OZONE INDUCED STRESS RESPONSE IN

MEDICAGO TRUNCATULA

By

MICHAEL PUCKETTE

Bachelor of Science in Biochemistry
Oklahoma State University
Stillwater, OK
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Dissertation Approved:

Dr. Ramamurthy Mahalingam

Dissertation Adviser

Dr. Ulrich Melcher

Dr. Andrew Mort

Dr. Stephen Marek

Dr. A. Gordon Emslie

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Reactive oxygen species (ROS) are a consequence of aerobic metabolism. These are small reactive molecules that can be rapidly generated by several different mechanisms. They can also be efficiently removed by several different mechanisms. These characteristic features make them serve as signaling molecules in all aerobic life forms.

ROS are a common feature of many plant stress interactions. Traditionally ROS have been viewed as detrimental molecules because of their cytotoxic effects. The role of ROS in plant signaling has recently received heightened attention as perception has shifted towards the view that ROS are essential for many plant systems. ROS play pivotal roles in diverse aspects of plant life including stress interactions, stomatal opening, root development, programmed cell death, and cell wall lignification. Consequently the plant must finely regulate the levels of ROS to maintain normal plant growth and development. This fine control is provided by the enzymatic and non-enzymatic antioxidants in the cell. Important antioxidant enzymes include catalase, superoxide dismutase, and ascorbate preoxidase. Ascorbate, glutathione, polyamines, gamma-amino butyric acid, mannitol, and flavonoids are examples of non-enzymatic antioxidants. The subtle balance between ROS levels and antioxidants is termed as redox homeostasis. Maintaining redox homeostasis is critical for cell survival because of the role of ROS as signaling molecules. A shift in the balance to favor ROS can induce

programmed cell death while a shift favoring antioxidants influences the cell cycle progression.

Inside plants there are several different types of ROS – singlet oxygen, peroxide, superoxide, and hydroxyl radicals. Singlet oxygen is mostly produced in the chloroplasts by energy transfer reactions while a series of electron transfer reactions during the conversion of triplet oxygen to water lead to the sequential production of superoxide, hydrogen peroxide, and hydroxyl radicals [1-4]. Different ROS molecules are produced in different cellular compartments and the type of ROS molecule produced is in fact dependent on the inciting stimulus.

We used ozone, O_3, as a model abiotic inducer of ROS. Ozone is most widely known for its role in the upper layers of the stratosphere blocking ultraviolet light. When ozone is present in the troposphere it acts as a chemical oxidant with detrimental effects on living cells. The usage of ozone for studying the role of ROS in plants has some characteristic benefits. Most notably, as a gas, ozone enters through the stomata of plants inducing the formation of ROS within the plant tissue. Methods of application for other ROS require the infiltration of the leaf tissue by physical force. This can result in secondary physical damage to plant tissue independent of ROS. This physical damage can initiate signal transduction much like that of ROS. This unwanted side-effect can complicate the interpretation of results. Additionally, physical application by infiltration does not treat the whole plant simultaneously. Different volumes of the treatment ROS are often injected into each treated leaf and have the potential to influence how the plant responds. Application of the gaseous ozone circumvents these difficulties and allows for

an in planta activation of ROS providing a useful tool for analyzing oxidative signaling pathways.

I chose to study the effects of ozone induced oxidative stress on the model legume *Medicago truncatula*. *M. truncatula*, barrel medic, is a close relative to the economically valuable alfalfa, *Medicago sativa*. Like alfalfa it is a trifoliate, or a plant which produces a set of three leaflets. However, unlike alfalfa, it has a diploid genome making it more suited to genetic experimentation. It has been used extensively to study the process of nodulation [5-11]. A whole genome sequence of this model legume is available. Genomic resources such as whole genome affymetrix gene chips, oligonucleotide arrays, transposon insertion lines, and the ability to perform transformation has made this a popular plant model.

While *M. truncatula* is of moderate economic importance as a forage crop in some parts of the world, a great deal of interest in it, and legumes as a family, are for its ability to synthesize diverse groups of metabolites. These metabolites can play positive roles in human and animal health. While flavonoids are common throughout the plant kingdom, iso-flavonoids are much more restricted to the legume family [12]. These compounds are synthesized from flavanones and have been found to have estrogenic, antiangiogenic, antioxidant, and anticancer activities in humans. The compound medicarpin is synthesized in the flavonoid pathway and has been found to be a phytoalexin. Additional compounds like triterpene saponins are toxic to monogastric animals and can hinder ruminant digestibility negatively impacting forage efficiency.

In an effort to expand our knowledge of ROS signaling in plants we chose to apply acute ozone to *M. truncatula*. Analysis of a resistant and a sensitive variety

simultaneously allowed us to examine the role of ROS as an oxidative stressor and also identify signaling mechanisms that lead to resistance. Legumes contain a variety of useful chemical compounds produced by unique secondary metabolic pathways. Analyzing the responses to ozone of secondary metabolic pathways has the potential to provide insight into novel legume oxidative stress response pathways. This understanding is important not only for the purpose of identification of response pathways but also for identification of novel antioxidant metabolites that may be harnessed for developing nutritional supplements to improve the health of those suffering with hypertension, diabetes, and cancer.

CHAPTER II

REVIEW OF LITERATURE

Plant stress interactions are a complex and broad field encompassing many different stressors. Typically stress is divided into two categories based on the inciting stimulus: biotic and abiotic. Biotic stress is caused by living organisms such as bacteria, fungi, viruses, and nematodes while abiotic stress is typically caused by changes in the physical environment.

ROS are a common feature in plant stress, Figure 1. ROS have been found to result from stress caused by wounding, light, drought, pathogen, heat, cold, and heavy metal. Plants are capable of producing ROS in various organelles, the most prolific ROS producer being the chloroplast. The Mehler reaction occurs at photosystem I (PSI) and involves an electron being passed onto O_2 instead of ferridoxin [1]. The passing of an electron onto O_2 results in the creation of superoxide [1]. This reaction is also called the water-water cycle because the electron starts from water in photosystem II and is reduced back to water by ascorbate peroxidase (APX), Figure 2 [2]. This cycle serves to protect the chloroplast from photo-oxidative damage and has been suggested to serve as a mechanism for electron flow around PSI under conditions where normal electron acceptors are not available [3].

An additional method by which chloroplasts can produce ROS is a result of photo-excited chlorophyll in the triplet state reacting with molecular oxygen, resulting in the formation of singlet oxygen [4]. The formation of singlet oxygen can result in degradation of the D1 protein and quenching of photoreceptors [4]. The half-life of

singlet oxygen produced by this method has been calculated as 200 nanoseconds with an ability to diffuse up to 10 nm [13, 14].

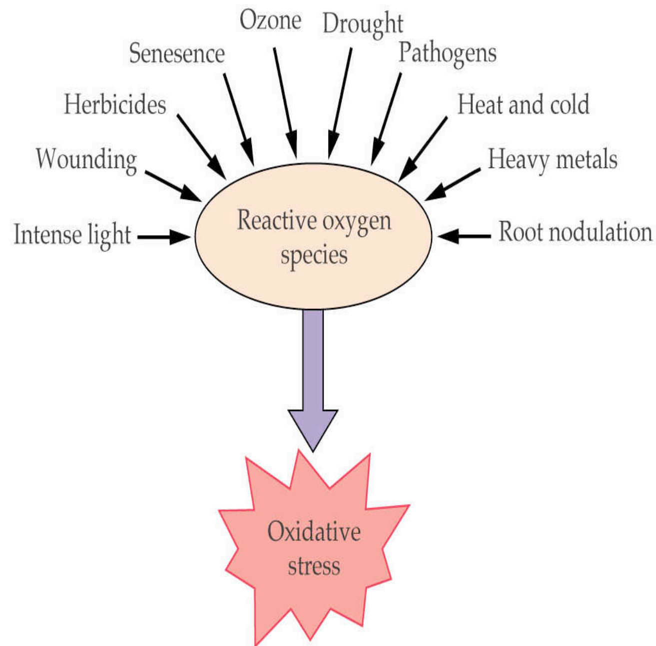

Figure 1: Role of ROS in plant stress interactions published in Buchanan B., Gruissem W., and Jones R. (2000) Biochemistry & Molecular Biology of Plants. Rockville Maryland: American Society of Plant Physiologists [15]

Plant produced ROS are not an unimportant by-product of plant-stress interactions. ROS can act as signaling molecules leading to differential gene expression through changes in the redox homeostasis. Transcription of chloroplast encoded genes for *psaAB* can be altered by the redox state of plastoquinone [16]. In *Escherichia coli* and yeast ROS have been shown to use redox-sensitive transcription factors, OxyR and Yap1 respectively, to alter gene expression [17, 18]. The interactions between Yap1 and

ROS are particularly novel as ROS not only slightly increases DNA binding efficiency but also induces a profound change in cellular localization through interactions with the protein's cysteine-rich domain [17]. Under non-oxidative stress conditions Yap1 is localized in the cytoplasm, but under oxidative stress conditions it localizes to the nucleus, allowing ROS to regulate its function through nuclear import and not through dramatic alterations to DNA binding affinity [17]. Examples such as Yap1 show that ROS does not have to be present in a specific cellular compartment such as the nucleus to have a direct effect on transcription factors.

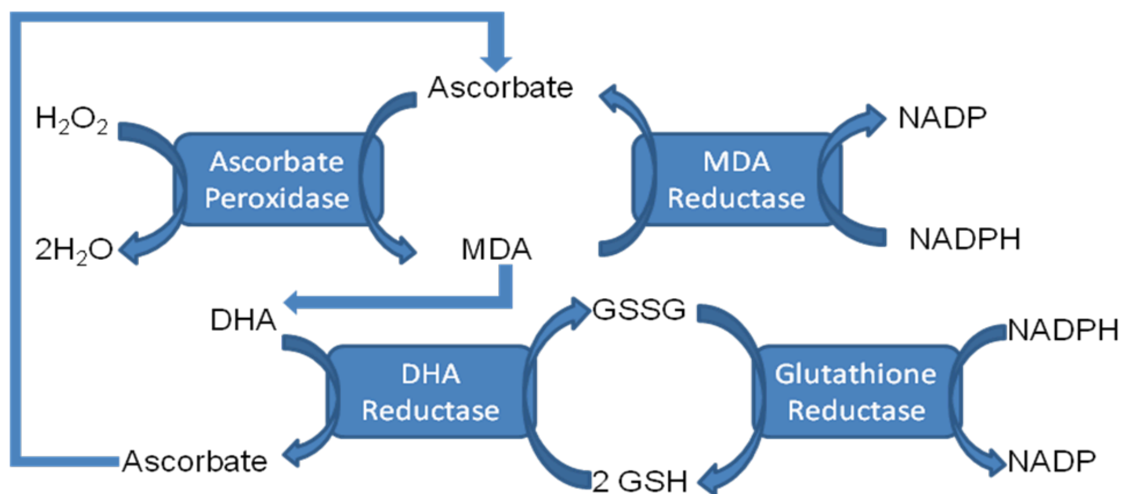

Figure 2: Ascorbate-Glutathione Cycle also called the Halliwell-Asada pathway MDA: monodehydroascorbate, DHA: dehydroascorbate, GSH: glutathione, GSSG: glutathione disulfide

A major control on ROS is antioxidants which function to protect an organism from oxidative damage. Ascorbate and glutathione comprise the ascorbate-glutathione cycle, Figure 2, and are considered the first line of defense in response to oxidative stress [19]. The ascorbate-glutathione cycle uses enzymatic reactions to recycle oxidized

ascorbate and glutathione back to their reduced state. The enzymes comprising this cycle are ascorbate peroxidase (APX), monodehydroxyascorbate (MDA) reductase, dehydroascorbate (DHA) reductase, and glutathione reductase (GR), Figure 2. The pathway is most notable for its role in reducing hydrogen peroxide in the chloroplast and is called the Halliwell-Asada pathway [20, 21].

The ability of the Halliwell-Asada pathway to efficiently recycle ascorbate and glutathione also has an impact on ozone sensitivity [22]. Arabidopsis mutants deficient in ascorbate have a heightened sensitivity to ozone [23]. In soybean, *Glycine max*, plants with higher GR and APX enzymatic activities had enhanced ozone tolerance [22]. While a critical component of the ozone response, ascorbate and glutathione do not account for all differences between ozone sensitive and resistant cultivars [24].

Ascorbate and glutathione are examples of non-enzymatic antioxidants. Enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT) can also play key roles in the regulation of ROS in the cell. SOD converts superoxide into hydrogen peroxide. The reaction is carried out by metal ions present at its reactive site and SODs are categorized based on the metal ions present. SOD is a highly ubiquitous enzyme with a high reaction rate and is considered the primary plant defense against superoxide [25]. CATs are enzymatic scavengers of hydrogen peroxide converting it into water and O_2. In plants CATs are predominantly localized in the peroxisomes where the primary function of CATs is to scavenge H_2O_2 produced by glycolate oxidase [26]

Adaptation to a stress is usually carried out by a sequential set of events called signal transduction. A stress signal is received and initiates a downstream cascade leading to an eventual response. Plant hormones, or phytohormones, play a critical

signaling role in plant stress responses. Research into the role of phytohormones in ozone induced stress has focused on those involved in programmed cell death (PCD), namely ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) [27-33]. These three phytohormones comprise a signaling cycle which controls PCD, Figure 3 [34]. The role of these three hormones in conjunction with each other is a complicated since they can have both pro-PCD and anti-PCD properties.

SA has been shown to play a dual role in ozone response having both a protective effect through initiation of systemic acquired resistance (SAR) but also playing a role in the initiation of lesion formation [35-38]. SA interacts with ROS in a positive feedback loop [34, 39, 40]. This results in a self amplifying signal where an ROS signal leads to the production of more ROS through SA [34].

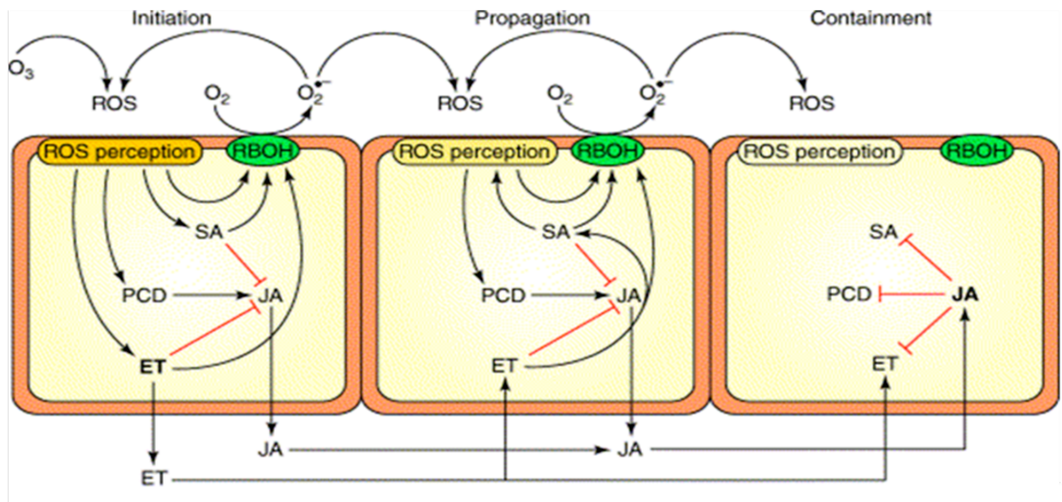

Figure 3: Programmed cell death (PCD) pathway showing the initiation of PCD by ROS, the propagation of PCD through plant produced ROS induced by SA and ET, and the eventual containment of PCD by JA, Overmyer et al (2003) [34]

A large amount of work examining the role of SA in antioxidant control and subsequent antioxidant dependent regulation of PCD was performed in the *NahG* Arabidopsis mutant [41]. The *NahG* mutant was created by introducing the bacterial salicylate hydroxylase, *NahG* gene into transgenic plants to degrade SA thereby preventing its accumulation [41]. Several years after the introduction of the *NahG* Arabidopsis mutant additional research brought to light questions towards the validity of using this mutant. It was found that in plants *NahG* degraded SA into catechol which induces the formation of hydrogen peroxide [42]. This revelation does not completely negate the importance of the work performed with this mutant. However, it makes accepting conclusions made about the role of SA in regulating antioxidant levels from work done in this mutant suspect [33].

Research on tobacco, *Nicotiana tabacum*, showed a unique relationship between SA and ozone when both resistant and sensitive cultivars were examined [43]. This research found that both the resistant tobacco cultivar Bel-B and the sensitive tobacco cultivar Bel-W3 had increases in free SA and conjugated SA in response to ozone [43]. While both cultivars had a SA response, the temporal pattern of the SA response was different between cultivars, with the resistant cultivar having an earlier response than the sensitive [43].

Ethylene is one of the most diverse phytohormones in terms of the biological processes it is involved in, a sharp contrast to its incredibly simple chemical structure, C_2H_4. In PCD, ET induces propagation of the lesion, Figure 3 [29, 34]. ET plays a similar role in controlling PCD in various aspects of plant development [44, 45].

Application of ET to Arabidopsis plants undergoing ozone stress increased the spread of PCD lesions [29]. Research using ethylene overproducing mutants, *eto1* and *eto3*, showed that these mutants produced lesions in response to ozone levels normally not associated with lesion formation, as well as establishing that ozone induced ethylene production was dependent upon SA [31]. Sensitivity of Arabidopsis cultivars to ozone stress has been linked to differences in ethylene production between cultivars even allowing classification of 20 different Arabidopsis accessions for ethylene production and correlating ethylene production to the degree of leaf injury [46]. Correlation between ethylene and sensitivity to ozone was also seen in birch, *Betula pendula* Roth [47]. Birch research also highlighted the complexity that can exist as it showed ET being involved in both resistance and sensitivity to ozone [47]. Researchers found that a decrease in ET sensitivity when coupled with high ET levels resulted in PCD [47]. However, a functional ET signaling pathway was also required for ozone resistance [47].

Once the PCD signal has been initiated by SA and spread by ET, it is eventually contained by JA, Figure 3 [29, 30, 32, 34]. JA is a 12 carbon fatty acid cyclopentanone that is a member of the jasmonate family of compounds [48]. The usage of JA as the signal for lesion containment can take advantage of ROS induced lipid peroxidation which results in the generation of substrates needed for JA biosynthesis [27]. The production of substrates for JA biosynthesis from ROS induced lipid peroxidation establishes a cycle in which the spread of ROS induced damage resulting in lipid peroxidation results in a larger substrate pool for synthesis of JA to contain the lesion [27]. Pretreatment of plants with methyl jasmonate reduced lesion spread in tobacco [49] and Arabidopsis [32]. However, when treatment of JA was applied to JA-insensitive

poplar mutants no reduction in damage was seen, showing that signaling cascades triggered by JA impart lesion containment and this containment was not a result of any molecular attributes of JA [50].

JA and SA, while traditionally viewed as having an antagonistic response, have also been observed to produce a synergistic response at low concentrations leading to the suggestion that plants can use the balance of SA and JA to optimize responses for different needs [51]. Research on pathogen responses of Arabidopsis cultivars found that the negative interactions between JA and SA varied among different cultivars in response to the same stress [52]. Alterations in the balance between JA and SA may represent a mechanism by which two accessions of the same species can have drastically different phenotypes in response to stress.

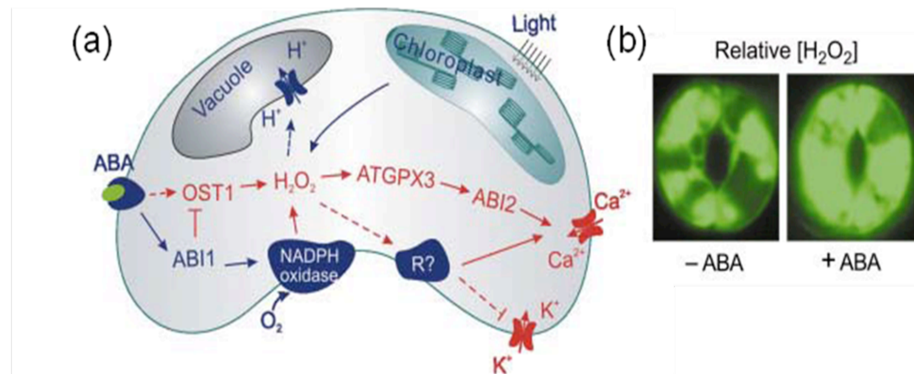

Figure 4. (a) ABA induced ROS production in guard cells leading to stomatal closure, Wang and Song (2008) [53] (b) Visualization of ROS production within the guard cells using the dye DCFDA in the absence of ABA treatment (-ABA) and in response to ABA treatment (+ABA), Pei et al (2000) [54]

ET, SA, and JA are not the only phytohormones involved in an ozone induced response in plants. ABA is known and well studied for its role in stomatal closure, Figure 4 [55]. This role allows it to have a direct interaction on ozone uptake into the plant. ABA induces stomatal closure through a ROS dependent process [53, 54, 56]. The presence of ABA initiates a signaling cascade that leads to the activation of NADPH oxidases which generate hydrogen peroxide [57, 58]. This leads to increased levels of ROS in the guard cells, Figure 4b [54].

Hydrogen peroxide in guard cells does not serve as a cytotoxic molecule but rather is part of the stomatal closure signaling cascade. Hydrogen peroxide perpetuates the signal leading to the activation of calcium channels as well as inhibition of potassium channels, Figure 4a [54, 59, 60]. This leads to the removal of potassium from the cell resulting in reduced osmotic potential which causes stomatal closure [54, 59-61]. Research on the ABA insensitive mutant 1, *abi1*, showed that in the presence of applied hydrogen peroxide ABA is not needed for inducing stomatal closure, Figure 5 [62]. This work was used to place *abi1* upstream of the hydrogen peroxide burst in the signaling pathway for stomatal closure, but it also shows that ROS are capable of inducing stomatal closure independent of ABA levels.

ABA interacts with many other phytohormone systems and is a general negative regulator of pathogen resistance [63]. Research into pathogen interactions in tomato mutants found that high ABA levels inhibited SA-dependent defense responses [64, 65]. ABA also plays an antagonistic role to SA-induced systemic acquired resistance with crosstalk possible at multiple points [66]. The interactions between ABA and SA is not completely one sided as the Arabidopsis protein SAZ, a repressor for a subset of ABA

responsive genes, was found to be repressed in response to the combined presence of SA and ABA providing an avenue by which SA could influence ABA pathways [67].

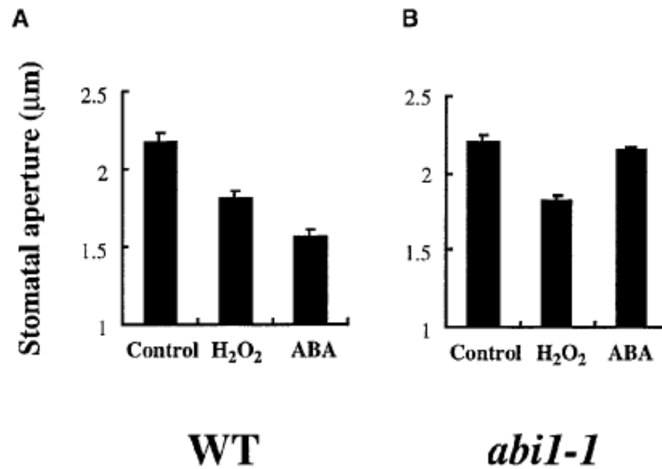

Figure 5. Effects of treatment of (A) wild-type and (B) ABA insensitive mutant *abi1-1* with ABA and hydrogen peroxide on stomata, Murata et al (2001) [62]

ABA and ET also have several well documented interactions most of which are antagonistic to each other [65]. ABA has been found to inhibit ET production in shoot growth [68] but able to induce ET production in developing flowers [69]. In pathogen resistance ABA serves an antagonistic role against JA-ET signaling pathways [70].

The interplay between the various phytohormones mediated by the ROS-antioxidant balance will eventually lead to fine tuning the repertoire of genes that are activated or repressed in response to oxidative stressors like ozone. Studying the transcriptional reprogramming in response to ozone can provide a better understanding of the mechanisms governing plant resistance or sensitivity to ozone. Microarray

technology provides an opportunity to examine the changes in thousands of genes or even the entire transcriptomes of an organism.

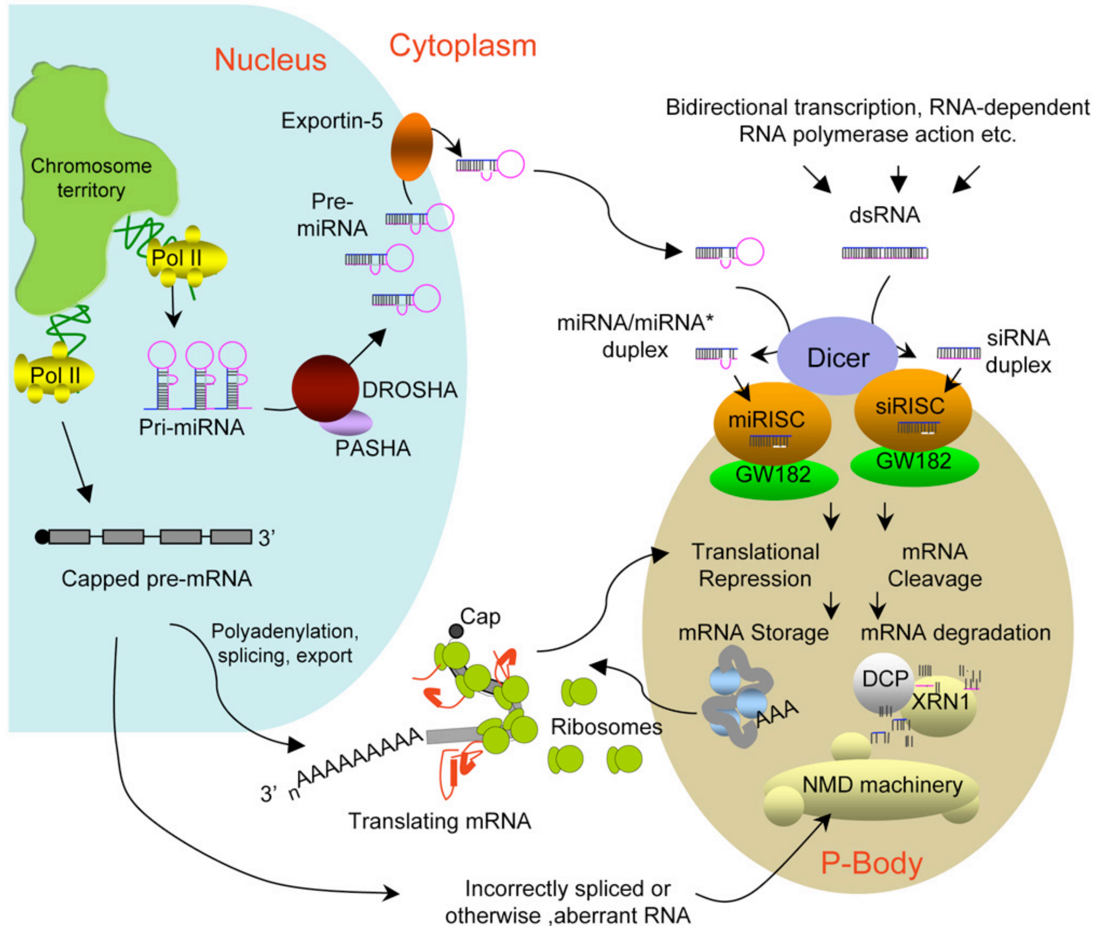

Figure 6: The path of a gene through the central dogma of biochemistry from translation to transcription with examples of controls on gene expression including miRNA and siRNA gene silencing, ribosomal loading, and P-body formation, Pontes and Pikaard (2008) [71]

Gene regulation is not a simple process of turning off and on transcription of certain genes. While transcriptional regulation plays a critical role in gene regulation it is not the sole method of control available to an organism. Genes can be controlled at all

levels leading to eventual protein synthesis in translation, with proteins having numerous control mechanisms beyond that.

Translation is performed when a ribosome binds to an mRNA transcript and then initiates protein synthesis, Figure 6. Since mRNA's are many nucleotides long a single mRNA can be translated simultaneously by multiple ribosomes. The degree to which a mRNA is loaded with ribosomes is a method of translational regulation. Examining which mRNAs are actively bound to ribosomes will give us a clearer view of changes in protein expression than a traditional total RNA examination. This is because not all mRNA is translated. Post-transcriptional regulatory mechanisms such as small interfering RNA (siRNA) and micro RNA (miRNA) dependent gene silencing can lead to mRNA degradation, Figure 6.

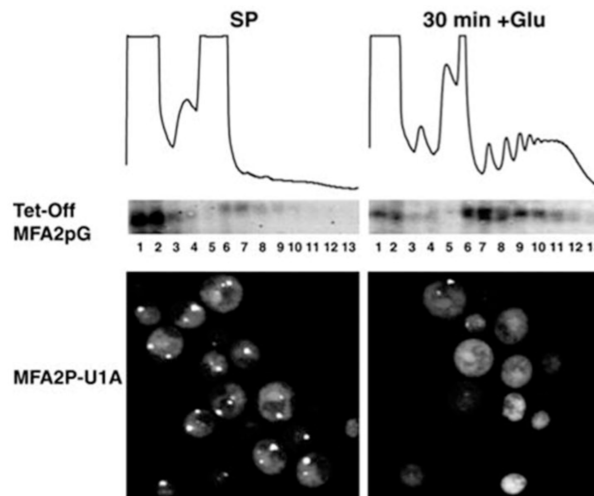

Figure 7: Induced differential ribosome loading of the reporter RNA MFA2P in yeast 30 minutes after the addition of glucose and the disappearance of MFA2P from P-bodies showing the transfer of MFA2P transcripts from P-bodies to the polyribosome complex, Brengues et al (2005) [72]

P-bodies play a role in both mRNA storage and mRNA degradation [71-73]. P-bodies have been implicated in both siRNA and miRNA based degradation, Figure 6 [71, 74]. While mRNA degradation serves an important role in gene regulation it is an irreversible one, in the sense that once an mRNA is degraded the only way it can be replaced is through synthesis of a replacement mRNA via transcription. Conversely, transcripts which are stored in P-bodies are not necessarily degraded. An important aspect of translational regulation are genes which are only temporarily stored in the P-bodies, Figure 7 [72]. The shuttling of mRNA between P-bodies and polyribosome complexes can result in the silencing and activation of a gene in a transcription independent manner, Figures 6 & 7 [72]. This shuttling represents an important form of regulation that is not taken into account when examining changes in gene regulation using total RNA.

Few papers have examined how differential ribosomal loading affects the transcriptome of plants in response to stress. These few papers examined the response to hypoxia stress [75, 76] and drought stress in Arabidopsis [77, 78] and tobacco [79]. Research on drought stress in Arabidopsis found that differential translation can completely negate the effects of differential transcription [77]. A microarray experiment looking at oxidative stress caused by H_2O_2 in *Saccharomyces cerevisiae* showed significant effects on ribosomal loading and concluded that translational control was key to the ability to adapt to oxidative stress [80]. Previous research has also revealed that polyribosome loading on mRNA is generally decreased in response to stress [75, 76, 81-85]. The transfer of mRNA from polyribosome complexes to P-bodies results in

translational repression despite the continued presence of the mRNA in the cell. In addition pre-existing mRNAs being stored in P-bodies could be returned to polyribosome complexes representing a potential key adaptation to a given stress.

The integration of various levels of gene regulation data on the genome scale in plants is still in its infancy. To the best of our knowledge this type of integrated analysis with reference to differences between resistant and sensitive accessions has not been done. A few studies have examined and integrated transcriptional and translational information, however, these in depth comparisons usually are not conducted on two plant varieties showing different phenotypes in response to stress [75-78].

My dissertation examines how both an ozone resistant and ozone sensitive ecotype of *M. truncatula* respond to ozone stress by analyzing both differential transcription and differential ribosome loading. In further analyses, I examine the translational regulation using polysomal RNA from these two accessions in microarray analysis. The integration of total RNA microarray, poly-ribosomal loading, and poly-ribosomal RNA microarray data should enable development of a coherent view of molecular events during oxidative signaling in two *M. truncatula* accessions showing contrasting phenotypic responses to ozone.

CHAPTER III

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO ACUTE OZONE-INDUCED OXIDATIVE STRESS IN MEDICAGO TRUNCATULA

In any naturally occurring population of organisms there is genetic diversity. This genetic diversity can cause different populations within the same species to respond differently to the same stimuli. Ecotypes are populations within a single species in nature that are localized to a geographic area. We examined 38 different *M. truncatula* ecotypes for their phenotypic response to acute ozone stress and classified the symptoms observed. An image of representative trifoliate leaves from all ecotypes screened in this study, ranking from most resistant to most sensitive is in the appendix (Figure A1). We identified only one ecotype, JE154, as resistant to ozone stress. The rest of the ecotypes showed varying levels of sensitivity based on ozone-induced symptoms on leaves.

For further biochemical and physiological analysis we selected eight ecotypes showing varying sensitivities to ozone. We examined levels of ascorbate (reduced and oxidized forms), glutathione (reduced and oxidized forms), lipid peroxidation, and total ROS to determine if any of these biochemical parameters had a direct correlation to the phenotypes seen in response to acute ozone fumigation. We also measured stomatal conductance, transpiration, and net photosynthesis rates for each of the eight ecotypes to determine if these physiological traits had a correlation to the ozone-induced phenotype. These physiological and biochemical assays revealed that there was no one parameter that could serve as a predictor of ozone sensitivity, highlighting the complexity of the

ozone resistance phenotype. This study suggested that ozone resistance is a complex trait probably governed by multiple loci.

We also examined the responses of the ozone-resistant JE154 and ozone-sensitive Jemalong to chronic ozone, drought stress, and a combination of ozone and drought stress applied simultaneously. JE154 was resistant to chronic ozone and drought stress individually and when applied together. Jemalong was found to be sensitive to both chronic ozone and drought stress.

The results from this study are published in the journal Plant Physiology and Biochemistry in January 2007 pages 70-79. Puckette performed all aspects of the experiments in this article. Hua Weng provided assistance for statistical analysis. Dr. Sathvanarayana Elavarathi provided technical support in the operation of the Li-Cor 6400 portable photosynthesis system. A copy of this publication is attached in the Appendix X.1.

CHAPTER IV

TRANSCRIPTOMIC CHANGES INDUCED BY ACUTE OZONE IN RESISTANT AND SENSITIVE MEDICAGO TRUNCATULA ACCESSIONS

The identification of the ozone resistant JE154 ecotype and the sensitive Jemalong cultivar led to the examination of differences in transcriptional regulation between these two lines. Microarrays containing more than 17,000 genes were used for analyzing temporal changes in the ozone-induced transcriptome for both JE154 and Jemalong. In order to determine time-points for transcriptome analysis we examined the ROS profiles in JE154 and Jemalong during and following ozone treatment. Both lines showed an ozone derived ROS burst at one hour. At the 12 hour time point, 6 hours after the end of ozone treatment, a higher ROS level was observed in Jemalong but not JE154. We selected the one hour and 12 hour time point representing a time point with an ozone derived ROS burst and a time point with a plant produced ROS burst in the sensitive cultivar. The end of the ozone-treatment period at six hours was selected as the intermediate time point for the microarray analysis.

RNA for microarray analysis was isolated from two independent biological treatments with technical replications for both the treatment and each of the time points. Data analysis utilized the bioinformatics software GENESIS to examine responses on a transcriptomic scale. A simple macro was compiled in Microsoft Excel to translate information on gene ontologies provided by the Noble Foundation into the proper format required for GENESIS.

This study showed that the same GO categories were differentially altered in both JE154 and Jemalong. However, the timing and repertoire of genes within the GOs were very different between the two lines. A large amount of differential expression was seen in the resistant ecotype at the one hour time point compared to relatively unaltered gene expression in the sensitive cultivar at one hour. At the two later time points the pattern was reversed with the sensitive cultivar having a greater amount of differential expression than the resistant ecotype.

Differential regulation at one hour in JE154 included many genes responsive to phytohormones as well as differential regulation of genes in the flavonoid pathway. JE154 not only rapidly up-regulated transcripts for the flavonoid pathway but also sustained their induced expression throughout all three tested time points. Jemalong up-regulated transcripts for the flavonoid pathway at the six hour time point but had a subdued response in comparison to the resistant ecotype at the 1 and 12 hour time points.

This research was published in the journal BMC Plant Biology in April 2008, Volume 8, article 46. Puckette performed the ROS analysis, RNA isolations, ozone treatment, data analysis, Real-Time PCR, wrote initial drafts of the manuscript, and assisted in the microarray hybridizations. Dr. Mahalingam performed the microarray hybridizations and Dr. Tang at the Noble Research Foundation supplied the microarray slides. A copy of this publication is attached in the Appendix X.2.

CHAPTER V

OZONE RESPONSIVE GENES IN MEDICAGO TRUNCATULA: ANALYSIS BY SUPPRESSION SUBTRACTION HYBRIDIZATION

Microarray technology is described as a close-ended technique, because it can only provide information on genes that are printed on the slide. Thus, the transcriptional gene regulation analysis of the response to ozone in resistant, JE154, and sensitive, Jemalong, accessions of *M. truncatula* by microarrays was restricted to only the 17,000 genes printed on these microarrays. There was a possibility that novel genes important to the ozone response were not represented on these microarrays. To identify potential ozone response genes not present on these microarrays an open-ended analysis was performed using the suppression subtraction hybridization (SSH) technique to analyze gene expression. As a PCR based strategy this technique simultaneously aids in the normalization of the cDNA population and exponential amplification of the differentially expressed genes. Conducting the subtraction analysis with the control RNA sample as the tester and the RNA from the stressed sample as the driver, enabled the identification of genes that were repressed in response to stress treatment. In this study a total of four subtracted libraries were constructed. The forward subtraction libraries from JE154 and Jemalong identified genes that are induced in response to ozone. The reverse subtraction libraries from the two lines enabled identification of ozone-repressed genes.

Sequence analysis of the clones from the four SSH libraries revealed that there were only 15 unknown sequences out of 455 sequences. This low number of unknown sequences shows that there is good coverage of ozone responsive genes in the existing *M.*

truncatula EST database. Examination of *M. truncatula* library sequences to determine their possible functions utilized a compendium approach using the GENEVESTIGATOR program. In order to use this program, Arabidopsis homologs of the *M. truncatula* genes were identified. These Arabidopsis locus identifiers were then fed into the program to retrieve the expression profiles seen in response to stressors and Arabidopsis mutations. This data highlighted the importance of phytohormones in the response to ozone, as well as displaying a hitherto unknown similarity between ozone stress and low nitrate stress.

This manuscript has been provisionally accepted for publication pending real-time PCR analyses and rewriting sections of the discussion. Puckette performed all aspects of the SSH technique, differential screening, macroarray analysis, sequence data analysis, and prepared drafts of the manuscript. The SSH procedure for the JE154 ecotype was done by Lila Peal. Sequencing of the SSH library inserts was done by Jarrod Steele and Yuhong Tang at the Noble Foundation in Ardmore OK. A copy of the submitted manuscript is attached in the Appendix X.3.

CHAPTER VI

REGULATION BY DIFFERENTIAL TRANSLATION IN RESPONSE TO OZONE IN MEDICAGO TRUNCATULA

Translation of a transcript into a protein involves three basic steps initiation, elongation, and termination. The final aspect of my thesis examines the effects of ozone-induced oxidative stress on the initiation step of translation by examining differential ribosome loading in *M. truncatula*. This represents an important site of regulation as research has found the initiation of translation to be the most important step in regulating protein synthesis [86]. Transcripts that are not associated with ribosomes will not be translated into proteins. A transcript with more ribosomes loaded will be more frequently translated than the same transcripts with a single ribosome loaded.

In the first step we examined the differences in ribosomal loading comparing non-polyribosomal and polyribosomal fractions by examining the polyribosomal gradient profiles generated by the gradient fractionator. Since the initiation step of protein synthesis is the most crucial regulatory step in protein synthesis we hypothesized that the RNA associated with poly-ribosomal fractions will better represent the proteome.

By coupling a poly-ribosome bound transcript isolation technique with microarray technology we gained insight into the constituents of the proteome. Not only can we better predict constituents of the proteome but this technique also allowed us to analyze which transcripts are either being enriched for or repressed against in the polyribosome fraction in response to ozone. A long standing criticism of transcriptional profiling is that it does not closely reflect changes in the proteome [87]. Conversely, methods used to

examine the proteome tend to be complicated, expensive, cumbersome, and/or lack sensitivity when compared to transcriptional profiling.

Coupling poly-ribosomal analysis with microarray technology provides a unique opportunity to combine the strengths of both transcriptomic and proteomic techniques. Research has found a close correlation between changes in ribosomal loading and protein synthesis in yeast and animal cells [86, 88, 89]. This type of examination is relatively inexpensive and easy to perform and only requires that microarray technology be available. Unlike a traditional total RNA based microarray experiment only transcripts that are actively associated with ribosomes and thus are entering translation are analyzed. Transcripts which are not being translated due to storage in P-bodies, mRNA silencing, or other inhibitors of the loading of ribosomes onto a transcript will not be represented. While this method can't account for all aspects affecting the potential activity of a protein, it does allow for a better view of how an organism responds on the proteomic level when compared with traditional transcriptional profiling, using total RNA or mRNA, or conventional 2D-PAGE analyses of the total proteome.

Integration of results from transcriptional and translational profiling provides a powerful method to identify highly expressed genes in the ozone response. Transcripts which are undergoing both transcriptional and translational regulation will likely represent key aspects of the ozone response.

It found that JE154 and Jemalong alter their polyribosome profiles differently after one hour of treatment with ozone. For both JE154 and Jemalong most transcripts with differential translational regulation are not differentially transcribed in response to ozone. JE154 had 283 genes that showed signs of differential regulation both in

transcriptional and translational analysis while Jemalong only had 13. This experiment and the data reported here demonstrate how differential ribosome loading can be used to augment the data derived from general differential gene expression in response to ozone induced oxidative stress.

CHAPTER VI.1

METHODOLOGY

VI.1.a Polyribosomal RNA isolation and purification:

Gradient preparation:

This procedure was originally developed by Dr. Riki Kawaguchi from the laboratory of Dr. Julia Bailey-Serres at University of California-Riverside [77, 79]. Open-top polyclear ultracentrifuge tubes (Seton Scientific Co; Los Gatos, Ca) of 13 x 51mm size were used for poly-ribosome isolation. Sucrose gradients were made in these centrifuge tubes and stored at -80°C until usage. Sterilized sucrose salts solution, pH 8.4, (40mM Tris base, 20mM KCl, 21mM $MgCl_2$) was pre-made for making the sucrose gradients. Gradients were constructed in a series of four layers and allowed to freeze at -80°C between additions of each layer. Layers were added into centrifuge tubes as follows: 0.75mL of layer 1 (88% 2M sucrose, 1% sucrose salts mix), 1.50mL of layer 2 (66% 2M sucrose, 1% sucrose salts mix), 1.50mL of layer 3 (44% 2M sucrose, 1% sucrose salts mix), 0.75mL of layer 4 (29% 2M sucrose, 1% sucrose salts mix).

A detergent mix (20% Triton X-100, 20% Brij 35, 20% Tween-40, 20% NP-40) was pre-made for isolations. Mixing of the detergent mix was facilitated by warming the solution at 42°C till all the ingredients were in solution. Gradients were placed into rotor buckets and thawed at 37°C for one hour then stored at 4°C for at least one hour before usage.

Tissue homogenization and ultracentifugation:

Leaf tissues were ground using liquid nitrogen and ground tissue was placed in 2mL tubes up to the 750µl marking. To each 2mL tube 1.25mL of polysome extraction buffer (0.2M Tris pH 9.0, 0.2M KCl, 1mM EGTA pH 8.3, 36mM 1M $MgCl_2$, 8% β-mercaptoethanol, 5.6mM cycloheximide, 6.5mM chloramphenicol, 50% detergent mix, 2% polyoxethylene 10 trydecyl ether (PTE), 1% deoxycholic acid, 1% heparin) were added and mixed by stirring with a spatula. Tubes were then placed on ice for 10 minutes with occasional inversion for thorough mixing of ingredients. Samples were then centrifuged for 2 minutes at a speed of 13,000rpm at 4°C using an accuSpin MicroR tabletop centrifuge (Fisher Scientific). Following centrifugation, 700µl of supernatant was layered on the thawed pre-made sucrose gradients. Four such leaf isolations would provide sufficient supernatant for six gradients. Tubes were balanced to within 0.001g using a Mettler Toledo balance using either excess isolation supernatant or extraction buffer. The tubes were then subjected to ultra-centrifugation using a Beckman Optima LE-80K ultracentrifuge (Beckman-Coulter) at 4°C for 2 hours and 20 minutes at 40,000 rpm with a Beckman 4182 rotor (Beckman-Coulter).

<u>Poly-ribosome fractionation</u>:

During centrifugation the ISCO model 640 density gradient fractionator (Teledyne-Isco) was turned on at least 30 minutes prior to usage. After completion of ultra-centrifugation, gradients were loaded onto the gradient fractionator and run at a flow rate of 1mL/minute while measuring the absorbance at 254nm. A characteristic profile for isolation from plant tissue was observed, Figure 8, allowing for identification of absorbance peaks. Based on the characteristic absorbance profiles fractions were collected into two pools representing the non-polyribosome bound fractions and the poly-

ribosome bound fractions. The split between the fractions occurred 22 seconds after a characteristic dip folowing the mono-ribosome peak. To the pooled non-polyribosome bound fraction, 7mL of DEPC treated 8M guanidine HCl was added. To the pooled polyribosome bound fraction 5mL of DEPC treated 8M guanidine HCl was added. Each tube was then vortexed for 3 minutes and the samples were split into two 13mL screw cap tubs (Sarstedt). This split was necessary to fit the total volume into tubes which would be usable in available centrifuge rotors. For each of the non-polyribosome bound tubes, 5.25mL of 100% ethanol was added and samples were vortexed for 1 minute and placed in -20°C overnight. For the tubes containing polyribosome fractions 3.75mL of 100% ethanol was added and then vortexed for 1 minute before being stored at -20°C overnight.

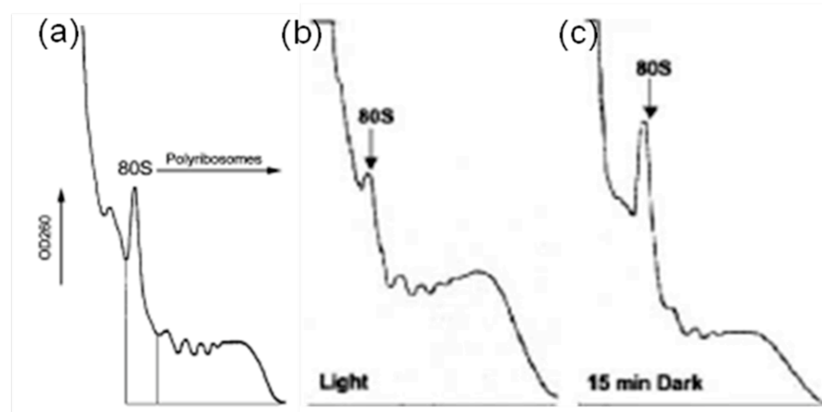

Figure 8: Examples of poly-ribosome profiles isolated from plant tissue (a) published in Combe et al [84], (b) and (c) published in Bhat et al [85]

Poly-ribosomal RNA purification:

The following day tubes were centrifuged at 4°C for 50 minutes at a speed of 9600rpm in a Beckman GS15R table top centrifuge (Beckman-Coulter). Supernatant was poured off, tubes inverted and allowed to dry for 20 minutes. Extraction buffer was prepared by adding 10µL β-mercaptoethanol to 1mL of the RLT buffer from the Qiagen RNeasy Plant mini kit (Qiagen). For each pair of split tubes 450µL of the extraction buffer was added and mixed in by vortexing. The solution was then transferred to the second solution tube and vortexed again. After vortexing, 250µL of 100% ethanol was added to the first tube and mixed by pipeting. This solution was also transferred to the second tube. All fractions from the same sample were pooled into a single tube resulting in a total volume of around 4mL. From these pooled fractions, 700µL was placed onto an RNeasy mini spin column and let sit for 3 minutes. Columns were then centrifuged for 18 seconds at 13,000 rpm. Flow through was then reapplied to the column and allowed to sit again for 3 minutes. Columns were again spun for 18 seconds at 13,000 rpm with the flow through discarded this time. This was repeated until all of the pooled fractions had been run through a column. Once all of the pooled fractions had been run through the spin column, 700µL of wash buffer RW1 from the Qiagen kit was added to the column and spun for 18 seconds at 13,000 rpm and the flow through was discarded. Then 500µL of buffer RPE (with ethanol added as per kit instructions) was added to the column and spun for 18 seconds at 13,000 rpm again discarding the flow through. Wash buffer RPE (500µL) was added again to the column and centrifuged for 2 minutes at 13,000 rpm. Columns were transferred to new 2mL tubes and spun for 1 minute at 13,000 rpm to ensure the removal of all buffer RPE. Columns were then placed in 1.5mL collection tubes and 50µl of RNase free water was added to the column and allowed to sit

for 5 minutes. Columns were centrifuged for 1 minute at 13,000 rpm to elute the RNA from the column. To ensure all the RNA was retrieved, another 50µl of RNase free water was added and allowed to sit for 5 minutes. Centrifugation was again performed for 1 minute at 13,000 rpm resulting in a 100µl final sample of poly-ribosome bound RNA.

To this, 20µL of DEPC treated 3M sodium acetate (pH 5.3) and 500µL of 100% ethanol were added and the mixture was allowed to sit at -80C overnight. The next day tubes were centrifuged at 4°C for 45 minutes at a speed of 13,000rpm. The resulting supernatant was aspirated off and 250µL of 80% ethanol was added. Tubes were again centrifuged at 4°C for 5 minutes at 13,000rpm. The resulting supernatant was aspirated off and the pellet allowed to air-dry for 2 minutes. The pellet was dissolved in 21µL DEPC treated water and was stored at -80°C till usage. The concentration and quality of the RNA was analyzed on the BioAnalyzer (Agilent).

VI.1.b Evaluation of polyribosome isolation gradient profiles

Gradient traces reporting the absorbance at 254nm were scanned into digital files using an EPSON perfection 3170 photo scanner. ImageJ version 1.40g software available from NIH was used to analyze the scanned area of the 80S and mono-ribosome peaks as well as the area of the poly-ribosome peaks. The ratio of these numbers was then taken to reflect any change in translational regulation by ribosomal loading in response to ozone.

VI.1.c Microarray slide printing and analysis of print quality

The 17,000 oligonucleotide set of *M. truncatula* (originally from the Operon company) was a kind gift from Dr. Yuhong Tang of the Samuel Roberts Noble Foundation. These oligos were resuspended in 25mM DMSO and were in 384 well plates. Oligos were printed on amino-silane coated GAPS slides (Corning) using the GeneMachines Omni Grid Robot at the OSU Microarray Core Facility. The oligos were printed in a format of 32 rows x 16 columns per grid and in total there were 24 grids place in 4x6 pattern.

In order to assess the quality of the print job we used the Syto61 stain, a DNA specific stain. This procedure for Syto-61 staining was kindly provided by Dr. Peter Hoyt, OSU microarray core facility. Microarray slides were placed in the staining solution (1% 100x TE, 10% of 95% ethanol, and 0.02% 5mM Syto-61) for 5 to 10 minutes at room temperature. Slides were then rinsed by brief placement into the washing solution (0.1% Tween 20). After the initial rinse, slides were placed in the washing solution for 10 minutes at room temperature. Slides were rinsed in ddH_2O and then placed in ddH_2O for 10 minutes at room temperature. Finally, slides were dried by centrifugation at 500rpm for 5 minutes using a Beckman GS15R centrifuge (Beckman) and subsequently scanned using the Cy5 or Alexa 647 channel on the ScanArray Express array scanner (PerkinElmer Life Sciences, Boston MA).

VI.1.d Microarray hybridization

cDNA synthesis:

For each hybridization, 40μg of poly-ribosomal RNA from the control sample and the corresponding one-hour ozone treated tissue samples was used. Polyribosomal RNA was mixed with 2μL of amino-oligo dT (1μg/μL) in a final volume of 28μL. This mixture was heated for 10 minutes at 65°C. During this time a reverse transcription master mix was prepared (10μL 5X 1st strand buffer, 5μL 0.1M DTT, 1.5μL 10X amino allyl dNTP mix, 1.5μL RNaseOUT) and incubated at 42°C. Samples were allowed to cool at room temperature for 10 minutes then centrifuged for 1 minute. After centrifugation 18μL of the reverse transcription master mix was added per tube along with 2μL (200U/μL) Superscript II reverse transcriptase. Samples were allowed to incubate at 42°C for 1 hour. Samples were then centrifuged for 30 seconds and 2μL of fresh reverse transcriptase added, samples were again allowed to incubate at 42°C for 1 hour. After the second hour of incubation samples were centrifuged for 1 minute.

RNA hydrolysis and cDNA purification:

To each tube 10μL of 0.5M EDTA (pH8.0) and 10μL of freshly prepared 1M NaOH were added and incubated for 15 minutes at 65°C. After incubation, 10μL 1N HCl was added to neutralize the reaction.

To each sample 500μL of binding buffer PB from the Qiagen PCR purification kit was added and the solution applied to a Qiagen PCR purification column. Columns were centrifuged for 1 minute at 13,000rpm and the flow through discarded. To each column 750μL of 5mM phosphate (KPO_4) buffer was added and columns centrifuged for 1

minute at 13,000 rpm and flow through discarded. Columns were again centrifuged for 1 minute and then transferred to new 1.5mL collection tubes. For each column 50µL of phosphate elution buffer, pH 7.0, (4µL 1M phosphate (KPO_4) in a total volume of 200 µL) was added and allowed to sit for one to two minutes then centrifuged for 1 minute at 13,000rpm. This step was repeated resulting in a total volume of 100µL in the collection tube. Samples were then dried down in a SC110A SpeedVac Plus (ThermoSavant) on the medium heat setting and stored at -20°C over night.

Microarray slide pre-hybridization:

Slides were hydrated before usage using warm tap water in a humidifier chamber and snap dried on a 65°C heating block. The hydration and drying of the slides was repeated a total of three times after which slides were UV cross linked at 1800µJ x 100. After cross linking, slides were rinsed in 0.1% SDS for 30 seconds followed by autoclaved ddH_2O for 30 seconds and finally placed in 95% ethanol for 1 to 2 minutes. Slides were then dried by centrifugation at 500rpm for 5 minutes. After drying slides were then allowed to incubate at 42°C in pre-hybridization solution (5x SSC, 0.1% SDS, 1% BSA) until needed.

To each dried down cDNA sample 6.5µL of 0.2M $NaHCO_3$ pH 9.0 was added and allowed to sit for 10 minutes at room temperature. Then 6.5µL of appropriate NHS-ester Cy-dye was added and the reaction incubated for 1.5 hours at room temperature in the dark. Following incubation 6.5µL of 4M hydroxylamine was added to each tube and allowed to incubate for 15 minutes in the dark at room temperature. After incubation 35µL of 100mM sodium acetate pH 5.2 was added to each tube. To each tube 500µL of

binding buffer PB from the Qiagen PCR purification kit to the Cy3 labeled probe and then mixed with the Cy5 labeled probe. The combined probes were then loaded onto a Qiagen PCR purification column and spun at 13,000 rpm for 1 minute. After discarding the flow through 750µL wash buffer PE was added to the column and centrifuged at 13,000 rpm for 1 minute. After discarding the flow through tubes were centrifuged again at 13,000 rpm for 1 minute to remove all of buffer PE. After centrifugation columns were transferred to new 1.5mL tubes and 35µL of elution buffer EB was added. Columns were spun for 1 minute at 13,000 rpm and another 35µL of elution buffer EB added to the column. After centrifuging again for 1 minute at 13,000 rpm samples were dried down in a speed vac at room temperature.

Microarray slides were removed from the pre-hybridization solution and washed in autoclaved ddH_2O for 30 seconds. After washing in water, slides were washed in 95% ethanol for 30 seconds and spun dry by centrifugation at 500 rpm for 5 minutes. Microarray slides were placed in slide cassettes and 10µL of hybridization buffer added to each well. Cover slips were washed in 95% ethanol then rinsed 3 times in autoclaved ddH_2O and allowed to air dry before being applied to the microarray slide. The dried probes were resuspended in 45µL of hybridization buffer (50µL formamide, 25µL 20x SSC, 5µL 2% SDS, 5µL salmon sperm DNA, 15µL ddH_2O) and mixed well. Probes were then denatured by boiling for 2 minutes then centrifuged for 2 minutes. Probe was then applied to the slide underneath the cover slip and allowed to hybridize at 42°C over night.

After the overnight hybridization, slides were washed with rocking in a solution of 2x SSC and 0.1% SDS till the cover slips fell off. After cover slips fall off slides were

transferred to a new solution of 2x SSC and 0.1% SDS and washed with rocking for 5 minutes. Slides were then put though the following series of washes with rocking: 1x SSC for 3 minutes, 0.2x SSC for 1 minute and 40 seconds, and finally 0.05x SSC for 15 seconds. Slides were then dried by centrifugation at 500 rpm for 5 minutes and scanned using a ScanArray Express scanner (PerkinElmer Life Sciences).

VI.1.e Slide analysis

Scanned microarray slide images were loaded into the GenePix image analysis software (Molecular Devices) for analysis. The resulting analysis was then submitted to the GPAP 3.2 microarray analysis program for analysis. GPAP 3.2 pre-processing and normalization was performed using R-project statistical environment (http://www.r-project.org) and Bioconductor (http://www.bioconductor.org) through the GenePix AutoProcessor (GPAP) website (http://darwin.biochem.okstate.edu/gpap, Weng and Ayoubi, 2004).

VI.1.f MapMan analysis

Data from GPAP analysis was analyzed by the MapMan program using the Medicago 16k mapping file downloaded from the MapMan server.

VI.1.g Validation by Real Time PCR

The expression of select genes was validated using real time PCR. Reverse transcription was performed using superscript III reverse transcriptase (Invitrogen) as per manufacturer's instructions. Real time PCR was performed using Maxima qtPCR SYBER Green master mix (Fermentas) in a GeneAmp 7500 Real Time PCR machine

(GeneAmp) using the cycling profile of 95°C 10 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 40 seconds. Analysis of real time data used Chl A/B binding protein real time results as the standard to adjust for differences between samples. Primer sequences used were Chl A/B binding protein: F-GAAGGCCTCAGGAATGATGA, R-ACCGCAACCAACTCTGAGAC; WRKY: F-TGGATACCACAACGCAGGAAA, R-GTGTCTGGAAAAGACTGCTCAA; OSUunk106: F-CCAAGCATGTTCAATCAACC, R-GCTGCTTGTCCAAAACCTGT; Auxin regulated protein: F-GGAAATTGTGAATCCCAAGG, R-TGGGACATAGTCGGAGCTGT; Vesitone Reductase primers have been previously reported [90].

CHAPTER VI.2

FINDINGS

A gradient trace was generated during each run of the gradient fractionator for isolating the polyribosome fractions. This gradient trace (example shown in Figure 9) served as validation for the effectiveness of the poly-ribosome isolation technique. We were able to successfully identify the 80S peak as well as the poly-ribosomal peaks in generated traces based on the similarity of these profiles to other profiles in published literature, Figure 8&9.

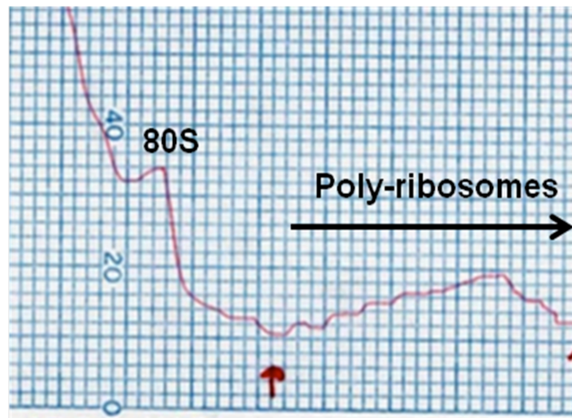

Figure 9: Example of a gradient trace isolated showing the 80S peak and poly-ribosome peaks. Vertical arrows represent the point on the trace where a change in fraction collection tubes took place.

In order to examine the effects of ozone stress on poly-ribosome loading we measured the area on the gradient traces comprising the 80S and monoribosome (NP) peaks and the area comprising the polyribosome (P) peaks. A minimum of eight and a maximum of 20 trace replicates were used to calculate the ratio of areas between the NP and P peaks. As the p-value from a student t-test shows in Table 1 it was highly unlikely that the NP to P ratios for control and ozone samples were from the same data set in JE154. However, this likelihood was fairly strong that no significant change in the area comprising the polyribosome fraction occurred in the Jemalong samples, Table 1. This ozone-induced decrease in the number of transcripts in the polyribosome fraction in JE154 was in agreement with other reports on plant stress [75, 76, 81, 82, 84, 85, 91]. The decrease in the area comprising the polyribosome peak was not a figment of *de-novo* synthesis of ribosomes as the average area comprising the NP area was relatively unaltered between control and ozone samples for both JE154 and Jemalong, Table 1.

Table 1: The average number of pixels contained under the 80S/monoribosome peak (NP) and polyribosome peak (P) and the ratio between NP and P in ozone treated, 1 hour, and control samples of JE154 and Jemalong.

JE154	Avg NP Area	Avg P Area	Ratio NP/P
1hr Control	5633	12034	0.47
1hr Ozone	5500	6909	0.80
p-value	0.391	0.006	0.0001

Jemalong	Avg NP Area	Avg P Area	Ratio NP/P
1hr Control	4105	6389	0.64
1hr Ozone	4249	5942	0.72
p-value	0.362	0.357	0.155

We used the polysomal RNA from control plants and ozone-treated samples in microarray experiments using the *M. truncatula* 17,000 oligonucleotide microarrays. Before microarray analysis could proceed we validated the usability of the *M. truncatula* microarray slides printed at the OSU microarray core facility by Syto-61 staining. Syto-61 staining confirmed that the oligonucleotide spotting quality was uniform and the slides represented a usable resource, Appendix Figure A2.

Microarray analysis compared one hour ozone treated and corresponding control polyribosome bound RNA samples for both JE154 and Jemalong. This experiment identified a greater number of differentially loaded transcripts in JE154 in comparison to Jemalong, Figure 10. This is presented in the form of an average M vs average A plot where M equals the log two transformed ratio of cy5 to cy3 ($\log_2(cy5/cy3)$) while A equals the half of the log two of the intensities ($0.5 \log_2(cy5*cy3)$), Figure 10. A total of 882 genes were differentially loaded in JE154 compared to a total of 574 in Jemalong. This suggests that in addition to a general repression of translation in JE154 there are also alterations to ribosomal loading of specific transcripts. If a general repression of translation were the only form of translational regulation we would have seen very little differences in transcript abundance between the polyribosome fractions of control and ozone samples. Jemalong also showed transcript specific alterations in ribosomal loading but to a lesser extent than JE154. However differential ribosome loading in Jemalong serves as a very important validation of translational regulation. The total number of differentially translated transcripts, 574, dwarfs the number of differentially transcribed transcripts we found previously, 61 [90]. If transcriptional regulation alone was responsible these numbers would be much closer to each other.

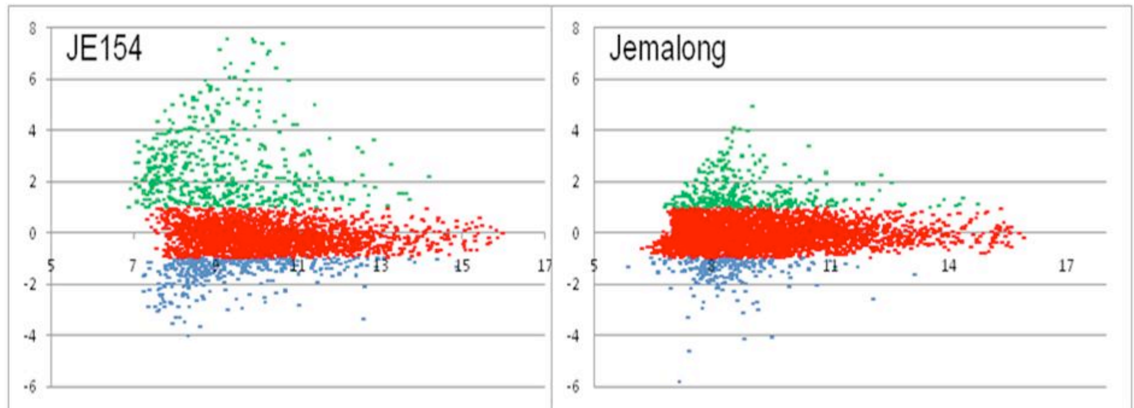

Figure 10: Average M vs Average A plots of microarray data from JE154 and Jemalong experiments. Average M on the Y-axis represents the magnitude of the response while average A on the X-axis represents the abundance of the transcript. Data points with an M value greater than 1.5, green, or less than -1.5, blue, represent differentially expressed genes.

Among transcripts with altered ribosomal loading were those associated with the phytohormones ET and ABA, Figure 11. Both JE154 and Jemalong show an up-regulation of genes associated with the ET response. However JE154, showed a larger number of genes (9 of 19 detected) with enhanced ribosomal loading than Jemalong (6 of 29 detected), Figure 11.

Genes associated with ABA showed a much more dramatic difference between the two accessions. In JE154, four of ten detected ABA responsive genes showed repressed ribosomal loading and only one gene, identified as an ABA responsive protein, had enhanced ribosomal loading, Figure 11. None of the genes associated with ABA showed altered ribosomal loading in Jemalong. Most notable of the transcripts which had

repressed ribosomal loading in JE154 was nine-cis-epoxycarotenoid dioxygenase. Nine-cis-epoxycarotenoid dioxygenase is the rate limiting step of ABA biosynthesis [92-94]. By repressing ribosomal loading of this rate limiting enzyme, JE154 could exert a major impact on ABA biosynthesis.

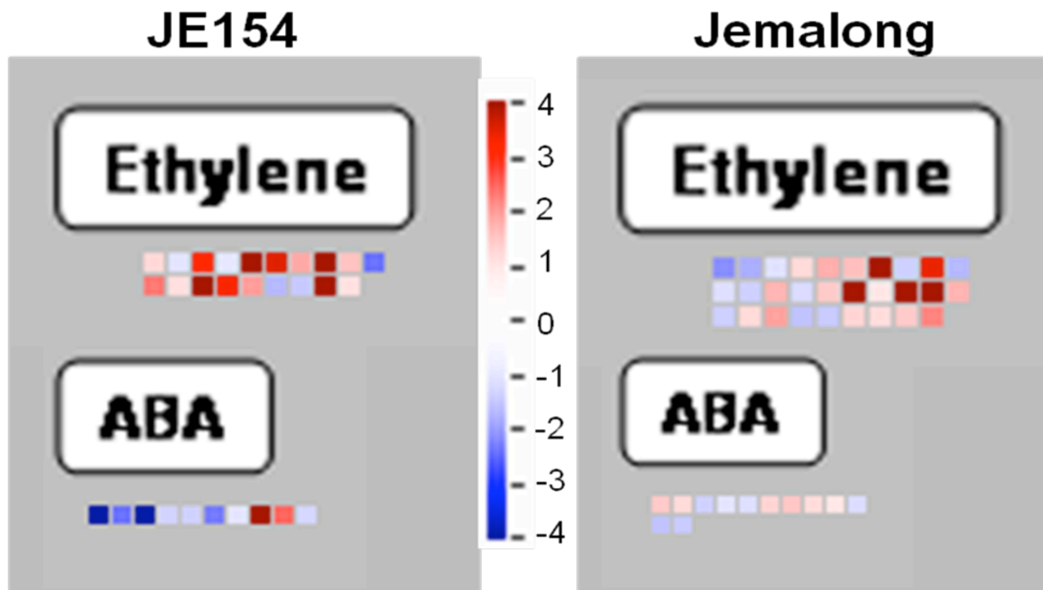

Figure 11: Change in expression of genes identified as involved in ethylene and abscisic acid (ABA) signaling for JE154 and Jemalong between control and treated polyribosome samples displayed as a heat map where the color is dependent upon the degree of differential expression. Red color indicates induction while blue color represents repression.

Ribosomal loading of transcripts associated with calcium regulation showed significant differences between JE154 and Jemalong, Figure 12. Despite repression of ribosomal loading for ABA associated genes there is significant enhancement of genes associated with calcium regulation in JE154, Figure 11 & 12. As previously mentioned

hydrogen peroxide is able to induce stomatal closure in both wild-type and ABA insensitive mutants, Figure 5 [62].

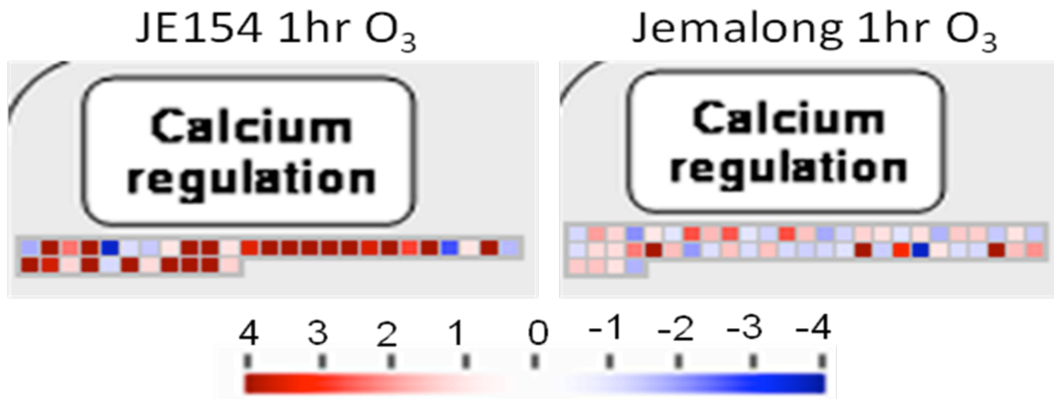

Figure 12: Heat map of genes associated with calcium regulation in JE154 and Jemalong in response to ozone.

An important finding from the transcriptome analysis with total RNA from Chapter IV was the differential expression of genes associated with the flavonoid pathway. The flavonoid pathway was also found to be the subject of differential translational regulation, Figure 13. Overall JE154 showed enhanced ribosomal loading for transcripts in the flavonoid pathway in response to ozone than Jemalong. Transcripts coding for chalcone synthase showed enhanced ribosomal loading in both JE154 and Jemalong, Figure 13. This enhancement was particularly punctuated in JE154 where all but one chalcone synthase had enhanced ribosomal loading. The resulting products of chalcone synthase, namely isoliquiritigenin and naringenin chalcone, are key metabolic feed-stocks for the rest of the flavonoid pathway. An enhancement of translation for the genes associated with their synthesis would provide a potentially key substrate resource for synthesis of other types of flavonoids.

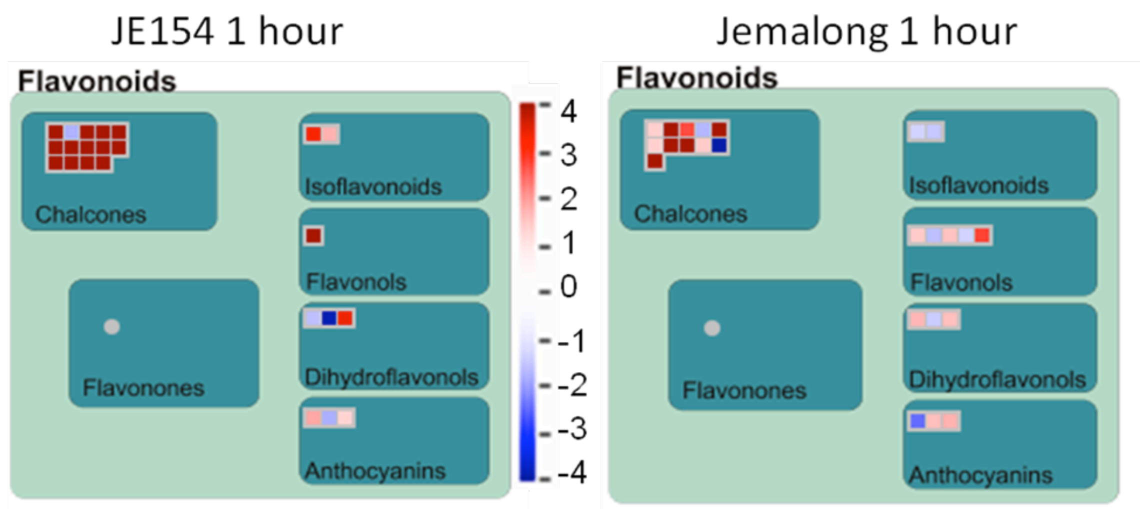

Figure 13: Heat map of genes associated with members of the flavonoid family of compounds for JE154 and Jemalong.

To confirm that observed differences were not dependent upon changes in transcriptional regulation a real time PCR was performed using total RNA, polyribosome RNA, and RNA isolated from the non-polyribosome fraction. Four genes were tested vesitone reductase, WRKY32, auxin-regulated protein, and OSUunk106. Vesitone reductase and WRKY32 had previously been identified as differentially expressed in

Table 2: Real Time PCR results for selected genes in Jemalong samples. NPF: Non-polyribosome fraction, P: Polyribosome fraction, NC: No Change.

	ID	Transcriptional Fold Change	Control NPF/Control P	Ozone NPF/Ozone P	Control NPF/Ozone NPF	Control P/Ozone P
Jemalong	Vesitone Reductase	6	4.5	6.7	3.1	5.3
	WRKY32	12.6	-2	4.3	7.4	13.8
	Auxin-Regulated Protein	NC	2.9	4.5	NC	NC
	OSUunk106	NC	2.5	NC	NC	NC

Table 3: Real Time PCR results for selected genes in JE154 samples. NPF: Non-polyribosome fraction, P: Polyribosome fraction, NC: No Change, ¥: previously reported in Puckette et al (2008), *: Transcript abundance was too low to be detected in control NPF samples.

JE154 ID	Transcriptional Fold Change	Control NPF/Control P	Ozone NPF/Ozone P	Control NPF/Ozone NPF	Control P/Ozone P
Vesitone Reductase	7.9 ¥	3	NC	10.1	7.1
WRKY32	157 ¥	*Higher in Control P	NC	*Higher in O3 NPF	13
Auxin-Regulated Protein	NC ¥	2.2	NC	NC	NC
OSUunk106	NC	NC	-3.5	1.9	-2.5

experiments using total RNA while auxin-regulated protein had previously been identified as having no change in transcript abundance [90]. OSUunk106 was a sequence generated in the SSH library construction that showed no homology to any existing *M. truncatula* ESTs. Results confirmed that translational regulation does occur, Tables 2 & 3. This is exemplified in Jemalong by WRKY32 which shows a change from having more transcripts in the non-polyribosome fraction to having more in the polyribosome fraction in response to ozone. In JE154 OSUunk106 shows signs of only translational regulation with a repression of ribosomal loading resulting in less transcripts in the polyribosome fraction in response to ozone in spite of no change in overall transcriptional expression, Table 3.

Differential translation for phytohormone related transcripts, calcium regulated transcripts, as well as transcripts involved in secondary metabolism show us that rapid changes in translational regulation can influence multiple aspects of signal transduction. Further these changes were more pronounced in the ozone-resistant JE154 compared with the sensitive Jemalong. This leads us to speculate that ozone, ROS, or a derivative from the reaction between ROS and the cell constituents is more rapidly perceived in JE154

and in turn triggers multiple signaling cascades. A lack of such early perception in Jemalong could contribute to its relatively passive response to ozone resulting in more severe cellular injuries.

CHAPTER VII

DISCUSSION

One of the most evident differences to ozone fumigation between JE154 and Jemalong is the speed of the response to ozone. At the one hour time point, JE154 showed massive differential transcriptional and translational regulation in comparison to a relatively inert response from Jemalong. This was also carried over into a general down-regulation of translation seen only in JE154, Table 1. This leads to the suggestion that a rapid and early response to ozone stress may be a key aspect of the resistant phenotype. By rapidly inducing changes in gene regulation JE154 is capable of inducing adaptations to help it cope with ozone stress. The general down regulation of translation coupled with the transcriptional and translational up-regulation of specific genes would allow JE154 to optimize usage of available resources for effectively combating the stress. Conversely a delayed initial response to ozone by Jemalong results in a massive oxidative damage to the cells.

Analysis of ROS levels within JE154 and Jemalong show that ozone is inducing a ROS build-up during treatment in both JE154 and Jemalong [90]. This buildup would mean that many of the proteins synthesized during ozone stress would subsequently be oxidatively damaged. This would represent a substantial drain of cell resources as these damaged proteins must be recycled and replaced. The general down regulation of translation observed in JE154 would minimize the amount of resources wasted and

provide a greater supply of cellular resources for an adaptive response or post-stress recovery.

ROS measurements for JE154 and Jemalong also showed innately higher levels of ROS in JE154 compared to Jemalong [95]. This innately higher level of ROS in control plants could represent a key aspect to JE154s resistance and might provide the mechanism by which JE154 is able to rapidly respond to ozone. An innately higher level of ROS in JE154 could serve to prime the plant for a rapid ozone response.

Table 4: Comparison of transcriptional regulation (change in the abundance of a transcript) with translational regulation (change in ribosomal loading) in JE154 in response to acute ozone.

JE154		Alterations in Ribosome Loading		
		Repressed	Enriched	No Difference
Alterations in RNA abundance	Down Regulated	16	0	19
	Up Regulated	1	266	28
	No Difference	316	192	1906
	Not Detected	41	34	149

An examination of individual genes found that translational regulation rarely negates transcriptional regulation. Out of the thousands of genes examined only one in JE154 and two in Jemalong showed a potential negation effect between transcriptional and translational regulation, Table 4 & 5. Interestingly, 508 genes in JE154 and 545 in Jemalong that showed changes in translational regulation showed no change in

transcriptional regulation, Table 4 & 5, and this is significantly larger than the number of genes that showed changes in both transcriptional and translational regulation.

Table 5: Comparison of transcriptional regulation (change in the abundance of a transcript) with translational regulation (change in ribosomal loading) in Jemalong in response to acute ozone treatment.

Jemalong		Alterations in Ribosome Loading		
		Repressed	Enriched	No Difference
Alterations in RNA abundance	Down Regulated	0	0	2
	Up Regulated	2	11	27
	No Difference	228	317	3323
	Not Detected	3	3	53

For JE154 examination of genes with differential translational regulation in Table 4 should be interpreted in the context of the overall repression of translation, Table 1. In order to perform microarray analysis concentrations of RNA are equalized so that equal volumes of RNA are applied for both ozone and control samples. Our results in Table 1 however tell us that in the case of JE154 this is not truly representative of physiology as fewer transcripts are being translated. This means that genes which show a repression of ribosomal loading are in effect receiving two forms of down regulation simultaneously. Not only are they being down-regulated by the overall decrease in translation but the ribosomal loading of these transcripts is also being selectively repressed.

The number of transcripts showing both down-regulation of transcription and selectively repressed ribosomal loading is only 16 in JE154. While this is a small number in comparison to the total number of transcripts examined one of these is nine-cis-epoxycarotenoid dioxygenase, the rate-limiting enzyme of ABA biosynthesis. This shows that JE154 is taking multiple regulatory actions to inhibit ABA biosynthesis. This seems somewhat contradictory as we see a large number of genes transcriptionally induced that are identified as responsive to ABA in the SSH library, Chapter V. As mentioned earlier, Chapter II, the answer to the apparent contradiction may lie in ozone-induced hydrogen peroxide bypassing ABA in the ABA-dependent stomatal closure signal transduction pathway. An ozone derived hydrogen peroxide burst could have the same effect as direct application of hydrogen peroxide [62]. Ozone dependent activation of the ABA signaling pathway would lead to many of the same down-stream effects as treatment with ABA including the differential expression of ABA-dependent genes and activation of calcium signaling pathways. Calcium is an important secondary messenger during plant stress signal transduction. As previously mentioned changes in calcium transporter activity is important in the closure of stomata guard cells, Figure 4a [54, 59, 60]. JE154's repression of ABA biosynthesis may be brought about by a feedback mechanism. The plant could be mistaking the sudden and rapid rise in ozone induced ROS as an ABA related event and is attempting to remedy what it perceives as an ABA response run rampant.

In JE154, transcripts with enriched ribosomal loading represent two possibilities. JE154 could be attempting to maintain a constant level of protein synthesis for a key transcript in the wake of a general down regulation of translation. The second possibility

is that JE154 is translationally upregulating a specific transcript to maximize the potential for protein synthesis. We surmise that maximization of translation for a given transcript is a greater probability for the 266 genes that are both up-regulated transcriptionally and also have enriched ribosomal loading, Table 4.

The flavonoid pathway genes showed substantial transcriptional up-regulation in JE154 across all three time points peaking at the six hour time point [90]. In microarray examination of transcriptional regulation one of the key genes up-regulated at the early one hour time point was chalcone synthase, Chapter IV. A chalcone synthase gene was also detected in the JE154 forward library, Chapter V. Examination of translational regulation showed flavonoid pathway transcripts having enriched ribosomal loading, particularly chalcone synthases, in JE154 at 1 hour, Figure 13. JE154 was maximizing the potential for protein synthesis of the flavonoid genes by not only increasing the number of transcripts available but also by ensuring that large numbers of them have multiple ribosomes loaded.

Translational regulation in Jemalong differs from that in JE154. Jemalong shows very little transcriptional regulation at the one hour time point [90]. Consequently there are very few genes which show both transcriptional and translational regulation. However, Jemalong has a much more profound translational response than transcriptional response to ozone stress. Since Jemalong does not show a general decrease in ribosomal loading, genes which show enrichment for ribosomal loading most likely have enhanced translation. The presence of translational regulation in the absence of transcriptional regulation in Jemalong supports the view that translational regulation is one of the earliest responses to stress [96]. More interesting, this data suggests a change in genes

specifically selected for differential ribosomal loading precedes any general repression of translation in response to stress.

CHAPTER VIII

CONCLUSIONS

Analysis of several biochemical and physiological parameters relevant to oxidative stress response across several *M. truncatula* accessions showing varying sensitivities to ozone led us to conclude that ozone resistance is a complex trait probably controlled by multiple genes. This leads to the speculation that perhaps sensitivity to ozone is the default and resistance the result of the cumulative effect of multiple traits. The spectrum of sensitivity seen among the screened *M. truncatula* ecotypes should represent the diversity of these traits. The more resistant ecotypes would have a larger number of resistance traits than the more sensitive ecotypes.

Chief among these traits is, most likely, the rapid response seen in JE154 at multiple levels of regulation. The root cause of this rapid response may lie in the differences in innate cellular redox balance between JE154 and Jemalong. Since JE154 and Jemalong have different levels of endogenous ROS [90, 95], there exists the potential that this would induce ozone resistant traits which result in innate differences in the proteome and metabolome. Without the presence of these ozone resistant traits innately higher levels of ROS may be ineffective as a predictor of resistance.

These differences could result in rapid responses to ozone stress in JE154 compared with sensitive Jemalong. It is possible that an ROS receptor could be present innately at higher concentrations in JE154 compared to Jemalong. Upon exposure to ozone derived ROS the receptor is activated resulting in the initiation of a signaling cascade. Triggering of the signaling cascade is directly related to the expression of the

receptor. The second potential mechanism involves translational control. An innately higher level of ROS could cause JE154 to have a pool of ROS responsive mRNAs transcribed and stored in P-bodies. Upon the reception of an appropriate signal these transcripts are then transferred out of P-bodies and onto ribosomes. In yeast such a transfer of mRNAs from P-bodies to ribosomes was found to occur within 30 minutes in response to glucose [72]. By having mRNAs pre-transcribed and stored in P-bodies we speculate JE154 is able to skip transcription of ozone responsive genes and proceed directly to translation. These mechanisms are not mutually exclusive and it is possible that a combination of both is taking place in JE154. Activation of an ROS receptor could be responsible for the initiation of translational regulation through movement of transcripts between P-bodies and ribosomal binding.

Our results examining transcriptional regulation over a time course found that while the overall responses of both JE154 and Jemalong were very similar, the timing of these responses differed. It is possible that this pattern is reflected in translational regulation as well. The translational response seen in Jemalong could be a mirror to an earlier JE154 response to precedes the 1 hour time point. It is also possible that innately higher levels of ROS in JE154 results in jump-starting ozone resistant traits and pathways rapidly.

This leads to the speculation that the initial responses to ozone in *M. truncatula* involve the detection of ozone derived ROS followed by a rapid alteration in ribosomal loading for certain genes, Figure 14. This initial selective translational regulation is followed by a general repression of translation. This does not mean that all genes are translationally repressed but rather that, in general, translation is repressed. Shortly after

or potentially simultaneously, the plant responds through transcriptional regulation. This transcriptional response is further fine-tuned by additional translational regulation. This allows the plant to enrich translation of key transcripts by providing a mechanism by which both the transcripts as well as the number of ribosomes loaded onto them are increased simultaneously.

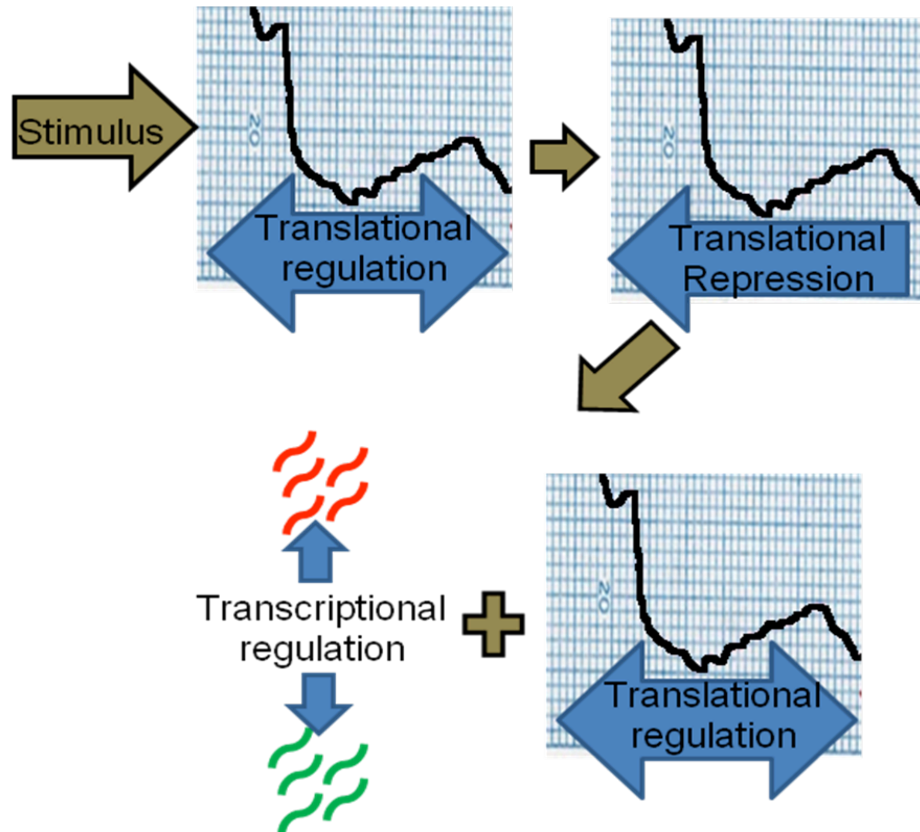

Figure 14: Proposed hierarchy of response to ozone induced stress. An initial response by translational regulation selectively alters key genes. This is followed by a general repression of translation. The final level of response involves the use of transcriptional regulation to adapt to the stress while translational regulation is used to fine-tune the transcriptional response.

Global alterations of transcription and translation provides important insight into the ozone response. However, the genes whose expression is being altered play critical roles in the phenotype displayed. Key players in the oxidative cell death cycle, phytohormones associated genes were found to be altered both transcriptionally and translationally. It was found that JE154 very rapidly transcriptionally up-regulates cell death containment mechanisms, specifically the phytohormone JA. If alterations in these genes lead to increases in JA levels it could potentially contribute to the rapid containment of lesions. This response was present in Jemalong but only at later time points. The potential delay of PCD containment mechanisms would give time for the lesions to spread.

Compounds with antioxidant properties such as key flavonoids could play a role in restoring redox balance or serve as protective conjugates to proteins similar to that of glutathione conjugation. A systematic metabolite analysis that includes measurements of various phytohormones in these two accessions will facilitate the integration of the metabolome, proteome, and transcriptome.

Using the microarray platform, this work sheds light into the advantages of integration of transcriptional and translational gene regulation and how some pathways may confer resistance to ozone in JE154. This work has identified several factors that can contribute to resistance, notably rapid overall differential regulation, the potential priming of JE154 for rapid response through innately higher levels of ROS, and the differential regulation of specific genes that facilitate resistance.

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X. APPENDICES

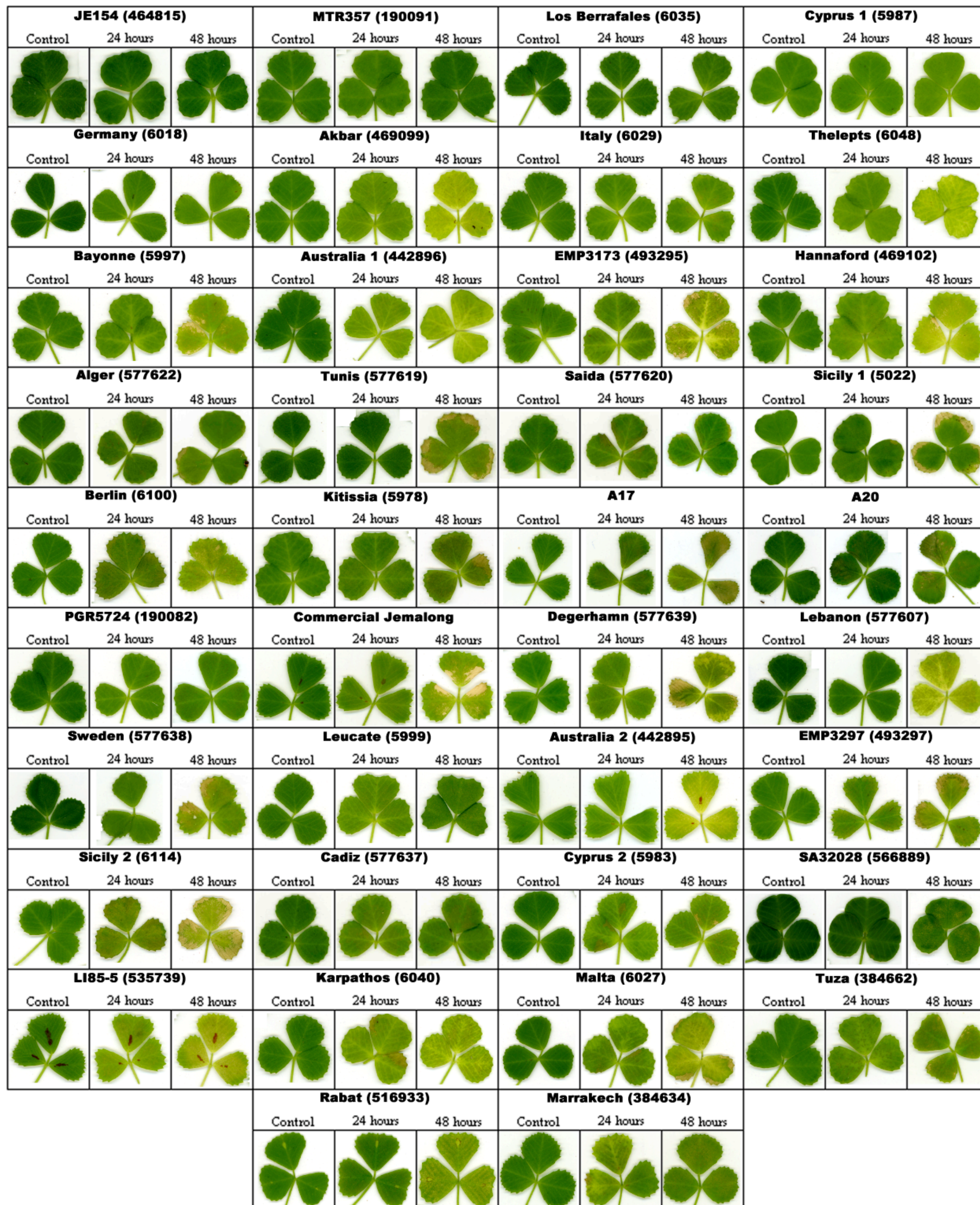

Figure A1: Leaf examples at 24 and 48 hours after the end of treatment

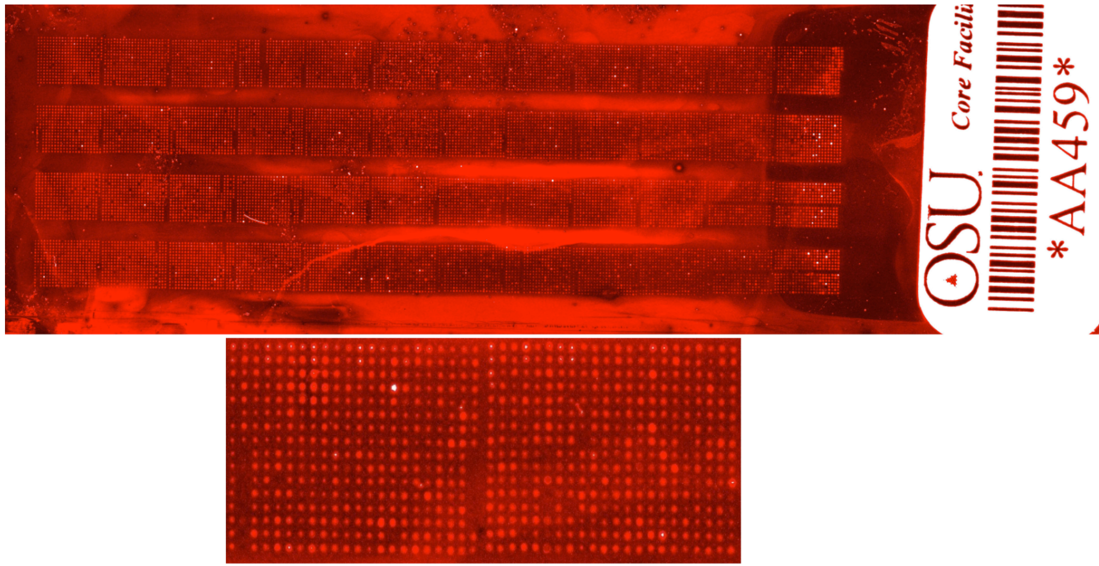

Figure A2: Syto-61 staining of microarray chips to ensure a successful printing.

X.1 APPENDICES: PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO ACUTE OZONE-INDCUED OXIDATIVE STRESS IN MEDICAGO TRUNCATULA

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Research article

Physiological and biochemical responses to acute ozone-induced oxidative stress in *Medicago truncatula*

Michael C. Puckette, Hua Weng, Ramamurthy Mahalingam*

Department of Biochemistry and Molecular Biology, Oklahoma State University, 246 Noble Research Center, Stillwater, OK 74078, USA

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Abstract

Oxidative signaling mediated by reactive oxygen species (ROS) is a central component of biotic and abiotic stresses in plants. Acute ozone (O_3) fumigation is a useful non-invasive treatment for eliciting endogenous ROS *in planta*. In this study, 38 different accessions of the model legume, *Medicago truncatula*, from various geographical regions were fumigated with 300 nmol mol^{-1} of O_3 for a period of six hours. Phenotypic symptoms were evaluated 24 and 48 h after the end of treatment. A majority of the accessions showed distinct visible damage. Eight accessions showing varying sensitivities to ozone were subjected to biochemical analysis to evaluate correlations between ozone damage and levels of ROS, antioxidants, and lipid peroxidation. Two-way analysis of variance indicated highly significant interactions between O_3 damage and levels of ROS, ascorbate, glutathione and lipid peroxidation. There were significant differences among the accessions for these traits before and after the end of O_3 fumigation, as indicated by equal variance Student's *t*-test. This study suggests that multiple physiological and biochemical mechanisms may govern O_3 tolerance or sensitivity. Surveying a large collection of germplasm led to identification of multiple resistant and sensitive lines for investigating molecular basis of O_3 phytotoxicity. The most resistant JE154 accession also showed enhanced tolerance to chronic O_3 and dehydration stress, suggesting germplasm with increased tolerance to acute O_3 can be a useful resource for improving resistance to multiple abiotic stressors.

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Keywords: Ascorbate; Glutathione; Lipid peroxidation; *Medicago truncatula*; Ozone; Oxidative stress; Peroxidation; Reactive oxygen species

1. Introduction

Tropospheric ozone (O_3) is the most abundant component of photochemical smog and is a major threat for crop plants and forest ecosystems [26,31]. Exposure of plants to high concentrations of O_3 results in visible injury due to collapse of mesophyll cells [34]. Ozone that enters plants through the stomata is rapidly broken down into various reactive oxygen species (ROS) at the cell-wall interface [30,36,52]. Two mechanisms of injury can be brought about by O_3 or ozone-induced ROS. The detrimental oxidation of lipids, proteins, and DNA [38,48], or by activation of programmed cell death pathway, similar to the pathogen-induced hypersensitive response [28,43,49,53]. It has been postulated that O_3 or ozone-induced ROS may locally cause necrotic cell death that in turn, can trigger the PCD pathway in surrounding tissues [29].

Susceptibility to O_3 varies from species to species and among cultivars within a species. Physiological basis for the variability of O_3 response among plant species is not clear. It has been shown that stomatal limitation of ozone uptake [6,44], metabolic differences in detoxification, and phytohormone levels are important factors governing plant responses to this pollutant [2,5,8,9,11,25,37,41,50,51].

Abbreviations: AA, reduced form of ascorbic acid; ANOVA, analysis of variance; DHA, dehydroascorbic acid; F_v, variable fluorescence; F_m, maximal fluorescence; GSH, reduced form of glutathione; GSSG, oxidized glutathione; MDA, malonialdehyde; O_3, ozone; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

* Corresponding author. Tel.: +1 405 744 6207; fax: +1 405 744 7799.
E-mail address: mali@biochem.okstate.edu (R. Mahalingam).

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The complex interactions of legumes with beneficial microorganisms, as well as pathogens, have led to the evolution of a plethora of unique natural product biosynthetic pathways, especially the isoflavonoid pathway, absent in other plant species [17,19,20,24]. Isoflavones like genistein exhibit estrogenic, antiangiogenic, antioxidant and anticancer properties [17,18]. *Medicago truncatula* is closely related to the commercially valuable forage legume, alfalfa, and has been chosen as a model species for genomics studies owing to its diploidy, small genome size, fast generation time and transformation efficiency [13]. The extensive biodiversity in *M. truncatula* is a valuable resource for identifying germplasm with enhanced tolerance to oxidative stress.

A few earlier studies of O_3 responses in Medicago were conducted using alfalfa cultivars and were focused on photosynthetic parameters and photoassimilate partitioning [14,45,46]. This study was aimed at examining 38 *M. truncatula* accessions collected from different geographical locations in order to: 1) assess their responses to acute ozone-induced oxidative stress; 2) examine the correlations between changes in quantifiable oxidative stress related biochemical markers and O_3 damage; and 3) test if lines showing extreme responses to acute O_3 followed similar response patterns to other abiotic stresses.

2. Results

2.1. Phenotypic analysis of ozone induced responses in *M. truncatula*

The 38 accessions selected for the O_3 screening experiment originated from various geographical locations (Table 1). A wide range of leaf morphologies, pigmentation patterns and differences in growth rates among these accessions were observed. Fifty days after planting all the accessions had sufficient foliage (at least 4–5 leaves per plant) to monitor the responses to O_3 fumigation and hence we decided to use plants of this age for this analysis.

The phenotypic symptoms of plants in response to acute O_3 treatment were monitored at 24 h and also at 48 h. These plants exhibited a vast array of symptoms that were of two major categories – chlorosis, and both chlorosis and necrosis. An accession from Turkey, JE154, was resistant to this high concentration of O_3 and showed the least visible damage. Chlorotic plants exhibited a range of symptoms – slight discoloration (MTR357, Los Berrafales, Cyprus 1, Italy 1, PGR5724), inter-veinal chlorosis (Australia 1, Rabat), patchy chlorosis (Thelepts, Akbar, Leucate, SA32028, Marrakech) and complete bleaching (Lebanon). A vast majority of the accessions showed both chlorosis and necrosis (Germany, Bayonne, EMP3173, Hannaford, Sicily 1, Saida, Tunis, Alger, Berlin, Kitissia, A17, A20, Degerhamn, Jemalong, Sweden, Australia 2, EMP3297, Cyprus 2, Cadiz, Sicily 2, LI85-5, Malta, Tuza, Karpathos). Some of these necrotic lesions were punctate and small in size, while in a few cases the lesions were present as patches on the leaves. A representative collection of the diverse phenotypes exhibited by different accessions in response to O_3 fumigation is shown in Fig. 1.

We arbitrarily classified the accessions into three groups based on the percentage of leaves showing visible symptoms. Quantitative analysis of the total number of leaves showing the symptoms from two independent rounds of screening indicated that the accession JE154 showed least damage (between 2–3%), followed by accessions Los Berrafales and MTR357 (Table 1). Ten accessions showed leaf damage that ranged between 25–50% and were considered as moderately resistant.

Table 1
Geographic origin and percent damage of *M. truncatula* accessions in response to ozone

Accession[a]	Name[b]	Country of origin	Damage (%)[c]	
			24 h	48 h
464815	**JE154**	**Turkey**	**2**	**3**
190091	MTR357	Australia	20	22
6035	**Los Berrafales**	**Canary Islands**	**15**	**25**
5987	Cyprus 1	Cyprus	24	37
6018	Germany	Germany	23	38
469099	Akbar	Australia	30	42
6029	Italy	Italy	27	43
6048	**Thelepts**	**Tunisia**	**38**	**43**
5997	Bayonne	France	32	44
442896	Australia 1	Australia	35	46
493295	EMP3173	Portugal	40	47
469102	Hannaford	Australia	28	47
577622	Alger	Algeria	48	49
577619	Tunis	Tunisia	46	51
577620	Saida	Algeria	39	53
5022	**Sicily 1**	**Sicily**	**33**	**54**
6100	Berlin	Germany	45	54
5978	Kitissia	Greece	36	56
A17[d]	A17	–	49	56
A20[d]	A20	–	59	56
190082	PGR5724	Australia	44	57
Jemalong	**Jemalong**	**Australia**	**55**	**57**
577639	Degerhamn	Sweden	33	58
577607	Lebanon	Lebanon	53	59
577638	Sweden	Sweden	52	60
6040	Karpathos	Greece	54	60
5999	Leucate	France	41	61
442895	Australia 2	Australia	55	62
493297	EMP3297	Portugal	59	63
6114	Sicily 2	Sicily	60	64
577637	Cadiz	Spain	55	64
5983	Cyprus 2	Cyprus	64	65
566889	SA32028	Turkey	57	65
535739	LI85-5	Libya	50	65
6027	Malta	Malta	53	67
384662	**Tuza**	**Morocco**	**60**	**69**
516933	**Rabat**	**Morocco**	**67**	**74**
384634	**Marrakech**	**Morocco**	**64**	**75**

[a] The accessions are arranged in order from most resistant to most sensitive based on damage, 48 h after the end of treatment. The accessions in bold were selected for physiological and biochemical analysis.
[b] Names refers to the identifiers for the accession numbers that are based on geographic origin or in some cases the common names assigned to the accessions.
[c] All the observations are based on averaged values from two independent replications.
[d] A17 and A20 are two mapping lines derived from Jemalong.

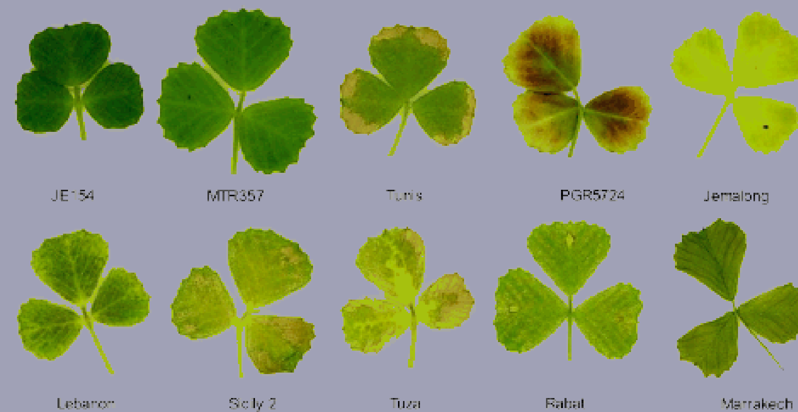

Fig. 1. Examples of ozone-induced phenotypes in *M. truncatula* accessions. A typical trifoliate leaf showing the most characteristic symptoms from each accession was scanned on a flatbed EPSON scanner, 48 h after the end of ozone fumigation (300 nmol mol^{-1} for six hours). The accessions are arranged in order of their relative ozone sensitivities, from the most resistant to most sensitive.

Nearly 65% (25) of the accessions screened suffered more than 50% leaf damage, 48 h after O_3 fumigation, suggesting that *M. truncatula* species in general is sensitive to oxidative stress.

2.2. *Physiological differences among M. truncatula accessions*

Based on the phenotypic responses to acute O_3 fumigation, eight accessions showing a range of sensitivities were selected – JE154, Los Berrafales, Thelepts, Sicily 1, Jemalong, Tuza, Rabat and Marrakech (Table 1). We measured stomatal conductance, transpiration and photosynthesis efficiency among these accessions grown under ambient ozone conditions to determine physiological basis underlying the differential responses to O_3. The most resistant JE154 had low stomatal conductance and transpiration while most sensitive Marrakech had high values for these traits (Fig. 2). The sensitive Tuza accession had a stomatal conductance and transpiration rate similar to that of JE154 and the moderately resistant Thelepts had extremely high values for these traits. This indicated that stomatal conductance can only partially explain ozone sensitivity among these accessions.

2.3. *Biochemical changes in response to ozone*

The eight selected accessions were subjected to biochemical assays to determine the levels of ROS, lipid peroxidation, and non-enzymatic antioxidants – total ascorbate, reduced ascorbate (AA), dehydroascorbate (DHA), total glutathione, reduced glutathione (GSH), and oxidized glutathione (GSSG) before and 24 h after O_3 fumigation.

Two-way analysis of variance to test the significance of interaction between the accessions and O_3 treatment indicated ROS levels, total ASA, AA, total glutathione, GSH, and GSSG were significant at $p < 0.001$. Levels of DHA and MDA were significant at $p < 0.01$.

2.3.1. *Reactive oxygen species*

Ozone-induced ROS burst is a common trait among many different plant species that are sensitive to O_3 [54]. Equal variance Student's *t*-test indicated that five of the eight accessions had significant differences in their ROS levels in response to O_3 treatment. One-way ANOVA analysis confirmed that mean ROS levels in accessions were significantly different. Ozone treatment further resulted in widening the differences in the mean ROS levels in these accessions (Table 2). Interestingly, the highest ROS levels were recorded for the most resistant JE154 before treatment. However, 24 h after fumigation the levels of ROS had dropped significantly in this accession. In the most sensitive accession, Marrakech, ROS levels were highest, 24 h after treatment (Table 2). This linear relationship between high ROS levels and increased O_3 sensitivity was tested by linear regression analysis and showed statistical significance ($p < 0.1$) (Table 4).

2.3.2. *Lipid peroxidation*

Equal variance *t*-test indicated that accessions Sicily 1, Tuza, and Marrakech exhibited significant differences in their lipid peroxidation levels before and following O_3 treatment. The changes were seen in the form of increased levels of peroxidation in these accessions following O_3 treatment. One-way ANOVA analysis indicated that the mean values of the lipid peroxidation were significantly different among the accessions and following O_3 treatment these differences were even more pronounced. Especially among the most sensitive accessions the increase in lipid peroxidation ranged from 2-fold (Rabat) to as high as 10-fold (Marrakech) (Table 2). Linear regression analysis indicated statistically significant

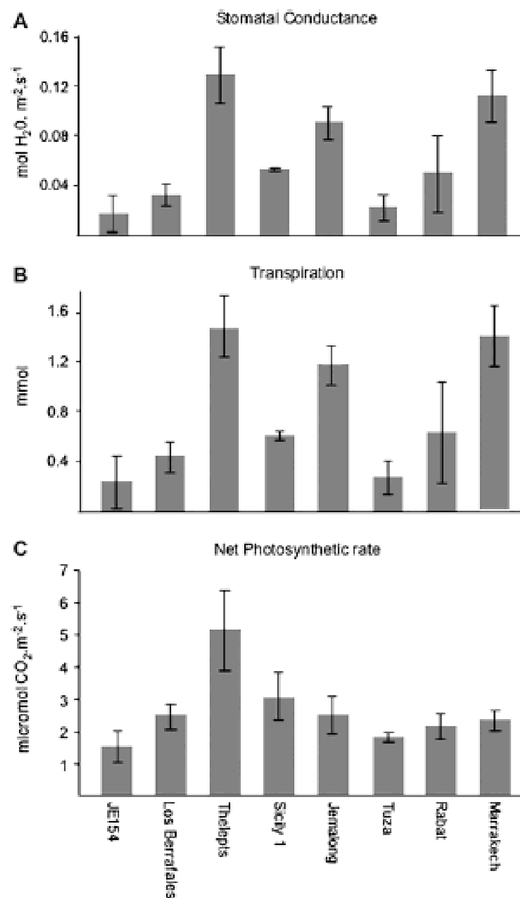

Fig. 2. Physiological analysis of *M. truncatula* accessions. A. Stomatal conductance. B. Transpiration rate. C. Net photosynthesis rates. Plants were grown and maintained under ambient ozone conditions. The accessions are arranged in order of their relative ozone sensitivities, from the most resistant to most sensitive.

correlation between O_3 sensitivity and lipid peroxidation ($p < 0.1$) (Table 4).

2.3.3. Antioxidants

Ascorbate and glutathione are the two major non-enzymatic antioxidants that are important for scavenging excess ROS in plants [39]. Total ascorbate, and its individual components, reduced and oxidized form of ascorbate, showed significant effects in response to O_3 treatment among the eight selected accessions. Equal variance *t*-test indicated that in seven accessions there were significant differences in the levels of total ascorbate before and 24 h after treatment (Table 3). One-way ANOVA confirmed that the levels of total ascorbate were significantly different among the eight accessions. Five accessions showed significant increase in total ascorbate levels following ozone fumigation, while one resistant accession (Los Berrafales) and a sensitive line (Marrakech) showed a significant decrease. Since reduced ascorbate is the actual scavenger of ROS we examined the changes of this metabolite more carefully. One-way ANOVA showed slight differences among the accessions (Table 3). However, following O_3 treatment the levels of AA showed pronounced differences among accessions. The resistant JE154 showed a 2-fold increase in the levels of reduced AA while the most sensitive Marrakech showed a decrease. However, the resistant Los Berrafales accession had lower levels of ascorbate while sensitive Jemalong had nearly 40% more ascorbate. This lack of correlation between AA levels and O_3 damage was also reflected in the linear regression analysis (Table 4). The ascorbate redox state did not show any significant correlation with the ozone damage index (data not shown).

Equal variance *t*-test indicated that four accessions JE154, Sicily 1, Jemalong, and Marrakech showed significant differences in total GSH before and 24 h after O_3 treatment. In the resistant JE154, increase in GSH and GSSG were significant, while in the sensitive Marrakech a 4-fold increase in GSSG levels were observed. In the accession Sicily 1, the levels of total GSH and reduced GSH were reduced by 50% following O_3 treatment. Changes in GSSG levels showed a statistically significant correlation with O_3 damage at $p < 0.1$ (Table 4). The glutathione redox state did not show any significant correlation with the ozone damage index (data not shown).

2.4. Responses of *M. truncatula* accessions to abiotic stressors

The resistant JE154 and sensitive Jemalong were analyzed for their responses to two different abiotic stressors, namely, chronic O_3 and dehydration stress. Exposure to chronic O_3 for a period of eight days clearly demonstrated that JE154 plants were resistant compared with Jemalong. At the end of the eight-day treatment, the number of leaves in Jemalong showing visible symptoms was more than 2-fold compared to JE154 (Fig. 3).

In JE154 accession, there was no apparent change in the F_v/F_m ratios in response to the dehydration stress. Significant decrease in the F_v/F_m ratios of Jemalong plants subjected to dehydration stress for a period of six days (Fig. 4A) supports the total collapse of these plants by the end of eight days of dehydration stress treatment. Moisture loss in sensitive jemalong was nearly three-fold higher compared to resistant JE154 (Fig. 4B). In JE154, the loss in moisture did not increase significantly between days 4 and 6, and was significantly lower compared to moisture loss observed in Jemalong (Fig. 4B).

3. Discussion

M. truncatula is native to the Mediterranean region, where the existence of numerous populations has provided an important resource for surveying phenotypic variation [3,4].

Table 2
Effects of ozone on ROS and lipid peroxidation levels in *M. truncatula* accessions

Accession[a]	Reactive oxygen species (RFU/mg of protein)[b]			Lipid peroxidation (μmol of MDA/g tissue)[b]		
	Control	O_3	p	Control	O_3	p[c]
JE154	2025 ± 143e	947 ± 174abc	***	1.51 ± 0.77a	2.41 ± 0.21a	NS
Los Berrafales	1251 ± 147bcd	1213 ± 247c	NS	0.79 ± 1.12a	0.66 ± 0a	NS
Thelepts	1631 ± 406d	867 ± 119a	*	3.28 ± 0bc	2.86 ± 1.27ab	NS
Sicily 1	1532 ± 84cd	1254 ± 182c	*	1.84 ± 0ab	2.83 ± 0.42a	¶
Jemalong	720 ± 83a	1182 ± 105bc	***	5.25 ± 2.04c	5.81 ± 1.173c	NS
Tuza	1192 ± 155bc	1042 ± 72abc	NS	1.75 ± 0.44a	3.74 ± 0.27b	*
Rabat	911 ± 82ab	842 ± 174a	NS	3.21 ± 1.06b	7.17 ± 2.51c	NS
Marrakech	1324 ± 651bcd	2836 ± 150d	**	0.39 ± 0.56a	5.17 ± 0.11bc	**
X	8.01	65.02		5.19	7.34	
P-Value	4.913e-05	4.66e-14		0.01686	0.005824	
Sig	***	***		*	**	
$LSD_{0.05}$	424.6	235.66		2.2629	2.5271	

¶ $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
[a] The accessions are arranged from the most ozone tolerant to most sensitive.
[b] Each value is the mean ± SE of three replications. Values within each column followed by the same letter were not significantly different using $LSD_{0.05}$ means analysis.
[c] The column heading p corresponds to significance of O_3 treatment effect with reference to the accession. NS, not significant.

Maximum variation in phenotypic responses to acute O_3 fumigation was observed among accessions from Mediterranean region (Table 1). Seven of the selected accessions from Australia, where it was spontaneously naturalized and bred as a fodder crop in ley-farming [15], showed varying levels of sensitivity to ozone. The identification of the most resistant accession from Turkey (JE154) and the most sensitive line from Morocco provides excellent biological material for initiating

Table 3
Effects of ozone on non-enzymatic antioxidants in *M. truncatula* accessions

Accessions[a]	Total ascorbate (μmol g^{-1})[b]			Reduced ascorbate (μmol g^{-1})[b]			Dehydroascorbate (μmol g^{-1})[b]		
	Control	O_3	p[c]	Control	O_3	p[c]	Control	O_3	p[c]
JE154	1.15 ± 0.01d	1.76 ± 0.03c	*	0.78 ± 0.00a	1.33 ± 0.05c	*	0.37 ± 0.01	0.43 ± 0.09c	NS
Los Berrafales	0.92 ± 0.02b	0.82 ± 0.01a	*	0.61 ± 0.02a	0.47 ± 0.01a		0.31 ± 0.04	0.35 ± 0.02bc	*
Thelepts	1.02 ± 0.02c	1.65 ± 0.09c	NS	0.69 ± 0.49a	1.67 ± 0.04d	NS	0.35 ± 0.49	0.01 ± 0.01a	NS
Sicily 1	1.45 ± 0.01f	1.29 ± 0.08b	NS	1.47 ± 0.02c	1.45 ± 0.09c	NS	0.00 ± 0.00	0.00 ± 0.00a	¶
Jemalong	1.15 ± 0.02d	2.03 ± 0.05d	¶	0.85 ± 0.04ab	1.16 ± 0.05b	*	0.30 ± 0.02	0.87 ± 0.10d	¶
Tuza	0.73 ± 0.03a	1.21 ± 0.03b	NS	0.68 ± 0.05a	1.04 ± 0.10b	NS	0.00 ± 0.00	0.18 ± 0.07ab	NS
Rabat	1.34 ± 0.02e	1.67 ± 0.02c	**	1.15 ± 0.06bc	1.37 ± 0.01c	¶	0.19 ± 0.07	0.29 ± 0.01	¶
Marrakech	1.63 ± 0.09g	1.23 ± 0.05b	NS	1.13 ± 0.01b	1.05 ± 0.12b	NS	0.50 ± 0.08	0.17 ± 0.16a	NS
X	115.85	103.17		5.58	53.21		1.96	25.77	
P-Value	2.095e-07	3.307e-07		0.01364	4.397e-06		0.1831	6.996e-05	
Significance	***	***		*	***		NS	***	
$LSD_{0.05}$	0.088112	0.1244		0.4124	0.1622		0.2020	0.18025	
Accessions[a]	**Total glutathione (nmol g^{-1})[b]**			**Reduced glutathione (nmol g^{-1})[b]**			**Oxidized glutathione (nmol g^{-1})[b]**		
	Control	O_3	p[c]	Control	O_3	p[c]	Control	O_3	p[c]
JE154	114 ± 3e	278 ± 9d	NS	105 ± 5e	236 ± 9c	NS	9 ± 1a	41 ± 0d	NS
Los Berrafales	92 ± 3cd	90 ± 16c	NS	76 ± 6c	72 ± 13b	NS	17 ± 3c	18 ± 3bc	NS
Thelepts	70 ± 1b	84 ± 3bc	*	60 ± 1b	73 ± 2b	¶	10 ± 0ab	11 ± 0a	NS
Sicily 1	107 ± 4de	55 ± 6a	NS	90 ± 4d	40 ± 4a	NS	17 ± 0c	16 ± 2ab	NS
Jemalong	78 ± 0bc	91 ± 1c	NS	66 ± 3bc	79 ± 1b	NS	13 ± 2b	13 ± 0a	NS
Tuza	67 ± 5b	89 ± 6c	¶	55 ± 6b	73 ± 5b	**	12 ± 0b	15 ± 1a	¶
Rabat	51 ± 16a	69 ± 3ab	*	39 ± 14a	52 ± 2a	***	112b ±	17 ± 6b	NS
Marrakech	71 ± 1b	95 ± 4c		64 ± 2b	72 ± 5b	NS	7 ± 0a	23 ± 0c	NS
X	21.84	174.08		21.04	182.90		9.33	32.11	
P-Value	0.0001296	4.19e-08		0.0001487	3.445e-08		0.002648	3.055e-05	
Significance	***	***		***	***		**	***	
$LSD_{0.05}$	15.03	17.41		14.7115	14.94		3.7863	5.5870	

¶ $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
[a] The accessions are arranged from the most ozone tolerant to most sensitive.
[b] Values within each column followed by the same letter were not significantly different using $LSD_{0.05}$ means analysis.
[c] The p column corresponding to each trait indicates significance of ozone treatment effect with reference to each accession. NS, not significant.

Table 4
Linear regression equations between O_3 damage and different physiological parameters in *M. truncatula* accessions

Parameter	Ozone damage (%)		
	Equation	*r*	*p*[a]
ROS	$Y = 0.020x - 0.934$	0.565	¶
Total ascorbate	$Y = -0.012x + 1.838$	−0.536	*
Lipid peroxidation	$Y = 0.053X - 0.216$	0.663	¶
AA	$Y = 0.078X + 9.46$	−0.196	NS
DHA	$Y = 0.006X + 1.502$	−0.222	NS
Total glutathione	$Y = -0.0004x + 1.273$	0.142	NS
GSH	$Y = -0.002x + 1.385$	0.129	NS
GSSG	$Y = 0.013x + 0.595$	0.539	¶

¶ $p < 0.1$; * $p < 0.05$.
[a] NS, not significant.

a genetic cross and developing a population for the purpose of mapping O_3 sensitivity trait. Since the commercial cultivar Jemalong and its derivative line A17 used for construction of high density linkage map [10], numerous EST libraries, sequencing [22] and large-scale mutagenesis [47] were also sensitive to O_3, these could also be used as a parental line for generating mapping population by crossing to JE154. Despite surveying 38 different accessions we identified only three accessions that can be considered as ozone-tolerant (Table 1), while most of them were moderately tolerant or O_3 sensitive. This supports earlier reports that legume species like soybeans, beans and peanuts are more sensitive to ozone-induced oxidative stress [1].

The primary screening aided in selecting eight accessions showing varying levels of O_3 damage for performing further analysis. Stomatal conductance in the plants maintained under ambient ozone conditions and sensitivity to acute ozone showed a good correlation for the most resistant and most

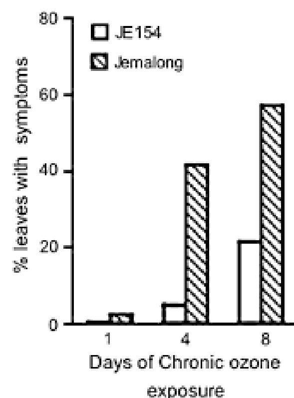

Fig. 3. Analysis of chronic ozone exposure on *M. truncatula* accessions. The acute-ozone resistant JE154 and sensitive Jemalong were subjected to 75 ppb of ozone for six hours every day for a period of eight days. The number of leaves showing visible symptoms (yellowing, necrosis) was recorded at the end of treatment of each day. Control plants maintained at ambient ozone conditions did not show any visible symptoms during the treatment period.

sensitive accessions (Fig. 2). The tolerant Thelepts accession had higher stomatal conductance, but also showed highest net photosynthesis rate that may be useful for counteracting the ozone damage. Such a mechanism has been previously reported for a common bean variety [25].

We chose to analyze the levels of ROS, lipid peroxidation, ascorbate and glutathione because each of these traits have been previously associated with oxidative stress [5,7,33,35,54]. We found an interesting inverse correlation between the levels of ROS in control plants and ozone sensitivity (Table 2). Accessions with higher levels of endogenous ROS were more tolerant compared to accessions with lower levels of ROS. This suggests that plants with higher ROS levels may be 'intrinsically primed' to tolerate extreme oxidative stress. This data also strengthens the role of ROS as a signaling molecule important during normal growth and stress conditions. Accessions with lower levels of ROS at the 24 h time point following O_3 treatment were in general more tolerant to ozone (JE154, Los Berrafales, Sicily 1 and Thelepts), while two of the four accessions classified as sensitive (Jemalong and Marrakech) showed significantly higher amounts of ROS (Tables 1 and 2). Linear regression between total ROS and lipid peroxidation was significant ($p < 0.1$, Table 4) confirming the reliability of these biochemical parameters of oxidative stress and provided a quantitative basis for supporting the subjective grading of ozone-induced leaf damage.

Statistical analysis were supportive for total ascorbate ($p < 0.05$), and GSSG levels at $p < 0.1$ (Table 4), implicating an important role for Ascorbate-Glutathione cycle [21,40] in determining the plant responses to O_3. AA is an important component of O_3 tolerance [9,12]. Arabidopsis mutants with lower ascorbate levels are extremely sensitive to O_3 [11]. When ROS values were examined in conjunction with antioxidants and O_3 damage we observed that in tolerant JE154, significant reduction in ROS level was accompanied by a significant increase in AA, GSH and GSSG levels (Tables 2 and 3) indicating the efficient ascorbate-glutathione cycle in maintaining the cell's redox balance. Though AsA and GSH levels showed a significant increase in Jemalong plants, it was probably not sufficient to counter the nearly two-fold increase in the ROS levels. In Marrakech, a significant doubling of ROS levels following O_3 exposure and a four-fold increase in GSSG levels suggests inefficiency of the AsA-GSH cycle leading to reduction in the levels of AA, ultimately resulting in its high O_3 sensitivity. The pattern of reduced levels of ROS and higher AA levels were observed in two other accessions (Tuza and Rabat), however, the plants were still sensitive. This suggested that increased ascorbate may not be the sole mechanism for countering ozone-induced oxidative stress [5,32]. The tolerant Los Berrafales accession identified in our screen showed a significant reduction in AA levels and no changes in GSH levels following O_3 fumigation suggesting a different mechanism for O_3 tolerance may be operating in this line. Analysis of the ascorbate and glutathione redox state did not show any significant correlations (data not shown).

Higher levels of tolerance to chronic ozone and dehydration stress in JE154 supports the view that plants capable of

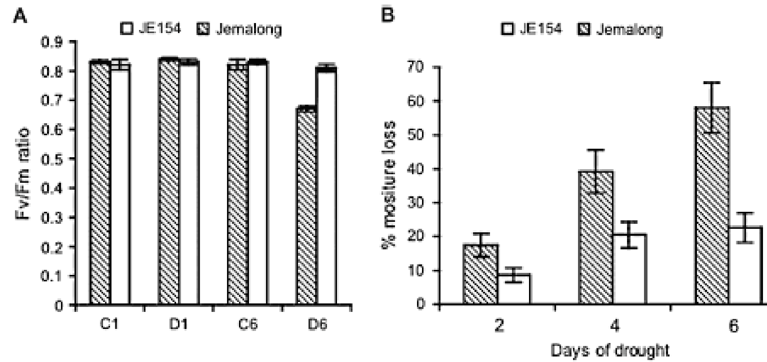

Fig. 4. Impact of dehydration stress on *M. truncatula* accessions. The acute-ozone resistant JE154 and sensitive Jemalong were subjected to a dehydration treatment by withholding watering for a period of eight days. Approximately 12 plants for each accession were used for dehydration treatment and 12 plants were maintained under control conditions. The F_v/F_m measurements are an average of 20 trifoliates, two trifoliates per plant for each day. A. The changes in F_v/F_m ratios were monitored every day during the dehydration regime. C1 – control plants at one day; D1 – one day after the start of dehydration treatment; C6 – control plants at day 6; D6 – six days after dehydration treatment. B. The weight of plants was monitored before and during dehydration treatment. Percentage of moisture lost was estimated based on the weights of the plants on day *x* and subtracting the values from the weights recorded on day *x*-1.

handling high levels of oxidative stress induced by acute O_3 can thrive well under sub-optimal environmental conditions. Determining the genes and metabolites that are differentially expressed in the resistant JE154 in conjunction with a sensitive line such as Marrakech or Jemalong should provide vital information about the molecular mechanisms during oxidative signaling. Identification and characterization of unique genes and gene products from accessions such as JE154 can provide a novel strategy for engineering crop plants with broad-based tolerance to multiple environmental stresses.

4. Materials and methods

4.1. Screening *M. truncatula* accessions for their ozone sensitivity

Thirty-eight different accessions of *Medicago truncatula* were used in this study. Thirty-six accessions were obtained from USDA ARS at Washington State University. Two mapping lines A17 and A20, and a commercial cultivar, Jemalong (Pogue Seed Company) were also tested. Seeds were planted in pots containing Metro Mix 200 soil (Scotts-Sierra Horticultural Products Company, Marysville, OH) and grown in a growth room under light source of 80 to 90 µmols s^{-1} m^{-2}, with 10 h of light and 14 h of darkness and ambient O_3 levels (25–30 nmol mol^{-1}) for fifty days. Plants were watered regularly with Peters Excel 15-5-15 fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH).

On the 50th day, 25 plants for each ecotype were fumigated with 300-nmol mol^{-1} of O_3 for six hours, from 10:00 AM to 4:00 PM, in an O_3 treatment chamber under a light intensity of around 80 µmols s^{-1} m^{-2}. Ozone was generated from an O_3 generator (Jelight Company Inc, Irvine CA) and was directed into an environmentally controlled and sealed growth chamber for the exposure. Levels of O_3 were monitored with an ozone monitor (Model 450, Advanced Pollution Instrumentation Inc, San Diego, CA). Same number of control plants was maintained at ambient O_3 levels (25–30 nmol mol^{-1}). At 24 and 48 h after the end of treatment, plants were examined for ozone-induced phenotypes. This included examining the individual leaflets on all the plants for any visible damage such as lesions, chlorosis, wilting, or total collapse. Two separate rounds of growth and O_3 treatment were performed for each ecotype and the results averaged. At least 200 trifoliate leaves were scored for each accession.

4.2. Stomatal conductance, transpiration and net photosynthesis measurements

Based on the results of phenotypic analysis following O_3 fumigation, eight accessions – JE154, Los Berrafales, Thelepts, Sicily 1, Jemalong, Tuza, Rabat and Marrakech were selected. Leaflets of 50-day-old plants from these accessions grown under ambient ozone conditions were sealed in a gas exchange cuvette of a Li-Cor 6400 Portable Photosynthesis system (Li-Cor, Lincoln, Nebraska). The following conditions were maintained during the measurements: leaf temperature, 24 °C,; CO_2 concentration, 350 µL L^{-1}; airflow, 0.1 L min^{-1}; white light illumination, 100 µmol m^{-2} sec^{-1}, RH, 34–35%. The plants had been exposed to the light conditions described above for 3–4 h before the beginning of the experiment. One leaflet was measured at a time. Measurements were taken five minutes after leaves were sealed in the cuvette under conditions described above, at which time the leaves had attained a steady state. Stomatal conductance, transpiration rates and net photosynthetic rates were recorded, using three different trifoliates from three different plants for each of the eight accessions.

4.3. Total ROS levels

All the assays were conducted 24 h after the end of ozone treatment. About 300 mg of frozen tissue was used for ROS measurements [27]. The leaf tissue was ground in liquid nitrogen and total proteins were extracted in 1 mL of Tris buffer (pH 7.2), centrifuged at 14,000 × g at 4 °C. About 800 µL of this extract were mixed with 1 mM 2′, 7′-dichlorodihydrofluroscein diacetate (Sigma Chemicals, St. Louis, USA) and incubated in the dark for 10 min at room temperature. Fluorescence was measured using a fluorometer (Versa Fluor model, Bio-Rad, Hercules, USA). Total protein amounts were quantified using Bradford dye (Bio-Rad, Hercules, USA). Data from three independent measurements were averaged and divided by the protein content and expressed as relative fluorescence units (RFU) per mg of protein.

4.4. Lipid peroxidation analysis

Lipid peroxidation was measured using a modified thiobarbituric acid (TBARS) method [16]. Approximately 200 mg of leaf tissue was homogenized in 4 mL of 0.1% TCA, centrifuged at 10,000 × g for 5 min. The supernatant (1 mL) was mixed with 2 mL of 20% TCA containing 2 mL of 0.5% TBA. The mixture was heated to 95 °C for 30 min and cooled on ice. After centrifugation at 10,000 × g for 10 min, the absorbance of the supernatant at 532 nm was recorded. Non-specific absorbance at 600 nm was measured and subtracted from the readings recorded at 532 nm. Concentration of malonaldehyde (MDA) was calculated using its extinction coefficient, 155 mM^{-1} cm^{-1}.

4.5. Antioxidant analysis

About 200 mg of leaf tissue was ground to a fine powder under liquid nitrogen. The powder was transferred to a tube containing 2 mL of 2% (w/v) meta-phosphoric acid and 2 mM EDTA, spun at 14,000 × g for 10 min at 4 °C. The supernatants were then split into two tubes, 900 µL each and neutralized with 600 µl of 10% sodium citrate. Total ascorbate was analyzed with 150 µL of neutralized extract in the presence of DTT by measuring the change in absorbance on a spectrophotometer (Model MBA 2000, Perkin Elmer) before and after adding the ascorbate oxidase enzyme [42]. Reduced ascorbate (AsA) was analyzed with 300 µL of neutralized extract (in the absence of DTT) by measuring the change in absorbance before and after adding the ascorbate oxidase enzyme [42]. DHA was calculated by subtracting reduced ascorbate values from total ascorbate.

A spectrophotometric assay was used [23] for measuring total glutathione and oxidized glutathione (GSSG). For determination of reduced glutathione (GSH), 250 µl of neutralized extract was mixed with 6.3 mM 5′, 5′-dithiobis-2-nitrobenzoic acid, 5 mM NADPH and 1 unit of glutathione reductase, incubated for six minutes and absorbance at 412 nm was recorded. For measuring GSSG, 600 µl of neutralized extract was mixed with 10 µl of 2-vinylpyridine and incubated at room temperature for one hour [23]. The other reaction components were the same as used for GSH. The GSH measurements were done using a UV-Vis spectrophotometer (Model MBA 2000, Perkin Elmer). The experiments were done in triplicates using the pooled leaflet samples from seven individual plants for each accession.

4.6. Statistical analysis

The R statistical package (http://www.r-project.org) was used for data analysis. Ozone damage refers to the percentage of damaged leaves following fumigation. Ozone damage data for the 38 accessions were pooled from two independent screening experiments and averaged results were subjected to arcsine transformation because of the large range in the values recorded for this trait. For ROS, lipid peroxidation, and antioxidant assays in the selected eight ecotypes, a 2 × 8 factorial experiment design, with two O_3 levels (ambient and elevated O_3) and eight ecotypes, was used. The following five statistical analyses were conducted.

1. Two-way ANOVA test was used for each assay to test interaction between O_3 levels (ambient and acute-ozone) and accessions.
2. If the interaction in (1) was significant, equal variance Student's t-test was used to test the means of control versus those after O_3 treatment for each ecotype by assay combination.
3. If the interaction in (1) was significant, a one-way ANOVA was conducted to test whether all the means of ecotypes in control O_3 level or elevated O_3 level were the same for each assay.
4. If the null hypothesis was rejected from one-way ANOVA in (3), Fisher's Least Significant Difference (F'LSD) test was used for ranking and pair-wise comparison among means for each assay by treatment combination.
5. To find the relationship between O_3 effect on each parameter, a linear regression $Y = a_0 + a_1X$ (Y is ROS, AA or GSH and X is O_3 damage) was tested on each assay.

4.7. Chronic ozone treatment

About 25 plants each of Jemalong and JE154 accessions were subjected to a chronic O_3 exposure of 75-nmol mol^{-1} for six hours per day for a period of eight days. Visual damage on the plants was analyzed at the end of the treatment of each day. F_v/F_m ratios were monitored during the course of treatment using a Fluorometer (Model OS-500, Opti Sciences Inc., Tygonsboro, MA).

4.8. Dehydration stress

Jemalong and JE154 (25 plants) were subjected to dehydration stress by withholding water for a period of eight days. During these eight days photosynthesis efficiency was monitored every day as described above and the number of leaves showing symptoms like yellowing, necrosis or withering were recorded. Amount of moisture lost from the plants

were determined by subtracting the dry weight of plants from their corresponding fresh weights.

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X.2 APPENDICES: TRANSCRIPTOMIC CHANGES INDUCED BY ACUTE OZONE IN RESISTANT AND SENSITIVE MEDICAGO TRUNCATULA ACCESSIONS

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Transcriptomic changes induced by acute ozone in resistant and sensitive *Medicago truncatula* accessions

Michael C Puckette[1], Yuhong Tang[2] and Ramamurthy Mahalingam*[1]

Address: [1]246 Noble Research Center, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK, USA and [2]The Samuel Roberts Noble Foundation Inc., Plant Biology Division, Ardmore, OK, USA

Email: Michael C Puckette - michael.puckette@okstate.edu; Yuhong Tang - ytang@noble.org; Ramamurthy Mahalingam* - ramamurthy.mahalingam@okstate.edu

* Corresponding author

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Abstract

Background: Tropospheric ozone, the most abundant air pollutant is detrimental to plant and animal health including humans. In sensitive plant species even a few hours of exposure to this potent oxidant (200–300 nL. L^{-1}) leads to severe oxidative stress that manifests as visible cell death. In resistant plants usually no visible symptoms are observed on exposure to similar ozone concentrations. Naturally occurring variability to acute ozone in plants provides a valuable resource for examining molecular basis of the differences in responses to ozone. From our earlier study in *Medicago truncatula*, we have identified cultivar Jemalong is ozone sensitive and PI 464815 (JE154) is an ozone-resistant accession. Analyses of transcriptome changes in ozone-sensitive and resistant accession will provide important clues for understanding the molecular changes governing the plant responses to ozone.

Results: Acute ozone treatment (300 nL L^{-1} for six hours) led to a reactive oxygen species (ROS) burst in sensitive Jemalong six hours post-fumigation. In resistant JE154 increase in ROS levels was much reduced compared to Jemalong. Based on the results of ROS profiling, time points for microarray analysis were one hour into the ozone treatment, end of treatment and onset of an ozone-induced ROS burst at 12 hours. Replicated temporal transcriptome analysis in these two accessions using 17 K oligonucleotide arrays revealed more than 2000 genes were differentially expressed. Significantly enriched gene ontologies (GOs) were identified using the Cluster Enrichment analysis program. A striking finding was the alacrity of JE154 in altering its gene expression patterns in response to ozone, in stark contrast to delayed transcriptional response of Jemalong. GOs involved in signaling, hormonal pathways, antioxidants and secondary metabolism were altered in both accessions. However, the repertoire of genes responding in each of these categories was different between the two accessions. Real-time PCR analysis confirmed the differential expression patterns of a subset of these genes.

Conclusion: This study provided a cogent view of the unique and shared transcriptional responses in an ozone-resistant and sensitive accession that exemplifies the complexity of oxidative signaling in plants. Based on this study, and supporting literature in Arabidopsis we speculate that plants sensitive to acute ozone are impaired in perception of the initial signals generated by the action of this oxidant. This in turn leads to a delayed transcriptional response in the ozone sensitive plants. In resistant plants rapid and sustained activation of several signaling pathways enables the deployment of multiple mechanisms for minimizing the toxicity effect of this reactive molecule.

Background

Ozone, a major component of smog in the troposphere, is hazardous to life due to its oxidative nature [1,2]. In ozone-sensitive plants acute exposure to this toxic pollutant leads to necrosis, caused by uncontrolled death of mesophyll cells [3,4]. In resistant plants of the same species there is no visible cell death on exposure to similar ozone concentrations. The physiological parameters especially stomatal conductance is regarded as an important factor determining plant sensitivity to ozone, since it determines how much ozone can enter into the cells [5,6]. However, several studies have indicated a poor correlation between ozone sensitivity and stomatal conductance in various plant species [7-9].

Ozone rapidly degrades into various ROS species at the cell wall interface [10-12]. A biochemical marker for plant sensitivity to ozone is an apoplastic ROS burst after ozone treatment, which is absent or reduced in ozone-resistant plants [12-14]. The role of ROS as a signaling molecule is gaining increasing attention [15-20]. Consequently, plant responses to ozone must be examined in the context that ROS can be cytotoxic at high concentrations and act as key signaling molecules at lower concentrations [21-24].

Alterations in ROS metabolism due to ozone will impact the overall plant redox status [24-26]. Changes in the antioxidants such as ascorbate and glutathione, key components of redox signaling, have been reported in response to ozone [4,8,27-34]. Given that apoplastic ascorbate forms the first line of defense against ozone, differences in ascorbate level have been suggested to be a major factor in determining plant resistance or sensitivity to ozone [35]. This is supported by Arabidopsis *vtc1* mutant, which has lowered ascorbate levels and is sensitive to ozone [29]. Since ascorbate is an important signal transducing molecule [36] extensive reprogramming of gene expression has been documented in plants with lower levels of this antioxidant [37]. Similarly, interaction of ozone with apoplastic ascorbate could lead to massive transcriptome changes.

The phytohormones salicylic acid (SA) and ethylene (ET) are important second messengers of oxidative stress signaling, involved in lesion initiation and propagation during ozone mediated cell death [5,38-44]. Jasmonic acid (JA) plays an important role in PCD containment by down regulating both SA and ET [40]. Arabidopsis ecotypes and mutants with higher intrinsic levels of ethylene have been reported to be sensitive to ozone [42,43]. Thus naturally occurring variability to ozone in various plant species may be due to intrinsic differences in phytohormone levels.

There are several reports examining changes in plant gene expression in response to ozone using microarrays [4,45-50]. Most of these studies were focused on either an ozone-tolerant or an ozone-sensitive line, or examined changes in gene expression at one time point. Cross-comparisons between these studies are hampered by the differences in ozone treatment conditions, microarray platforms and plant species. In this study we have performed time course analysis of changes in gene expression in response to acute ozone treatment using the ozone-sensitive Jemalong and ozone-resistant JE154 accession. Time points selected were one, six, and twelve hours after treatment initiation. We present the results of this microarray analysis and discuss important gene ontologies (GOs) that are unique to each *M. truncatula* accession and the possible roles of these genes in ozone/ROS signaling.

Results

ROS profiles in ozone treated M truncatula accessions

ROS analysis of both accessions showed "ozone-derived" ROS peaks at one hour during fumigation (Figure 1). Despite lower stomatal conductivity in JE154 compared with Jemalong [8] this data demonstrated that ozone entry and initial reactions were similar between the two accessions. A distinct "plant-derived" ROS burst was observed in Jemalong 12 hours after initiation of treatment (Figure 1). High levels of ROS were maintained in Jemalong up to 24 hours after treatment initiation. ROS levels in JE154 post-ozone fumigation were slightly higher than the corresponding controls but this was not statistically significant. An interesting observation was the innately higher levels of ROS in JE154 control plants compared to Jemalong. ROS profiling provided a basis for choosing time-points to conduct expression profiling. The one-hour time point was selected since ozone-generated ROS levels were significantly up in both accessions. The six-hour time point represented the end of ozone fumigation. The 12-hour time point marked the start of a sustained plant-derived ROS burst in Jemalong.

Ozone induced changes in gene expression patterns

Overall correlations of microarray data from the biological replicates ranged between 0.7–0.87. In JE154, 2102 genes were differentially expressed across all three-time points in response to ozone, while in Jemalong 2397 genes were classified as ozone-responsive [see Additional files 1, 2, 3, 4, 5, 6]. Though the total numbers of genes that were differentially expressed were similar between the two accessions, remarkable differences were observed at the three tested time points. The ratio-intensity plots showed the marked contrast between the two accessions in their temporal patterns of gene expression both in magnitude and direction (Figure 2). In Jemalong, the patterns of changes for both the up and down regulated genes were similar. Early during the ozone treatment there were few

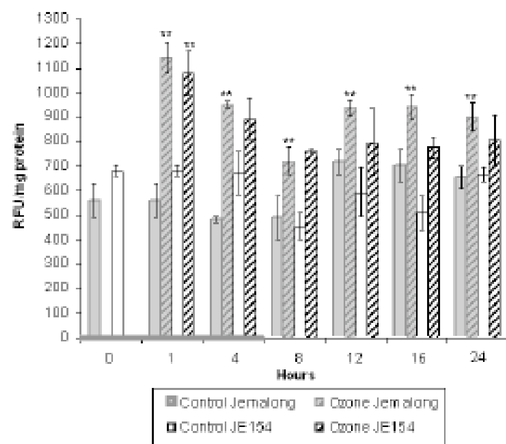

Figure 1
Total ROS levels in *M. truncatula* accessions. Leaf samples were taken at 0, 1, 4, 8, 12, 16, and 24 hours. Gray bar on x-axis represents ozone treatment for six hours. ROS levels are reported in Relative Fluorescence Units per mg protein. ** $P < 0.01$ for significance of total ROS change in ozone treated plants as determined by Student t-test.

changes, while maximal changes in transcriptome were observed at the end of treatment. Number of genes differentially expressed post-treatment was slightly lower compared to the six-hour time point (Figure 3). In JE154, the changes in number of induced genes was opposite to that in Jemalong, with maximal induced genes observed early in treatment and fewer changes at the end of ozone treatment. Like in Jemalong, the number of induced genes increased post-ozone treatment. These contrasting patterns for induced genes may be an important factor contributing to the differences in ozone related phenotypes between these accessions. In JE154 the number of down regulated genes was similar across the three time points (Figure 3).

Commonly differentially expressed genes between accessions were maximal at the end of treatment. A similar pattern was reported in a study of ozone responsive genes in sensitive and resistant pepper cultivars [46]. Despite larger numbers of induced and repressed genes at the 12 hour time point (in comparison with 6 h time point) in JE154, the overlap with the differentially expressed genes of Jemalong at this time point was very low (Figure 3). This suggested that post-ozone treatment different signaling pathways were altered in the two accessions and may be crucial for manifesting the ozone phenotype.

Gene ontologies of ozone responsive genes

In order to gain insight into the biological significance of genes differentially expressed between accessions we examined GOs using Cluster Enrichment Analysis (CLENCH) [51]. Non-overlapping GOs containing three or more genes are shown in Table 1. All the three levels of GO classification (Process, Function and Compartment) were important in identifying ozone responsive genes. In JE154, 17 GOs were identified as significant one hour into treatment (Table 1). At six hour there was 35 GOs that were significantly overrepresented in Jemalong. This was consistent with the fact that the largest number of differentially expressed genes was identified in this time point. At 12 hours in JE154, 14 unique GO's were upregulated and four were down regulated. In Jemalong the opposite pattern was observed at 12 hours, wherein 11 GOs were repressed and five were up regulated. Despite 33% more genes induced at 12 h in Jemalong compared with JE154, the number of identified GOs was very low, indicating an uncoordinated response in the sensitive plant. Four GOs in the set of induced genes (Response to wounding, response to SA, response to UV, multidrug transport) were common to all time-points in JE154, while none met this criterion in Jemalong. Five GOs in the set of induced genes (Defense response, Response to JA, Response to oxidative stress, Cellulose and pectin containing cell wall, ER) were common to two time points in JE154. Among genes repressed in Jemalong, the GO categories, response to JA and nutrient reservoir activity were enriched at six and 12 hours (Table 1).

GOs with contrasting responses in JE154 and Jemalong

Response to oxidative stress

Seven of the nine genes belonging to GO – response to oxidative stress, were oppositely regulated between the two accessions at the 12 h time point. This included HSP17.6, peroxidase73, calreticulin, isoflavone reductase, chalcone synthase, 2-alkenal reductase and SAC21, whose expression were up regulated in JE154 but were repressed in Jemalong. Induction of peroxidases and 2-alkenal reductase in JE154 indicates the resistant accession was pro-active in reducing oxidative damage. The down regulation of these genes in Jemalong could lead to the build up of ROS and subsequent oxidative cell death.

Response to SA and JA

CLENCH analysis identified several GOs associated with phytohormones in both JE154 and Jemalong. Significant up regulation of SA and JA responsive genes were observed at the one-hour time point only in JE154 (Figure 4). We speculate that JE154 may rapidly increase its SA levels due to higher intrinsic ROS compared with Jemalong (Figure 1). This is further supported by the observation that ROS levels in both accessions were similar one hour after ozone treatment, yet this GO did not

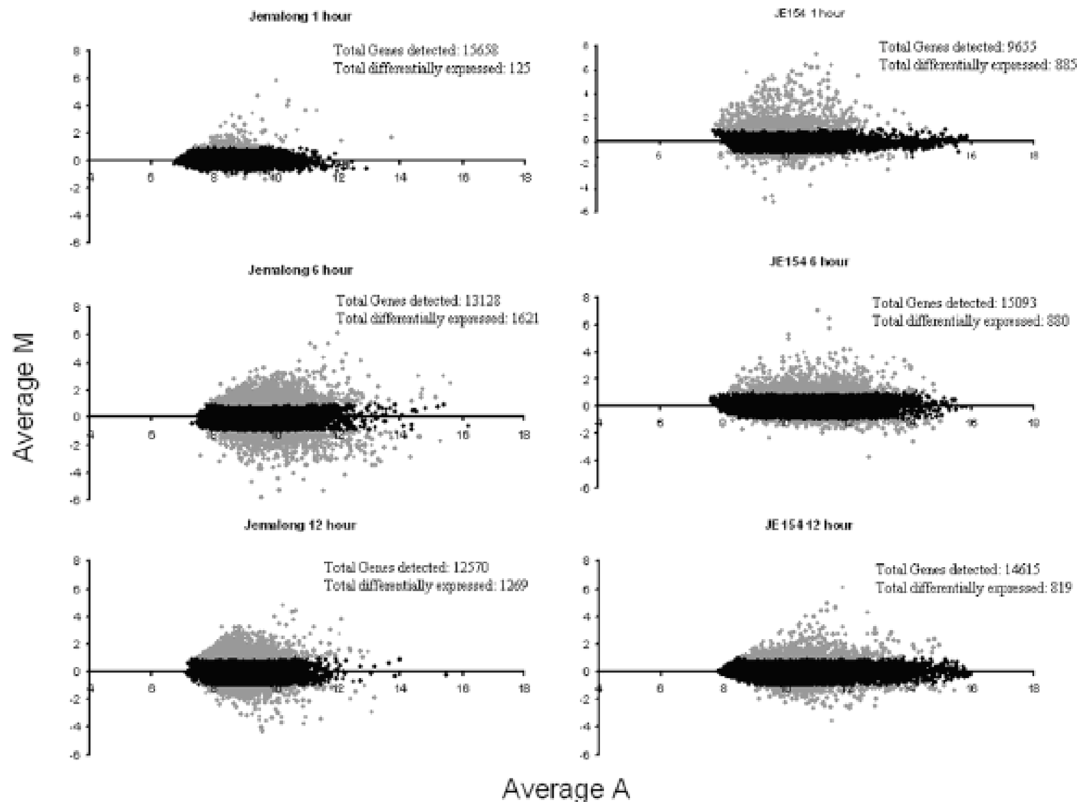

Figure 2
Ratio-Intensity plots of microarray data in *M. truncatula* accessions. The M (magnitude) value represents the log2 ratio of change in gene expression of ozone: control. The A (abundance) value represents the log10 value of the product of ozone and control background-corrected hybridization intensity values.

come up in Jemalong. It is also possible that higher levels of ROS in JE154 could lower the threshold level for SA-induced gene induction in this accession, since this GO was also up at six and 12 hour time points.

Genes associated with JA stimulus showed dramatic differences between accessions. JA responsive genes were up in JE154 at the one and 12-hour time points and were down regulated at the six-hour time point. Enrichment of JA biosynthesis genes at 12 hours in JE154 indicated that this phytohormone could be playing a protective role in containing ROS-induced cell death [52]. JA responsive genes were repressed in Jemalong at the six and 12-hour time points. Genes associated with ethylene signalling were down regulated at the 12 h time point in Jemalong. Thus in Jemalong, SA, JA and ethylene signalling pathways were repressed while in JE154, JA biosynthesis, and SA and JA pathway related genes were induced. The contrasting responses in the hormone signalling genes supports the view that phytohormones play an important role in determining plant responses to ozone [5,40,53].

Flavonoid Pathway

Increase in transcription of genes involved in flavonoid biosynthesis was observed in JE154 across all time points in response to ozone (Figure 5). Jemalong showed up regulation of flavonoid biosynthesis genes only at six hours. Further, levels of up regulation were less than that observed in JE154. We focused on identifying the subsets of flavonoid pathway that were differentially regulated

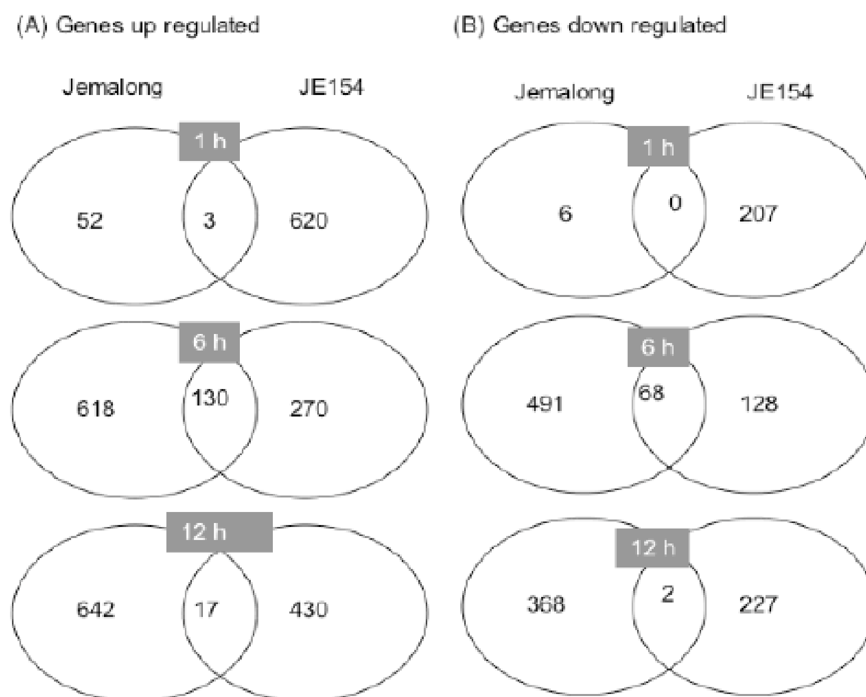

Figure 3
Venn diagram representation of differentially expressed acute ozone responsive genes at different time points in *M truncatula* accessions.

between the two accessions. At the one-hour time point, JE154 up regulated genes associated with pathways leading to medicarpin biosynthesis, while Jemalong down regulated expression of chalcone synthase homologues. At six hours both accessions up regulated a majority of the genes in flavonoid biosynthesis pathway (Figure 5). The notable difference was that JE154 had a greater degree of up regulation in several genes, most notably chalcone synthase homologues. Given the pivotal role of chalcone synthase to the entire pathway, a significant up regulation could eventually lead to a wider variety of flavonoid compounds [54,55].

At twelve hours, JE154 up regulated genes leading to medicarpin synthesis, as well as, dihydrokaempferol and dihydroquercetin (Figure 5). In addition, JE154 down regulated the enzyme flavonol synthase that uses dihydrokaempferol and dihydroquercetin as a substrate for flavonol synthesis [56,57]. With the up regulation of flavonoid-3-monooxygenase, the enzyme responsible for converting dihydrokaempferol to dihydroquercetin [58], at 12 hours in JE154, the data suggested a buildup of dihydroquercetin. In Jemalong we observed a general down regulation of the pathway particularly leading to medicarpin, and up regulation of leucoanthocyanidin dioxygenase homolog, involved in flavonol biosynthesis.

GOs unique to JE154

Isoprene biosynthesis

Isoprenoids have been shown to be important antioxidants in plants especially in response to ozone [59]. At the 12 hour time point in JE154 key genes in the isoprenoid biosynthesis pathway were strongly up regulated. Both cytosolic and plastidial pathway genes for isoprene bio-

Table 1: Significant gene ontology categories of ozone-responsive genes in *M truncatula* determined by Cluster Enrichment analysis

Gene Ontology		JE154			Jemalong		
	Cat.	1	6	12	1	6	12
Multidrug transport	P	+	+	+		+	
Response to SA	P	+	+	+			-
Response to UV	P	+	+	+			
Response to wounding	P	+	+	+		+/-	
Response to JA	P	+	-	+		-	-
Defense Response	P	+		+			
ATPase activity	F	+					
Calcium ion binding	F	+					
Calmodulin binding	F	+					
Hydrolase activity	F	+					
Kinase activity	F	+					
Sugar porter activity	F	+					
Transcriptional repressor	F	+					
Vacoular membrane	C	+					
Carboydrate metabolic process	P	-					
Transferase activity	F	-					
Translation initiation	P	-					
ER	C		+	+		+	
Response to oxidative stress	P		+	+		+	-
Cellulose and pectin containing cell wall	C		+	+			
Ubiquitination	P		+				
Chloroplast	C		-			-	
Peptidyl-pro-isomerase activity	F		-			-	
Photosynthesis	P		-			-	+
Response to light stimulus	P		-				
Cytosol	C		+			+	-
Phenylpropanoid biosynthesis	P		+			+	
Response to heat	P		+			+	
Senescence	P		+			+	
Copper ion binding	F		-			+	
Toxin catabolism	P		-			+	
AMP binding	F					+	
Cell wall constituent	F					+	
ESCRT III complex	C					+	
Lignin biosynthesis	P					+	
Peroxidase activity	F					+	-
Response to cold	P					+	
Response to fungus	P					+	
Response to salt stress	P					+	
Response to virus	P					+	
Peptidase activity	F			+		-	
Response to light	P			+		-	
Chlorophyll biosynthesis	P					-	
Embryonic development	P					-	
FK506 binding	F					-	
GA mediated signaling	P					-	
Glucan metabolic process	P					-	
Lipoxygenase activity	F					-	
Metal ion binding	F					-	+
Nutrient reservoir activity	F					-	-
Photorespiration	P					-	
Starch biosynthesis	P					-	
Flavonoid biosynthesis	P			+			
Isoprenoid biosynthesis	P			+			
JA biosynthesis	P			+			
Oligopeptide transport	P			+			
Protein folding	P			+			

Table 1: Significant gene ontology categories of ozone-responsive genes in *M truncatula* determined by Cluster Enrichment analysis

GO	Cat.	1	6	12
Sucrose biosynthesis	P		+	
UDP-glycosyltransferase activity	F		+	
Water deprivation	P		+	
Glutathione transferase activity	F		-	
Response to biotic stress	P		-	
Ca ion binding	F			-
Chromosome organization	P			+
Development	P			-
Light harvesting complex	C			+
Protein myristoylation	P			-
Response to ethylene	P			-
Ubiquitin thiolesterase activity	F			+
Unfolded protein binding	F			-

1,6 and 12 refers to the time points in hours at which the GO's were determined to be significant by CLENCH program. Only those GOs that had 3 or more genes are listed in this table.
Abbreviations: Cat. – GO category; P – process; F – function; C – compartment; + indicates that the genes belonging to that GO category were up regulated and - indicates genes in that GO category were down regulated.

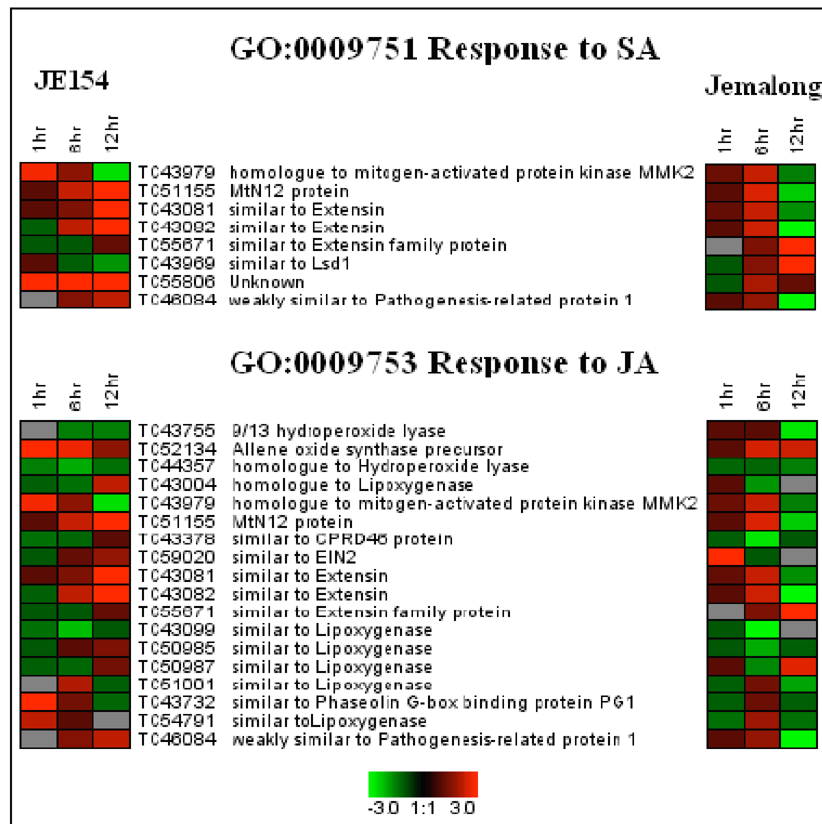

Figure 4
Heat map view of salicylic acid and jasmonic acid responsive genes differentially expressed in response to acute ozone in *M truncatula* accessions. Red indicates induction while green color represents repression of gene expression and black indicates no change in expression.

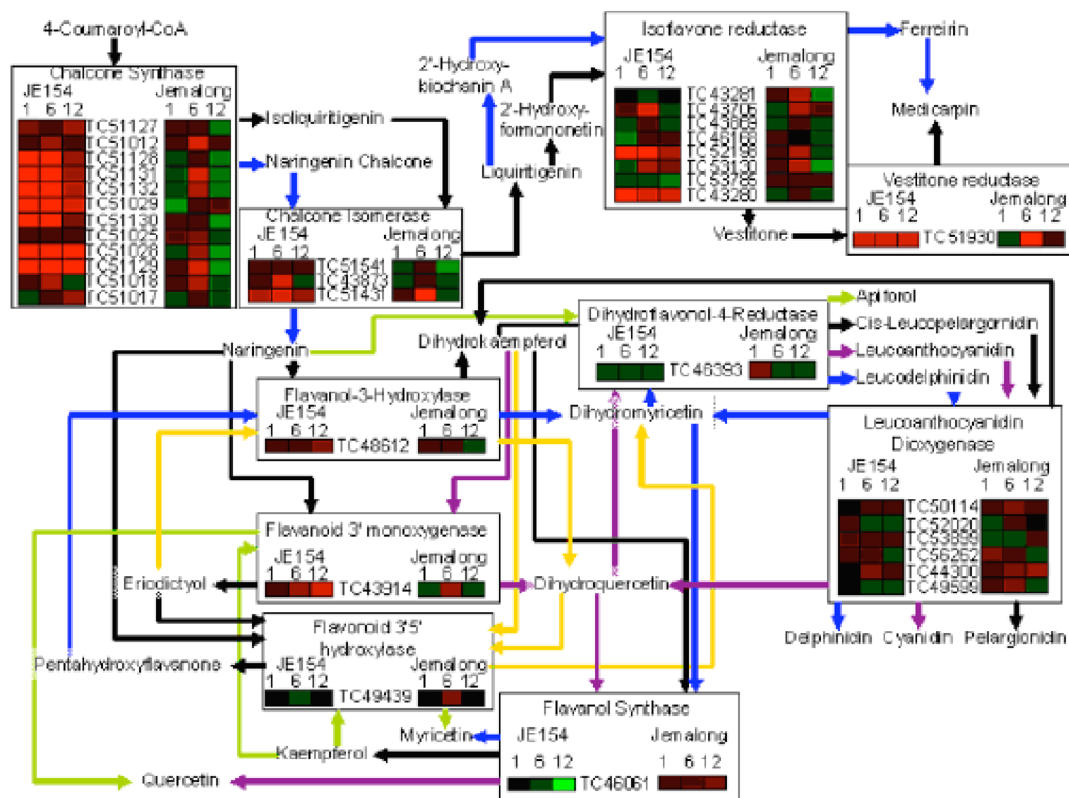

Figure 5

Representation of flavonoid pathway with cognate heat maps of genes from microarray analysis. For enzymes with multiple substrates and products, colored arrows connect substrates to their corresponding products.

synthesis are up regulated. The first committed enzyme in plastidial Deoxy-xylulose pathway, Deoxy-xylulose-5-phosphate reductase, and the penultimate enzyme, Isopentenyl-di-phosphate synthase, was up regulated. The first committed enzyme in cytosolic mavelonate pathway, 3-hydroxy-3-Methylglutaryl CoA reductase, was also up regulated. Activation of key genes involved in isoprene biosynthesis in both cytosolic and plastidial compartments suggests an important role for isoprenes in resistant response of JE154 to ozone.

GOs unique to Jemalong

Peroxidase activity

At the six-hour time point Jemalong up regulated six different peroxidases suggesting it was taking active measures to reduce the amount of ROS. Interestingly, this GO (comprising of five of the six genes) was down regulated at the 12-hour time point. Consistent with the down regulation of peroxidases, a concomitant increase in ROS levels were observed in Jemalong that was sustained up to 24 hours (Figure 1).

Ascorbic acid biosynthesis

At the six hour time point in Jemalong, two important genes in the ascorbic acid biosynthesis, GDP-mannose-1-phosphate guanyl transferase, and GDP-mannose epimerase, were down regulated. At the same time a copper ion binding protein encoding L-ascorbate oxidase was up regulated. In tobacco plants a homolog of this enzyme is localized to apoplast wherein it oxidizes ascorbate leading to enhanced ozone sensitivity [34,36]. We speculate that oxidation of apoplastic ascorbate due to higher levels of ROS and concomitant reduction in the biosynthesis of

ascorbate, may contribute to the increased ozone sensitivity in Jemalong.

Real-time PCR analysis

We examined the changes in gene expression of 15 genes using real-time PCR. Genes were selected based on the changes in gene expression at the 1, 6 or 12-hour time point. Genes unique to Jemalong or to JE154 were selected for this analysis. Four genes from jemalong and 11 genes from JE154 gave consistent results compared with the microarray data (Table 2).

Discussion

ROS bursts are common in plants in response to environmental perturbations and are generated utilizing multiple cellular processes [60-65]. Based on the similarity of the ROS profiles in response to ozone and avirulent pathogens, ozone has been described as a model abiotic elicitor of ROS in plants [66]. Previous work has shown that in ozone sensitive plants there is a plant produced ROS burst post ozone treatment not present in resistant plants of the same species [12-14,41]. Our studies confirm that there is a sustained ROS in sensitive *M truncatula* accession Jemalong, post-ozone treatment. There was a large variation in ROS levels observed at the 12 h time point in resistant JE154. This may be due to sporadic microscopic cell death previously reported in resistant accessions of other plant species [5,67].

Temporal transcriptome analysis of *M truncatula* accessions provided insights into differences in expression and type of genes that are important for ozone resistance or sensitivity. The massive transcriptome changes, especially the large number of transcription factors [see Additional table 7] and signaling genes differentially regulated early could assist JE154 in adapting to stress rapidly. Down regulation of translation initiation process could potentially allow JE154 to tweak its proteome to limit the extent of damage caused by ozone induced ROS (Table 1). On the contrary, a subdued transcriptional response in Jemalong at the early time point indicates a weakness in the perception and/or signaling of ozone-derived ROS, even though

Table 2: Real Time PCR analysis of select ozone responsive genes in *M truncatula*

Acc-time	Gene name	Primer sequences	RT FC/Array FC
Jemalong 1 h	Isoflavone Reductase	F-CTGCTAACCTCTTTGCTGGA	1.7/1.1
	TC52198	R-CCAAAGAGATGAACCTTGTC	
Jemalong 6 h	CA dehydrogenase	F-GGGTGAAAGTAGCAAAGGC	16.2/10.5
	TC51691	R-TGTCAGCTCCAAGATCCTCA	
	Vestitone Reductase	F-GAGCTGATCCAGAACGTAA	4.7/7.9
	TC51930	R-GTTGCTTAGATCGGCGTTG	
Jemalong 12 h	SOD	F-GCTGTGGCAGTTCTTGGTAA	3.1/4.1
	TC51579	R-ACAGTGGTTGGACCATTTCC	
JE154 1 h	Vestitone Reductase	F-GAGCTGATCCAGAACGTAA	7.2/4.0
	TC51930	R-GTTGCTTAGATCGGCGTTG	
	GST 14	F-CATCACTGCATGGATGACCA	10.3/11.6
	TC44882	R-CATGAAATGCCTCTCTGCGA	
	Chitinase	F-TTGTGATCCAGCCTCAAACG	1.9/1.5
	TC45276	R-ACCTCCGAGAGATAGCAAC	
JE154 6 h	Vestitone Reductase	F-GAGCTGATCCAGAACGTAA	9.4/3.6
	TC51930	R-GTTGCTTAGATCGGCGTTG	
	GST 14	F-CATCACTGCATGGATGACCA	3.2/10.1
	TC44882	R-CATGAAATGCCTCTCTGCGA	
	Chitinase	F-TTGTGATCCAGCCTCAAACG	6.8/4.2
	TC45276	R-ACCTCCGAGAGATAGCAAC	
JE154 12 h	Vestitone Reductase	F-GAGCTGATCCAGAACGTAA	6.0/4.0
	TC51930	R-GTTGCTTAGATCGGCGTTG	
	GST 14	F-CATCACTGCATGGATGACCA	7.0/3.2
	TC44882	R-CATGAAATGCCTCTCTGCGA	
	Chitinase	F-TTGTGATCCAGCCTCAAACG	18.8/20.3
	TC45276	R-ACCTCCGAGAGATAGCAAC	
	NAD Dehydrogenase	F-TTCGTTGGGACTGGTTCTTG	-3.6/-2.9
	TC47869	R-GCTCCCACGTAAATAAGTAG	
	Peroxidase 2	F-CTGGTGGTCCATCCTATACA	8.5/8.6
	TC43707	R-GAGGGAGCCTTCCATTTAC	

Abbreviations: Acc-time: accession name – time points. F and R in front of the primer sequences refer to the forward and reverse primers, respectively. CA – cinnamyl alcohol; SOD – superoxide dismutase. RT-FC refers to fold change observed in the real time PCR analysis. Array-FC refers to fold change observed in microarray analysis.

ROS levels were similar to that of JE154 at one hour (Figure 1).

We speculate that the early up regulation of kinase activity especially homologs of OXI1 kinase [68], MAPKKK [69] and several calcium/calmodulin dependent protein kinases facilitated reprogramming JE154 transcriptome towards defensive. The consistent up regulation of defense and hormone responsive genes in JE154 may contribute to the observed resistance to ozone. On the contrary, the down regulation of defense response genes and hormone responsive genes in Jemalong, especially at the 12 hour time point when the ROS levels are significantly high, could lead to its susceptibility to ozone induced oxidative stress.

Increased SA accumulation in Arabidopsis and tobacco plants is associated with ozone sensitivity [41,70]. SA potentiates the generation of ROS in photosynthetic tissues during stresses and ROS in turn triggers the production of SA in a feed-forward self-amplifying loop [41,70-72]. Recent studies have shown that in Arabidopsis plants pathogen-induced NADPH oxidase-derived ROS suppressed cell death and was dependent on SA [73]. It is tempting to speculate that in JE154 the ozone-induced ROS and SA could similarly be suppressing the cell death. Down regulation of peroxidase genes in Jemalong at 12-hour time point may lead to build up of ROS. Coupled with the repression of JA signaling pathway, this can potentially lead to spreading of cell death lesions in Jemalong.

Another aspect of the ozone-induced phenotype in Jemalong was extensive chlorosis. In a comparative study of ozone responses in several bean cultivars, high stomatal conductance accompanied by chlorophyll loss was a common feature of sensitive cultivars [74]. Higher stomatal conductance in Jemalong compared with JE154 [8], and down regulation of genes involved in chlorophyll biosynthesis at six hours in Jemalong supports the observed phenotype and is consistent with report of ozone sensitivity in bean cultivars.

Based on comparisons of microarray data, transcriptional responses to ozone and UV-B have been reported as similar [75]. This shared response may be attributable to the production of antioxidant isoflavonoids [76-78]. Given the importance of these legume natural products [79,80], we examined our microarray data focusing on the flavonoid pathway (Figure 5). Differential expression profiles of the flavonoid pathway at the 12 hour time point is vital in view of the differences in properties of dihydroquercitin and quercitin. Dihydroquercetin has been studied for its antioxidant properties in the animal system where it was able to act as an antioxidant with low toxicity [81-84]. It has also been found to inhibit lipid peroxidation in rats [82]. Since JE154 up regulated flavonoid-3-monoxygenase we speculate it may be able to lower the ROS levels at 12 hours through the antioxidant effects of dihydroquercetin. In addition, down regulation of flavonol synthase may aid in preventing buildup of quercetin. Interestingly, dihydroquercetin and quercetin differ in structure by a double bond, but have very different properties in response to cellular stress [85]. In human endothelial cells exposed to heat stress and chemical treatment, quercetin dramatically increased cell death while dihydroquercetin treatment resulted in cell death rates similar to those of control cells [85]. This suggested that the resistance to ozone in JE154 might be aided by its ability to tweak the flavonoid pathway for maximizing antioxidants promoting cell survival.

In JE154 the up regulation of isoprene biosynthetic pathway could be an important factor for limiting the ROS levels post-ozone fumigation. Isoprenes have strong antioxidant role in plants exposed to ozone and reduce lipid peroxidation of cellular membranes, especially the chloroplast membranes [59,86]. Thus the ozone resistance phenotype of JE154 can be attributed to its swiftness in responding to the stress and the diversity of defense, hormone and antioxidant genes it recruits. Initial tardy response to ozone in Jemalong, exacerbated by down regulation of ascorbate biosynthesis and hormone signaling pathways may be contributing to its sensitivity.

Conclusion

Naturally occurring genetic variation in M truncatula to acute ozone provides a useful resource for molecular genetic analysis of the oxidative signalling pathway. There were significant differences in the ROS levels in response to acute ozone fumigation between the sensitive Jemalong and resistant JE154. The ozone-generated ROS burst during the treatment was similar in both the accessions. However, the ozone–induced ROS burst, six hours after the end of the treatment were different between the two accessions. ROS profiling showed that similar to tobacco, in M truncatula the bi-phasic ROS burst in response to ozone is observed in resistant JE154 but not in the sensitive Jemalong. Secondly, significant differences in the physiologically relevant, in-planta induced ROS in these two accessions provided a compelling rationale for examining the differences in gene expression at this time point.

Using a ozone-resistant accession and a sensitive accession simultaneously for time-course expression profiling was extremely useful for understanding the temporal dynamics of changes in gene expression in response to ozone. The most striking finding was the seemingly naïve response in Jemalong at the early time point compared to massive changes in gene expression in JE154. We have

observed a similar response in the ozone-sensitive Ws-0 ecotype of Arabidopsis [4]. This suggests that in the resistant plants, ozone or ozone-induced signalling molecules are perceived rapidly, that in turn leads to activation of the down stream signalling events. A mapping based strategy using these two accessions will aid in the identification of such ozone resistance genes. Further, examination of pairs of ozone-resistant and sensitive lines in other plant species will aid in determining if ozone sensitivity is due to a passive response with reference to changes in gene expression.

In the resistant JE154, transcriptome analysis indicated early and sustained activation of multiple signalling pathways such as phytohormones, antioxidants, defense response and secondary metabolism. Induction of multiple signalling pathways has been reported in the ozone-resistant Columbia ecotype of Arabidopsis [47,49,50]. As suggested above, examination of pairs of resistant and sensitive lines in other plant species will assist in identifying a common set of ozone responsive genes that can be useful for screening germplasms of crop plants for introgressing ozone resistance trait in plant breeding programs.

Methods

Plant growth conditions

JE154 and Jemalong were selected for this study based on their divergent responses to ozone stress [8]. Plants were cultivated in Metro Mix 200 (Scotts-Sierra Horticultural Products Company, Marysville, OH) in a growth room under a light source of 100 μmols $s^{-1} m^{-2}$, with 10 hours of light and 14 hours of darkness and ambient O_3 levels (25–30 nmol mol^{-1}) for fifty days. Plants were maintained in 2-inch pots and each pot had 1–2 plants. Pots were placed on a plastic tray and were watered at 3–5 day intervals with Peters Excel 15-5-15 fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH).

Ozone treatment

Fifty-day-old plants of both JE154 and Jemalong were exposed to 300 nL L^{-1} of ozone for six hours, between 10:00 AM and 4:00 PM. Control plants were maintained in the growth room under ambient ozone conditions and identical lighting and temperature settings. Plants were selected randomly from three different pots at each time point. At least 3–4 trifoliates were sampled from each plant. Both young and old trifoliates were harvested at 0, 1, 4, 6, 8, 12, 16, and 24 hours after the start of treatment, frozen in liquid nitrogen, and stored at -80°C. Two biological replications were conducted and the leaf samples from corresponding time points were pooled for ROS analysis and microarray experiments.

ROS quantification

ROS were measured in control and ozone treated leaf samples using a Versa-Fluor Fluorometer (Bio-Rad) as previously described [8]. The experiment was repeated four times and the results averaged.

Microarrays and hybridizations

Microarray slides containing 70-mer oligonucleotide were obtained from the Samuel Roberts Noble Foundation and are described previously [87].

Trizol reagent (Invitrogen, Carlbad, CA) was used for total RNA isolation from leaf samples at one, six, and 12 hours after the start of ozone treatment. RNA quality was assessed by northern blot analysis using a GAPDH gene specific probe.

Samples were labeled with Cy-3 and Cy-5 dyes by amino-allyl labeling method [49]. In most of the hybridizations the control cDNA was labeled with Cy3 and the ozone-treated sample was labeled with Cy-5. For one technical replicate for each time point in both accessions, control cDNA was labeled with Cy5 and ozone treated sample was labeled with Cy-3 dye. Hybridizations were carried out overnight at 42°C. Slides were washed with 2 × SSC, 0.2% SDS for five minutes, followed by sequential washes in 1 × SSC for three minutes, 0.5 × SSC for two minutes, 1 × SSC for one minute and 0.05 × SSC for 15 seconds. The slides were dried by spinning in a centrifuge at 500 rpm for five minutes and were scanned in an array scanner (ScanArray Express, PerkinElmer Life Sciences, Boston, MA).

Microarray data analysis

A total of 19 microarray slides were used in this study. For Jemalong four biological replicates were conducted for the one and six-hour time points and three replicates for the 12-hour time point. There were three biological replicates of JE154 hybridizations for the one and six hour time points, and two replications for the 12-hour time points. Scanned images were analyzed using GenePix software (Molecular Devices, Toronto). Gridding was performed utilizing map files supplied by Noble Foundation (Ardmore, OK). Spots showing signal saturation on both dye channels were flagged and removed from analysis. Mean values of signal intensities were used. A background value of 300 was chosen based on the average fluorescence intensities in non-spotted areas of the slides considered for this analysis.

Pre-processed results from GenePix analysis were fed into GenePix auto processor (GPAP) [88], a web-based application utilizing R (language for statistical computing and graphics) and Bioconductor (freely available software for genomics data sets). Local LOWESS normalization was

used to balance the mean fluorescence intensities between the green and red channels. Genes showing a two or more fold change in gene expression (P-value < 0.05) were selected based on this program for further detailed analysis.

Cluster Enrichment analysis

CLENCH 2.0 [51] was used to analyze the microarray data for enriched gene ontology clusters. Since the *M. truncatula* genome is not as well characterized as *A. thaliana*, genes were examined by BLAST search for *A. thaliana* homologues and used in CLENCH analysis. The total gene list comprised of all the genes that were reliably detected on the microarray for each accession and at each time point. Statistically significant up and down regulated genes were analyzed separately in each accession for each time point. Slim-Terms (provided by TAIR), a coarser level view of the complete GO terms, was used in this analysis.

The template for the flavonoid pathway was retrieved from the KEGG database [89]. The pathway was modified and color-coded using Microsoft Paint program. The heat maps derived from the microarray analysis were generated using the Genesis program [90].

Real-Time PCR analysis

Total RNA isolated for microarray analysis was diluted to approximately 200 ng/uL. One microgram of this total RNA was used for cDNA synthesis, using Superscript reverse transcriptase (Invitrogen), carried out according to the manufacturer's instructions. A 1:10 and a 1:20 dilution of the cDNA were used for the real-time PCR analysis.

Up and down regulated genes in JE154 and Jemalong at different time points were selected for this analysis. Four genes that were equally expressed in both the lines were used as controls. Primers were designed using Primer express program (Applied Biosystems) to amplify 80–100 base pair fragment and the primers used are listed in the Table 2.

Real-time RT-PCR was performed using the iQ SYBR Green kit (Bio-Rad) as follows: for a 25 μL reaction, 12.5 μL of Master mix, 0.75 μL of 10 μM forward and reverse primers and 1 uL of cDNA template were added. Samples were run and analyzed using GeneAmp 7500 Real Time PCR machine using the cycling protocol 95°C 10 min followed by 95°C 15 sec, 52°C 20 sec, and 72°C 45 sec for 40 cycles.

Normalization was done with the control genes using the delta delta-Ct method. The experiment was repeated twice using different cDNA preparations and the average delta delta Ct values of the two replicates were plotted with standard deviations. In order to determine the delta Ct value, averaged Ct values of ozone treated samples were subtracted from the average Ct value of control samples. Fold change was computed by multiplying the delta Ct value by two. To adjust for differences in cDNA concentrations, real time PCR was performed on genes expressed similarly for each of the three time points. To adjust for differences in cDNA concentrations the fold change value generated from these experiments was then subtracted from the fold change of the samples. For Jemalong 1 hour, Jemalong 12 hour, and JE154 6 hour, gene TC56786 was used to establish differences in cDNA concentrations. Gene TC51792 was used to normalize differences in cDNA concentrations in Jemalong 6 hour. Gene TC46232 was used to normalize differences in cDNA concentrations in JE154, 1 hour. Finally an average of two genes, TC46973 and TC52875, which did not change in expression, was used to establish differences in cDNA concentrations in JE154 12 hour time point.

Authors' contributions

MCP conducted the ROS analysis, RNA isolations, ozone treatment, microarray hybridizations, data analysis, Real-time PCR and drafted the manuscript. YT provided the microarray slides, protocols for hybridizations and provided comments on the manuscript. RM designed the study, conducted the microarray hybridizations and edited the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1
Microarray data of JE154-1 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S1.xls]

Additional file 2
Microarray data of JE154-6 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S2.xls]

Additional file 3
Microarray data of JE154-12 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S3.xls]

Additional file 4

Microarray data of Jemalong-1 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S4.xls]

Additional file 5

Microarray data of Jemalong-6 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S5.xls]

Additional file 6

Microarray data of Jemalong-12 h time point. The M and A-values for each gene on the array for the multiple replicates are given. The average M and A-values from the multiple replicates and the associated p-values are also given.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2229-8-46-S6.xls]

Additional file 7

Ozone responsive transcription factor gene families in M truncatula.
Click here for file
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X.3 APPENDICES: OZONE RESPONSIVE GENES IN MEDICAGO TRUNCATULA: ANALYSIS BY SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Ozone responsive genes in *Medicago truncatula*: Analysis by suppression subtraction hybridization

Puckette M[1], Peal L[1], Steele J[2], Tang Y[2], Mahalingam R[1]

[1] 246C Noble Research Center, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA

[2] The Samuel Roberts Noble Foundation Incorporated, Plant Biology Division, Ardmore, OK 73401, USA

Ramamurthy Mahalingam (Corresponding Author)
Email: ramamurthy.mahalingam@okstate.edu
Phone: 405-744-6207
Fax: 405-744-7799

Michael Puckette
Email: michael.puckette@okstate.edu

Jarrod Steele
Email: jrsteele@noble.org

Lila Peal
Email: lila.peal@okstate.edu

Yuhong Tang
Email: ytang@noble.org

Summary

Acute ozone is a model abiotic elicitor of oxidative stress in plants. In order to identify genes that are important for conferring ozone resistance or sensitivity we used two accessions of *Medicago truncatula* with contrasting responses to this oxidant. We used suppression subtraction hybridization (SSH) to identify genes differentially expressed in ozone-sensitive Jemalong and ozone-resistant JE154 following exposure to 300 nl. L^{-1} of ozone for duration of six hours. Following differential screening of more than 2500 clones from four subtraction libraries, more than 800 clones were selected for sequencing. Sequence analysis identified 239 unique contigs. Using the Genevestigator program we compared the ozone responsive genes against genes differentially expressed in other stresses and mutants of Arabidopsis. Similarity between ozone response genes and those induced in response to abiotic stresses supports the view that ROS act as signaling molecules in coordinating the plant stress/defense responses. This digital northern analysis suggested that apart from ABA, SA, and ethylene, other phytohormones such as brassinosteroids, cytokinins, auxins and gibberllins play a role in determining plant responses to ozone. Enrichment of genes associated with carbon fixation in the reverse libraries and strong similarities of ozone induced genes with those responding to low nitrate indicates lowered carbon-nitrogen balance in plants subjected to oxidative stress.

Key words:

Gene expression, *Medicago truncatula*, ozone, phytohormones, suppression subtraction hybridization

Abbreviations:

ABA- abscisic acid, CK-cytokinins, ET-ethylene, GA-gibberellins, GO-gene ontology, PCD - programmed cell death hybridization, ROS-reactive oxygen species, SA-salicylic acid, SSH- Suppression subtraction hybridization

Introduction

Ozone is an atmospheric pollutant generated by the consumption of fossil fuels. Chronic exposures to low ozone concentration reduce photosynthesis efficiency and hence yield or biomass, while acute exposures produces symptoms similar to the hypersensitive response (HR) induced by pathogens (Kangasjarvi et al. 2005). The physiognomy, molecular and biochemical features of the ozone-induced lesions are similar to the HR cell death (Baier et al. 2005; Kangasjarvi et al. 2005; Rao and Davis 2001). Ozone enters the plant cell through open stomata and is rapidly converted into various reactive oxygen species (ROS) (Kanofsky and Sima 1995; Mehlhorn et al. 1990; Schraudner et al. 1998). In addition to ROS generated from ozone break down, sensitive plants exhibit a post-exposure burst of ROS (Pellinen et al. 1999; Puckette et al. 2008; Schraudner et al. 1998; Wohlgemuth et al. 2002). ROS play an important signaling role in numerous other stressors including drought (Xiong et al. 2002), ultraviolet radiation (Mackerness et al. 2001; Rao and Ormrod 1995a), pathogens (Doke 1983), excess light (Karpinski et al. 1999), metal toxicity (Schutzendubel and Polle 2002), low temperature (Prasad et al. 1994), salt stress (Hernandez et al. 1993), and wounding (Orozco-Cardenas and Ryan 1999). ROS regulate diverse signaling pathways including hormone signaling, defense, and programmed cell death (Apel and Hirt 2004; Desikan et al. 2004; Mahalingam and Fedoroff 2003; Miller et al. 2008).

Comparison of resistant and sensitive genotypes of plant species shows that phytohormones play a major role in determining the responses to ozone (Puckette et al. 2008; Rao and Davis 1999; Rao et al. 2000; Tamaoki et al. 2003; Vahala et al. 2003). Ozone intake through the stomata is probably the most important factor that determines plant resistance or sensitivity to this pollutant. Stomatal opening and closure are regulated by several factors including abscisic acid, ROS, nitric oxide, calcium fluxes (Hetherington and Woodward 2003; MacRobbie 1998; Murata et al. 2001) and anion channels (Pandey et al. 2007; Schroeder and Hagiwara 1989; Vahisalu et al. 2008). The phytohormones ethylene and salicylic acid are important for initiation and spread of lesions while jasmonic acid is important for lesion containment (Kangasjarvi et al. 2005; Overmyer et al. 2003). Intrinsic differences in these phytohormone levels among genotypes within a plant species may be an important factor contributing to ozone tolerance or sensitivity.

Few studies have compared the resistant and sensitive genotypes of plant species to examine the molecular changes in response to ozone (Lee and Yun 2006; Puckette et al. 2008).

We previously identified an ozone resistant accession, JE154, and an ozone sensitive, Jemalong, cultivar in the model legume *Medicago truncatula* (Puckette et al. 2007). Temporal transcriptome analysis in response to acute ozone in these two accessions showed massive rapid alterations in gene expression in resistant JE154, contrasting with delayed changes in gene expression in Jemalong (Puckette et al. 2008). Though this study gave an insight into how these two accessions differed in their transcriptional reprogramming in response to ozone, it is limited to only the genes present on the microarray slide. To complement this microarray analysis and to identify novel genes in *M. truncatula*, we undertook an open-ended analysis using the suppression subtraction hybridization (SSH) technique to identify ozone responsive genes in these two accessions. We have conducted digital northern analysis to examine the overlap in ozone response genes with the genes induced in response to other stresses and mutants in Arabidopsis. This analysis led to the identification of several new phytohormones and nutrient signals that impinge upon the oxidative signaling pathway.

Materials and Methods

Plant material and ozone treatments

Seeds for the ozone-sensitive Jemalong cultivar and ozone-resistant JE154 accession of *M. truncatula* (Puckette et al. 2007) were obtained from the Pogue seed company and the USDA ARS at Washington State University, respectively. Plants were grown for about eight weeks, under conditions previously described (Puckette et al. 2007). Approximately 50 days-old (eighth week) plants were exposed to 300 nL. L^{-1} of ozone, for six hours. Leaf samples were collected at four time points, 1 h after treatment initiation, 1h after the end of ozone treatment, 15 h after the end of treatment, and 24 h after the end of treatment. Leaf samples from control plants maintained under ambient ozone conditions were simultaneously harvested for each of the four time points.

RNA isolations

Samples were homogenized in liquid nitrogen and total RNA was isolated using Trizol (Invitrogen) as previously described (Puckette et al. 2008). Messenger RNA was isolated using the Oligotex mRNA midi kit (Qiagen) following the instructions in the manual. The quality of

the mRNA was assessed by traditional northern blot analysis using the GAPDH probe (data not shown).

Suppression Subtraction Hybridization

Four different libraries were constructed. For the two forward libraries, Jemalong and JE154, mRNA from the ozone treated plants were used as the tester and mRNA from control plants served as driver. For the reverse libraries, mRNA from control plants were used as tester while mRNA from ozone-treated plants served as driver.

Equal concentrations of the mRNA from the four time points were pooled together to construct subtraction libraries using the Suppression Subtraction Hybridization (SSH) library kit (Clontech) as described in kit instructions with minor modifications. About 2.5-5 µg of mRNA was used for each of the libraries. Validation of subtraction efficiency was done using primers specific to *M truncatula* GAPDH in a PCR containing 16µl water, 2.5µL 10x PCR buffer, 2µL 2.5mM dNTP, 1.2µl $MgCl_2$, 1µL 10µM GAPDH-F (GATCCTTTCAGCACCACTGA), 1µL 10µM GAPDH-R (CCTTCAACAATGCCAAATCT), 1µL diluted template, 0.5µL Taq polymerase (Takara). PCR was performed in an Eppendorf Mastercycler (Eppendorf) using the profile 94° C 30s followed by 33 cycles of 94 ° C for 30s, 52 ° C for 1min and 72 ° C for 2 min.

Cloning and PCR

PCR amplified subtracted cDNAs were ligated into either a T/A vector (Invitrogen) or a pGEM-T Easy vector (Promega) and cloned into DH5-alpha *E. Coli* cells. Ligation reactions were performed according to manufacturer's instructions. After overnight growth on LB-Ampicillin-X-gal plates white colonies were picked from the plates using sterile toothpicks and placed into 96-well plates containing LB ampicillin and grown overnight. About 10µl of culture colonies were diluted in 100ul of nano-pure water and used as template for amplification in 96 well PCR plates. PCR took place in a total volume of 10µL using 1µL of 10x PCR buffer (0.1M Tris pH 8.0, 0.5M KCl, 25mM $MgCl_2$), 1µL 2.5mM dNTPs, 1.3µL 2.5µM Nested Primer 1 (TCGAGCGGCCGCCCGGGCAGGT), 1.3µL 2.5µM Nested Primer 2R (AGCGTGGTCGCGGCCGAGGT), 0.2µL Taq Polymerase, and 5.2µL autoclaved distilled water. PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research Inc) using the profile 94° C for 5 min followed by 20 cycles of 94 ° C for 30s and 68 ° C for 1min and

a final extension at 72 ° C for 5 min. The PCR products were analyzed on 1% TAE-agarose gels. More than 100 colonies that gave two different amplification products that could be separated under these electrophoresis conditions were re-plated and screened again to identify colonies that gave single discrete amplifications.

Macroarray preparation

PCR products were diluted with 15µL of autoclaved nanopure water and printed on charged nylon membranes (Millipore, Bedford Ma) with a Seiko D-TRAN robot (Seiko). Membrane printing was done using a 96 pin head that spotted approximately 0.2µl per spot on the membrane. Each PCR product was spotted in duplicate. In between printing different samples, pins were dipped in 5% bleach followed by 95% ethanol and finally washed in autoclaved nanopure water while being sonicated. Pins were dried over a vacuum before proceeding to the next cycle. Following printing, membranes were cross linked in a UV Stratalinker 1800 (Stratagene) utilizing autocross link option. Membranes were stored at 4 ° C till usage.

Macroarray Hybridization

The secondary PCR amplification reaction products from the forward and reverse subtracted and unsubtracted cDNAs were used to generate P^{32} labeled probes. Probes were labeled as described in the PCR select differential screening kit (Clontech) with minor modifications. After labeling, probes were cleaned using sephadex columns and the amount of labeled product was determined on a scintillation counter (Beckman Coulter). Equal counts of labeled PCR products from the subtracted and unsubtracted cDNAs were used for hybridizations. Probes were hybridized to membranes over night at 72 ° C in a rotary hybridization oven. Post-hybridization washes of membranes were performed as described in the PCR select differential screening kit (Clontech). Washed membranes were exposed to a phosphoimager screen (GE Healthcare) over night and then scanned on a Typhoon Trio Variable Mode Imager (GE Healthcare).

Macroarray data analysis

Image files generated by the Typhoon system were imported into the ImageMaster 2D Platinum (GE Healthcare) software system. Images were gridded using the auto-spot function with size set to five. In the cases of a spot with a very strong signal, spot size was increased to encompass

the entire spot. Volume and intensity of each spot was then retrieved and used to identify clones which had intensity or volume greater than 120% in the hybridization from subtracted library probe over the unsubtracted library probe (Figure 1). These colonies were then selected and grown on plates for sequencing analysis. Before sequencing, all the selected colonies were analyzed on agarose gels as described above.

Sequencing

Clones selected by differential screening were grown in 96-well plates in liquid media (Terrific Broth) for 20 hours at 470 rpm in the HiGro microwell plate growth system (Genomic Solutions, Ann Arbor, Michigan). Plasmid DNA isolation was performed by standard procedures using solution I, II and III on a Biomek FXP (Beckman Coulter, Fullerton, California). Bi-directional sequencing was conducted using BigDye Terminator v3.1 standard procedures and with the following primer sequences: 5'-AGCGTGGTCGCGGCCGAGGT-3' and 5'-TCGAGCGGCCGCCCGGGCAGGT-3'. Sequencing reactions were purified using Agencourt ® CleanSEQ ® dye-terminator removal system (Beckman Coulter, Beverly, MA) following manufacturer's protocol using the Biomek FXP (Beckman Coulter, Fullerton, California). Purified sequencing samples were analyzed using the 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) following manufacturer's suggested protocol.

Sequence Data Analysis

Sequence data was processed using an EST pipeline by removing contaminating vector sequences, low quality sequence and poly (A)s and assembled into unigene set.
Sequences were subjected to a BLAST search analysis using The Gene Index Project from the Computational Biology and Functional Genomics laboratory *M. truncatula* EST database, http://compbio.dfci.harvard.edu/tgi/. Gene ontology (GO) numbers for the identified sequences that were present in this database were used with the Genesis program (Sturn et al. 2002) to generate figures showing the distribution of GOs for each library. If a gene had 90% or greater homology to a gene in a closely related organism, those GOs were then used. For sequences that did not give any significant hits we assigned 'unknown' for the three GO categories.

We identified the Arabidopsis homologs for 192 ozone responsive Medicago ESTs. The stress response profiles of these Arabidopsis homologs were retrieved using the Genevestigator

program (Zimmermann et al. 2004). The locus identifiers of Arabidopsis genes were entered into this program. Along with the stress response profile, gene expression profiles of various Arabidopsis mutants were also retrieved. All the available ATH1 gene chip data (3110) were included for this analysis. We selected the mutants and stresses that showed more than 10% overlap with the ozone responsive genes identified in this study.

Real Time PCR validation

For validation of the differential expression, several genes were selected for Realtime PCR analysis. This was performed using Maxima SYBR Green qPCR master mix (Fermentas) in a 7500 Realtime PCR system (Applied Biosystems). Sequences of PCR primers are given in Supplemental Table 2. PCR was performed with the following conditions, 94° C for 10 min followed by 40 cycles of 94 ° C for 15s, 55 ° C for 20s, and 72 ° C for 40s.

Results and Discussion

Based on a survey of 38 *Medicago truncatula* accessions for ozone related phenotypes we selected two accessions, JE154 and Jemalong, which exhibit contrasting responses to acute ozone fumigation (Puckette et al. 2007). The ozone-sensitive Jemalong cultivar shows yellowing and localized necrosis by 24 hours after the end of ozone treatment. Resistant JE154 accession does not show any visible symptoms in response to acute ozone fumigation. Recently, we have analyzed the changes in the gene expression in these two accessions using 17K oligonucleotide arrays (Puckette et al. 2008). We are using the SSH based analysis to complement the previous study. *M. truncatula* oligonucleotide arrays were based mostly on the ESTs derived from plants grown under control conditions. Further, larger proportions of these ESTs were derived from roots. We hypothesized that genes from the leaf tissues induced in response to stresses are under-represented in the existing *M truncatula* EST collection. Secondly, in the current analysis we have included the late time points (15 h and 24 h) to capture a wide spectrum of differentially expressed genes in response to ozone. Conducting reverse subtractions wherein the tester cDNA is from control samples while the driver cDNA is from ozone treated samples further enabled the identification of genes that were repressed in response to ozone stress. For both the resistant and

the sensitive accessions, forward subtractions to identify ozone-induced genes and reverse subtractions to identify repressed genes were undertaken in this study.

Approximately 4600 colonies were picked from the four subtraction libraries (Supplementary Table 1). Nearly 10% of these colonies failed to grow in the 96 well plates containing LB ampicillin. Each of the colonies that grew on the 96 well plates was subjected to colony PCR followed by agarose gel electrophoresis to determine the number and sizes of inserts. About 6% of these colonies failed to give amplification products that were more than 100 bp while about 5% of the colonies resulted in multiple amplifications. Colonies from the forward subtracted libraries from the two accessions that gave multiple amplification products were re-streaked on ampicillin plates followed by colony PCR analysis. This enabled us to retrieve 111 independent clones that gave discrete amplifications. Given that the procedure uses a 4-cutter enzyme to digest the cDNA it was not surprising that nearly 95% of amplified products were between 100-400 bp. About 30 clones were larger than 400 bp, with the largest amplified fragment being close to 1500 bp. Overall this initial screening to determine growth and amplification efficiencies of clones identified 2638 colonies that were printed on nylon membranes for differential screening (Supplementary table 1).

A unique benefit of using the combination Typhoon scanner and Image master software was the ability to set a minimum threshold to determine differential expression following the hybridizations with radioactively labeled probes (Figure 1). This greatly increased the speed, sensitivity and consistency of the differential screening analysis. Based on this analysis we selected 817 clones as being differentially expressed from the four different libraries. Differential expression of several of these clones was confirmed by real-time PCR (Supplementary table 2).

The 817 clones selected by differential screening analysis were sequenced from both directions finally resulting in 871 unambiguous sequences that were assembled into 239 contigs. Of the 239 contigs, 227 sequences gave homologies to genes of known functions while 12 sequences were identified as genes of unknown functions (Supplementary Table 3). The genes of known function were sorted into 11 primary functional categories (Figure 2) that had similar distributions in the two accessions for seven categories. Differences in the two accessions were identified for a few gene categories. A larger fraction of ozone-induced genes in JE154 were involved in metabolic processes and energy pathways while higher percentage of genes induced

in Jemalong were associated with translation and cell growth (Figure 2). This suggested that JE154 had adapted to the oxidative stress early while Jemalong was tweaking its proteome and morphome to encounter the massive oxidative damage caused by ozone-induced ROS.

A large scale transcriptomic meta-analysis identified significant overlapping gene expression profiles between ROS response genes and those in various abiotic stresses (Gadjev et al. 2006) and our in silico analysis of ozone response genes is in agreement with this study. Subtleties in oxidative signaling were easy to identify because of the inclusion of two accessions with contrasting ozone responses. For example, even when similar gene ontologies were over represented in the two accessions, genes that were identified within were very different. One of the significant differences among the ozone responsive genes between Jemalong and JE154 was genes differentially regulated in response to stress (Figure 3). Within this category JE154 showed enrichment for genes associated with dehydration stress and cold, while Jemalong showed favoritism to genes associated with oxidative stress and hypoxia. The latter observation is not a surprise considering the importance of oxidative stress in the form of a ROS burst seen particularly in ozone sensitive plants including *M. truncatula* (Pellinen et al. 1999; Puckette et al. 2008; Schraudner et al. 1998; Wohlgemuth et al. 2002). Generation of ROS is characteristic for hypoxia (Blokhina et al. 2003). In fact, a statistically significant increase in ROS levels 24 h after the end of ozone treatment in Jemalong has been reported (Puckette et al. 2007). The overlap in ozone responsive genes with those responsive to hypoxia, cold and heat stresses supports a role for ROS as mediators for co-ordinating the expression of a sub network of genes involved in oxidative signaling. The larger number of genes in Jemalong belonging to the GO, programmed cell death may contribute to the ozone-induced lesions seen visibly in sensitive plants (Pasqualini et al. 2003; Puckette et al. 2007). The presence of dehydration stress associated genes in JE154 is another interesting result in light of previous research finding that JE154 is more tolerant to drought stress than Jemalong (Puckette et al. 2007). The genes identified as dehydrins in the JE154 forward libraries, has also been reported in a chronic ozone stress library in *Phillyrea latifolia* (Paolacci et al. 2007). Dehydrins have been found to accumulate at the plasma membrane in wheat in response to cold stress and hypothesized to play a role in tolerance to cold induced drought stress within the cell due to the observed correlation between increased dehydrin levels and increased tolerance (Danyluk et al. 1998; Hara et al.

2003). Dehydrins can act as antioxidants (Desikan et al. 2000) and thus may contribute to the lower levels of ROS in JE154, 24 hours after the end of treatment (Puckette et al. 2007).

Supporting the over-representation of the dehydration stress responsive genes in JE154, we observed a significant overlap with genes differentially expressed in response to ABA treatment (Figure 4). The role of ABA in drought stress has been well documented (Nishimura et al. 2007; Seki et al. 2007; Zhu 2002). Further, a high degree of overlap was identified in ozone responsive genes of Jemalong and JE154 to genes differentially expressed in ABA hyper-accumulating mutants, *ahg1-1* and *ahg3-1* (Nishimura et al. 2007; Yoshida et al. 2006) suggesting a key role for this phytohormone in ozone stress signaling. Both AHG1 and AHG3 are negative regulators of ABA signaling pathway (Nishimura et al. 2007; Yoshida et al. 2006). We speculate ABA may play a role in determining the plant response to ozone directly via its regulation of the stomatal aperture (Adedipe et al. 1973; Jeong and Ota 1981; Tingey and Hogsett 1985; Vahisalu et al. 2008) and/or its interactions with ROS (McAinsh et al. 1996; Neill et al. 2002; Overmyer et al. 2008; Pei et al. 2000).

Ozone-induced genes in JE154 showed the highest degree of similarity to the genes altered in Arabidopsis *brevix radix (brx)* mutant (Figure 4) based on Genevestigator analysis (Zimmermann et al. 2004). The BREVIS RADIX gene encodes a transcription factor that controls cell proliferation and elongation at the root tip growth zone (Mouchel et al. 2004). A feedback mechanism mediates the interaction between auxin and brassinosteroids through regulation of brassinosteroid biosynthesis and causes a global decrease in auxin-responsive transcription via BRX protein (Mouchel et al. 2006). Based on these observations and the enrichment of auxin response genes only in JE154 forward library we speculate that auxins play a role in resistance to ozone induced oxidative stress in JE154. The significant difference in extent of overlap between JE154 and Jemalong forward libraries with reference to genes differentially expressed in *brx* mutant suggest that auxin/brassinosteroid interactions play a critical role in determining resistance or sensitivity to ozone. This was further supported by the observation that significant fraction of genes in the Jemalong reverse library was differentially expressed in the *brx* mutant (data not shown).

The double mutant *mpk4:ctr1* contains mutations in both the MAP kinase 4, a central component of the systemic acquired resistance pathway (Petersen et al. 2000) and the constitutive triple response 1 , negative regulator of the ethylene response pathway (Kieber et al.

1993). Microarray analysis comparing the single mutants with the double mutant indicated that MPK4 signaling pathway could have a repressive effect on the ethylene signaling pathway (Brodersen et al. 2006). The high degree of similarity in the ozone responsive genes of JE154 with not only the *mpk4* mutant but also the *mpk4:ctr1* double mutant leads us to speculate that JE154 may repress the ethylene signaling pathway that could contribute to its resistance to ozone induced oxidative stress (Figure 4).

It has been reported that plants that maintain a low nitrogen level are more resistant to ozone stress than plants grown in high nitrogen levels (Brewer et al. 1961; Pell et al. 1990; Tjoelker and Luxmoore 1991). The high degree of similarity of ozone responsive genes with genes differentially expressed under low nitrate conditions suggests that ozone stress lowers N levels in the plants. Down regulation of anabolic processes supported by enrichment of photosynthesis genes in the reverse libraries suggests that the levels of carbon are lowered in response to ozone. To maintain a proper carbon-nitrogen balance plants may down regulate nitrate responsive genes. This may also be a strategy to lower the levels of nitric oxide that can interact with ozone-induced ROS to trigger the oxidative cell death cycle (Delledonne et al. 2001; Overmyer et al. 2003; Zaninotto et al. 2006).

Nitrogen availability, transport, and stress in the absence of nitrogen are communicated through several hormones including auxin, gibberellins (GA), and cytokinins (CK) (Sakakibara et al. 2006; Tamaki and Mercier 2007). Genes responsive to auxin were induced only in resistant JE154 while CK responsive genes were induced in sensitive Jemalong (Supplementary Table 4). GA responsive genes were induced in JE154 and repressed in Jemalong. The differences between the two accessions with reference to genes responsive to GA, CK, and auxin indicate that these phytohormone pathways could be a point of divergence leading to ozone resistance or sensitivity. For example, CKs and auxins have been shown to inhibit ABA induced stomatal closure by increasing ethylene synthesis in Arabidopsis (Tanaka et al. 2006). Our analysis of ozone response genes in Medicago in conjunction with other microarray analyses of various hormonal mutants indicates that along with the well established players of the oxidative cell death cycle, namely, SA, JA, ET and ABA (Kangasjarvi et al. 2005; Overmyer et al. 2003; Overmyer et al. 2008; Overmyer et al. 2000) other phytohormones such as GA, CK and auxins may also be important in determining the plant responses to ozone. Analysis of ozone response phenotypes using mutants of auxins, CK, and GA will aid in establishing these interconnections.

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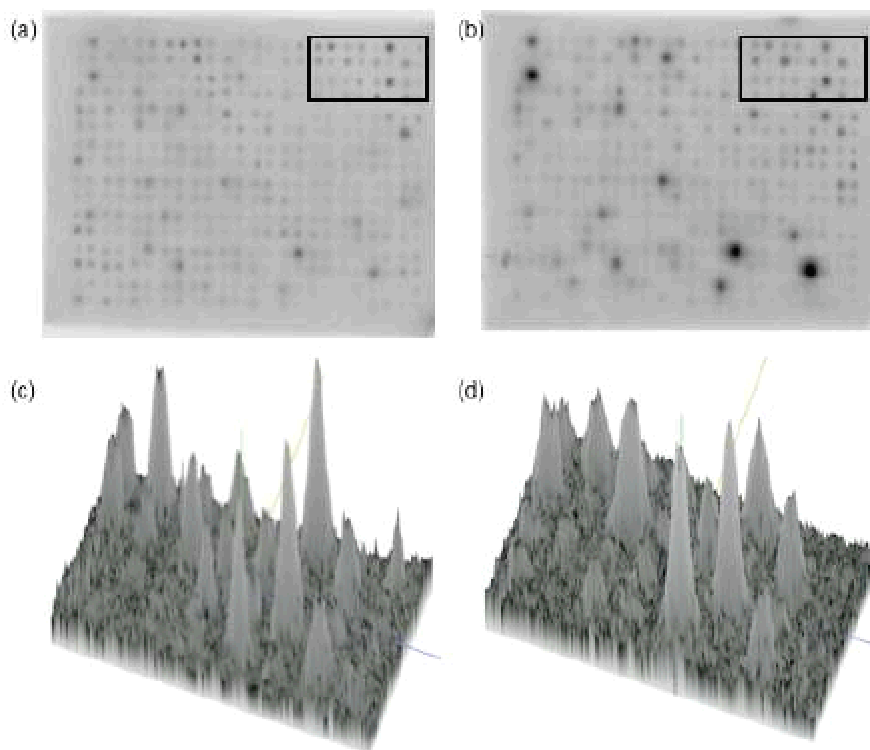

Figure 1: Macroarray differential screening. Membranes were hybridized with P^{32} labeled forward subtracted cDNA probe (a) or unsubtracted cDNA probe (b) and subjected to phosphorimaging. The resulting images were gridded using ImageMaster Pro software. ImageMaster Pro was then used to measure intensity and volume of the signals. A cross sectional view of the peaks and valleys generated by the software is shown (c,d) for a small portion of the hybridized memebrane, shown as a rectangular box (a,b).

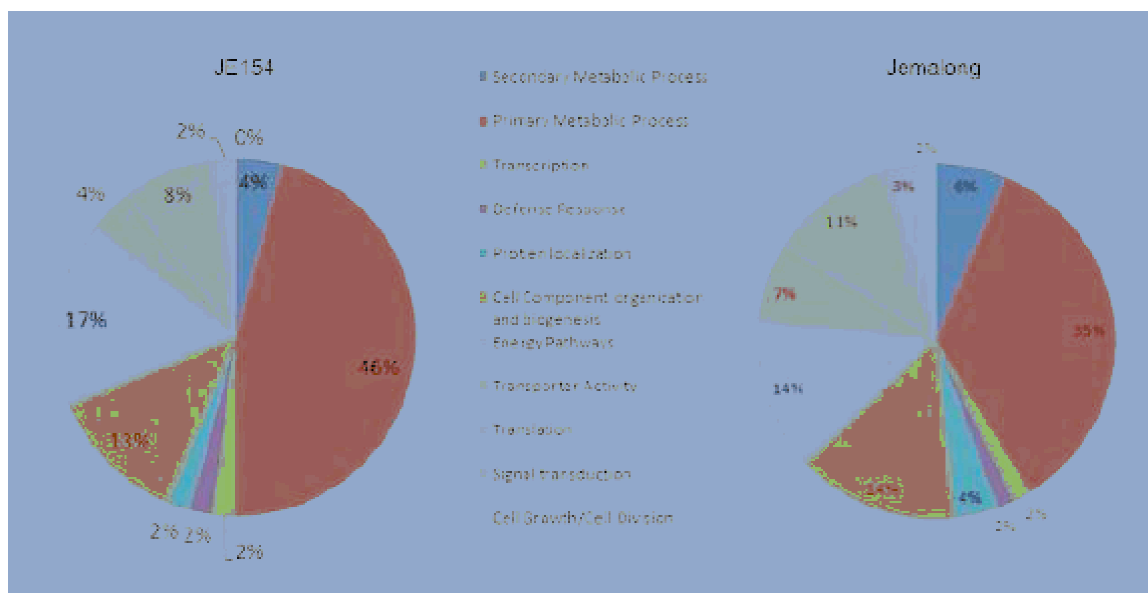

Figure 2. Pie chart showing the distribution of gene ontologies of ozone induced genes identified by suppression subtraction hybridization in *M truncatula*.

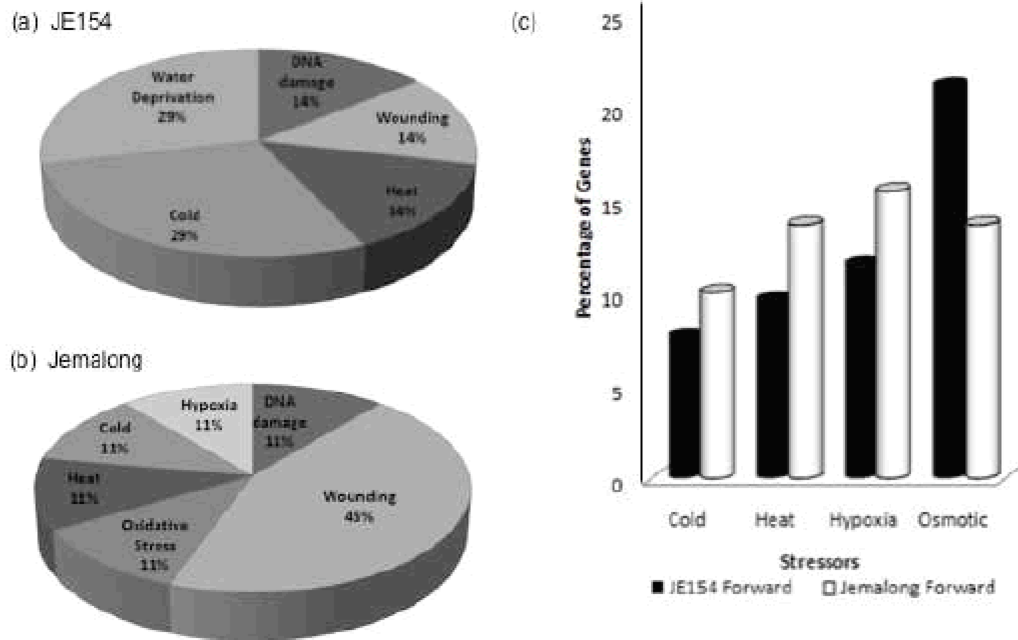

Figure 3: Distribution of ozone induced genes that belong to the GO:0006950 "Response to Stress" in M truncatula accessions (a,b). (c) Digital northern analysis showing the overlap between of ozone induced genes in M truncatula with homologous Arabidopsis genes identified in response to various stresses.

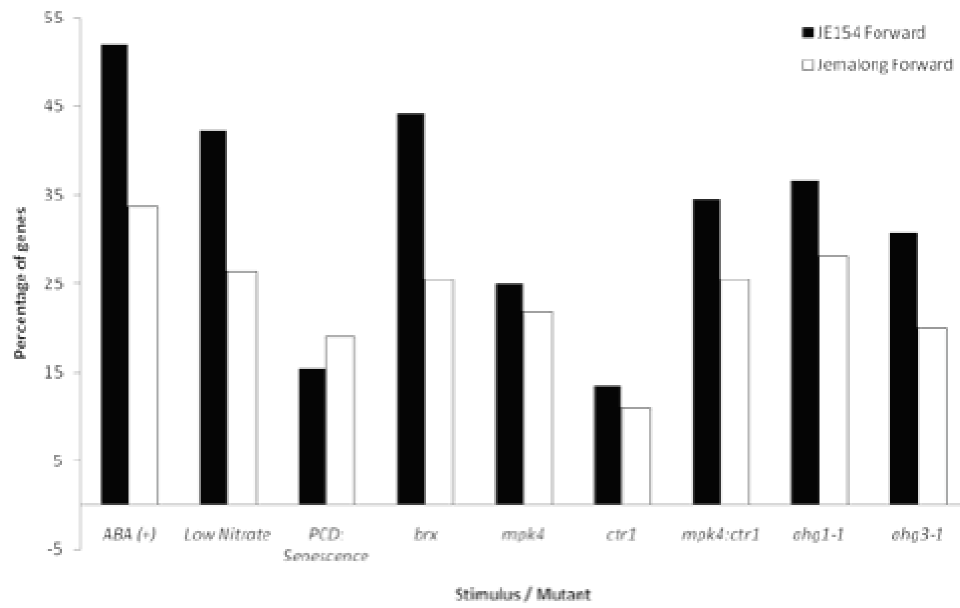

Figure 4: Genevestigator based analysis showing the overlap between of ozone-induced genes in M truncatula with homologous genes identified in response to various stimuli and mutants of Arabidopsis.

VITA

Michael Carroll Puckette

Candidate for the Degree of

Doctorate of Philosophy

Dissertation: OZONE INDUCED STRESS RESPONSE IN MEDICAGO TRUNCATULA

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data:
I was born on May 1st 1981 in Longview Texas. I am the son of Dr. Thomas Allen Puckette and Deborah Lee Puckette. I am a member of the Choctaw Nation of Oklahoma. I came to Oklahoma State University in the Fall of 1999 for my bachelors degree which I obtained in 2003. After completion of my bachelors degree I stayed at Oklahoma State University and entered the doctorate program for Biochemistry and Molecular Biology.

Education:
Completed the requirements for the Bachelors of Science in Biochemistry at Oklahoma State University, Stillwater, Oklahoma in August 2003.

Name: Michael Carroll Puckette Date of Degree: December, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: OZONE INDUCED STRESS RESPONSE IN MEDICAGO TRUNCATULA

Pages in Study: 114 Candidate for the Degree of Doctorate of Philosophy

Major Field: Biochemistry and Molecular Biology

Scope and Method of Study:

This study used ozone to induce ROS stress in *Medicago truncatula*. Techniques used include microarray analysis, suppression subtraction hybridization, differential screening of EST libraries, real time PCR, lipid peroxidation assay, ascorbate-glutathione assays, ROS assays, and gradient fractionation to isolate polyribosome bound mRNA.

Findings and Conclusions:

It was found that within the naturally occurring *Medicago truncatula* population there exists great diversity for the symptoms seen in response to ozone. Both resistant and sensitive accessions up regulated many of the same gene networks but at different times and to magnitude. There was a rapid general decrease in polyribosome loading in response to ozone in resistant plants. Coupled with the massive transcriptional changes this could provide for an early reprogramming of the cells to an adaptive response in resistant plants. The initial passive response, or a lack of active response, in sensitive plants leads to oxidative stress via ROS buildup and ultimately oxidative cell death.

ADVISER'S APPROVAL: Dr. Ramamurthy Mahalingam