthase	FORWARD	CAG GTT GAG TTT GAT GAG CT	59.4
	REVERSE	CTT GAT AAG CAT CGG GTT	57.6

Synthesis of cDNA

Total RNA from infected tobacco plants was quantified and used for cDNA synthesis. For primer annealing, 1µg of RNA was mixed with 1µg of Oligo dT-(14) (Fisher Scientific), diluted to 10µl with DEPC treated-water, incubated at 75°C for 10 minutes, and cooled to room temperature. For the cDNA synthesis reaction, 4.0µl reverse transcriptase (RT)-buffer, 1.0µl RNasin, 2.0µl M-MLV reverse transcriptase (200µ/µl), 1.0µl of 2.25mM dNTP mix, and 2.0µl DEPC-treated water were added to 10µl RNA. This mixture was incubated for 60 minutes at 42°C followed by 10 minutes at 70°C. The final 20µl product was diluted by adding 20µl DEPC-treated water and stored at -20°C until further use.

In order to determine if synthesized cDNA was equal in concentration for all the time points, a PCR reaction was done using primers of a housekeeping gene *EF1 α* . This housekeeping gene (~500bp long) is needed for basic cell functioning and it is expressed constitutively in the cells. To amplify the *EF1 α* using the synthesized cDNAs, the reactions were done in the PCR Mastercycler with cycling conditions shown in Table 3.

A typical PCR reaction mix contained 2.0 μ l of PCR buffer (10X), 2.0 μ l dNTP (2.25mM), 0.5 μ l Taq polymerase (5U/ μ l), 0.8 μ l forward and 0.8 μ l reverse *EF1 α* gene primers, and 12.9 μ l sterile water. To this mix, 1.0 μ l cDNA was added and mixed. The tubes were placed in PCR mastercycler for amplification using the conditions listed in Table 3. The amplified PCR products were analyzed by agarose gel electrophoresis.

Table 3 PCR Conditions used to Determine the Quality of Synthesized cDNA

Condition	Time
1. 94°C	3 minutes
2. 94°C	30 seconds
3. Primer Annealing Temp. 50°C	30 seconds
4. 72°C	30 seconds
5. Repeat (steps 2-4)	30 cycles
6. 72°C	5 minutes
7. 10°C	Hold

Gel Electrophoresis

Agarose (SeaKem ®) was used to make the gels for analyzing PCR products. A 1.5% agarose gel was prepared by adding 0.75g agarose in 50ml 1X TAE buffer and

heated in a microwave until all the agarose melted. It was then cooled to ~50°C and 2.5µl ethidium bromide (10µg/µl) was added. The agarose solution was then poured in a gel casting tray with appropriate comb. Once solidified, the combs were removed. From the 20µl PCR reaction products, 10µl was taken and mixed with 3µl DNA loading dye and loaded into the wells. A 100bp DNA ladder was also loaded in the first lane to determine the size of the amplified products. TAE (1X) (refer to appendix B) was used as a running buffer. Constant voltage (100 volts) was applied for about 25 minutes. The amplified DNA bands in the agarose gel were recorded using a UV photo imager (Epi Chem II Darkroom). The amplified DNA bands were compared to verify equal loading of cDNA.

Expression Profiling of Salicylic Acid Biosynthetic Genes with RT-PCR

For analyzing the expression of *DAHPh synthase*, *chorismate mutase*, *isochorismate synthase*, *shikimate kinase*, and *chorismate synthase* genes, a PCR reaction mix for each time point was made with 2.0µl PCR buffer (10X), 2.0µl 2.25mM dNTP, 0.5µl Taq polymerase, 0.8µl forward and 0.8µl reverse gene primers (10pmol stock), and 12.9µl sterile water. To each mix, 1.0µl cDNA from each time point was added to corresponding tubes and mixed. The tubes were then placed in the PCR mastercycler and subjected to the conditions in Table 4 with step 3 being specific for different genes. The PCR products were separated on a 1.5% agarose gel as previously described. The profile for all the genes was then analyzed by comparing the levels of amplification in resistant plant lines to levels in susceptible lines.

Measuring Aromatic Amino Acid Levels in Plants Using HPLC

To measure the aromatic amino acid levels, plant extracts were prepared from TMV infected resistant and susceptible plants. The concentration of TMV used for infection was increased to 3.1µg/ml for HPLC analysis. In preparing the plant extracts, 12 leaf discs (~200mg) from each time point (0, 1, 2, and 4 hours) were homogenized using the FastPrep®-24 tissue homogenizer for 20 seconds in 1ml extraction solvent (60% v/v methanol, 0.1% v/v phosphoric acid with 2% w/v 3-methylsalicylic acid).

Table 4 PCR Conditions for Genes of SA Biosynthetic Pathway

Condition	Time
1. 94°C	3 minutes
2. 94°C	30 seconds
3. Primer Temp (A-B)	30 seconds
4. 72°C	30 seconds
5. Repeat (steps2-4)	35 cycles
6. 72°C	5 minutes
7. 10°C	Hold

A – (55°C) used for *shikimate kinase* and *chorismate synthase* genes

B – (60°C) used for *DAHP synthase*, *isochorismate synthase* and *chorismate mutase* genes

The homogenate was then centrifuged for 3 minutes at 11,300 rpm and each supernatant was filtered through a 0.45µm iso-disc N-4-4 nylon filter. Thereafter, 10-50µl of the filtrate was injected into C-18 HPLC column for separation.

The C-18 column was first prewashed with 100% methanol at a flow rate of 0.7ml/min for 30 minutes, followed by wash with 80% acetonitrile for another 30 minutes and then with the solvent system (5% acetonitrile, 0.1% (v/v) phosphoric acid) for an additional 30 minutes to equilibrate the column. The solvent system used was a linear gradient (5%-80%) of acetonitrile in 0.1% (v/v) phosphoric acid made in distilled water. The acetonitrile concentrations increased with time: 0min: 5%; 50min: 55%; 51min: 80%; 60min: 80%; 65min. Each run was completed in 90 minutes. This was, however, later modified for efficiency while measuring the standards and actual samples. The run time was reduced to 40 minutes and the solvent system was maintained throughout at 10% acetonitrile in 0.1% (v/v) phosphoric acid-water.

The Varian Pro Star (210 model) HPLC system used was equipped with a 325 LC dual wavelength UV-Vis Detector. The Absorbance was recorded at 254nm and chromatographic data acquired and analyzed using Galaxie chromatography data system software (Varian Inc.).

Aromatic amino acids in plant extracts were compared to and identified by similar retention times of purified standards (tyrosine, phenylalanine, and tryptophan). The reference amino acids (tyrosine, phenylalanine, and tryptophan) were dissolved in 0.1M HCl and injected into the C18 HPLC column to determine their retention times. The levels of amino acid at various time points before and after TMV infection in resistant and susceptible plants were compared. The protocol used was adapted from Janzik et al. (2005).

CHAPTER 3

RESULTS

The primary goal of this research was to determine which molecules in the salicylic acid biosynthetic pathway might be responsible for causing the SA accumulation that correlates to systemic acquired resistance in TMV infected tobacco plants. The resistant plants inoculated with TMV displayed necrotic lesions while the susceptible plants did not. The susceptible plants did not have the 'N' (resistance) gene and were therefore incapable of mounting a hypersensitive resistance response. This necrotic response highlights the restriction of pathogen growth and represents a form of programmed cell death (Lam et al. 2001).

The leaves of the susceptible plant showed mosaic (mottled) areas with alternating yellowish and dark green areas (Figure 8). The size and coloration of lesions are indicative of the concentration/virulence of the infection and also the capability of the plant to combat the pathogen (Pfelger and Zeyen 1991). Figures 6-9 show plants before infection and plants infected with a tobacco mosaic virus at a concentration of 1.4µg/ml.

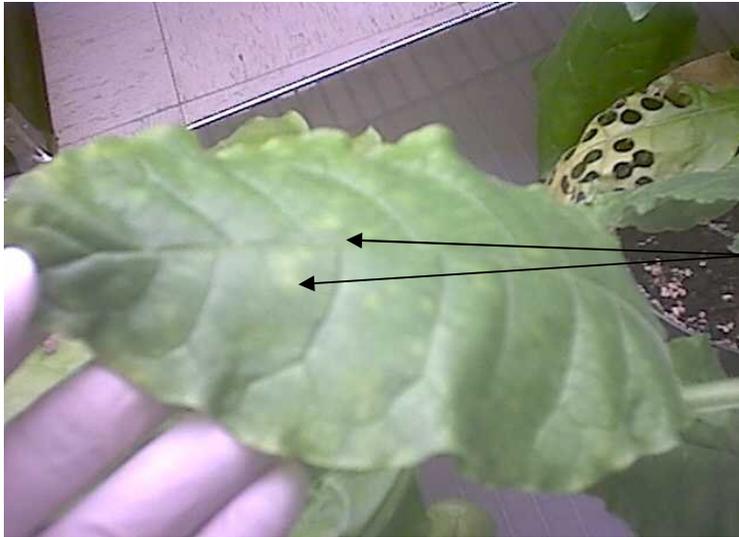
Plants Used for Infection



Figure 6 Normal Resistant Tobacco Plants Prior to TMV Infection

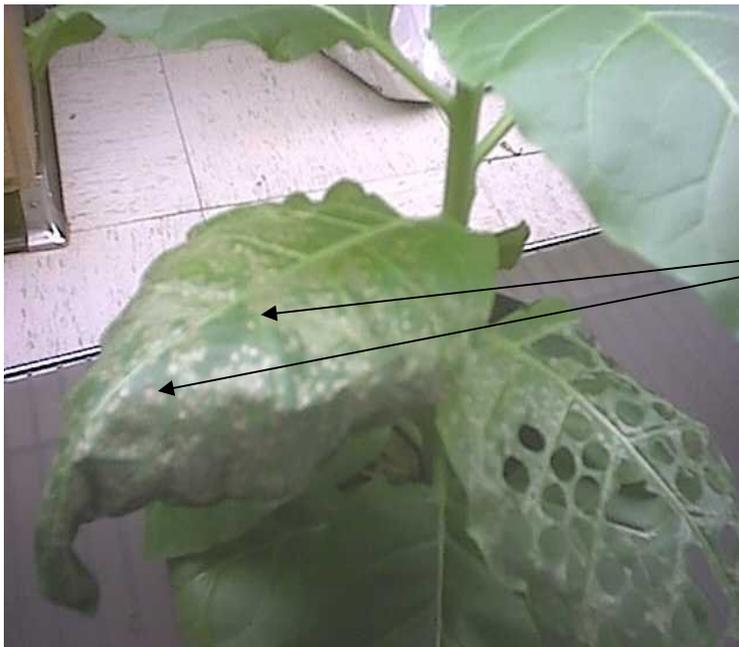


Figure 7 Normal Susceptible Tobacco Plants Prior to TMV Infection



Alternating yellow and green spots

Figure 8 Leaf of a TMV Infected Susceptible Tobacco Plant Showing Mosaic Symptoms



Necrotic Lesions

Figure 9 Resistant Tobacco Plant Showing Hypersensitive Response

Total RNA Extraction

Extraction of total RNA was the most important procedure for the gene expression study because from this total RNA cDNA was synthesized and used for PCR amplification of SA biosynthetic genes. Part of the challenge, apart from possible DNA contamination and RNA degradation, was to purify high quality total RNA from all 6 different time points and for 2 different infected plant lines (resistant and susceptible). Therefore, because of sample size, this particular step in the study demanded extra precautions. It was necessary to obtain good quality undegraded RNA for all time points. To determine the quality of isolated RNA, electrophoresis was performed on a formaldehyde-agarose gel. The gel picture (Figure 10) shows that isolated total RNA did not have detectable degradation. Intact total RNA separated on a denaturing gel would have two sharp 28s and 18s ribosomal RNA as seen in Figure 10. Degraded RNA will not show sharp bands but, instead, the bands will look smeared. This gel also served to compare the quantity of isolated RNA in each sample.

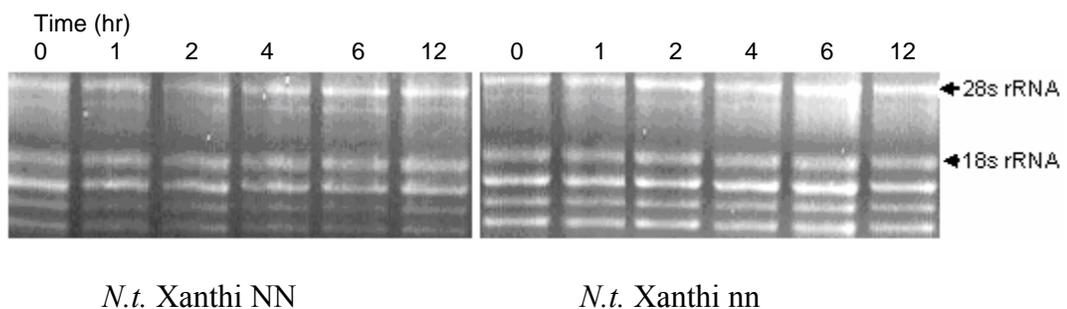


Figure 10 Agarose Gel Electrophoresis of Total RNA from Resistant (NN) and Susceptible (nn) Plant Lines

RNA Quantitation by UV Spectrophotometer

For cDNA synthesis, 1µg of total RNA was used from each time point. This was to ensure that no variation in product concentration was due to unequal amounts of RNA used for cDNA synthesis. To calculate the volume of RNA solution needed to get 1µg of RNA, the following formula was applied and the data are shown in Table 5.

$$1 \text{ OD}_{260} = 40\mu\text{g/ml} (0.04\mu\text{g}/\mu\text{l})$$

1.6µl of RNA sample diluted to 0.4ml with DEPC-treated water

$$\text{OD}_{260} \times 250 (\text{dilution factor}) \times 0.04 = \text{RNA}/\mu\text{l}$$

$$1/(\text{RNA}/\mu\text{l}) = \mu\text{l}/1\mu\text{g}$$

Table 5 Quantitation of Total RNA

	Sample	OD260nm	RNA (µg /µl)	µl/1µg
1	TXN 0hpi	0.055	0.550	1.818
2	TXN 1hpi	0.058	0.580	1.724
3	TXN 2hpi	0.062	0.620	1.613
4	TXN 4hpi	0.051	0.510	1.961
5	TXN 6hpi	0.054	0.540	1.852
6	TXN 12hpi	0.042	0.420	2.381
7	TXS 0hpi	0.065	0.650	1.538
8	TXS 1hpi	0.085	0.850	1.176
9	TXS 2hpi	0.133	1.330	0.752
10	TXS 4hpi	0.066	0.660	1.515
11	TXS 6hpi	0.193	1.930	0.518
12	TXS 12hpi	0.040	0.400	2.500

TXN – TMV infected resistant plant line (*N.t. Xanthi NN*); TXS – TMV infected susceptible plant line (*N.t. Xanthi nn*); OD – optical density (Absorbance)
hpi – hours post inoculation

RT-PCR Amplification of *EF1α*

EF1α is one of the three elongation factors that are responsible for achieving accuracy during translation of the mRNA code into an amino acid sequence (McDowall

2006). This gene is constitutively expressed in all cells and is commonly used to determine equal loading/use of RNA in Northern hybridization and RT-PCR amplifications (Kumar and Klessig 2003). Therefore, *EF1 α* was used to verify equal amount of cDNA for all time points used for RT-PCR analysis (Figure 11).

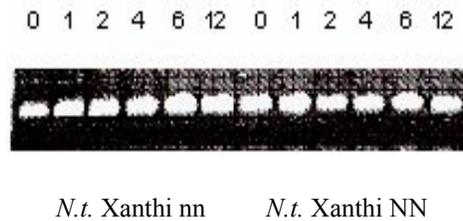


Figure 11 RT- PCR Amplification of *EF1 α* . The synthesized cDNA (1 μ g) from each time point was used for PCR amplifications. Time indicated: 0hr; uninfected control, 1-12hr; time points following TMV infection, Xanthi mn; susceptible plant, Xanthi NN; resistant plant. The number of cycles used for the PCR reaction was 28 cycles

RT-PCR Amplified Fragments of Gene Specific Primers

The gene-specific primers were first tested with synthesized cDNA to ensure they worked properly. They were designed to amplify fragments of approximately 150 to 300bp long. PCR amplifications for each of these genes were performed at an annealing temperature of 55°C for 35 cycles (determined based on the linear range of amplification). The amplified products were separated by agarose gel electrophoresis visualized by staining with ethidium bromide (Figure 12).



Figure 12 Amplification of Gene-Specific Primers at 55°C. M; 100bp DNA marker, 1; *DAHP synthase*, 2; *Shikimate kinase*, 3; *Chorismate synthase*, 4; *Chorismate mutase*, 5; *Isochorismate synthase*

RT-PCR Amplification of SA Biosynthetic Genes

Once the amount of cDNA template was normalized, it was possible to begin expression profiling of the SA biosynthetic genes. Five genes were used for PCR amplification using cDNA synthesized from TMV-infected resistant and susceptible plants. The PCR reaction mix (made as previously described) used for each gene differed only by inclusion of the primer set specific for each gene.

Gene Expression of *DAHP Synthase*

In comparing the time points to the uninfected control sample (0hr), expression of *DAHP synthase* increased in both plant lines but an earlier response is seen in the resistant plant compared to susceptible plant (Figure 13). By the 1 hour (hpi) time point the gene expression is significantly higher in resistant plant then it starts to decrease a little for the later time points though it still remained high compared to uninfected control. In the susceptible plant the 2hpi time point shows the highest level and then begins to level off.

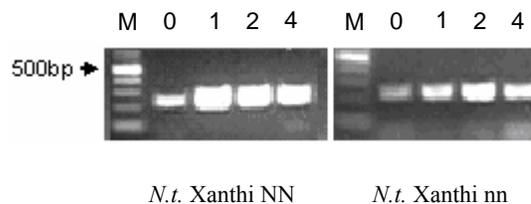


Figure 13 Gene Expression of *3-Deoxy-D-Arabino-Heptulosonate 7-phosphate (DAHP) Synthase* in TMV Infected Plants. M; 100bp DNA marker, 0hr; uninfected control, 1, 2 and 4hr; time points following TMV infection, Xanthi nn; susceptible plant, Xanthi NN; resistant plant

Gene Expression of *Chorismate Mutase*

Next, we analyzed the expression of gene encoding chorismate mutase, which catalyzes the production of phenylalanine and tyrosine from chorismate. By 1 hour, gene expression was significantly higher in the resistant plant (Figure 14). At 2hpi the expression level was already decreasing in resistant plants but in susceptible plants the same time point shows the highest gene expression which then decreased by 4hpi.

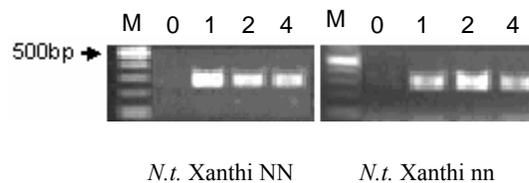


Figure 14 Gene Expression of *Chorismate Mutase* in TMV Infected Plants. M; 100bp DNA marker, 0hr; uninfected control, 1, 2 and 4hr; time points following TMV infection, Xanthi nn; susceptible plant, Xanthi NN; resistant plant

Gene Expression of *Isochorismate Synthase*

Expression of *Isochorismate synthase* increased by 1hr postinoculation compared to an uninoculated control for the resistant plant (Figure 15). There was slight increase of expression in the susceptible plant at 2 hour postinoculation. The increase in expression of *ICS* in TMV-infected resistant plants occurred earlier than in the susceptible plants.

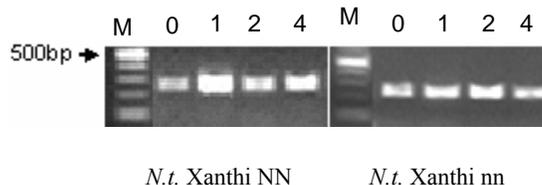


Figure 15 Gene Expression of *Isochorismate Synthase* in TMV Infected Plants. M; 100bp DNA marker, 0hr; uninfected control, 1, 2 and 4hr; time points following TMV infection, Xanthi nn; susceptible plant, Xanthi NN; resistant plant

Gene Expression of *Chorismate Synthase*

The expression of *chorismate synthase* and *shikimate kinase* was analyzed up to 12hr post TMV inoculation (Figure 16A). Differential expression of chorismate synthase was not very clear in resistant plants compared to susceptible plants. A replicate experiment (Figure 16B) confirmed that there was expression at 1 hpi in the resistant plant. The lack of expression in Figure 16A at this time point was therefore an experimental error.

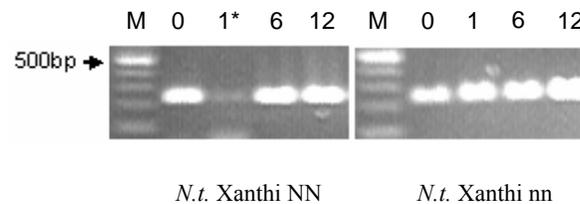


Figure 16A Gene Expression of *Chorismate Synthase* in TMV Infected Plants. M; 100bp DNA marker, 0; uninfected control, 1, 6 and 12; time points following TMV infection, 1*; may be an experimental error, Xanthi nn; susceptible plant, Xanthi NN; resistant plant

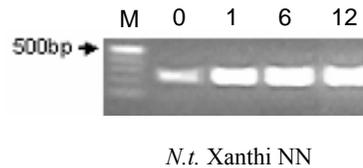


Figure 16B Replicate Gene Expression of *Chorismate Synthase* in TMV Infected Plants. M; 100bp DNA marker, 0; uninfected control, 1, 6 and 12; time points following TMV infection, Xanthi NN; resistant plant

Gene Expression of *Shikimate Kinase*

Shikimate kinase showed increased expression compared to the 0hr uninfected control in both resistant and susceptible plants. Susceptible plants also showed early induction by 1hpi as seen in resistant plants (Figure 17). Gene expression was still significantly higher at 12hpi in both plant lines when compared to the un-infected control.

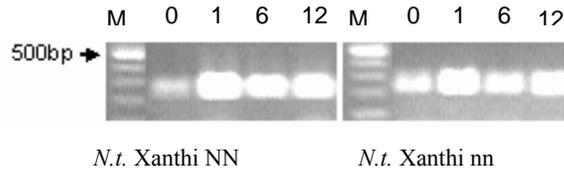


Figure 17 Gene Expression of *Shikimate Kinase* in TMV Infected Plants. M; 100bp DNA marker, 0; uninfected control, 1, 6 and 12; time points following TMV infection, Xanthi nn; susceptible plant, Xanthi NN; resistant plant

Analysis of Aromatic Amino Acid Levels in TMV Infected Tobacco Plants

The 3 aromatic amino acids, phenylalanine, tryptophan, and tyrosine, share chorismate as a common precursor with salicylic acid. It is quite possible that synthesis of these amino acids is reduced to allow availability of more chorismate for conversion into SA or the synthesis of chorismate increases to meet the higher demand for SA. Peak areas (measured in milli absorbance unit (mAU) x minutes) and retention times of the reference compounds (tyrosine, phenylalanine, and tryptophan) were first determined from HPLC chromatograms. These retention times corresponding to pure aromatic amino acid standards were used to identify amino acid compounds in the extracts (Figures 18-21). Chromatograms of the extracts prepared from the infected resistant and susceptible plants are shown below (Figures 22-29). Four different time points (0hr, 1hr, 2hr, and 4hr) were used for analysis. The integrated area under the peaks was used to calculate the relative amounts of amino acids (mAU x min) present in the samples.

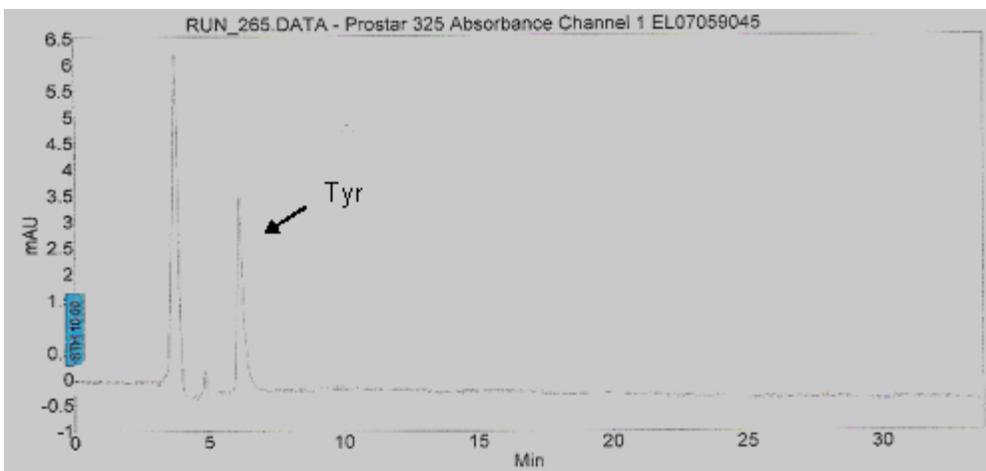


Figure 18 HPLC chromatogram of 0.38mM Tyrosine Standard (10 μ l) showing a retention time of 6.11minutes and peak value of 1.4mAUxmin run in 10% acetonitrile in 0.01% phosphoric acid-water at flow rate of 0.7ml/min

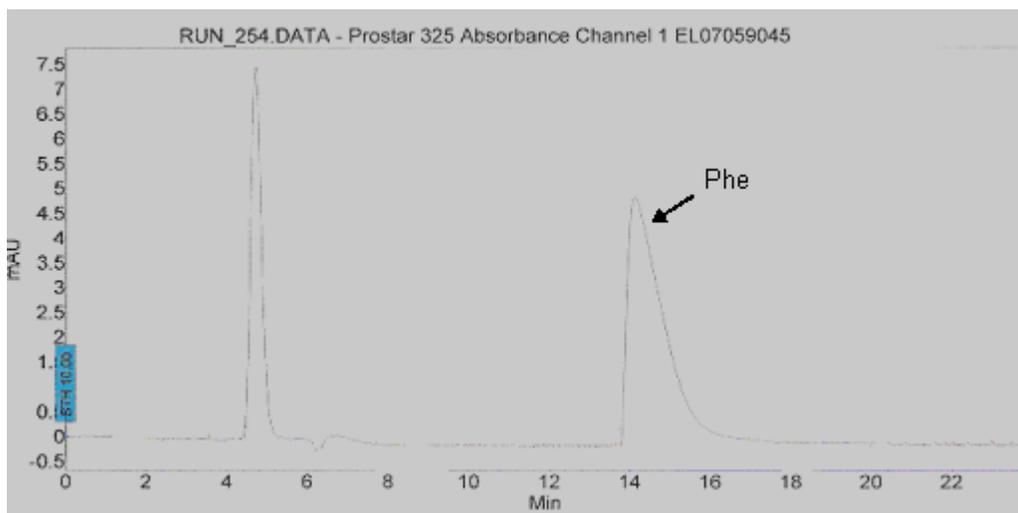


Figure 19 HPLC chromatogram of 2.45mM Phenylalanine Standard (5 μ l) showing a retention time of 14.11minutes and peak value of 5.0mAUxmin run in 10% acetonitrile in 0.01% phosphoric acid-water at flow rate of 0.7ml/min

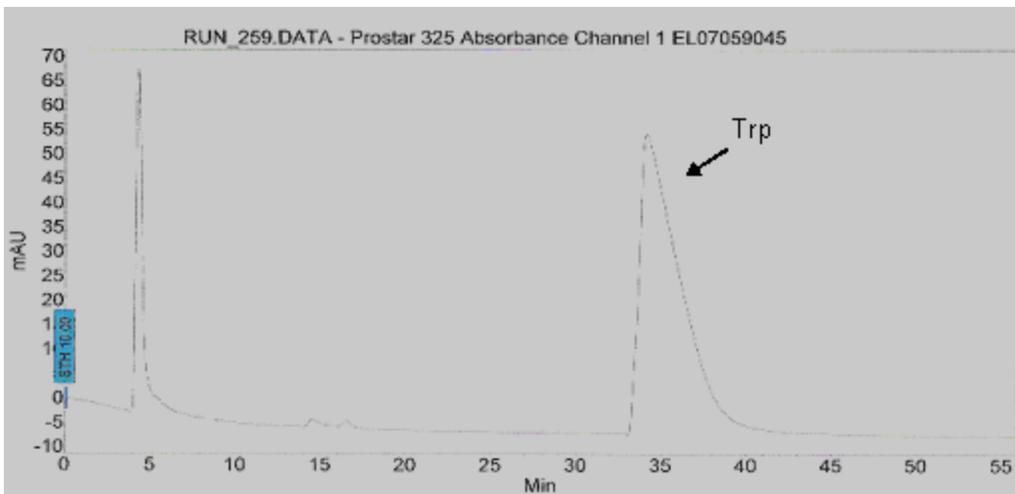


Figure 20 HPLC chromatogram of 2.45mM Tryptophan Standard (10 μ l) showing a retention time of 34.15minutes and peak value of 164.9mAUxmin run in 10% acetonitrile in 0.01% phosphoric acid-water at flow rate of 0.7ml/min

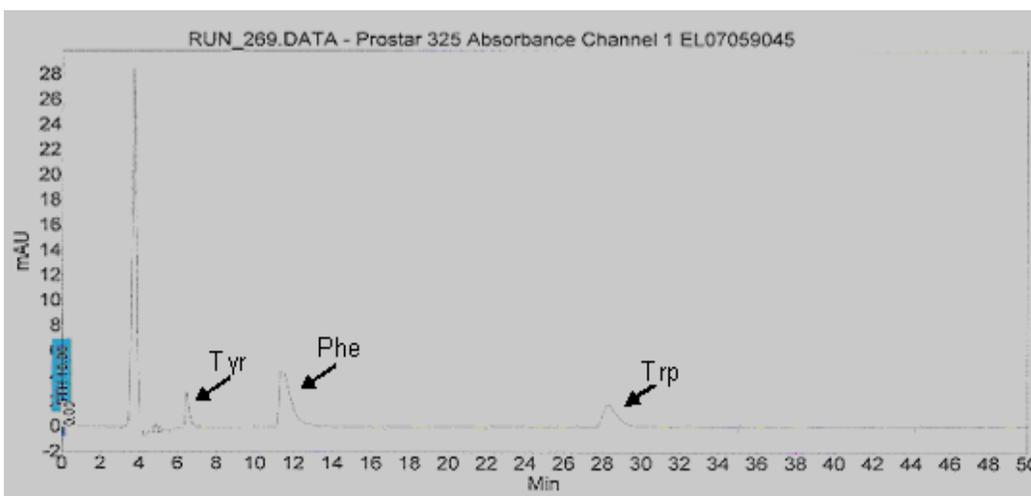


Figure 21 HPLC chromatogram of all three amino acid standards (20 μ l) showing retention times for tyrosine: 6.47min; phenylalanine: 11.39min; tryptophan 28.29min run in 10% acetonitrile in 0.01% phosphoric acid-water at flow rate of 0.7ml/min

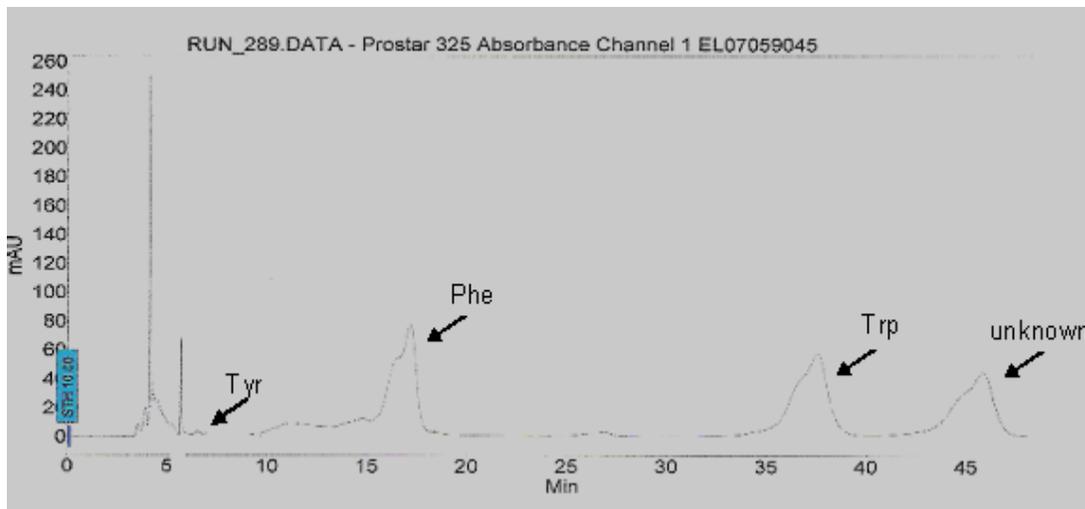


Figure 22 HPLC chromatogram of 50µl clarified leaf extracts from uninoculated (0h) susceptible plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.49min, 17.17 and 37.53 min respectively. The solvent system used consisted of 10% acetonitrile containing 0.01% phosphoric acid in water with a flow rate of 0.7ml/min

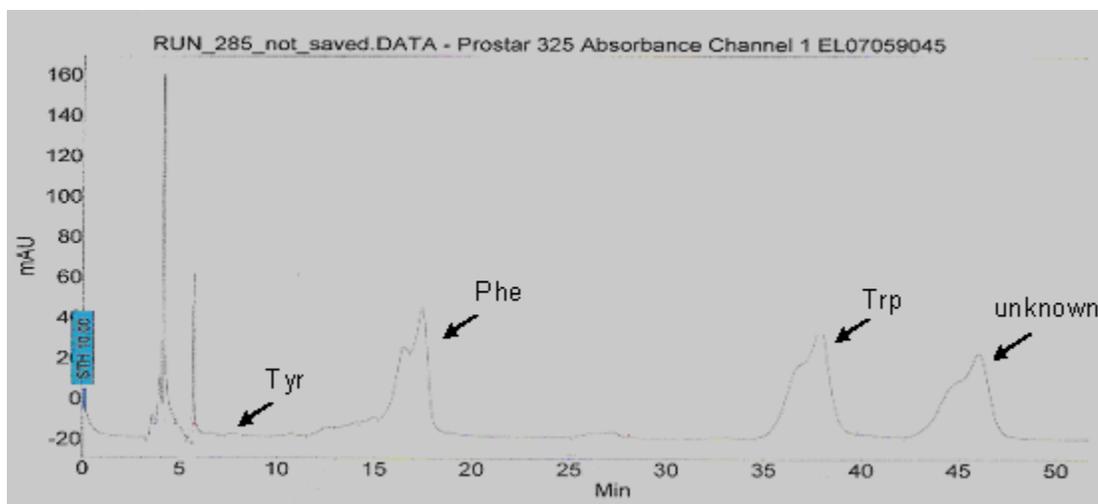


Figure 23 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (1h) susceptible plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.52min, 17.42 and 37.83 min respectively

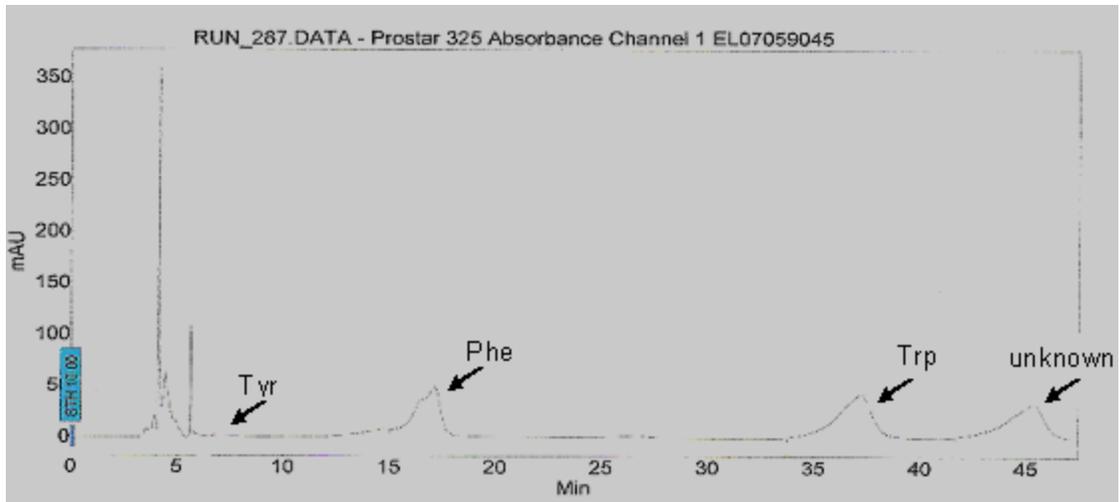


Figure 24 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (2h) susceptible plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.47min, 17.22 and 37.19 min respectively

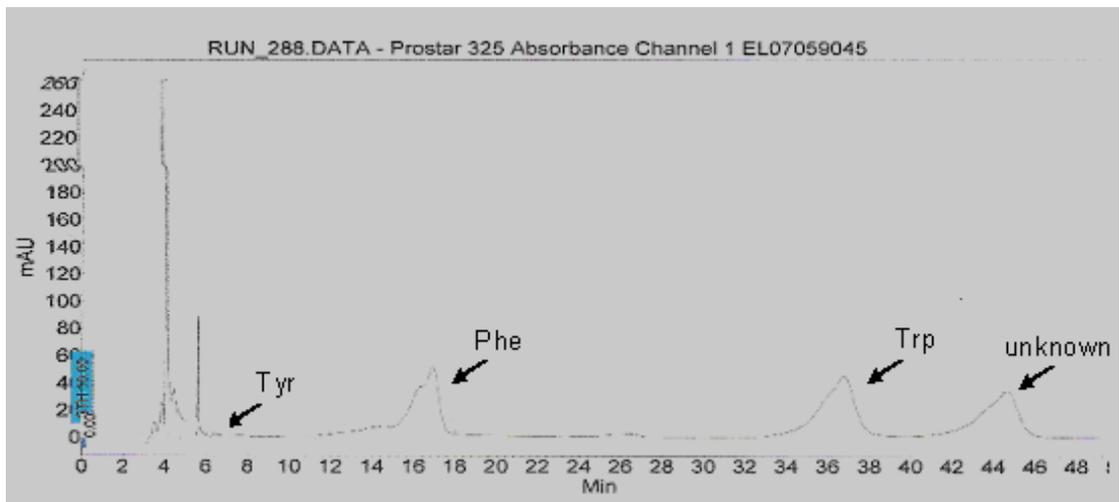


Figure 25 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (4h) susceptible plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.38min, 16.93 and 36.75min respectively

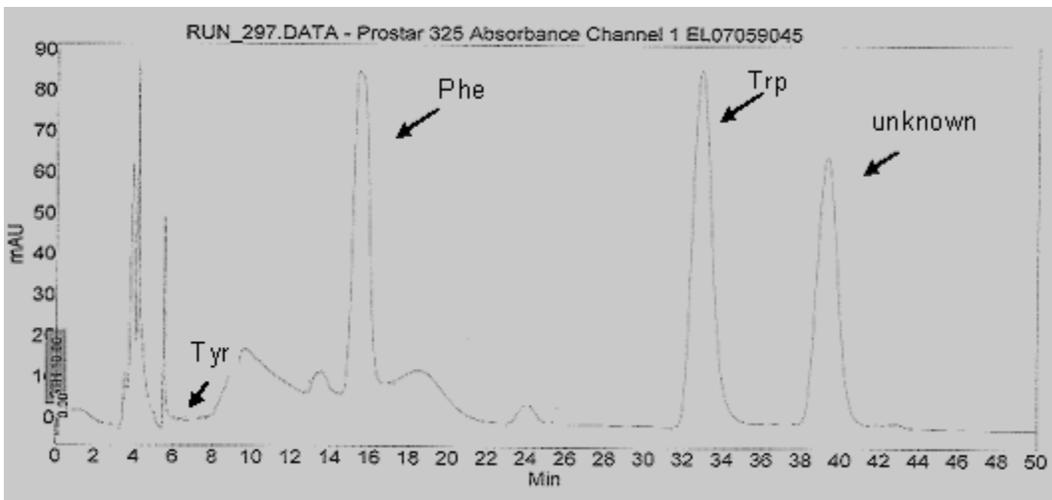


Figure 26 HPLC chromatogram of 50µl clarified leaf extracts from uninoculated (0h) resistant plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.22min, 15.38 and 32.85min respectively

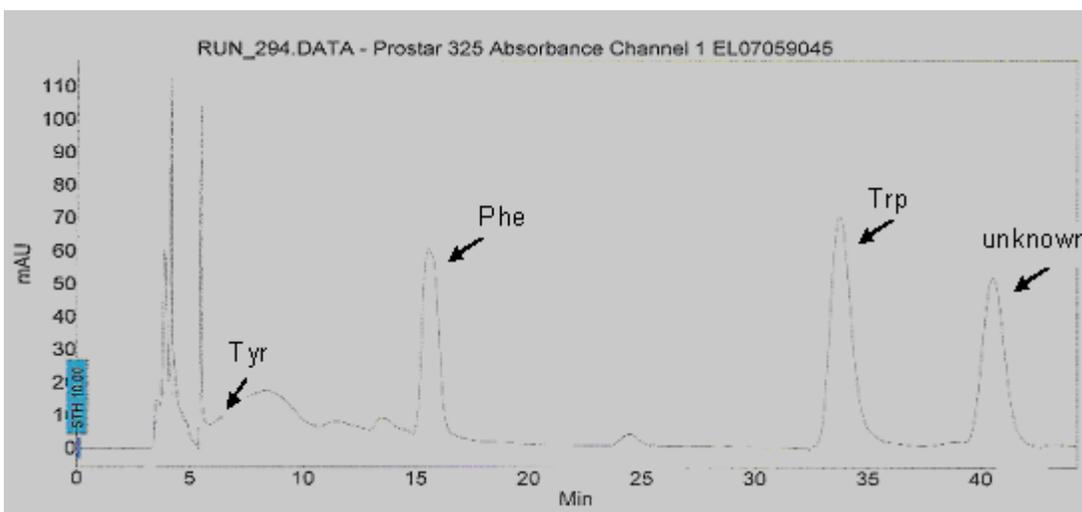


Figure 27 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (1h) resistant plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.33min, 15.52 and 33.71min respectively

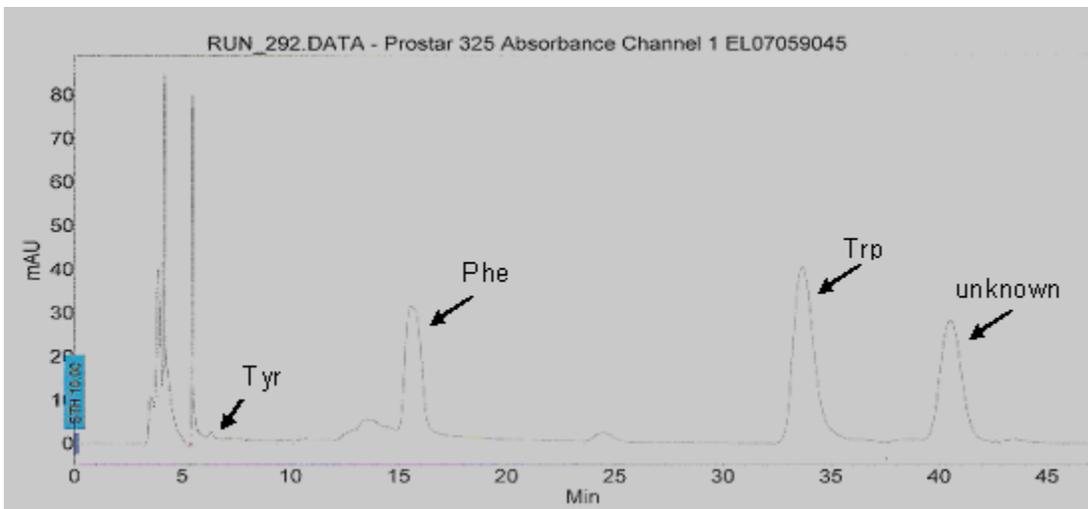


Figure 28 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (2h) resistant plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.29min, 15.53 and 33.65min respectively

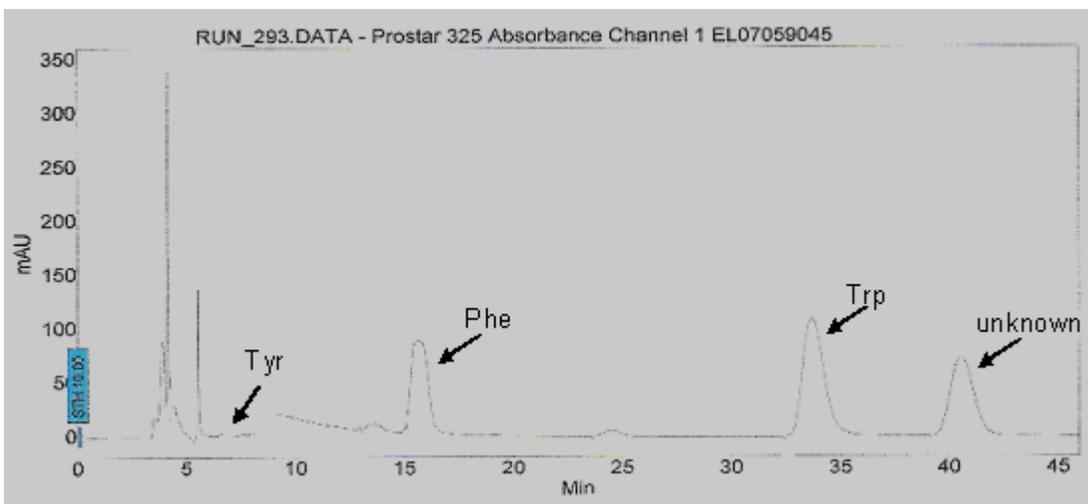


Figure 29 HPLC chromatogram of 50µl clarified leaf extracts from inoculated (4h) resistant plant sample. Tyrosine, phenylalanine, and tryptophan eluted at 6.31min, 15.43 and 33.69min respectively

Tables of Aromatic Amino Acid Levels

The peak values obtained for tyrosine noted in Table 6 show very minimal amounts and as a result tyrosine was excluded from further analysis due to a higher probability of inaccurate readings. Although the HPLC system is highly sensitive, measurements of very low amounts of compound could be unreliable because no room is left for any margin of error. Peak values for tryptophan and phenylalanine are shown in Table 7 and 8 respectively.

Table 6 Tyrosine Levels in TMV Infected Plants

	Peak Values (Susceptible)	% Increase/decrease	Peak Values (Resistance)	% Increase/decrease
Time in Hrs				
0	0.9	0	0.2	0
1	0.6	-33.3	4.4	2100
2	0.3	-66.6	1.2	500
4	0.6	-33.3	0.2	0

Table 7 Tryptophan Levels in TMV Infected Plants

	Peak Values (Susceptible)	% Increase/decrease	Peak Vaules (Resistance)	% Increase/decrease
Time in hrs				
0	106.7	0	103.4	0
1	104.3	-2.2	76	-26.5
2	77	-27.8	43.9	-57.5
4	81.6	-23.5	122.1	18.1

Table 8 Phenylalanine Levels in TMV Infected Plants

	Peak Values (Susceptible)	% Increase/decrease	Peak Values (Resistance)	% Increase/decrease
Time in hrs				
0	95.8	0	70.7	0
1	53.2	-44.4	50.3	-28.8
2	60.3	-37.0	23.9	-66.1
4	66	-31.1	77.82	10.1

Amino acid levels shown in the Tables were measured in mAU x min.

Considering first tryptophan (Table 7), by 1hpi there was only a 2.2% decrease compared to the 0hr un-inoculated control in the susceptible plant where as resistant plant had a 26.5% decline (Figure 30). Then by 2hpi susceptible plant had decreased by 27.8% while resistant plant 57%. The last time point at 4hpi, tryptophan level in the resistant plant had been restored above initial amount with 18.1% increase while in the susceptible plant; the amino acid was still at a 23.5% decline. Similar results were seen for phenylalanine levels (Figure 31). A greater decrease of 66.1% was seen in the resistant plant with a 10.1% increase above normal levels by 4hpi. Data from a representative set of samples and a replicate experiment showed similar trend.

Graphs of Aromatic Amino Acid Levels

Figure 30 Tryptophan Levels in TMV-Infected Resistant and Susceptible Tobacco Plants

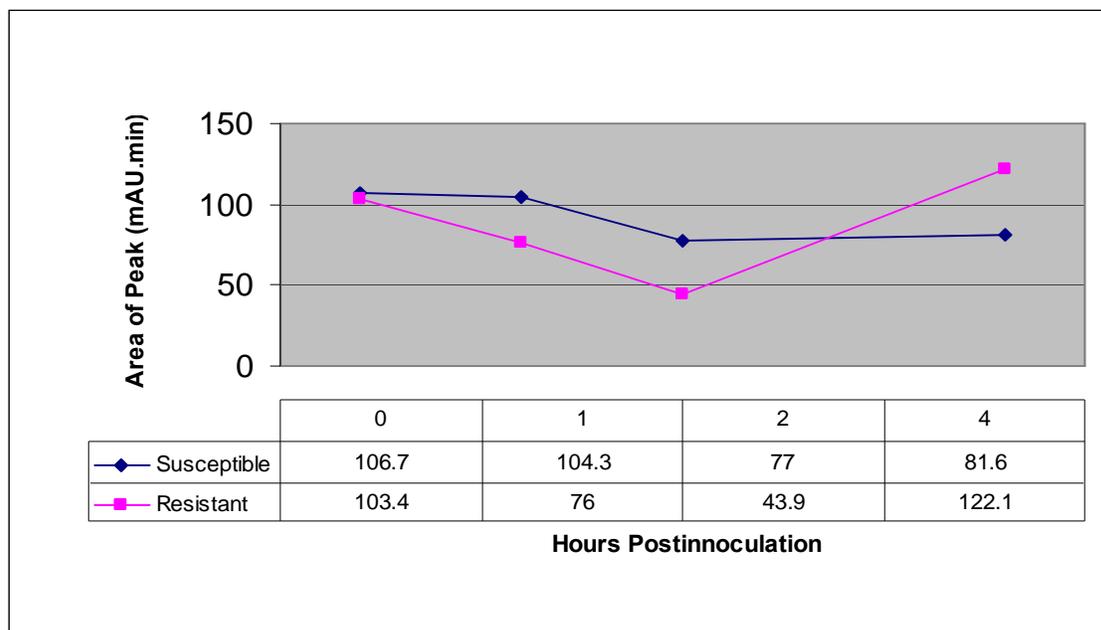
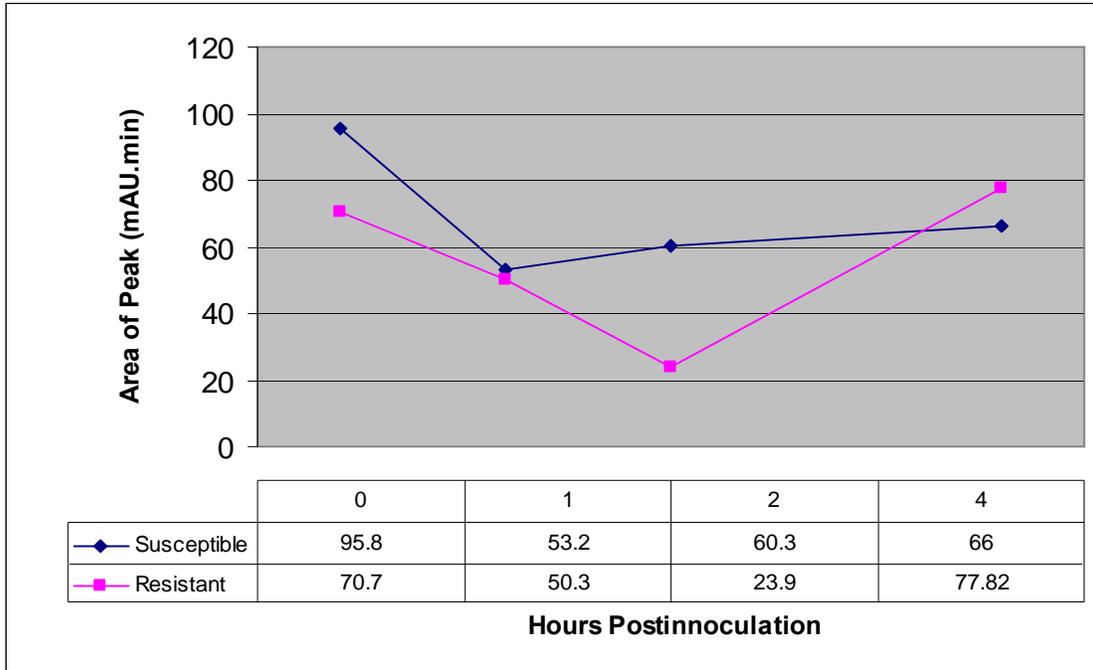


Figure 31 Phenylalanine Levels in TMV-Infected Resistant and Susceptible Tobacco Plants



CHAPTER 4

DISCUSSION

Expression of Genes of Salicylic Acid Biosynthetic Pathway

Our results from this study show that the enzymes of the shikimate pathway are up-regulated following plant pathogen interaction in resistant and susceptible plants. Increased expression of these genes occurred earlier in resistant plants for three genes included in this study (refer to Figures 13-15). *DAHPh synthase*, *chorismate mutase*, and *isochorismate synthase* all showed a greater increase in expression by 1hpi in the resistant plant. This makes it possible to attribute the differential expression as a possible cause of increase in salicylic acid levels in virus infected resistant tobacco plants. Free and bound SA levels increased from 0.1 and 0.25 $\mu\text{g/g}$ fresh weight to 1.0 $\mu\text{g/g}$ and 20 $\mu\text{g/g}$ fresh weight respectively in *Arabidopsis* plants infected with *Pseudomonas syringae* (Summermatter et al. 1995). The shikimate enzymes might also be up-regulated to cause increase in the production of other metabolic products in the post chorismate pathway, e.g. lignin and camalexin which are also needed in plant defense.

It was previously shown by Janzik et al. (2005) that ozone affected expression levels of some of these genes in tobacco line Bel W3 with *DAHPh synthase* showing the earliest induction by 1hpi. Salicylic acid, known to be associated with the ozone triggered signaling pathway (Overmyer et al. 2003), is highly induced in the Bel W3 tobacco line in response to ozone (Pasqualini et al. 2002). This provides further evidence that induction of genes of the SA biosynthetic pathway might contribute to its accumulation.

Figures 13 to 17 show earlier increase in expression of SA biosynthetic genes in the infected resistant compared to susceptible plant lines. *DAHPSynthase*, being the first gene in the pathway, showed high level of up-regulation supporting its likely role in redirecting more phosphoenolpyruvate (PEP) from glycolysis and erythrose 4-phosphate (E4P) from the pentose phosphate pathway into the shikimate pathway (Hermann and Weaver 1999). The biochemistry of plants shows that several pathways are often intertwined. For example, salicylic acid can cause resistance via a NPR1 dependent or independent pathway (Shah 2003 and references therein). The NPR1 independent pathway involves other defense signals such as jasmonic acid that has been shown to be involved in cross-talk with salicylic acid (Beckers and Spoel 2005 and references therein). In fact, both synergistic interactions that lead to enhanced resistance and pathway trade-off from antagonistic interaction have been reported between the 2 molecules to fine-tune the resistance response (Beckers and Spoel. 2005 and references therein).

Flavonoids, a class of plant secondary metabolites, are most commonly known for their antioxidant properties, among other vital properties. They use as a precursor, phenylalanine made from a branch (phenylpropanoid pathway) of the post Shikimate pathway, to produce 4-coumaroyl-CoA (Ververidis et al. 2007). The compound 4-coumaroyl-CoA can then be combined with carbons of 3 molecules of malonyl-CoA to produce chalcones and further modifications produce other products such as anthocyanins and flavanoid-derived compounds. All these compounds have crucial functions in plants including pigmentation in flowers and even protection against microbes and insects

(Ververidis et al. 2007). This further highlights how different pathways are interrelated and are often dependent on one another to be functional.

Interestingly also is the regulation of the *isochorismate synthase* gene which is believed to be the mainly responsible for SA accumulation in plants such as Arabidopsis and Tobacco (Nugroho et al. 2001). Recent reports by Ogawa et al. (2006) suggests that SA made through the PAL enzyme (refer to Figure 3) is the main route for SA synthesis in infected tobacco plants. The *ICS* gene shows up-regulation in the infected tobacco plants when compared to an uninfected control (refer to Figure 15). Tsai et al. (2006) also reported a characterized *ICS* gene in *Populus* that was fully expressed in green leaves after wounding where phenolic glycosides accumulate. A number of other plants seem to use the *ICS* route to synthesize SA as plastid localized *ICS* has been identified in Madagascar periwinkle. Also, *ICS* expressed sequence tags in soybean and wild tomato have been identified (Wildermuth et al. 2001).

According to a recent review by Whitham et al. (2006), it was reported that generally the increased production of SA is required for the increased accumulation of pathogenesis-related (*PR*) transcripts and proteins during resistance response to viruses but no accumulation is seen in susceptible interactions. Unlike those in the incompatible (resistant) interaction, SA levels are not altered in compatible virus-host interactions (Dempsey et al. 1999 and references therein) and thus the amplification of a signal needed for robust *PR* gene expression does not occur. None the less basal SA levels mediate the induction of defense related genes in a compatible host-virus interaction (Ishihara et al. 2004). In fact, it was shown that cucumber mosaic virus (CMV) induces the expression of SA-mediated defense related genes such as *PR-1* and *PR-4a* in

compatible tobacco plants suggesting that a SA-dependent signaling pathway is also regulated by compatible viruses in tobacco (Whitham et al. 2006 and references therein). It is possible that although the shikimate genes are up-regulated in the susceptible plant line, a specific signal for SA accumulation remains absent.

It has been shown that during the hypersensitive response in cotton against *Xanthomonas campestris pv malvacearum* (Xcm), H₂O₂ is required both for local and systemic accumulation of SA, which may locally control the generation of superoxides (O₂⁻) (Martinez et al. 2000). Detaching cotyledons at regular intervals after inoculation demonstrated that the signal leading to systemic accumulation of SA was triggered around 3 hours post-inoculation (Martinez et al. 2000). The signal was associated with an oxidative burst followed by accumulation of H₂O₂ between 4 and 6h post-inoculation (Martinez et al. 1998). The fact that the appearance of these reactive oxygen species (ROS) in infected cotyledons preceded SA accumulation suggested that H₂O₂ and or O₂⁻ could induce SA accumulation (Martinez et al. 2000). Work done by Summermatter et al. (1995) also reported a direct effect of H₂O₂ on the accumulation of SA.

Transgenic potato plants expressing a bacterial gene encoding glucose oxidase exhibited constitutively elevated, sub-lethal levels of H₂O₂, leading to increased disease resistance (Wu et al. 1997). The constitutively elevated levels of H₂O₂ activated an array of host defense mechanisms, including a several-fold increase in total SA that was mostly conjugated SA as free SA levels were unaffected (Wu et al. 1997). Conjugated SA is the storage form of SA that can be hydrolyzed to its free form when a plant is challenged by a pathogen. Bearing this in mind, it could be that along with increased expression of SA biosynthetic genes, ROS are also needed to signal increase in SA accumulation. This is

something that is absent in susceptible plants lacking the 'N' gene that are unable to mount an oxidative burst (Allan et al. 2001).

The fact that these SA biosynthetic genes increased in expression in both resistant and susceptible plants shows that they are involved in the plant's line of defense except that susceptible plants eventually succumb to the invasion. According to Ishihara and associates (2004), gene expression profiling in the *Arabidopsis* resistant (C24) and susceptible (Col-0) ecotypes using micro array analysis showed that similar number of genes (50-80) was modulated significantly in the 2 plant lines through the first 24 hrs. They analyzed 9000 expression sequence tags in *Arabidopsis* plants at 6, 12, 24, and 48hrs after infection with Cucumber mosaic virus (CMV-Y). However, by 48hr the response to (CMV-Y) was much more robust in resistant C24 (788 significant genes) compared to 150 genes in susceptible plants. Similar studies on compatible *Arabidopsis*-Virus (CMV-Y) interactions revealed that SA biosynthetic genes are among defense genes that are induced (Huang et al. 2005).

Additionally, results by Huang and colleagues (2005) suggested that expression of several defense-related genes in compatible host interactions might also use components of signaling pathways involved in incompatible host-pathogen interactions, but their increased expression has no negative effect on viral infection. It was proposed that the susceptible plant lacks the ability for specific recognition that could localize the virus to prevent replication, spreading, and host suppression (Huang et al. 2005).

In this study, it is interesting to note that in comparison to the uninfected control sample, a significant increase in gene expression levels was evident as early as 1hr post inoculation indicating how quickly plants act against the invading viral pathogen. Any

lag in response could prove detrimental to the plants as the virus seeks to overtake and control the cells' metabolic machinery. In fact Dempsey et al. (1999) suggested that the difference between plants that are resistant or susceptible to a specific pathogen usually is not determined solely by the presence or absence of the defense machinery, but rather by the ability to rapidly activate it following infection. The early significant increase in expression by lhpi was more evident in *DAHPh synthase* and *Shikimate kinase* (refer to Figures 13 and 17).

Protein kinases are involved in transferring phosphate groups from molecules that are high in energy, such as ATP, onto target substrates via a process called phosphorylation. Phosphorylation can cause conformational changes in proteins that can activate or deactivate them (Cozzon 1988). This type of post translational modification is used in cells for the transduction of signals and to alter enzyme activity, cellular location, and association with other proteins (Cozzon 1988). High expression of *shikimate kinase* in the infected plants might further implicate *shikimate kinase* in the signal transduction for SA accumulation. It is possible that shikimate kinase activity might help in the phosphorylation of other molecules not directly involved in the shikimate pathway. These molecules might be required for the signal transduction leading to SA accumulation.

Contrary to our prediction that the genes *anthranilate synthase* and *chorismate mutase* might be down-regulated to decrease the amount of chorismate available to be used for synthesis of the aromatic amino acids, *chorismate mutase* was up-regulated in the resistant and susceptible plants. There was however an earlier increase seen in the resistant plants (Figure 14). Work reported by Niyogi and Fink (1992) showed that

anthranilate synthase transcript levels increased in response to bacterial infection and wounding in *Arabidopsis*. This increase in mRNA accumulation presumably allows enhanced synthesis of secondary metabolic products such as phytoalexins (Niyogi and Fink 1992). Phytoalexins are plant compounds that accumulate in infected sites to confer resistance against microorganisms and some examples include camalexin, anthocyanins, and isoflavonoids (Zook and Hammerschmidt 1997).

Camalexin accumulation inhibits the growth of pathogenic fungi and bacteria and is synthesized from an intermediate of the tryptophan pathway between anthranilate and indole (Zook and Hammerschmidt 1997). Lignin, which helps to strengthen cell walls to prevent further invasion of surrounding cells, is also made by using phenylalanine as a precursor. Both of these secondary metabolites are said to function in local acquired resistance and in the hypersensitive response (Baker et al. 1993).

In the current study, analysis of gene expression of the SA biosynthetic pathway showed that there is an earlier increased-expression response in resistant tobacco plants compared to susceptible plants. In 3 of the genes, *DAHPh synthase*, *isochorismate synthase*, and *chorismate mutase*, resistant plants showed highest level of expression by 1hr post inoculation compared to susceptible plants showing highest level an hour later. This suggests that resistant plants are able to respond much more quickly to pathogen infection when compared to susceptible plants. The differential gene expression seen in this study could possibly be linked to why resistant plants are able to accumulate high amounts of SA when resisting pathogen infection.

Aromatic Amino Acid Analysis

In considering the second hypothesis of this study, that the use of chorismate for production of phenolic compounds other than SA might be limited allowing more substrate availability for conversion into SA, we measured the aromatic amino acid levels in the 2 plant lines. The results obtained from HPLC analysis of the aromatic amino acids phenylalanine and tryptophan (tyrosine was excluded due to lower levels) showed that the levels of these compounds decreased after plant infection (refer to Figures 30-31). There was an immediate decrease by 1hpi in both resistant and susceptible plants for both compounds. However, a more pronounced decrease was seen in the resistant plants as seen by as much as 66.1% decrease in phenylalanine levels by 2hpi compared to only 37% in susceptible plants (Table 8).

It is possible that phenylalanine, along with tryptophan, was being used upon plant pathogen interaction to help synthesize other defense compounds such as lignin and camalexin as previously stated. If that is the case, it would explain the decrease in these amino acid levels observed within early hours of infection. Aromatic amino acids are also used in basic protein synthesis and this could be linked to the decrease as well.

If the amino acids were being consumed upon pathogen interaction for defense purposes, then replenishing previous pools could be crucial to maintaining the defense response and restoring normal plant processes. As viruses spread across plant tissues from an infected site, the infection caused by the attack is continuous and progressive. The inability of the susceptible plant to quickly restore initial aromatic amino acid levels could have contributed to it eventually succumbing to the viral attack.

Another possibility is that the early decline in aromatic amino acids was a direct response to cause an increase in chorismate made available for the accumulation of salicylic acid. Malamy et al. (1990) reported a 20 fold increase in salicylic acid above basal level of 0.01 μ g/g fresh weight in TMV infected resistant tobacco (Xanthi NN) plants. Future studies measuring chorismate level and or measuring chorismate synthase activity could confirm this possibility. Expression of the *chorismate synthase* gene did not clearly show differential expression between the plant lines during early hours although there was slight up-regulation. Janzik et al. (2005) also reported expression of *chorismate synthase* in ozone-treated tobacco. They found no increase in transcript level at 2hpi but by 4hpi there was up-regulation that reached a maximum at 10hpi. The increase was still high at 36hpi. This later expression might be due to chorismate synthase being the last enzyme in the shikimate pathway.

These results provide valuable insight into the resistance response in plants resisting viral infection. Not only can study in this area help in the creation of more resistant crop plants but it can also help in discovering new pharmaceutical drugs. The shikimate pathway is the common route leading to production of the aromatic amino acids and because it is found only in microorganisms and plants, it makes the pathway an important target for herbicides, antibiotics, and live vaccines. The penultimate step in this pathway is inhibited by N-[phosphonomethyl] glycine, the active ingredient of the broad spectrum, non-selective herbicide glyphosate (Steinrucken and Amrhein 1980). Chemical compounds that interfere with any enzyme activity in this pathway are considered “safe” for humans when handled in reasonable concentrations.

In fact, glyphosate has been tested successfully in mice as a therapeutic agent against pathogenic protozoa that cause diseases like toxoplasmosis or malaria (Roberts et al. 1998). In mice infected with *Toxoplasma gondii*, 1mM of glyphosate inhibited the enzyme activity of 5-enolpyruvyl shikimate 3-phosphate (EPSP) synthase and 3.12mM inhibited growth of the pathogen. The herbicide at this concentration had no effect on the growth of mammalian cells (Roberts et al. 1998).

Because salicylic acid proves so crucial especially for systemic resistance in plants, numerous studies are being done to determine how probable it is to use it directly on field plants as pretreatment against later infection (Hayat and Ahmad 2007). One of the major problems in commercial application of SA directly to field plants is that SA has been shown to have adverse effects on plants especially if used in unfavorable amounts. Out of various concentrations of SA used, application of 10^{-5} M SA to the leaves of *Brassica juncea* resulted in increased biomass accumulation, but concentrations above that had inhibitory effect (Fariduddin et al. 2003).

Similarly, Pancheva et al. (1996) observed an inhibition in the growth of leaves and roots of barley seedlings treated with salicylic acid and an increase in the SA concentration enhanced it further. However, SA analogs such as benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and acibenzolar-S-methyl (Actigard®, manufactured by Syngenta) are being commercialized to induce host plant resistance without direct activity against target pathogens (Shuman 2003).

It must be noted that this research is part of a larger study attempting to unlock and identify the specific components of the SA biosynthetic pathway responsible for the initial surge in SA production necessary for a pathogen-infected plant to mount a

systemic resistance response. Future studies of protein-protein interaction with DAHP synthase and shikimate kinase might prove informative to this study and to the field in general. DAHP synthase is up-regulated after TMV infection and because it is the first enzyme in the shikimate pathway it is possible that signaling molecules interact directly with it to redirect more substrate into the pathway. Also, because protein kinases are commonly involved in signal transduction, shikimate kinase might also interact with other proteins. The eventual goal would be to have the capability to map out a confirmed sequence of signaling molecules involved in the accumulation of this crucial plant hormone.

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APPENDICES

Appendix A: Abbreviations

aa	amino acids
avr	avirulence protein
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CMV	cucumber mosaic virus
CP	coat protein
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DEPC	diethyl pyrocarbonate
DHQ	3-dehydroquinate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E4P	erythrose 4- phosphate
EDTA	ethylenediaminetetraacetic acid
EST	expression sequence tags
EtBR	ethidium bromide
EtOH	ethanol
FA	formaldehyde agarose
HPLC	high performance liquid chromatography
HR	hypersensitive response

HSP	heat shock protein
ICS	isochorismate synthase
LAR	local acquired resistance
LRR	leucine rich repeat
M	molar
MeSA	methyl salicylate
mg	milligram
ml	milliliter
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
NBS	nucleotide binding site
NPR1	none-expressor of pathogenesis-related gene
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PEP	phosphoenol pyruvate
pmol	pico mole
PR	pathogenesis related
RNA	ribonucleic acid
R-protein	resistance protein
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
SA	salicylic acid

SABP2	salicylic acid binding protein 2
SAMT	salicylic acid methyl transferase
SAR	systemic acquired resistance
TAE	tris-acetate EDTA buffer
TE	tris-EDTA buffer
TMV	tobacco mosaic virus
μg	microgram
μl	microliter

Appendix B: Recipes

1.5% Formaldehyde Agarose Gel

1.87g Agarose

12.5ml 3-[N-morpholino] propanesulfonic acid (MOPS) (0.2M)

3.75ml Formaldehyde

110ml distilled water

Heat to melt agarose

1X Formaldehyde Agarose Gel Running Buffer

100mL 10X MOPS (0.2M)

900mL RNase-free sterile water

20X RNA Loading Buffer

40 μ l 10X MOPS

60 μ l Formaldehyde

200 μ l Formamide

50X Tris Acetate EDTA Buffer

121.0 g Tris base

28.55mL glacial acetic acid

50.0mL 0.5 M EDTA (pH 8.0)

Add distilled water to bring to 500mL volume

1X Tris Acetate EDTA Buffer

20mL 50X TAE buffer

980mL distilled water

Staining Solution (0.5 µg/mL)

250mL 1X TAE Buffer

12.5µL EtBR (10 mg/mL stock concentration)

10X 3- (N-morpholino) propanesulfonic acids (200ml)

8.4g MOPS

1.36g Sodium Acetate

4ml (0.5M) sodium EDTA

Adjust the pH to 7.0 with NaOH

1.5% Agarose gel

0.75g agarose

50ml distilled water

2.5µl ethidium bromide (10 mg/mL stock concentration)

100mM Phosphate (sodium) buffer

39ml Stock solution A (27.6g/L monobasic Na-phosphate)

61ml Stock solution B (28.4g/L dibasic Na-phosphate)

Diluting to total volume 200ml (pH 7)

50mM was made by diluting 50ml in 100ml distilled water

75% ethanol

37.5 ml 100% ethanol

12.5ml distilled water

Diethyl pyrocarbonate treated water

0.1ml DEPC

100ml distilled water

Shake vigorously

Incubate overnight at 37 C

Autoclave 15min

Appendix C: Gene Sequences from Public Database NCBI

3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHPh) synthase

Accession # M64261

```
1 GGCTCAATTT CAGGTACCCA AAAAACAAGA AAATCACATA CCAAAAAACA CTCACATTTT
61 TCATAAAAAGT ATTAAATTCA ATGGCTCTTT CAAGCAGTAG CACTACCAAC TCCCTTCTTC
121 CCAACAAATC TCAACTGGTT CAAAATCAAT CCCTTTTACC TTCTCCTCTA AAGAATGTAT
181 CTTTCACCAC CAACTCAACC AAACCCGTTA GATTTGTTCA ACCAATCTCA GCCATTCATT
241 CCTCTGACTC TTCCAAGAAC CCCATTGTCT CCGACAAGCC CTCCTCCAAG CCTTCACCGC
301 CGGCGGCCAC TGTTACGGCG GCGGCTACGA CGGTGACAAA AACAGAATGG ACAGTGGAGA
361 GCTGGAAATC CAAAAAGGCT CTTCAGTTAC CCGAATACCC AAATCAAGAG GAGCTTCAAT
421 CTGTTCTTAA GACGATTGAA GAGTTCCTC CTATCGTGTT TGCTGGTGAG GCGAGAAGTC
481 TTGAGGAGCG TCTCGGTGAG GCTGCTATGG GCCGGGCTTT CTTGTTACAA GGAGGTGATT
541 GTGCTGAGAG TTTTAAGGAA TTTAATGCCA ATAATATTAG GGATACTTTT AGAATCCTTC
601 TTCAAATGGG TGCTGTTCTC ATGTTTGGTG GTCAGATGCC TGTTATCAAG GTTGGAGGA
661 TGGCTGGGCA ATTTGCAAAG CCAAGATCAG ATAATTTTGA GGAGAAGAAT GGAGTGAAGC
721 TGCCGAGTTA CAGGGGAGAC AACGTGAACG GAGATGCATT TGATGCCAAG TCAAGAACTC
781 CTGACCCTCA GAGTTGATC AGGGCGTATT GTCAATCTGC AGCTACTTTG AATCTATTGA
841 GGGCTTTTTG TACAGGAGGA TATGCTGCCA TGCAGAGGAT CAACCAATGG AACTTGGATT
901 TCACAGAGCA CAGTGAGCAG GGTGATAGGT ATCGTGAAC AGCTAATAGA GTGGATGAGG
961 CCCTTGTTTT CATGGCTGCT GCTGGACTTA CAGTGGATCA TCCTATTATG AAAACCACAG
1021 AGTTCTGGAC TTCTCATGAG TGCTTACTTT TGCCCTATGA GCAGTCACTA ACACGATTGG
1081 ATTCAACTTC TGGCCTTTAC TATGATTGTT CCGCCCATTT TATTTGGGTT GGAGAAAGAA
1141 CTAGGCAGTT GGATGGTGCC CATGTTGAGT TCTTGAGAGG AGTTGCCAAC CCCCTTG GTA
1201 TTAAGGTGAG TGACAAGATG GATCCAAGTG CATTGGTCAA GCTCATTGAG ATTTTGAACC
1261 CTGATAACAA AGCTGGGAGG ATTACAATAA TTACCAGAAT GGGAGCAGAG AACATGAGGG
1321 TTAAGCTTCC TCATCTTATC AGAGCAGTCC GAAGAGCAGG GCAAATCGTC ACTTGGGTAT
1381 CTGATCCTAT GCATGGGAAT ACCATCAAAG CTCCTTGCGG TCTAAAAACT CGACCTTTTG
1441 ATTCCATCAG GGCTGAAGTA AGAGCATTCT TTGATGTTCA TGAGCAAGAA GGAAGCCACC
1501 CAGGAGGAGT ACACCTGGAG ATGACAGGCC AAAACGTCAC AGAGTGCATT GGTGGATCAC
1561 GAACTGTGAC CTTTGATGAT CTGAGCTCAC GTTACCACAC CCACTGTGAT CCTAGGCTCA
1621 ATGCATCTCA ATCCCTTGAG CTCGCCTTCA TTATTGCAGA ACGCCTAAGA AAGAGGAGGC
1681 TTGGATCACA AAACGTATTA GGTCAATAGA TATTTGGAAA GGTCCAAAAT GCTGCTTTTC
1741 TTGATTTACT CCGCTTAAAT TATGAATGAT TGTATCAGTG TTCACATTTT TTTTTAAAAA
1801 CCATATTGGA GGATATGTTA TGTTTGCTTA TGTGCATACT AGAAGAGTGC TATGTTGTAT
1861 GAGAGTTTGG TGACTAAGGA ATAAAGCAAG GAAGTTACTT TGTCTTAAAA AAAAAAAAAA
1921 AAAAAAAA
```

Chorismate mutase

Accession # AB182997

```
1 CTTCAATCTA AGGTTGGTAG ATACCTATCA TCAGAAGAAA ATCCTTTCTT CCCAGATAAG
61 TTGCCTGCCT CAATCATACC ACCTAGCAAA TGCACACCAG TTTTGCATCC TGCAGCAGAA
121 TGTGTGAACG TAAATGAGAA GATATTGGAT GTTTATAAAA AGCAGTTGCT TCCACTATTT
181 TGTACTGATC AGGCTGACGA TGAAGAAAAC TTTGCAACTA CTGCTTCCTG TGATATTCAG
241 TTATTGCAGG CACTCTCTAG AAGGATTCAT TATGGGAAAT TTGTTGCTAA GGTTAAATTC
301 AGGGATTGTA CTGATCAATA TAAACCACTT ATTCTTGCTA AGGATAGGGA TGCTCTAATG
361 AAGCTATTGA CATTTGAAGC AGTTGAAGAG GTGGTAAAAA AGAGAGTTGC AAAGAAAGCA
421 TTTGTGTTTCG GGCAACAAGT GACCCTAAAT ATTGATGATA ACACCAAAGA AGCAAAGTAC
481 AAGGTTGATC CATCACTGGT TTCGCGCTTA TATGATGAAT GGGTTATGCC TTTGACTAA
```

Isochorismate synthase

Accession # AY740529

```
1 CAGGTTGAGT TTGATGAGCT TGAAGGAAGT TCAGTTATTG CTGCAACAGT CGCATGGGAT
61 AATGCTGTCT CTTGCACGTA CCAGAGGGCA ATAGAAGCAC TTCAGACCAC AATATGGCAG
121 GTTTCCTCCG TTCTTATGAG GGTGCGGAAA AAAATATCTC GTTCGCATAT ACTCGCAAGT
181 ACTCATGTCC CGGGTAAAGC ATCTTACGAC CAAGCTGTTA AGCGTGCTTT GCAAATAATA
241 AGAAGAAACA ACCCGATGCT TATCAAGGTG GTACTTGCTC GTAGCACCAG AGTTGTGACA
301 GCTGCGGACA TTGATCCTTT AACATGGTTA TCTTGCTTAA AGGTTGAAGG AGAAAATGCA
361 TATCAGTTCT GTTTGCAACC TCCCCAGTCA GCGGCATTCA TTGGAAACAC TCCAGAGCAG
421 CTATTTTCATC GGGACTGCCT CAGCATTTGT AGCGAGGCTT TAGCTGGAAC ACGGGCTAGG
481 GGTGGATCAG AGCTTCTGGA TCTTAAGATA GGACAGGATT TACTATCCAG TGCTAAGGAC
541 CATAATGAGT TTGCTATAGT ACGGGAGTGC ATAAGAAGAA AATTGGAGGC TGTATGTTCC
601 AGCGTTTTTAA TTGAACCAA GAAAGCAATA AGAAAATTTT CAAGAGTTCA ACATCTTTAT
661 GCTCAATTGA GGGGGAGACT CCAGACTGAA GATGATGAGT TTAAGATCTT GTCGTCCATT
721 CACCCTACTC CAGCAGTTTG TGGGTATCCT ACAGAAGATG CACGGGCTTT TATTTTCAGAA
781 ACCGAAATGT TTGACCGAGG AATGTATGCT GGTCTGTTG GTTGGTTTGG AGGGGAAGAG
841 AGTGAATTTG CTGTTGGAAT AAGGTCAGCT TTGGTTGACA AGGGTCTTGG TGCATTAATT
901 TATGCGG
```

Chorismate synthase

Accession # EB427412

1 ATCTTCCAAT CTTTCATATAC TTTTTTCGTCC GTCACACCCC AAACGGTTAG AAATACAGGC
61 TGCTGGTAAT ACATATGGAA ATTACTTCCG TGTTACAAC TTCGGAGAAT CACATGGTGG
121 TGGAGCTGGT TGTGTTATTG ATGGATGTCC CCCTCGTCTC CCACTTTCTG AATCTGATAT
181 GCAGGTTGAA CTTGACAGGA GGAGGCCAGG TCAAAGCCGA ATTACCACAC CTAGGAAAGA
241 GACTGACACT TGCAAAATAT CATCAGGCAT TGCCGATGGG CTGACTACTG GATCCCCAAT
301 CAAGGTTGAA GTACCTAACA CTGATCAGAG AGGAAATGAC TACAGTGAAA TGTCCCAGGC
361 TTATAGGCCA TCTCATGCAG ATGCTACTCA TGACTTCAAG TATGGAGTAA GATCCGTACA
421 GGGGGTGGT AGATCATCAG CAAGAGAAAC CATTGGGAGA GTTGCTGCTG GAGCCGTTGC
481 TAAGAAAATT CTCAAAC TTT ATTCTGGAAC TGAGGTTCTT GCTTATGTTT CTCAAGTTCA
541 CCAAGTTGTA CTTCTGAGG ATTGGATTGA TCATCAGACT GTGACTCTAG AGCAGATAGA
601 AAGCAATATT GTCCGATGCC CGGATCCAGA ATATGCAGAG AAGATGATTG CTGCTATTGA
661 TGCTGTACGA GGAGAGGGGA CTCTGTTGGT GGAGTTGTAA CTGTATTGTT AGAAATGTGT
721 CCCACGAGGT CTTGGTACGC CAGTCTTCGA TAAACTTGAA GCTGAGCTAG CTAAAGCTTG
781 GGGGTGGTAG ATCATCAGCA AGAGAAACCA TTGGGAGAGT TGCTGCTGGA GCCGTTGCTA
841 AGAAAATTCT CAAACTTTAT TCTGGAAC TG AGGTTCTTGC TTATGTTTCT CAAGTTCACC
901 AAGTTGTA CTCTGAGGAT TGGATTGATC ATCAGACTGT GACTCTAGAG CAGATAGAAA
961 GCAATATTGT CCGATGCCCC GATCCAGAAT ATGCAGAGAA GATGATTGCT GCTATTGATG
1021 CTGTACGAGT GAGAGGGGAC TCTGTTGGTG GAGTTGTAAC TTGTATTGTT AGAAATGTCC
1081 CACGAGGTCT TGGTACGCCA GTCTTCGATA AACTTGAAGC TGAGCTAGCT AAAGCTTGCA
1141 TGTCATTACC TGCAACAAAG GGTTTTGAGT TTGGAAGTGG CTTCCGAGGC ACATTCATGA
1201 CTGGTAGCAA GCATAATGAC GAATTCTATA TGGATGAGCA TGGCCGAATC AGGACAAGAA
1261 CCAACAGATC TGGTGGTATC CAGGGCGGTA TATCAAATGG AGAAGTTATC AATATGAAAA
1321 TAGCTTTCAA GCCAACTTCA ACTATTGCTA GGAAGCAGCA AACTGTGACG AGGGATAAAC
1381 ATGACACAGA ACTCATTGCT AGGGGTCGCC ATGATCCTTG TG TAGTTCCC CGAGCTGTTT
1441 CTATGGTTGA AGCAATGGTA GCCCTGGCGC TTGTGGATCA GTTAATGGCT CATTATGCAC
1501 AGTGTATGCT GTTCCCAATT AATCCTGCAT TGCAGGAACC TTTGCAGTCA TCGACACCCG
1561 AGTCAGCTGA GGTTACCCTC TGAATGGAGC TGAGCAGCTT TTATGTGTAA TTTTATCAA
1621 CACCTTAGTA GTTTTACTGT TTAGAGGAGA CGGATATCTC CTTTTTGCCT CTTTATCACT

Shikimate kinase

Accession # EB425065

1 CCCCTCACCT ACCTCTCTCT CAAATCTCAC AACAGCTTCC CTATTTTCCC CCTTCACCTA
61 ATTCTTCGTC TTCATCTTCT TGATTTCATAC GTATATGGTA ATCTCTCTTC CAATTTTTGT
121 TGCTCTTCTG CTCAATAAGG CAACAGAAGC TTGGTGACAA GATAGAGTAT TGTTTCAGCT
181 CTTTCTGATT TTA AAAAGGAC GAAGTTTTTC TTGTGGTGT TGGTGGAGTA GAAACTGTAA
241 ATGGAGGCTA GAGTTTCGCA GAGCCTTCAG TTGTCGTCAT GGATTAATC GGATAAGTA
301 GTAAGGAAAC CCGGTGGTTG CTTACGTTTT TCTGTGAAAT GGAATGAAAA ACTGATGCAT
361 CGTACAGTCA TATCCTGCCA TTTACAGCCT AGAAAAGCTA ATAGACGAGT AGCATTGAAG
421 GTTTCATGTT CTTCTCATAA TGTTCAAGCT TCAGTTCTGG AGTCTGAATG TATTACTGCA
481 TCAACTGATG AAATCGAGAC ATTGAAGAAT AAATCAGAAG AGGTTGAAGA ATATCTAGAT
541 GGACGATGTA TTTACCTCGT TGGAATGATG GGCTCAGGCA AAACAAC TGT GGGCCGGATT
601 TTGGCAGAAA CACTGGGATA TTCCTTTTTT GACTGTGATA GGCTGATAGA GCAGGCTGTT
661 GGTGGAAC TA CTGTAGCTGA AATCTTCAAG CTCCATGGAG AGAGCTTCTT TAGGGACAAT
721 GAGACGGAGG TATTGCACAA GTTGTCTGCG ATGCATCGGC TTGTTGTTTT AACAGGTGGA
781 GGTGCAGTTG TTCGTCCCAT TAATTGGAGA CATATGCA

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CoSIDA Academic All-District IV College Division II
Men Soccer First Team; as part of the Academic All
American Program by ESPN Magazine