The Role of Glutathione in the Defense Pathway Response of *Arabidopsis Thaliana* to the Pathogen *Pseudomonas Syringae* Pathovar Tomato DC3000 AvrB

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THE ROLE OF GLUTATHIONE IN THE DEFENSE PATHWAY RESPONSE OF 
ARABIDOPSIS THALIANA TO THE PATHOGEN PSEUDOMONAS SYRINGAE 
PATHOVAR TOMATO DC 3000 AVR B

by

Allison Patrice McKenna

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment for the
requirements of the
Masters of Science Degree
Department of Biological Sciences

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Acknowledgements—continued

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Allison Patrice McKenna
Inoculation of *Arabidopsis thaliana* with *Pseudomonas syringae* pv. *tomato* DC3000 AvrB (Pst) results in a series of biochemical changes, including an oxidative burst, accumulation of salicylic acid, (de)phosphorylation of proteins, localization of transcription factors to the nucleus and ultimately expression of pathogenesis-related genes. The redox status within plant cells, which is maintained by a cycle of antioxidants that are largely dependent on the reduced form of glutathione (GSH), because of its ability to donate electrons that are necessary to quench reactive oxygen species (ROS). This study focuses on the role of glutathione in the early defense responses of *Arabidopsis* to Pst. It is hypothesized that (1) a decrease in the overall [GSH] or (2) an increase in [GSSG] (a decrease in the [GSH]:[GSSG]), will affect salicylic acid accumulation and pathogenesis-related gene expression in *Arabidopsis* tissue inoculated with Pst.
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Plants come into frequent contact with pathogens such as bacteria, fungi, and viruses resulting in a variety of responses. Disease results if the pathogen is able to attach to the cell surface, degrade the host’s barriers, produce toxins, and inactivate plant defenses (Dixon and Lamb 1990). In some cases, the microbe is unable to establish itself either due to an inability to activate pathogenicity functions or due to an effective plant defense response (Delaney 1997). Plant defenses are often activated due to a gene-for-gene resistance where the plant has a resistance (R) gene and the bacteria has an avirulence (Avr) gene (Bent 1996). The interaction between a plant R gene and a bacterial Avr gene gives rise to biochemical reactions that make up the systemic acquired resistance (SAR) response (Bent 1996). Avr genes are expressed by the bacteria upon contact with the plant cell. Through the use of a syringe-like projection that inserts into the plant cell through the plant cell wall and membrane, these avirulence genes are released into the cell in an attempt to make the plant a more hospitable environment for the bacteria. Upon recognition of the avr genes by
the plant cell, that has a set of resistance genes, the plant cells react. The reactions by the plant cells include the hypersensitive response (HR), an oxidative burst, pathogenesis-related (PR) gene expression, salicylic acid (SA) accumulation, and the formulation of a mobile signal that moves to the rest of the plant through the phloem (Hammond-Kosack and Jones 1996, Levine et al. 1994; Rajasekhar et al. 1999; Cameron et al. 1999; Lamb and Dixon 1999).

SAR is a pathogen defense mechanism in plants that allows for the ability of a host plant to express defense gene products rapidly after a secondary infection (Cameron et al. 1999). SAR is established during an initial pathogen attack after three stages: 1) initiation/immunization, 2) perception of a mobile signal in uninfected leaves, and 3) manifestation of SAR during a secondary challenge (Cameron et al. 1999). The initiation/immunization stage consists of a HR (if gene-for-gene resistance between the plant and the pathogen exists) or disease induced necrosis (if the pathogen is not recognized by the plant). The HR (Levine et al. 1994) is associated with an oxidative burst and leads to the induction of PR gene products (Cameron et al. 1999), SA accumulation in the local tissue (Enyedi et al. 1992), and a as of yet to be identified phloem mobile signal that moves to the rest of the plant to establish SAR (Vernooij et al. 1994). The perception of the mobile signal in the uninoculated
leaves leads to low occurrence microscopic HRs (Alvarez et al. 1998, microHRs), PR gene products and SA accumulation in the distant tissue from the site of inoculation (Ryals et al. 1996). PR gene products are defined as those proteins that are induced by a pathogen in tissues that do not normally express the proteins and can be induced in at least two different plant-pathogen combinations (Van Loon and Van Strien 1999). These proteins are used by the plants during adaptation to biotic stress conditions with functions as chitinases and β-1,3-glucanases (Legrand et al. 1987). During the Arabidopsis/Pseudomonas syringae pv. tomato interaction, PR1 is strongly expressed during the initiation/immunization stage, one day after inoculation (Cameron et al. 1999). PR1 gene expression during the manifestation stage correlates strongly with the ability to exhibit a SAR response. Manifestation occurs when the plant is challenged with a second, normally virulent pathogen and the plant responses as if it were an avirulent pathogen (Cameron et al. 1999).

The oxidative burst in plant cells under attack by a pathogen produces H$_2$O$_2$ originating from the superoxide generated by a plasma membrane-associated NADPH oxidase (Fig. 1) (Lamb and Dixon 1997). The oxidative burst is due to recognition of the pathogen by plant cells, which in turn causes an increase in Ca$^{2+}$ influx, thus increasing the activity of the plasma membrane-bound NADPH oxidase complex.
Figure 1. Signal networks in the hypersensitive response. The recognition of pathogens leads to a influx of calcium ions that initiates a cascade of biochemical events that leads to the accumulation of hydrogen peroxide and salicylic acid. Hydrogen peroxide and salicylic acid upregulate cellular protectant genes, such as PR genes (modified from Lamb and Dixon 1997).
The NADPH oxidase complex evolves H$_2$O$_2$ by transferring electrons from cytoplasmic NADPH to O$_2$ to form superoxide. Superoxide is dismutated by superoxide dismutase to form H$_2$O$_2$. H$_2$O$_2$ can move across the plasma membrane into the cell therefore altering the redox status of the cell. This alteration of redox status can activate transcription factors directly or indirectly through the work of antioxidants in the cytoplasm (Levine et al. 1994, Bent, 1996, Alvarez et al. 1998, Bauerle 1996). The oxidative burst takes place in two phases: 1) immediately after inoculation with a weak transient accumulation; and 2) a massive and prolonged oxidative burst beginning 3 and 6 hours after initial inoculation (Lamb and Dixon 1997). The second oxidative burst depends on Avr expression in the pathogen, the expression of the HR in the plant, and the presence of the pathogen (Lamb and Dixon 1997). H$_2$O$_2$ from the oxidative burst can be generated either from the membrane-bound enzyme NADPH oxidase, or by an apoplastic peroxidase that releases H$_2$O$_2$ at an elevated pH (Bolwell et al. 1998).

The oxidative burst is closely associated with the HR. In cells undergoing the HR, defense responses are induced including those encoding for enzymes of the phenylpropanoid pathway such as phenylalanine ammonia lyase (PAL). PAL is an important component in plant defense due to its involvement in the biosynthetic
pathway providing scopoletin deposition into the cell wall, which can slow pathogen spread. PAL is also a key enzyme involved in the synthesis of SA, which is essential for local and systemic induction of PR proteins thus enhancing resistance to pathogens (Enyedi et al. 1992, Vernooij et al. 1994, Malamy et al. 1996). SA is an important signal amplification molecule in defense responses by enhancing the $\text{H}_2\text{O}_2$ accumulation and hypersensitive cell death by a pathogen (Shirasu et al. 1997). Shirasu et al. (1997) were able to block induction of the $\text{H}_2\text{O}_2$ accumulation and hypersensitive cell death by using a phenylpropanoid synthesis inhibitor, $\alpha$-aminooxy-$\beta$-phenylpropionic acid. By adding SA exogenously, the oxidative burst and cell death were rescued. Experiments with UV and ozone treatments of tobacco resulted in production of ROS that can induce SA accumulation by increasing benzoic acid hydroxylase activity, and induce PR protein accumulation (Yalpani et al. 1994).

The oxidative burst leads to several consequences including direct antimicrobial activity through the inhibition of bacterial growth by superoxide and $\text{H}_2\text{O}_2$ (Lamb and Dixon 1997). It also increases the cross-linking in the cell wall thus slowing pathogen spread while waiting for the activation of transcription-dependent defenses and trapping the pathogens in host cells that are programmed to die (Lamb and Dixon 1997). Gene activation of transcription-dependent defenses is another
consequence of the oxidative burst. Cellular protectant gene products such as glutathione-S-transferase (GST), phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) are induced by exogenously applied \( \text{H}_2\text{O}_2 \), as well as, the presence of microbial elicitors (Tenhaken et al. 1995). Glutathione-S-transferase is a cellular protection gene enzyme that works to neutralize the products of membrane lipid peroxidation and other cellular stresses. Due to the nature of GST and the induction of the GST gene at low \( \text{H}_2\text{O}_2 \) levels, suggests that \( \text{H}_2\text{O}_2 \) works as a local signal for hypersensitive cell death (Levine et al. 1994). \( \text{H}_2\text{O}_2 \) is a key component in phytoalexin synthesis which are involved in plant defense in the absence of the gene-for-gene response (Jabs et al. 1996). \( \text{H}_2\text{O}_2 \) is related to systemic responses and interactions with SA accumulation (locally and systemically, downstream of \( \text{H}_2\text{O}_2 \)) (Hammond-Kosack and Jones 1996). SA can also act upstream of PAL transcription leading to an autoamplification of SA, thus helping integrate transcription-dependent and oxidant-dependent responses (Fig. 2) (Lamb and Dixon 1997). It was thought that the oxidative spike in \( \text{H}_2\text{O}_2 \) was due to an inhibition of catalase by SA (Chen et al. 1993). However, there has been no data to suggest that catalase activities are reduced following local pathogen infection. Bi et al. (1995) show that \( \text{H}_2\text{O}_2 \) is a weak inducer of PR proteins but both Neuenschwander et al. (1995) and Bi et al. (1995) suggest
Figure 2. Sequence of events during a gene-for-gene recognition between a resistant plant and an avirulent pathogen. When plants and pathogens interact there is an initial oxidative burst that leads to cell death in the immediate tissue surrounding the point of interaction (called a hypersensitive reaction). Salicylic acid and hydrogen peroxide begin to accumulate in the area immediately surrounding the HR which initiates the expression of PR genes in the local tissue. During the initial steps of recognition and reaction by the plant there is a mobile signal which is formed that travels to distant, non-infected tissue. This mobile signal is recognized by that uninfected tissue and begins show micro-oxidative bursts, as well as, an accumulation of salicylic acid. The accumulation of salicylic acid then initiates PR gene expression in the uninfected tissue leading to the acquisition of SAR (figure modified from Ryals et al. 1996).
that $\text{H}_2\text{O}_2$ is not a secondary messenger working downstream of SA in the SAR response. This can be questioned by the findings of Alvarez et al. (1998), where primary oxidative bursts were measured at the local infection and secondary micro-oxidative bursts were measured in distant leaves. These micro-oxidative bursts were required for the *Arabidopsis* to systematically respond to the pathogen. Micro-oxidative bursts were measured by $\text{H}_2\text{O}_2$ accumulation and were typically found in distant tissue adjacent to veins suggesting exposure to a mobile signal. SA was originally speculated to be such a signal due to its accumulation in local and distant tissues (Enyedi et al. 1992). However, Vernooij et al. (1994), using grafting experiments with *nahG* (a mutant tobacco that is unable to accumulate SA) and Xanthi (control) grafted plants, found that SA is not the mobile, systemic resistance-inducing signal but that it is required in systemic tissues for long-distance signaling.

The unknown mobile signal travels via the phloem tissue to uninfected parts of the plant to induce SAR in distant tissue. Other possible systemic signals include ethylene, systemin and jasmonates, none of which share a common signaling pathway but may utilize cross-talking mechanisms with the SA pathway (Dempsey and Klessig 1995).
It has been suggested that pretreatment of plants with antioxidants can inhibit the activation of PR genes. Green and Fluhr (1995) attempted to prove this by pretreating tobacco with N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) prior to SA treatment and found no change in PR gene activation between the control treatment and that with the antioxidants. This suggests that ROS would play a role in PR gene expression prior to SA accumulation (Hunt and Ryals 1996). Enyedi (1999) found that the ROS generator, rose bengal (RB), induces SA in a dose-dependent manner, as well as, inducing PR mRNA expression and activating SAR. Enyedi (1999) also found that pretreatment with the antioxidant compounds, NAC and PDTC, diminished the effects of RB and greatly reduced the induction of SA.

Cameron et al. (1999) found that SA may play different roles in the HR compared to the SAR response pathway and suggests that the Rps2 resistance gene product may act to downregulate SA production. In fact, they indicate that it seems in Arabidopsis, unlike tobacco mosaic virus-tobacco model, the level of necrosis is not correlated with higher levels of SA accumulation and SAR.

Much of the role of SA and ROS in the SAR pathway has been discovered through the use of mutants. Two SAR compromised mutants, nim1 and npr1, that were later found to be allelic, have been identified and characterized (Delaney et al.
1995, Cao et al. 1997). The nim1 mutants are not responsive to exogenous application
of SA or synthetic SAR activators. This indicates that SAR signaling is blocked
before SAR gene expression but after SA accumulation (Ryals et al. 1996). The
Arabidopsis NIM1 protein shows approximately 80% homology to the mammalian
Iκβ (Ryals et al. 1997). Iκβ is an inhibitor of the nuclear transcription factor NFκβ.
Iκβ binds the heterodimer of NFκβ (p50/p65) thus deactivating NFκβ’s ability to
translocate to the nucleus and bind DNA. Iκβ dissociates from NFκβ either by being
degraded by a protease or by a modification of Iκβ (Sen and Packer 1996). The
phosphorylated serine residues that are important in Iκβ degradation functions are
conserved in NIM1 within a large contiguous block of conserved sequence from
amino acids 35-84. Based on the structural homology and the presence of elements
known to be important in Iκβ function, NIM1 may function like the Iκβ class of
proteins (Ryals et al. 1997). This is interesting because in mammalian systems, ROS
act as secondary messengers for several cytokines and growth factors. H2O2 is an
intracellular secondary messenger activating NFκβ in the induction of inflammatory
and immune responses (Bauerle and Baltimore 1996). NFκβ is the prototype of a
family of dimeric transcription factors made from monomers that have 300 amino
acid Rel regions. These Rel regions bind DNA, interact with each other, and bind the
inhibitor of NFκβ (Ikβ). Ikβ has 5-7 ankyrin repeat domains (approximately 30 amino acids long) which form a unit that is able to interact with Rel regions (Sen and Packer 1996). The activation of NFκβ involves the phosphorylation of Ikβ at its regulatory N-terminus on serine 32 and 36, which leads to a conjugation with ubiquitin and ultimately the proteosome degradation of the Ikβ inhibitor (Bauerle and Baltimore 1996). ROS are thought to be common messengers in the activation of NFκβ which is primarily an oxidative stress-responsive transcription factor.

Glutathione exists as an antioxidant-redox couple in both reduced (GSH) and oxidized forms (glutathione disulfide, GSSG) and is found in animals and plants. Both the reduced and oxidized states of glutathione (GSH and GSSG respectively) have an essential role in maintenance of the redox state within the cell (Dröge et al. 1994; Alscher 1989; Galter et al. 1994). GSH functions in the cell as a proton donor and is used by several antioxidant enzymes to reduce damage caused by free radicals (Meister and Anderson 1983, Alscher 1989, Rennenberg 1982). Glutathione-S-transferase (GST) works to rectify lipid peroxidation by having GSH donate a proton and accept a hydroxyl group (GSOH). The GSOH is highly reactive and GST uses another GSH to donate a second proton to form water and glutathione disulfide (GSSG). Glutathione peroxidase also works as an antioxidant enzyme that uses GSH
as a proton donor to detoxify H$_2$O$_2$ (Rennenberg 1982, Meister and Anderson 1983).

GSH is also used in the ascorbate/dehydroascorbate cycle by dehydroascorbate reductase (DHAR) to reestablish ascorbate (which is used to dissociate H$_2$O$_2$) (Noctor and Foyer 1998a). Glutathione reductase (GR) is the key enzyme involved in reducing GSSG to GSH (Alscher 1989). All of these enzyme functions contribute to the modulation of the GSH:GSSG ratio.

The balance of the ratio of GSH:GSSG has an important role in cellular functions including optimal protein synthesis by influencing protein folding (Alscher 1989; Rennenberg 1982), as well as, a sensory mechanism that helps cells monitor and adapt to environmental changes (May et al. 1998a). The GSH:GSSG ratio may also be involved in regulating plant cell division and development (Earnshaw and Johnson 1985). The GSH:GSSG ratio is also important in cellular signaling in mammals. There is an indication that a certain amount of intracellular GSSG may be required for the activation of NFκβ, while an excess of GSSG can inhibit NFκβ activation at the level of DNA binding (Galter et al. 1994). The positive mediation of NFκβ by H$_2$O$_2$ and GSSG indicates that there is an oxidative induction of NFκβ activation and nuclear translocation. The redox modulation on a nuclear level shows
that oxidative inhibition by mixed disulfide formation at a redox-reactive cysteine in the DNA binding region may be the negative mediator by GSSG concentrations.

Wingate et al. (1988) found GSH to be involved in signal transduction during the initiation of PAL in legumes by a fungal elicitor. Exogenously applied GSH (0.01-1 mM) to legumes induced hydroxy-proline rich glycoproteins and enzymes PAL and CHS in a manner similar to that by the fungal elicitor (Wingate et al. 1988). However, the same was not true for the exogenous application of GSSG where PAL activity did not increase (Wingate et al. 1988).

Glutathione functions primarily in the H$_2$O$_2$-scavenging pathway in chloroplasts and other peroxide generating organelles, such as the mitochondria and peroxisomes, where glutathione shifts from an oxidized to a reduced state as part of the ascorbate-dehydroascorbate pathway (Rennenberg 1982). Glutathione works to reduce physiological redox stresses in plants that accumulate during photosynthesis, low- and high-temperature exposure, as well as water stress (all of which are affected at the membrane level) (Smith et al. 1989).

Due to the importance of glutathione in maintaining the reductive/oxidative state of the cell, it may play an additional role in plants by functioning as a signal transduction trigger or as a messenger in the defense responses of the plant. It is
possible that ROS produced during the response to a pathogen attack will cause a short-term increase in the concentration of GSSG due to glutathione peroxidase, glutathione-S-transferase, and the ascorbate-glutathione cycle, all of which work to quench the oxidative burst in the cell. This short-term rise in GSSG concentration may cause NIM1/NPR1 (IκB-homolog) dissociation from a yet to be isolated NFκB-like transcription factor that initiates transcription of SAR genes. The aim of my research is to examine the transient changes in GSH:GSSG during the early phase of pathogen attack. The following are the research questions I am going to address:

1. Is there a shift in GSH:GSSG in whole *Arabidopsis thaliana* plants when inoculated with *Pseudomonas syringae* pv *tomato* DC3000 AvrB?

2. If so, can a shift in the GSH:GSSG ratio trigger the accumulation of free SA, as well as, PR mRNA in whole *Arabidopsis thaliana* plants?

3. Can pretreatments with BSO (a glutathione synthesis inhibitor) or BCNU (a glutathione reductase inhibitor), both of which modify glutathione concentrations, cause an accumulation or depression of free SA and PR mRNA in whole *Arabidopsis thaliana* plants?

The purpose of this project is to characterize the role of glutathione and its oxidation state during the very early response in the *Arabidopsis/Pseudomonas*
syringae pv. tomato DC3000 AvrB SAR model system and to establish whether chemical modification of the GSH:GSSG ratio changes SA levels and PR mRNA accumulation and ultimately affects the ability of Arabidopsis to recognize and respond to Pseudomonas and its avirulence gene AvrB. This will lead to a better understanding of the role of ROS in the early events of plant-pathogen interaction.
MATERIALS AND METHODS

Plant growth conditions and treatments

*Arabidopsis thaliana* (ecotype Columbia) seeds were sown (approximately 25 seeds/pot) in 216 pots (12 flats of 18 pots) containing 360 Metro Mix growing media and placed in a Percival Environmental Growth Chamber that was programmed to provide 150 µmoles/m²/sec of light in an 18h light: 6h dark cycle with a constant temperature of 24°C. Five days prior to inoculation with *Pseudomonas syringae* pv *tomato* DC3000 AvrB, 72 randomly selected pots were root drenched with a 1 mM buthionine sulfoximine (BSO) solution, a known inhibitor of γ-glutamylcysteine synthetase (Griffith, 1979; Griffith, O.W. & Meister, A. 1979), each pot receiving approximately 20 ml of BSO solution every other day up until inoculation (three treatments total). Twenty-four hours prior to inoculation, 72 different pots were exposed to a single foliar spray of a 100 µM BCNU solution (1,3-bis(2-chloroethyl)-
1-nitrosourea; carmustine; an inhibitor of glutathione reductase) until the leaves were wetted completely. The remaining four flats were treated with \( \text{H}_2\text{O} \).

Bacterial growth and inoculation procedure

*Pseudomonas syringae* pv. *tomato DC3000* containing the *avrB* plasmid (supplied courtesy of Ms. Cathy Worley, University of Delaware) were grown in 50 ml of liquid NYG medium containing 100 mg/ml rifampicin and 25 mg/ml kanamycin (to select for the bacteria and the *avr* plasmid respectively) in a 25°C incubator for two days. The suspension was centrifuged and the medium was decanted. The bacterial culture was washed twice, resuspended and diluted in 10 mM MgCl₂ solution to an optical density of 0.07 at 600 nm. The bacterial suspension was infiltrated in approximately 20 µl aliquots into the abaxial surface of two leaves per plant using a 1 ml graduated syringe without a needle. All plants in half of the BSO-treated pots, half of the BCNU-treated pots, and half of the \( \text{H}_2\text{O} \)-treated pots were inoculated (approximately 25-50 plants per pot).
Tissue harvesting

Tissue samples (1 gram fresh weight) were harvested for the GSH:GSSG ratio determination at 0, 1, 3, 6, 9, 12, and 24 hours post-inoculation. Samples were placed into 16x100 mm borosilicate glass test tubes sealed with parafilm and frozen in liquid nitrogen. Tissue samples (0.1 gram fresh weight) were collected for mRNA isolation and placed into 13 ml polypropylene centrifuge tubes (17x95 mm) capped and placed into liquid nitrogen immediately. Tissue samples (0.5 gram fresh weight) were collected for free SA concentration determination, placed into 13x100 mm borosilicate glass test tubes. All samples were placed into a -80°C freezer for long-term storage.

Measurement of non-protein thiols by HPLC

Extraction of thiols procedure

Glutathione was extracted from Arabidopsis plant tissue (0.1 g fresh weight) using the method described by May et al. (1996) and later modified by this researcher using techniques described by Anderson et al. (1999) and Getz et al. (1999). The frozen plant tissue, in the presence of 80 mg of polyvinylpyrrolidone (PVP-40) was
added to a 2 ml tube. 1.5 ml of 0.1 M HCl containing 1 mM EDTA was added to the plant tissue and vortexed. The tissue and HCL were allowed to incubate on ice for 15-20 minutes before being centrifuged at 4°C for 15 minutes. After centrifugation, two separate aliquots of 120 µl supernatant were added to two separate 1.7 ml centrifuge tubes containing 180 µl 0.1 M potassium phosphate buffer (pH 7.3). One sample was used to measure reduced glutathione (GSH) and the other sample was used to measure oxidized glutathione (GSSG). In order to determine the concentration of GSSG, 10 µl of 1 mM N-ethyl-maleimide (NEM) was added and the tube was placed in the dark to incubate for 10 minutes at room temperature. After 10 minutes, 30 µl of 1 mM tris-(2-carboxyethyl)phosphine (TCEP) was added to both the GSH and the GSSG tubes. The samples were allowed to incubate for 20 minutes. After the 20 minute incubation, 20 µl of 30 mM monobromobimane (MBB) was added to both sets of tubes and placed in the dark to incubate for 20 minutes. After 20 minutes, 250 µl 5% acetic acid was added to the tubes to stop the MBB reaction. The samples were filtered using spin filter vials (0.2 µm pore size) and 300 µl of the solution was transferred to a 300 µl TPX microvial (SRI) and maintained at 10°C while the samples were running. Samples are stable up to 5 days (G. Gullner, personal communication).
Glutathione HPLC procedure

300 µl samples were applied to a 250 x 4.6 mm reversed-phase C-18 column (5 µm diameter) at 40°C using solvent A (10% HPLC grade methanol with 0.25% acetic acid in HPLC grade water, pH 4.3) and solvent B (90% HPLC grade methanol with 0.25% acetic acid in HPLC grade water, pH 3.9) at a flow rate of 1.0 ml/min. The concentrations of the solvents started at 96% A and 4% B and was changed over 20 minutes along a gradient to 82% A and 18% B. Another gradient was applied over the next minute so that the final solution was 100% B. This 100% B was maintained for 7 minutes upon which time the gradient was returned to 96% A and 4% B over one minute. These concentrations were maintained for the remainder of the 35 minute run time (6 minutes). The HPLC method used the fluorescence detector set at 380 nm excitation and 480 nm emmission. The spike for glutathione typically came off the column and was detected around 9.2 minutes. The concentration of GSH was determined by generating a standard curve using known concentrations of GSH (0, 10, 20, 30, 40, 50 µl of 1mM stock GSH (0, 407, 814, 1221, 1628 µg GSH).
Arabidopsis leaf tissue (0.9-1.0 g fresh weight) was homogenized with a tissue tearer in a volume of 5 ml of 0.1 M potassium phosphate (pH 7.5) containing 0.5 mM EDTA. The homogenate was centrifuged for ten minutes at 20,000g and the supernatant was decanted to a clean test tube and stored on ice. The supernatant was considered a crude extract. Glutathione reductase activity was measured using a method described by Smith et al. (1988). The reaction mixture was maintained at room temperature and was constituted with the following: 1.0 ml 0.2 M potassium phosphate (pH 7.5) containing 1 mM EDTA, 0.5 ml 3 mM dithionitrobis(2-nitrobenzoic acid (DTNB) in 0.01 M phosphate buffer, 0.25 ml H₂O, 0.1 ml 2 mM NADPH (reduced nicotinamide adenine dinucleotide phosphate, Type I, tetrasodium salt), 0.05 ml of the glutathione reductase extract, and 0.1 ml 20 mM GSSG. Standards were determined using stock glutathione reductase (1 U/ml, Type III from Baker’s yeast). The reaction mixture was added to the cuvette in the order listed and the reaction was initiated by the addition of GSSG. The increase in absorbance at 412 nm was monitored for five minutes using a Beckman spectrophotometer. The rate of glutathione reductase activity (units/min) was calculated from the linear curve and was normalized by dividing that value by the fresh weight of the sample.
Salicylic acid extraction and concentration determination via HPLC

Extraction procedure

500 mg of plant tissue was frozen in liquid nitrogen ground to a fine powder. 2.5 ml of 90% methanol was added and the sample was centrifuged for 3200 rpm (2800g) for 15 minutes. The supernatant was decanted into a clean tube and set aside. The pellet was resuspended in 95% methanol and centrifuged at 3200 rpm (2800g) for 15 minutes. The supernatants were combined and dried under vacuum (without heating) using the Savant SpeedVac system. After drying overnight, the remaining pellet was resuspended in 2.5 ml of 5% trichloroacetic acid (w/v, TCA) using a glass rod. This step was done quickly so as to minimize the exposure of the sample to the TCA and degrading the yield of SA. The resuspended pellet was centrifuged at 3200 rpm (2800g) for 15 minutes and the supernatant was decanted into a clean borosilicate glass tube. The supernatant was partitioned twice with 2.5 ml extraction medium (ethylacetate/cyclopentane (50:50)) and the upper organic phase containing SA was collected and transferred to a clean tube. The organic phase was evaporated overnight in a SpeedVac (no heat). The resulting pellet was resuspended in 55% HPLC Grade Methanol at a rate of 1 µl/1 mg fresh weight of the original tissue sample. The
resuspended sample was passed through a 0.2 μM nylon filter, transferred to an HPLC vial, and stored at −20 °C until HPLC analysis.

Salicylic acid HPLC procedure

A spectrofluorescence detector at an excitation wavelength at 315 nm and an emission wavelength of 405 nm is used to quantify SA. The samples were applied to a 250 x 4.6 mm reversed-phase C-18 column (5 μm diameter) at a flow rate of 1.5 ml/min at 40 °C with the degasser on normal. The solvents that were used were 100% HPLC grade methanol (C) and 20 mM sodium acetate at pH 5.0 (D). The concentrations of the solvents were as follows: 23% C and 77% D for the first five minutes; 77% C and 23% D for 3.5 minutes (until 11 minutes run time); and back to 23% C, 77% D for the remaining run time (15 minutes total run time). To determine the recovery rate of the extraction method, known concentrations (30 and 60 μM) of salicylic acid were added to a series of untreated tissue samples. The spike for salicylic acid typically came off the column and was detected at about 4 minutes. The concentration of SA was determined by generating a standard curve using known concentrations of SA (0, 25, 50, 100, 250, 500, 1000 μM SA).
RNA isolation and northern blot hybridization

Fresh leaf tissue (100 mg) was ground in liquid nitrogen and 1.25 ml TRI Reagent (MRC, Cincinnati, OH) was added to the tube and ground on ice using a Tissue Tearer/Polytron. The sample was incubated for 5 minutes at room temperature (25 °C) and then centrifuged at 3400 rpm at room temperature for 10 minutes to pellet cellular debris. The supernatant was transferred into a RNAse-free 2.0 ml microcentrifuge tube and centrifuged at 12,000 rpm for 5 minutes at 4°C. After centrifugation, the supernatant was transferred to a 2 ml microcentrifuge tube and 125 µl of BCP (Phase separation reagent, MRC, Cincinnati, OH) was added and the tube was mixed vigorously for 5 seconds using a vortexer. The tube was incubated for 10 minutes at 25 °C and centrifuged for 10 minutes at 12,000 rpm at 4 °C to complete the phase separation. The upper aqueous phase containing the RNA was transferred to a sterile RNAse-free 1.5 ml microcentrifuge tube and 500 µl isopropanol (molecular biology grade) was added. The tube was incubated at room temperature for 5 minutes and centrifuged for 10 minutes at 12,000 rpm at 4 °C to pellet the RNA. The supernatant was carefully removed and the RNA pellet was washed in 1 ml 75% ethanol made up in diethyl pyrocarbonate (DEPC) treated water. The tube was centrifuged for 5 minutes at 4 °C at 12,000 rpm to pellet the RNA. The ethanol wash
was discarded and the pellet was dried briefly under vacuum (Savant SpeedVac) and then dissolved in 20 µl of DEPC treated water. The RNA concentration was determined using optical density at 260 nm and stored at -80 °C. Equal amounts of RNA (10 µg determined from OD_{260} values) were subjected to electrophoresis on a 1.2% (w/v) formaldehyde agarose gel, and blotted onto Nytran membranes (Schleicher and Schuell, Keene, NH) as described by Ausubel et al. (1995), using the Turbo-Blotter system (Schleicher and Schuell, Keene, NH). Filters were UV cross-linked using an UV Crosslinker (Fisher Scientific, Pittsburgh, PA). The membranes were prehybridized and hybridized as described previously (Ausubel et al. 1995) with 32P-labeled (10^6 - 10^7 cpm·µg^{-1}) cDNA using Quik-Hyb Solution (Stratagene, La Jolla, CA). The 32P-probes used were an ITS probe (a 700 to 900 bp fragment with part or all of 18s, 5.8s, and 26s ribosomal subunits present), a 392 bp fragment amplified from the PR1 gene (757 base pairs long, Uknes et al. 1992), and a 629 bp fragment amplified from the PR2 gene (1181 base pairs long, Uknes et al. 1992) amplified from *Arabidopsis* DNA. Following hybridization with ITS to confirm the presence of RNA, membranes were washed twice for 20 minutes at 65° C in low stringency wash buffer (2X SSC, 0.1% SDS). An additional high-stringency wash was performed with 0.1X SSC containing 0.1% SDS for 20 min at 65 °C. The
membranes were exposed to X-ray film at -80 °C in the presence of an intensifying screen for two to five days. The membranes were then stripped with 55% formamide, 2X SSE buffer, and 0.1% SDS for one hour at 65°C, according to manufacturer’s instructions (Schleicher and Schuell, Keene, NH). The hybridization steps were repeated using a 392 base pair fragment of PR 1. After membranes were exposed to X-ray film at -80°C in the presence of an intensifying screen for two to five days, the membranes were stripped with the 55% formamide wash. The membranes were then hybridized with a 629 base pair fragment of Arabidopsis PR2. Primers and gene sequences are available in Appendix B.

Statistical Analysis

To determine whether the presence of Pseudomonas syringae pv tomato DC3000 AvrB modified the levels of reduced or oxidized glutathione in Arabidopsis, one way analysis of variance (ANOVAs) was performed on reduced glutathione and oxidized glutathione data separately. These data were compared to the mock inoculated (those inoculated with 10 mM MgCl₂ solution instead of Pseudomonas) samples from the same time periods. Due to problems with the glutathione sample analysis, some samples did not render data points, therefore, Kruskall-Wallis one-way
ANOVA were used. If the results of the analysis showed a difference between samples, Student’s t-test was used to determine at which time point there was a difference. All ANOVA and t-tests were executed with a level of confidence of $p<0.05$.

To determine whether the presence of *Pseudomonas syringae pv tomato* DC3000 AvrB in *Arabidopsis thaliana* caused a modification in free salicylic acid concentrations, one way ANOVAs were performed. Again, due to unequal sample sizes, the Kruskall-Wallis one-way ANOVA was performed for mock and *Pseudomonas* treated samples. These one-way ANOVAs were also performed to determine if the pretreatments of BSO or BCNU caused a modification in salicylic acid concentrations compared with the pretreatment control of water. Student’s t-test was used to determine which treatment and at which time points were different from the control.
CHAPTER III

RESULTS

Effects of *Pseudomonas syringae* pv *tomato* DC 3000 AvrB inoculation on glutathione (total, GSH, GSSG) levels in *Arabidopsis thaliana* (ecotype Columbia) tissue over a 24 hour period post-inoculation

**Total glutathione**

The HPLC analysis of the tissue inoculated with *Pseudomonas syringae* pv *tomato* DC3000 containing the *avrB* plasmid (Pst) revealed a significant shift in total glutathione from 1 to 6 hours after inoculation (Fig. 3). One hour post-inoculation, there was a significant increase in total glutathione concentration compared to the mock inoculated samples at the same time points (3.2X, 2.6X, and 2.4X at 1, 3, and 6 hours post-inoculation respectively). At 9 hours post-inoculation, there is no significant difference in total glutathione concentrations between the *Pseudomonas*- and mock-inoculated treatments. After 12 hours, the *Pseudomonas*-inoculated tissue has total glutathione concentrations that are in the same range as those samples from
the 1 hour through the 6 hour time points. In mock inoculated plants total glutathione levels remained constant for the first 12 hours of the experiment (around 1000 µg/g fresh weight) and then declined by 50% at 12 hours post-inoculation (417.18 µg glutathione/g fw).

T-tests showed that the Pst treated tissue had a significantly higher total glutathione concentration compared to that of the mock inoculated tissue at 1, 3, 6, 12, and 24 hour time points (2.4X, 3.2X, 2.0X, 5.6X, and 4.5X respectively). These results indicate that the presence of Pst in Arabidopsis markedly enhances the glutathione concentration compared to mock inoculated leaf tissue. When the total glutathione concentration was examined in terms of its components (GSH vs. GSSG), typically the shifts in concentrations could be attributed to the changes in amounts of the reduced form of glutathione (GSH) (Table 1).

Reduced glutathione

Data for the GSH concentration exhibits a bimodal response to infection with Pst. Within one hour of inoculation of Arabidopsis leaves with Pst, there was a significant increase in the foliar GSH concentration (3.2X) compared to the mock inoculated tissue (Fig. 4). At 3 hrs and 6 hours post-inoculation, GSH concentration was still elevated compared to control, however, this difference in concentration was
not significantly different compared to the mock inoculated leaf tissue. At 9 hours post-inoculation, GSH concentration was not significantly different than the mock-inoculated control plants. At 12 hours, the GSH concentration was significantly higher in Pst-inoculated tissue (4.9X) compared to mock-inoculated tissue. The elevated GSH concentration was still present 24 hours post-inoculation (4.7X increase in GSH concentration). Overall, there was a 2.8X increase in GSH concentration in the first 24 hours after inoculation.

**Oxidized glutathione**

In contrast to the GSH levels seen post-inoculation, oxidized glutathione (GSSG) concentration remained relatively unchanged in both the Pst- and mock-inoculated *Arabidopsis* tissue for the first 9 hours following inoculation (Fig. 5). At 12 hours, there was a 4.9X increase in GSSG concentration in Pst inoculated tissue compared with mock inoculated tissue. The increase in GSSG observed at 12 hours post-inoculation was transient and returned to levels that were similar to mock inoculated tissue by 24 hours post-inoculation.
GSH:GSSG ratio

The GSH:GSSG ratio is an established indicator of environmental challenge or oxidative stress in plant tissue (May et al. 1998a, Bielawski and Joy 1986, Kranner and Grill 1996). When the concentration of GSH increases (with no apparent change in the GSSG concentration), the GSH:GSSG ratio will increase (a positive shift). When the concentration of GSH decreases, or the GSSG concentration increases, typically due to oxidative stress (May et al. 1996), there will be a drop in value of the GSH:GSSG ratio (a negative shift). Arabidopsis leaf tissue inoculated with Pst, exhibits a positive shift in the GSH:GSSG ratio in the first 3 hours post-inoculation (Fig. 6). This change corresponds to the increase in the reduced glutathione increases (Fig. 4). There is a downward shift in the GSH:GSSG ratio beginning at 6 hours, which is sustained until 12 hours post-inoculation. These changes in GSH:GSSG can be directly attributed to a decrease in the reduced glutathione concentration accompanied by a transient increase in GSSG concentration. After 12 hours, there is a second upward shift in the GSH:GSSG ratio to 24 hours post-inoculation which is related to the second increase in reduced glutathione.
Glutathione reductase activity

Glutathione reductase (GR) plays a major role in the maintenance of the homeostatic redox status of the plant cell (May et al. 1998a, Smith et al. 1989) by reducing the disulfide bonds of GSSG, glutathione reductase, enables the reducing enzymes of the cell to use the necessary reduced form of glutathione (GSH) to perform their functions in quenching reactive oxygen species (Noctor and Foyer 1998b). Leaf tissue showed no significant increase in glutathione reductase activity for the first 9 hours post-inoculation compared to the mock control (Fig. 7). However, at 12 hours the activity of glutathione reductase in the Pst-inoculated tissue increased 2.5X compared to mock inoculated tissue. The elevated GR activity increase was still present after 24 hours post-inoculation compared to mock inoculated tissue. It should also be noted that the activity of GR increases at the same time as the transient increase in GSSG concentration at 12 hours. These data indicate that the initial increase in GSH concentration observed (Fig 4) due to de novo synthesis of GSH by γ-glutamylcysteine synthetase and glutathione synthetase (two enzymes responsible for glutathione synthesis, Noctor and Foyer 1998a, May and Leaver 1993) and are not a result of the action of glutathione reductase. The downward shift in the GSH:GSSG ratio, as well as, the increase of GSSG concentration at 12 hour post-inoculation was
likely caused by the initiation of increased glutathione reductase activity in Pst inoculated tissue compared to the mock treatment.
Figure 3. Total glutathione concentration in *Arabidopsis thaliana* leaves following inoculation of *Pseudomonas syringae pv tomato* DC3000 avrB compared to mock inoculation with 10 mM MgCl₂. There is a significant increase in total glutathione one post-inoculation and a second increase in concentration 12 hours post-inoculation.

*indicates significant difference from mock inoculation at the same time point, p<0.05
Figure 4. Reduced glutathione (GSH) concentrations in Arabidopsis thaliana leaves following inoculation with Pseudomonas syringae pv tomato DC3000 avrB compared to mock inoculation with 10 mM MgCl₂. There was a significant increase in GSH concentrations at 1, 12 and 24 hours post-inoculation.

*indicates significant difference from mock inoculation at the same time point, p<0.05
Figure 5. Oxidized glutathione (GSSG) concentrations in *Arabidopsis thaliana* leaves following inoculation of *Pseudomonas syringae pv tomato* DC3000 avrB compared to mock inoculation with 10 mM MgCl$_2$. There is a significant increase in GSSG concentration at 12 hours post-inoculation.

*indicates a significant difference from mock inoculation at the same time point, p<0.05
Figure 6. Changes in GSH:GSSG ratio in *Arabidopsis thaliana* leaves after inoculation with *Pseudomonas syringae* pv *tomato DC3000* avrB compared to mock inoculation with 10 mM MgCl₂. Pseudomonas inoculated leaves show a rise in GSH:GSSG ratio within one hour of inoculation with it peaking at three hours. After three hours GSH:GSSG ratio begins to fall and reaches control levels at 12 hours. By 24 hours the GSH:GSSG ratio begins to increase again, presumably due to an increase in glutathione reductase activity (Fig. 7).
Figure 7. Glutathione reductase activity in Arabidopsis thaliana leaves inoculated with Pseudomonas syringae pv tomato DC3000 avrB compared to mock inoculation with 10 mM MgCl₂. There is no significant difference in glutathione reductase activity over the first nine hours post-inoculation. At twelve hours, there is a significant leap in activity which continues on at 25 hours. This increase in activity coincides in time with the increase of GSSG concentration.

*indicates a significant difference from mock inoculation at the same time point, p<0.05
Table 1. Changes in glutathione (total, reduced, oxidized, and ratio) and glutathione reductase activity in *Arabidopsis thaliana* plants after inoculation with *Pseudomonas syringae* pv *tomato* DC3000 avrB compared to mock inoculation with 10mM MgCl₂.

<table>
<thead>
<tr>
<th>Inoculant</th>
<th>time (h)</th>
<th>Total glutathione (µg/g fw)</th>
<th>average [GSH] (µg/g fw)</th>
<th>average [GSSG] (µg/g fw)</th>
<th>GSH:GSSG</th>
<th>GR activity (U/min/g fw)</th>
</tr>
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<tr>
<td>pst</td>
<td>0</td>
<td>1243.01 +/- 288.11</td>
<td>1329.70 +/- 293.99</td>
<td>62.69 +/- 19.43</td>
<td>21.21</td>
<td>0.01728 +/- 0.00085</td>
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Effect of *Pseudomonas syringae* pv *tomato* DC3000 avrB inoculation on whole plant salicylic acid concentration in *Arabidopsis thaliana* (ecotype Columbia) tissue

Salicylic acid accumulation in plant tissue typically indicates that a pathogen has initiated an oxidative burst and cellular necrosis (Enyedi et al. 1992, Silverman et al. 1993, Lawton et al. 1995). An accumulation of salicylic acid is required for systemic acquired resistance to be activated (Lawton et al. 1995). Inoculation with Pst caused no significant change in the free salicylic acid concentration in the control *Arabidopsis* whole plants within 48 hours of inoculation (Fig. 8). To determine whether a modification in glutathione would affect salicylic acid concentration, plants were pretreated with BSO (Fig. 9) or BCNU (Fig. 10) and later inoculated with Pst to elicit an oxidative burst. The treatments of BSO (glutathione synthesis inhibitor) and BCNU (glutathione reductase inhibitor) exhibited no accumulation in salicylic acid. Furthermore there was no significant difference in the salicylic acid concentration of those plants inoculated with Pst compared to those mock inoculated (Table 2). It is interesting that there was no significant change in salicylic acid concentration in *Arabidopsis* inoculated with Pst. There may be several explanations for this. The extraction yield for salicylic acid was less than 40 ng SA/ g fresh weight (normal yield are typically around 2.5 µg/ g fresh weight in elicited *Arabidopsis* tissue,
Sharma et al. 1996) and may not be truly representative of what is actually present. A more plausible explanation of reduced salicylic acid concentration is the fact that inoculation of the tissue was performed on just two leaves per plant. However, the harvest of the tissue included the entire plant, which likely diluted the assay to detect salicylic acid. Salicylic acid concentrations are highest in the tissue directly affected by pathogen attack (Enyedi et al. 1992). By harvesting the whole plant, whatever subtle change in salicylic acid concentration that may occur would be lost.
Figure 8. Changes in salicylic acid concentration in *Arabidopsis thaliana* for 48 hours post-inoculation with either *Pseudomonas syringae pvs* *tomato* DC3000 avrB or the mock inoculant, 10 mM MgCl$_2$. There is no significant difference in salicylic acid concentration in *Pseudomonas* or mock inoculations.
Figure 9. Changes in salicylic acid concentration in *Arabidopsis thaliana* for 48 hours post-inoculation with either *Pseudomonas syringae* pv *tomato* DC3000 avrB or mock inoculant, 10 mM MgCl₂, after pretreatment with BSO for five days. There was no significant difference in salicylic acid concentration between the *Pseudomonas* and the mock inoculation.
Figure 10. Changes in salicylic acid concentration in *Arabidopsis thaliana* for 48 hours post-inoculation with either *Pseudomonas syringae pv tomato* DC3000 avrB or mock inoculant, 10 mM MgCl$_2$, after pretreatment with BCNU for 24 hours. There was no significant difference in salicylic acid concentration between the *Pseudomonas* and the mock inoculation.
Table 2. Changes in salicylic acid in *Arabidopsis* whole plants pretreated with glutathione concentration modifiers (BSO and BCNU) and control (H$_2$O) and inoculated with *Pseudomonas syringae* pv *tomato* DC 3000 avrB

<table>
<thead>
<tr>
<th>pretreatment</th>
<th>time (h)</th>
<th>inoculant</th>
<th>average [SA] (ng/g fw)</th>
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<td>pst</td>
<td>12.91 +/- 1.54</td>
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<td>15.38 +/- 1.88</td>
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<tr>
<td></td>
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<td></td>
<td>mock</td>
<td>13.87 +/- 2.79</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>pst</td>
<td>14.98 +/- 4.00</td>
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<td></td>
<td></td>
<td>mock</td>
<td>12.12 +/- 1.30</td>
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<tr>
<td></td>
<td>48</td>
<td>pst</td>
<td>27.86 +/- 10.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mock</td>
<td>30.29 +/- 8.22</td>
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Effects of BSO and BCNU in the presence of *Pseudomonas syringae* pv *tomato* DC3000 avrB inoculation on the accumulation of PR 1 and PR 2 mRNA in *Arabidopsis thaliana* (ecotype Columbia)

Total mRNA from *Arabidopsis* treated with H₂O (control), BSO, and BCNU and inoculated with Pst was transferred to Nytran membranes and hybridized with the inton spacers probe (ITS) to confirm uniform loading and the presence of RNA. The hybridization with the ITS probe (a 700 to 900 bp fragment with part or all of 18s, 5.8s, and 26s ribosomal subunits present) resulted in positive results for all of the treatment Nytran membranes. The control tissue and inoculated with either Pst or the control (10mM MgCl₂) showed three bands in ten lanes (Fig. 11) corresponding to 5.8s, 18s, and 26s ribosomal RNA subunits.

With the BSO treatment, there were also three bands in ten lanes, corresponding to 5.8s, 18s, and 26s ribosomal RNA subunits (Fig. 12). There were also three bands on ten lanes with the BCNU treatment lanes, corresponding to 5.8s, 18s, and 26s ribosomal RNA subunits (Fig. 13).

After ITS verification that the Nytran membranes contained RNA, the control, BSO and BCNU membranes were stripped of the ITS probe and reprobed with an amplified 392 bp fragment of PR1. The PR 1 gene sequence is 757 bp long. The control blot showed a very thin, faint band corresponding to the position of lane nine...
in the control (H$_2$O+pst 72h, Fig. 14A). No other bands corresponding to PR1 gene were discernable. In the BSO treatment blot, there is a faint set of single bands that correspond with the positions in all ten lanes (Fig. 14B). Expression of the PR1 gene may be in response to the BSO treatment that is blocking the production of glutathione. In the BCNU treatment, there are no discernable bands corresponding to PR1 present (Fig. 14C).

After stripping the PR1 probe, the control, BSO and BCNU membranes were reprobed with an amplified 629 bp fragment of PR2. The PR2 gene sequence is 1181bp long. The control (H$_2$O treatment) blot showed no obvious bands that corresponded to any of the lanes (Fig. 15A). The BSO treatment induced the expression of PR2 gene mRNA in a variety of intensity (Fig. 15B). Lanes 3, 5, 7, 9 exhibit darker bands compared to lanes, 1, 2, 4, 6, 8 and 10, suggesting that there is a slight amplification of PR2 gene expression when *Pseudomonas* is present in the leaf tissue. The BCNU treatment did not cause the expression of PR2 mRNA (Fig. 15C).
Figure 11. Expression of 18s, 5.8s, and 26s ribosomal subunits in *Arabidopsis thaliana* control leaves. The blot shows equal loading of RNA in the lanes. (+, *Pseudomonas syringae pv tomato* DC3000 avrB inoculated; -, mock inoculated; lanes 1 & 2, 0 h post-inoculation; lanes 3 & 4, 12 h post-inoculation; lanes 5 & 6, 24 h post-inoculation; lanes 7 & 8, 48 h post-inoculation; lanes 9 & 10, 72 h post-inoculation)
Figure 12. Expression of 18s, 5.8s, and 26s ribosomal subunits in *Arabidopsis thaliana* leaves pretreated with BSO for five days prior to inoculation. The blot shows equal loading of RNA in the lanes. (+, *Pseudomonas syringae pv tomato* DC3000 avrB inoculated; -, mock inoculated; lanes 1 & 2, 0 h post-inoculation; lanes 3 & 4, 12 h post-inoculation; lanes 5 & 6, 24 h post-inoculation; lanes 7 & 8, 48 h post-inoculation; lanes 9 & 10, 72 h post-inoculation)
Figure 13. Expression of 18s, 5.8s, and 26s ribosomal subunits in Arabidopsis thaliana leaves that were pretreated with BCNU for 24 hours prior to inoculation. The blot shows equal loading of RNA in the lanes with considerable background nonspecific binding. (+, Pseudomonas syringae pv tomato DC3000 avrB inoculated; -, mock inoculated; lanes 1 & 2, 0 h post-inoculation; lanes 3 & 4, 12 h post-inoculation; lanes 5 & 6, 24 h post-inoculation; lanes 7 & 8, 48 h post-inoculation; lanes 9 & 10, 72 h post-inoculation)
Figure 14. Expression of PR1 mRNA in Arabidopsis thaliana. Control tissue (A), BSO pretreated tissue (B), and BCNU pretreated tissue (C) (+, Pseudomonas syringae pv tomato DC3000 avrB inoculated; -, mock inoculated; lanes 1 & 2, 0 h post-inoculation; lanes 3 & 4, 12 h post-inoculation; lanes 5 & 6, 24 h post-inoculation; lanes 7 & 8, 48 h post-inoculation; lanes 9 & 10, 72 h post-inoculation). There is a band in all the lanes of the BSO-treated tissue blot, all the bands are showing an approximately equal expression of PR1 mRNA. There is a faint band in lane 9 of the control tissue, and no apparent bands in the BCNU-treated tissue blot.
Figure 15. Expression of PR2 mRNA in *Arabidopsis thaliana*. Control tissue (A), BSO pretreated tissue (B), and BCNU pretreated tissue (C) (+, *Pseudomonas syringae pv tomato* DC3000 *avrB* inoculated; -, mock inoculated; lanes 1 & 2, 0 h post-inoculation; lanes 3 & 4, 12 h post-inoculation; lanes 5 & 6, 24 h post-inoculation; lanes 7 & 8, 48 h post-inoculation; lanes 9 & 10, 72 h post-inoculation). There is a band in all the lanes of the BSO-treated tissue blot; the bands in lanes 3, 5, 7, and 9 are showing a slight amplification in intensity of expression of PR2 mRNA, presumably due to the presence of *Pseudomonas* in the tissue. There are no apparent bands in the control or BCNU-treated tissue blots (the bands in A and C are non-specific binding).
CHAPTER IV

DISCUSSION

The overall goal of this study was to develop a better understanding of the role of glutathione during early pathogen defense responses in *Arabidopsis thaliana*. It is well established that glutathione is important in the maintenance of the oxidative state of the cell during immune defense responses in animals (Dröge et al. 1994, Galter et al. 1994, Meyer et al. 1993). Glutathione can also activate transcription of genes, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) both of which are related to defense against pathogens in plants (Edwards et al. 1991, Wingate et al. 1988). Due to glutathione's importance, it is hypothesized that a transient alteration of the GSH:GSSG ratio after inoculation with *Pseudomonas syringae pv tomato* DC3000 AvrB (Pst) in *Arabidopsis thaliana* tissue may play a role in accumulation of defense signaling molecules, such as salicylic acid, and ultimately initiation of gene transcription of pathogen-related genes (i.e. PR1 and PR2).

During this study, glutathione concentrations (reduced and oxidized) were
monitored in *Arabidopsis* tissue following inoculation with *Pseudomonas syringae* pv *tomato* DC3000 AvrB. The results of our study show that there was an immediate rise in GSH concentration, though not due to the action of the glutathione reductase enzyme (GR, which converts GSSG back to GSH). Therefore the initial rise in GSH concentration is likely due to *de novo* synthesis of GSH the tissue by the activity of γ-glutamylcysteine synthetase, a key enzyme in the biosynthesis of glutathione. The early increase in GSH concentration seen in this study is consistent with has been observed in response to chilling, heat shock, pathogen attack, reactive oxygen species accumulation, air pollution and drought—all due to the increase in the activity of γ-glutamylcysteine synthetase (May et al. 1998b).

A critical transient increase in GSSG concentration at 12 hours was correlated to a decrease in GSH concentration at 9 hours post-inoculation. This drop in GSH concentration at 9 hours post-inoculation and increase in GSSG, as well as, an increase in glutathione reductase activity, compared to the control at 12 hours, indicates that the activity of glutathione reductase is likely modifying the GSH:GSSG ratio at this time point. At 24 hours, glutathione reductase activity in *Pseudomonas*-inoculated tissue is still significantly higher as compared to the control. It is expected that the concentration of GSSG would rise in response to pathogen inoculation due to
the action of the reduction-oxidation (redox) cycles within the plant tissue (Dixon et al. 1998, Kranner and Grill 1996). Presumably, the glutathione reductase activity increased after a certain threshold of redox modification, which is reflected in the GSSG concentration data at 12 hours. As intracellular ROS increase in concentration due to the oxidative burst, the detoxification systems of the cell works to maintain function of enzymes and other proteins. Glutathione reductase along with glutathione transferase, glutathione peroxidase, ascorbate peroxidase and dehydroascorbate peroxidase all work together in a cycle with reduced glutathione being a key player in the function of all of these enzymes (Dixon et al. 1998, Kranner and Grill 1996, Eshdat et al. 1997, Alscher 1989, Rennenberg 1982). In our experiment, there was a 4X increase in GSSG concentration at 12 hours post-inoculation. This increase in GSSG is significant because a decline in the GSH:GSSG ratio (increased GSSG concentration) due to stress may inactivate enzymes vital to metabolism through the formation of mixed disulfides (Kunert and Foyer 1993). Due to this potential of deactivation of vital enzymes, the intracellular concentration of GSH is typically ten times higher than the concentration of GSSG. Consequently, any spikes in intracellular GSSG concentration is usually short lived and can therefore can be used as a “signaling molecule” by activating inhibitors which are kept in the inactive form

In mammalian cells, the concentration of GSSG has distinct effects on NFκβ. Galter et al. (1994) found that a critical concentrations of GSSG is required for NFκβ activation and nuclear translocation. They also found that an excess of intracellular GSSG also inhibits NFκβ at the level of DNA binding.

The redox reaction timing which occurred in this study corresponds with published oxidative burst data. When soybean cells are inoculated with *Pseudomonas syringae pv glycinea*, there is both a weak, transient burst of hydrogen peroxide within the first hour post-inoculation and a massive, sustained burst over several hours beginning 4 hours post-inoculation (Levine et al. 1994). In fact, Alvarez et al. (1998) found that inoculation of *Arabidopsis* leaves with *Pseudomonas syringae* induces secondary oxidative bursts in discrete cells in distant tissues, leading to low-frequency micro-bursts. The primary oxidative burst and the secondary microburst are required for SAR. This suggests that reactive oxygen species and their antioxidant counterparts are important in the ultimate expression of pathogenesis-related genes (PR1 and PR2).

Treatment of *Arabidopsis* with BSO, an inhibitor of glutathione synthesis, leads to a decrease in foliar glutathione concentration (Gussarson et al. 1996, Griffith
and Meister 1979, Griffith 1981). In our experiments, when *Arabidopsis* was treated with BSO, there was a reduction in total glutathione concentration (data not shown). In addition, when BSO-treated leaves were inoculated with *Pseudomonas*, glutathione concentrations had an even lower concentration. In our system, BSO-inhibition of glutathione synthesis caused cells to have a reduced redox capacity, resulting in a low-level of PR1 and PR2 mRNA accumulation in the tissues prior to inoculation (Figs. 14 and 15). This is an important result since PR genes were induced in the absence of a pathogen. By modifying the cellular redox capacity, a signal was generated and cells expressed PR genes to protect the plant from additional pathogen challenges. This signal is not elucidated in this study, but there are earlier mutant *Arabidopsis* studies that have found homology between NIM 1 (a gene found in *Arabidopsis* also known as NPR1, partly responsible for controlling the onset of SAR) and Ikβ (a mammalian inhibitor molecule of nuclear transcription factor, NFκβ) (Ryals et al. 1997). As described earlier, the protein-protein interaction between Ikβ and NFκβ is sensitive to shifts in the GSH:GSSG ratio (Galter et al. 1994, Dröge et al. 1994). NPR1 encodes a protein containing ankyrin repeats that are required for its function (Cao et al. 1997, Li et al. 1999). NPR1 has also been found to interact with a subclass of basic leucine zipper protein transcription factors (bZIP)
called AHBPl and TGA6 (Zhang et al. 1999). This interaction is required for salicylic acid induction of PR gene expression (Zhang et al. 1999). Upon activation of the NPR1 and the bZIP transcription factor the protein complex becomes localized in the nucleus (Kinkema et al. 2000) where there is a derepression of a negative regulator of SAR called SNII. SNII likely represses expression of PR genes by inhibiting transcription mechanically through indirect binding of the DNA through an interaction with a DNA-binding protein (Li et al. 1999). Interestingly, SNII was discovered to have a short homology with the mouse retinoblastoma protein (which is a tumor repressor) (Li et al. 1999). Once SNII is derepressed, the bZIP transcription factor, while interacting with NPR1, binds the as-1-like element found to be part of the PR-1 promoter (Jupin et al. 1996, Lebel et al. 1998, Zhang et al. 1999, Després et al. 2000).

Due to the homologies between many of the plant counterparts of SAR induction and mammalian immunological functions, it is interesting to find that a reduction of the glutathione concentrations in plant tissue after treatment with BSO, also causes expression of protective genes (PR genes). When plants were treated with BCNU, an inhibitor of glutathione reductase activity, there was an increase in GSSG concentration over a sustained period of time. In fact, in tissue that was also
inoculated with *Pseudomonas*, there was an additive effect which lead to a doubling or tripling of GSSG concentration throughout the time periods that were monitored (data not shown). PR genes were not expressed in plants treated with BCNU, even after 72 hours. These results indicate that a sustained increase in GSSG may lead to an inhibition of PR gene expression, likely due to inactivation of enzymes or proteins that are critical in the signal transduction required for PR gene expression.

One molecule that is required for PR gene expression is salicylic acid (SA). SA enhances both spontaneous and elicitor-induced production of $H_2O_2$ that enhances the oxidative burst leading to the hypersensitive response (Shirasu et al. 1997).

Interestingly, the production of SA also seems to be enhanced by ROS, especially $H_2O_2$, demonstrating that there is a positive feedback loop (Enyedi 1999, Rao and Davis 1999). SA is also involved in the regulation of the amount of NPR1 that accumulates in the nucleus (Kinkema et al. 2000) and is hypothesized to be involved in activating a phosphorylation event that allows for the release of SA response protein from an inhibitor protein (Després et al. 2000). It is likely that these proteins are involved in the signal transduction of PR gene expression. Després et al. (2000) suggest that a rise in the levels of SA concentrations could result in the modification of NPR1 and/or bZIP transcription factors either by (de)phosphorylation, altered
protein turnover, changes in subcellular localization, and or sequestration resulting in the modification of one or more of their functions. Rao and Davis (1999) also state that SA is required to maintain the redox state of glutathione defense responses. Arabidopsis fumigated with ozone show an accumulation of superoxide, $\text{H}_2\text{O}_2$, and hydroxyl radicals (ROS). In nahG plants, transgenic Arabidopsis plants that are unable to accumulate SA, ozone caused a marked decrease in the GSH:GSSG ratio, indicating that the redox maintenance in plant tissue requires SA. Spraying mustard seedlings with SA also reduced both GSH and GSSG levels by about 18% one hour after treatment (Dat et al. 1998).

It is obvious that SA is required by the plant tissue to induce PR gene expression that allows for SAR. Interestingly, in our system where whole plant SA concentrations were examined (meaning the entire plant was harvested and not just areas immediately around the HR) there was not significant accumulation of SA in response to inoculation with Pseudomonas, or treatment with either BSO or BCNU. Previously published data (where tobacco was inoculated with tobacco mosaic virus) show that the rate of SA metabolism immediately surrounding the HR (0-3.5 mm) in tobacco inoculated with TMV is the highest. Tissue surrounding the HR (3.5-6.5 mm and 6.5-10 mm) exhibits markedly less accumulation of SA over time (Enyedi et al.
1992). In fact the most increases in SA concentration were after 48 hours (Enyedi et al. 1992). In our study, it is likely that there is a dilution of the SA concentration that may have been present immediately surrounding the HR through the harvest of the whole plant and not individual leaves where HR were present. Since the primary focus of this project was the response of the whole plant to *Pseudomonas* in the early stages of the plant-pathogen interaction, it is not surprising that the subtle changes in SA were not detected in this experimental design.

Conclusions

This study focused on the early events during the interaction of *Pseudomonas syringae pv tomato* DC3000 AvrB and *Arabidopsis thaliana* and the role of glutathione in the first critical hours of plant-pathogen interaction when signal transduction is likely to be at its highest levels. *Pseudomonas* inoculation caused an increase in GSH concentration that can not be correlated to any increased activity of glutathione reductase. Twelve hours after inoculation, there was an increase in GSSG concentration that was coupled with a decrease in GSH concentration resulting in the lowest GSH:GSSG ratio observed during monitored time points. At twelve hours there was a concomitant increase in glutathione reductase (GR) activity that was
sustained to 24 hours post-inoculation. This increase in GR activity corresponds to an increase in the GSH:GSSG ratio. This is a critical time point in the activation of PR genes through the action of SA and the redox potential within the tissue. When *Arabidopsis* plants were pretreated with BSO, there was PR gene expression at all time points. The decrease in the redox buffering capacity in these plants may have lead to a signaling event that caused NPR1 to bind bZIP and be transported to the nucleus and turn on PR gene expression. This is a likely scenario, since the mammalian homologous counterpart of the NPR1 protein is also sensitive to changes in the GSH:GSSG ratio, leading to gene expression (Dröge et al. 1994). The results of this study indicate that the glutathione-mediated redox maintenance cycle is a powerful environmental sensing tool used by plant cells to detect changes in the environment and within its tissues and allow for appropriate protective gene responses.
Appendix A

Table of Abbreviations
# Table of Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BA</td>
<td>benzoic acid</td>
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<tr>
<td>BA2H</td>
<td>benzoic acid hydroxylase</td>
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<tr>
<td>BCNU</td>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea, carmustine</td>
</tr>
<tr>
<td>BSO</td>
<td>buthionine sulfoximine</td>
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<tr>
<td>bZIP</td>
<td>basic leucine zipper protein transcription factors</td>
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<tr>
<td>CHS</td>
<td>chalcone synthetase</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>DHAR</td>
<td>dehydroascorbate reductase</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>GR</td>
<td>glutathione reductase</td>
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<td>GSH</td>
<td>reduced glutathione</td>
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<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<td>HR</td>
<td>hypersensitive response</td>
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<tr>
<td>IκB</td>
<td>inhibitor κβ</td>
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<td>ITS</td>
<td>internal transcribed spacer</td>
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<tr>
<td>MBB</td>
<td>monobromobimane</td>
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<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<td>NEM</td>
<td>N-ethyl maleimide</td>
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<td>NFκB</td>
<td>nuclear transcription factor κβ</td>
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<td>PAL</td>
<td>phenylalanine ammonia lyase</td>
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<td>PCTC</td>
<td>pyrroldine dithiocarbamate</td>
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<tr>
<td>Pst</td>
<td><em>Pseudomonas syringae</em> pv <em>tomato</em> DC3000 AvrB</td>
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<td>PR</td>
<td>pathogenesis-related</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SA</td>
<td>salicylic acid</td>
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<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
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<td>TCA</td>
<td>trichloroacetic acid</td>
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<td>TCEP</td>
<td>tris-(2-carboxyethyl)phosphine</td>
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<td>TMV</td>
<td>tobacco mosaic virus</td>
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Appendix B

Primers and Sequences
Primers and Sequences

ITS primers

AB101
ACGAATTCATGGTCCGGTGAAAGGTGTTCG

AB102
TAGAATTCCCCGTTGCTCGCCGTTAC

PR1 gene sequence

CAACTTAGAAAAATGAATTTTACTGGCTATTCTCGATTTTTAATCGTCTTTT
GTAGCTCTTGTAGGTGCTCTTCTTCTTCCCTCGAAAGCTCAAGATAGCCCA
CAAGATTATCTAAAGGGTTCACAAACCAGGACAGGAGGAGGAGGAGGTAGGC
AGGTCCCATGCAGTGGGACGAGAGGGTTGCAGCCTATGCTCGGAGCT
ACGCAGAAACAACTAGAGGCAACTGCAAGACTCATACACTCTGGTGAGG
CTTTACGGGAAAAACTTAGCCTGGGTTAGCGGGTGACTCGTGCGCCT
CTCCGCCGTGAAACATGTGGGTTAGCGGAGAAGGCTAATCAAATACACTACG
CTGCGAACACGTGCAATGGAGTTTGTGGTCACTACACTCAAGTGGTT
TGGAGAAAGTCAATCGAGACTCGGATGTCACCAAGTGAGGAGGTGAACAA
TGGTGCGAACCATCATTGCCAATCTATGGAGATCTCGTGAGAATTATG
TGAACGAGAAGCCATACTAATGAAGTGTGATGATCATGCATACAACA
CGTACATAAAAGGACGTTATATGTATCAGTATTTCAAATAAGGACGATCAT
ATGCGAGAYGTATCATTATATATATCAATAATACAATAAATAAAGAGCTGGAGATT
ACGAGAATCTATTTAAATAAAGTGTACATCTAAATTATTATAGATTTATAT
ATGTAATATATGTGCGCTTTTTAAAAAGTTACATAAATTATATAGATTTAT
ATGTCATTCAAAAAAAAAAAAAAAAAAAAAAAA (757 bp, Uknes et al. 1992)
(Bold section indicates sequence that was amplified for PR1 probe)

PR1 primers

Sense
CCA CAA GAT TAT CTA AGG GTT C

Antisense
GGC TTC TCG TTC ACA TAA TTC C
PR-2 gene sequence

AATCAAGAAAATGTCTGAATCAAGGAGCTTACCCACCACCATGTTGA
TGATTCTCTCCGCTTTGTAATAGCTTTTCCTCACCACACAGCTGGAC
AAATCGGAGTGATGCAGAGGATGCTAGGCGATACCTTGCCAAGTTCCATCG
GACGTTGTGGCTCTTTACAAACAAACAAACATCCAGCGAATGCGCTCTAC
GGCCCTGACCCAGGCCTCCTTGCCGCTCTCCGTGCTGCCATCGAGCTC
ATCCTCGACGTTCCAGTTCAAGATCTTGAACGTCGCTCCAGTCAAA
CGGAGGCGCAAGTGAGGTTCAAGAAACAGTCACAGCTACAGAGAT
GGTGTCAGATTCCGGTACATCAACGTTGGAAATGAGGTGAAACCCTC
AGTTGGGCTTGTTCTCTTTACAAAGCAAATGCAGAACATCGAGAACCGG
TTTCTGGAGCGAGGCTTTGAAAGTCAGAGTCTCAACACAGCTATAGCCACT
GACACACCCACACTGATACCTCTCCGCTCTCCCTAAAGGAAGTTTCAGGG
TGAGTATAAGAGCTTCTCGAACAGTGATAGGTTTCTTTGCAAGCA
AGCAATCTCCCTTGCTCGTGAAATCCTACTCCCTACTTCAGCTACAGTGG
GAGACACGCCAACAATCCATCTAGACTACGCTCTTGCCAGCCAGAGGA
TCCACTGTGGATAAGACTCCAGGAGTTGACTCATACCAAAACCTATTCCGAC
GCAAATCTCCGACTCGGTCTTTATGCAAGCATTGGAGAAATCAGGGGGCGG
ATCGTTGGAAATCTGCTTGCTCGGAGACCGGTTGGGCAACAGAGGGG
GCAGTCGGGACGAGTGTTGGAACAGGCAAGACTTATGTAAAACAATTT
GATACACACATGTAAGAATGGATCACCAGAAGGCGCAGGAAAAAGCTA
TAGAGACTTATATATATCGTCATATGTTCGATGAAATAAGAAGGAAACAGC
TAGAGAAAGTTTTGGGACTGTTTTACTCCAGATCGACGTCTAAGGTATGA
AGTTAATTCTAACTTATTAGAGACTTGTGGGGTTTTATGTAAAGCGTA
TTTTAATTTACAAATCTCCAATAGAGACTTGTGGGGTAAAATGTAAAGC
ATGCCTGTAATGTATAAGTTTTAAGGGTATAAAATGAAAGCTGCAAGCAT
AAAATAAGGAATGAAA (1181 bp long)
(Bold section indicates sequence that was amplified for use as the PR2 probe)

PR2 primers

Sense

GAT CTT GAA CGT CTC GCC TCC AGT C

Antisense

GGC CTT CTC GGT GAT CCA TTC TTC


*Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a
subgroup of the TGA family of bZIP transcription factors. *Plant Cell, 12,* 279-
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S. & Gmünder, H. (1994). Functions of glutathione and glutathione disulfide in
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reaction to tobacco mosaic virus. *Proceedings of the National Academy of
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