SHORT TERM TEMPORAL TRENDS IN GENE EXPRESSION IN SENSITIVE AND TOLERANT SOYBEAN GENOTYPES EXPOSED TO OZONE

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Abstract

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Current ground-level ozone concentrations ([O_3]) are high enough to damage crops and are projected to increase. Soybean (*Glycine max*), one the four major global food crops, is particularly sensitive to O_3. Though considerable genetic variation exists in its sensitivity, the mechanistic basis for these differences remain poorly understood. My work aimed to identify molecular mechanisms associated with the differential tolerance to O_3 among tolerant (Fiskeby III) and sensitive (Mandarin Ottawa) soybean genotypes. Short-term changes in gene expression (< 48 hrs post O_3 exposure) were investigated using quantitative real-time polymerase chain reaction (qPCR) techniques to determine whether there were rapid differences between the tolerant and sensitive soybean genotypes in either amount or in which genes were expressed. Plants were exposed from 9 am to 5 pm either at 19.3 $\pm$ 0.43 ppb (target was 25 ppb) or 63.6 $\pm$ 0.43 ppb (target was 75 ppb) O_3. A single leaflet from the fifth mature leaf was collected just prior to exposure, and then at 1, 2, 4, 8, 12, 24, and 48 hours post-exposure. Visible leaf injuries were quantified according to a leaf bronzing score.

Sampled leaflets were immediately frozen in liquid N_2 and stored until processing for RNA extraction and purification, cDNA construction and qPCR. Gas exchange measurements under standard conditions were made on the third mature leaf on each plant at the end of the exposures to determine if there were changes due to O_3 exposure and/or genotype. The photosynthetic rate was not significantly different between the treatments or genotypes. However, stomatal conductance (g_s) was significantly higher in Mandarin than Fiskeby under both O_3 conditions. *Glutathione reductase 2* (GR2) expression in both genotypes under high O_3 treatment was upregulated in a similar fashion with the expression peaks both shifted an hour earlier, and to a similar extent. *Protein D1* (pD1) expression was downregulated in Mandarin but was not affected in Fiskeby III, maybe due to the larger g_s in Mandarin and subsequently higher dose. An improved understanding of the molecular factors influencing O_3 sensitivity could help guide breeders to develop O_3 tolerant genotypes so that yields can be maintained at or above current levels should ambient O_3 continue to increase globally.

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Dedication

To my loving parents, who patiently supported me through the adventure of my graduate studies. Words alone cannot express my gratitude.

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Foreword

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Introduction

O_3 is present in both the troposphere and stratosphere, but it is the tropospheric O_3 that is damaging to plants and human health. In fact, O_3 is known to be among the most phytotoxic air pollutants (Krupa and Manning, 1988; Chameides *et al.*, 1994; Ahmadov *et al.*, 2014). While stratospheric O_3 has a known role in protecting the earth from ultraviolet radiation, tropospheric O_3 can occur naturally from stratospheric incursions, lightning, and fires (Edwards, 2003). However, its abundance is augmented by being a secondary byproduct of photochemical reactions involving anthropogenic nitrogen oxides (NO_x), carbon monoxide (CO) and volatile organic compounds (VOCs) (Fishman and Crutzen, 1978). As emissions of O_3 precursors rise in many parts of the world there is an excessive increase in ambient O_3 concentrations (Dentener *et al.*, 2006) . This was originally thought to be limited to urban regions, nearby power plants, and locations downwind of these areas. However, the problem has increased in scale during the past few decades as a result of rising population densities, expanding industrialization, and ensuing pollution in large parts of the world, especially in the developing countries (Fiscus *et al.*, 2005).

Annual average tropospheric O_3 has more than doubled during the past century (Staehelin *et al.*, 1994), and it is the most damaging gaseous pollutant to crop yields, forest growth, and species composition (Ashmore, 2005). Crop losses from O_3 damage were estimated to be in the range of $1.8- $3.9 billion in the United States and $3.0- $5.5 billion in China (Dingenen *et al*., 2009). The projected 25% increase in background O_3 for the next 30 to 50 years may cause even greater losses to world agriculture (Vingarzan and Thomson, 2004; Meehl and Stocker, 2007). A meta-analysis combined from 406 experimental observations revealed that current [O_3] (compared to base [O_3]) has led to yield losses

ranging from 5% to 19% of the world major crops including potato, wheat, barley, rice, bean and soybean (Feng and Kobayashi, 2009). Therefore, understanding how crops respond to increasing O_3 pollution is essential for meeting the growing demands for sustainable food systems as the world faces increasing population, urbanization, and climate change.

In the 1970s through the 2000s, a number of world forest and crop research organizations, including the United States Department of Agriculture, conducted studies of the response of vegetation to O_3 using open-top exposure chambers (OTCs) (Amthor, 1988; Bortier *et al.*, 2000; Elagöz and Manning, 2005; Fuhrer *et al*., 1989; Heagle and Philbeck, 1979; Heagle *et al*., 1973; Krupa and Manning, 1988; Mulchi *et al*., 1992; Pleijel *et al*., 1991). Results from these studies showed significant reductions in yield at ambient levels of O_3 despite potential "chamber effects" that may have occurred in those experiments and which could have biased the results (Elagöz and Manning, 2005).

Soybeans are one of the world's staple food crops according to the world production ranking in 2012 (Food and Agriculture Organization, 2012). Soybeans serve not only as a good source of proteins for the human diet and for feeding livestock, but also increasingly as a biodiesel resource (Masuda and Goldsmith, 2009). Soybeans were, to a great extent, more sensitive to O_3 when compared to other major food crops such as wheat, corn and sorghum, (Heck *et al.*, 1983; Emberson *et al.*, 2009). MOZART (The Model for Ozone and Related Chemical Tracer) was used to simulate changes of O_3 and showed a reduction in global soybean yields of 8.5% to 14% in 2000 (Avnery *et al.*, 2011). Recent results from Free Air CO_2 Enrichment experiments on soybean with elevated O_3 (chamberless exposure systems) have found similar yield losses, thus validating the studies conducted in the OTCs (Ainsworth and Long, 2005; Betzelberger *et al.*, 2010).

O_3 damage to plants can occur without any visible signs macroscopically when given chronic exposure at low levels of O_3 ($[O_3]<100$ ppb), but there is visible injury microscopically. This is evidenced by the sparse distribution of dead cells among palisade mesophyll or around the stomatal cavity in an O_3-tolerant *Phaseolus vulgaris* L. genotype but which are not visible at the macroscopic level (Faoro and Iriti, 2005). In contrast, severely affected plants do show visible symptoms on leaves as a result of acute O_3 exposure (high O_3 dose exposure within a short time frame when $[O_3]>100$ ppb), including chlorophyll loss, adaxial leaf bronzing, and even development of necrotic spots, which is more generally an indicator of pathogen invasion, but which can be mimicked by exposure to O_3 (Robinson and Britz, 2000; Fiscus *et al.*, 2005; Chen *et al.*, 2009; Emberson *et al.*, 2009; Betzelberger *et al.*, 2010).

Previous research has indicated that there has been no selection for increased tolerance to O_3 in soybean genotypes over the past 30 years (Betzelberger *et al.*, 2010). However, there are significant genotype differences in the response to O_3, with some more affected by O_3 than others with respect to the development of foliar injury, reduced growth, and lowered seed yield (Lee *et al.*, 1984; Foy and Lee, 1995; Robinson and Britz, 2000; Cheng *et al.*, 2007). By analyzing hundreds of soybean genotypes, 30 ancestral lines of soybean were found that together represented 92% of the North American soybean genome (Gizlice *et al.*, 1994). These ancestor lines were then screened for tolerance to salt, aluminum, drought and high O_3 levels. Fiskeby III and Mandarin (Ottawa) were selected as two soybean genotypes classified as O_3-tolerant and O_3-sensitive, respectively (Burkey and Carter, 2009).

O_3 causes crop yield losses through a number of different mechanisms, such as variable stomatal uptake, antioxidant capabilities once inside the leaf, either or both of which may be modulated initially by differential gene expression. O_3 uptake occurs mainly through the stomata (Long and Naidu, 2002), so differences in stomatal conductance (g_s) can have large effects on the ability of species to tolerate O_3. Species with high g_s will take up more O_3 than those with lower g_s. At the cellular level, a larger stomatal aperture, higher stomatal density and prolonged stomatal opening during the day may all contribute to a higher g_s and greater uptake (dose), resulting in a higher O_3 sensitivity (Grulke *et al.*, 2007*a*). For example, increased O_3 sensitivity in new introductions of Greek wheat genotypes (*Triticum aestivum*) has been attributed in part to higher g_s (Pleijel *et al.*, 2006). Furthermore, multiple modeling studies show that plant injury is more closely associated with dose rather than measured concentrations (exposure) (Fuhrer *et al.*, 1997; Massman, 2004; Pleijel and Danielsson, 2004).

Mesophyll resistance (g_m) is another important limiting factor for O_3 diffusion into a leaf for the reason that the amount of cell surface available to interact with O_3 can alter its effectiveness at a given dose. Oksanen *et al.* (2001) found that palisade and spongy mesophyll thicknesses, as well as their ratios, were lower in O_3-sensitive aspen clones (*Populus tremuloides*) than in O_3-tolerant clones. In addition, cell wall thickness can influence the residence time of O_3 by increasing diffusional resistance and by providing larger pools of antioxidants (Turcsanyi, 2000; Cheng *et al.*, 2007).

Upon entering the leaves through the stomata, O_3 rapidly breaks down into various reactive oxygen species (ROS) (Fiscus *et al.*, 2005). ROS are chemically reactive molecules containing oxygen and typically include superoxide ($\cdot O_2^-$), singlet oxygen ($^1O_2$), hydroxyl

radical ($\cdot OH$), and hydrogen peroxide (H_2O_2). Both H_2O_2 and $\cdot O_2^-$ can act as molecular signals and cause damage to cells (Mühlenbock *et al*., 2008, Gordon *et al*., 2012). Depending on the nature of the ROS, some are highly toxic and therefore are rapidly scavenged by cellular antioxidant mechanisms. However, an excessive generation of ROS can overwhelm the antioxidant quenching capacity of both the symplast and apoplast, leading to known O_3 effects such as decreases in photosynthetic rates, chlorosis and eventual cell death (Fiscus *et al*., 2005). In a general sense, O_3 effects on plant cells and tissues are not the result of O_3 itself, which reacts almost immediately with internal cell surfaces and is destroyed, but are caused by the excessive amount of ROS produced by the plant after exposure to the O_3 (Tausz *et al*., 2007). This burst of ROS is known as the hypersensitive response and shares many of the biochemical pathways associated with pathogen attack on plants. Differential antioxidant capacity is directly associated with O_3 sensitivity (Iglesias *et al.*, 2006); therefore, it is another important factor affecting the sensitivity of plants to O_3.

Both non-enzymatic and enzymatic mechanisms are employed by the cell to scavenge excess ROS. Non-enzymatic antioxidants include carotenoids, tocopherol and flavonoids, ascorbic acid (AA) and reduced glutathione (GSH), the last two of which are believed to be the central redox buffering molecules that function to scavenge ROS (Foyer and Noctor, 2009, 2011). Availability and redox status of AA is controlled by ascorbate oxidase localized in the cell wall, which oxidizes AA to dehydroascorbate (DHA) (Pignocchi 2003; Pignocchi *et al*., 2006). Such dynamic inter-conversion between AA and DHA maintains the apoplast antioxidant capacity and has been found to correlate with O_3 sensitivity in many plant species including *Arabidopsis* low vitamin C mutants (Conklin, 1996), white clover (*Trifolium repens*) (D'haese *et al.*, 2005), wheat (*Triticum aestivum* L.)

(Feng *et al.*, 2010), snap bean (*Phaseolus vulgaris*) (Guri, 1983) and soybean (*Glycine max* L.) (Cheng *et al.*, 2007). An abnormal apoplastic ROS burst caused by elevated O_3 exposure may lead to perturbations in the extracellular redox balance and, consequently, in the intracellular redox metabolism. This includes alterations in the glutathione level and the ratio between GSH and glutathione disulfide (GSSG), which play essential roles in ROS mediated signaling pathways upon O_3 exposure (Foyer and Noctor, 2011). For example, mutants with decreased GSH content are hypersensitive to a variety of stressors (Creissen *et al.*, 1999).

GSH and AA act in accordance with other enzymes such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) to modulate the cellular redox status (Alscher and Hess, 1993; Chernikova *et al.*, 2000). Removing ROS also involves other well-defined enzymes such as superoxide dismutase (SOD), catalases (CAT) and peroxidases (PX). Multiple isoforms of SOD are distributed throughout the cell including in the cytosol, mitochondria and chloroplasts; and these organelles can convert superoxide radical into H_2O_2. PX has a similar distribution as SOD while CAT is primarily found in peroxisomes; however, they share functionality in further converting H_2O_2 to H_2O (Kangasjärvi and Talvinen, 1994; Inzé and Montagu, 1995). Glutathione S-transferase (GST) also displays peroxidase activity and plays a crucial role in detoxifying the products of lipid peroxidation by facilitating the conjugation of detrimental electrophilic compounds with GSH and then purging them out of the cytosol (Marrs, 1996). Of the numerous studies reporting effects of O_3 on the antioxidant defense systems, a majority observed increased concentrations of antioxidants or elevated defense enzyme activities upon O_3 exposure (Gupta *et al.*, 1991; Bortier *et al.*, 2000; Venkateswarlu *et al*., 2012; Kumari *et al.*, 2015).

Interestingly, a higher basal level of ROS and glutathione were both found to contribute to the greater oxidative stress tolerance of an *Arabidopsis* genotype atr7 (Mehterov *et al.*, 2012).

While a number of early researchers focused on the potential toxicity of ROS, recent studies have emphasized their signaling role in plants faced with oxidative stress such as O_3 exposure. The earliest O_3-derived signaling events, such as protein phosphorylation or dephosphorylation and calcium (Ca^{2+}) influx, can occur within a few minutes of exposure (Vaultier and Jolivet, 2014). These changes are followed by the production of several signaling compounds including various ROS, ethylene (ET), salicylic acid (SA) and jasmonic acid (JA) (Overmyer *et al.*, 2003). Mittler *et al*. (2011) summarized several advantages of ROS as signaling molecules. These include the rapid and dynamic changes in ROS levels, which allow the cell to respond quickly and specifically to stressors, the highly specific spatial control enabled by a tight regulation over the subcellular localization of ROS signals in cells, the ability of ROS to serve as rapid long distance signals travelling throughout the plant, and most importantly, the ability of ROS signaling to cross talk with the above mentioned signaling pathways including Ca^{2+}, phytohormones and protein phosphorylations as a part of the network.

ET and SA accumulate in O_3-exposed plants and high levels of these phytohormones appear responsible for the formation of leaf lesions and even programmed cell death (PCD) (Overmyer *et al.*, 2000; Overmyer *et al.*, 2005; Yoshida *et al.*, 2009). These studies also demonstrated that ET and SA likely amplify the oxidative signal generated by ROS, thereby promoting lesion formation. However, by analyzing the O_3-induced transcriptome of *Arabidopsis* mutants, Tamaoki *et al*. (2003a) indicated that low levels of ET production could stimulate the expression of defense genes, rather than promoting PCD which occurs

when ET production is high. As in the case of ethylene, O_3-induced SA seems to have a dual function that depends on its production level (Rao and Davis, 1999). In addition, plant exposure to O_3 not only results in activation of the biosynthetic pathways of ET, JA and SA, but also increases the expression of genes related to the signal transduction pathways (Tosti *et al*., 2006). These results suggest that depending on the concentrations, O_3-induced production of ET and SA may either activate the PCD pathway or serve to induce antioxidant defense responses.

By using JA-deficient mutants of various plants, JA was shown to be involved in the repression of ROS-dependent lesion development in O_3-exposed leaves, in contrast to the effects elicited by ET and SA (Overmyer *et al*., 2000; Ren *et al*., 2015; Sasaki-Sekimoto *et al*., 2005). Further illustrating the complexity of interactions involved in the O_3 response, SA and ET accumulation in response to O_3 is negatively regulated by JA signaling (Kanna, 2003; Rao, 2000).

Abscisic acid (ABA) has been investigated for its role in regulating stomatal aperture and also for its contribution to signaling pathways in plants. The role of ABA and the interaction between ABA and H_2O_2 in O_3-induced stomatal closure was described in the 2006 Ozone Air Quality Criteria Document (U.S. EPA, 2006), suggesting that the presence of O_3-derived H_2O_2 increases the sensitivity of guard cells to ABA and, therefore, results in more sensitive control of stomatal closure. Ludwików *et al*., (2009) used Arabidopsis ABI1td mutants, in which a key negative regulator of ABA action (abscisic acid insensitive1 protein phosphatase 2C) had been knocked out, to compare the transcription of O_3 responsive genes to the Arabidopsis O_3 tolerant Col-0 ecotype/genotype. They suggested a role for ABI1 in negatively regulating the synthesis of both ABA and ET in O_3-treated plants (350 ppb O_3 for

9 hours). Additionally, ABI1 may stimulate JA-related gene expression, providing evidence for an antagonistic interaction between ABA and JA signaling pathways.

Once the O_3 stress signal has been relayed and perceived at the cellular level, it is transmitted to the nucleus, which leads to transcription reprogramming. Changes in global gene expression in response to the primary and secondary signals eventually alter the metabolism and physiology of plants and lead to their response to the environmental stimuli. The rapid development in various analytical and biological approaches has facilitated the transcriptomic identification of plant gene expression profiles in response to O_3 stress (Mahalingam and Gomez-Buitrago, 2003). DNA microarrays including oligonucleotide and cDNA micro- and macro- arrays have been frequently used to identify O_3-inducible genes in different plant species including *Arabidopsis thaliana* (Li *et al*., 2006; Mahalingam and Shah 2005; Tamaoki *et al*., 2003a; Tosti *et al*., 2006), soybean (Whaley *et al.*, 2015), rice (Oryza sativa) panicle and seed tissues (Cho *et al.*, 2013), pepper (*Capsicum annuum*) (Lee and Yun 2006), clover (*Medicago truncatula*) (Puckette *et al*., 2008), *Phillyrea latifolia* (Paolacci *et al.*, 2007), poplar (*Populus*) (Street *et al.*, 2011) and European beech (*Fagus sylvatica*) (Olbrich *et al*., 2010). Species, O_3 concentration, exposure duration and sampling times considerably varied in these studies. However, particular expression profiles of several gene functional categories were identified under O_3 exposure and were roughly similar across this diverse set of plants. Genes with upregulated expression were linked to signaling and defense categories. Conversely, downregulated expression was related to photosynthesis and energy capture and use categories (Lee and Yun 2006; Li *et al*., 2006; Olbrich *et al*., 2010; Tamaoki *et al*., 2003b; Tosti *et al*., 2006). Furthermore, the sensitivity to O_3 stress is suggested to be a function of the differential regulation of these two classes of genes in plants (Lee and Yun

2006; Li *et al*., 2006; Puckette *et al*., 2008). However, relevant investigations at the molecular level are still in their beginning stages, especially with regard to soybeans, and more firm conclusions cannot be drawn at this time.

Differences in gene expression between sensitive and tolerant plants in response to O_3 have been evaluated by transcriptomic analysis (Lee and Yun, 2006; Puckette *et al*., 2008). Approximately two-thirds (67%) of the 180 O_3 stress-related genes in pepper were differentially regulated in the sensitive and tolerant genotypes. At 0 and 48 hours into a 3-day exposure (8 hrs/day) at 150 ppb, O_3 responsive genes were either upregulated or downregulated more significantly in the sensitive than in the tolerant genotype (Lee and Yun, 2006). Differences in timing and extent of changes were also shown in gene expression between contrasting clover genotypes. Acute exposure (300 ppb O_3 for 6 hours) led to the production of an oxidative burst in both clovers (Puckette *et al.*, 2008). However, the sensitive Jemalong genotype showed a downregulation of defense response genes 12 hours after the onset of exposure, while the tolerant JE 154 accession exhibited much more rapid and large-scale transcriptome changes (Puckette *et al.*, 2008). The inability for Jemalong to upregulate defense response genes might have contributed to its O_3 sensitivity.

Chronic O_3 concentration (1.2X ambient for 8-12 days) was used to study the changes to gene expression in *Arabidopsis* sensitive genotype WS and tolerant genotype Col-0 (Li *et al.*, 2006). The WS genotype showed a significantly greater number of changes in gene expression than the Col-0. In another study using an acute O_3 exposure (300 ppb for 6 hours), a rapid induction of genes such as proteases led to cell death in the WS genotype, and again, downregulation of cell signaling genes, indicating an ineffective defense response (Mahalingam, 2006).

The Arabidopsis Col-0 genotype was also used to study the temporal response to acute O_3 exposure (350 ppb during 6 hours and for 6 hours after the exposure) by conducting a time course microarray experiment (Mahalingam and Shah, 2005). Two hundred genes were identified and significantly expressed and grouped into three different gene expression profiles: early upregulated (81 genes), late upregulated (60 genes), and downregulated (59 genes). The results demonstrated that genes associated with signaling pathways and transcription regulation were in the early regulated group, while genes linked with redox homeostasis and defense were in the late regulated class (Mahalingam and Shah, 2005).

Transcriptomic changes of woody plant species in response to long term chronic O_3 exposure have also been conducted. Long-term exposures (100 ppb O_3 for 90 days), in contrast to short-term exposures, resulted in the upregulation of genes associated with secondary metabolites, such as isoprenoids, polyamines and phenylpropanoids in 2-year-old seedlings of the Mediterranean shrub *Phillyrea latifolia* (Paolacci *et al.*, 2007). In 3-year-old European beech saplings exposed to O_3 for 20 months (with the monthly average twice ambient O_3 concentrations ranging from 11 to 80 ppb), O_3-induced changes in gene expression were similar to those observed for herbaceous species (Olbrich *et al.*, 2009). Genes encoding proteins associated with plant stress response, including ET biosynthesis, PR proteins and enzymes detoxifying ROS, were upregulated. Some genes associated with primary metabolism, cell structure, cell division and cell growth were also reduced (Olbrich *et al.*, 2009). A similar study using adult European beech trees determined that the magnitude of the transcriptional changes described above was far greater in the saplings than in the adult trees exposed to the same O_3 concentrations for the same time period (Olbrich *et al.*, 2010). This suggests that changes occur at the molecular level in O_3 sensitivity between

seedling/sapling sized trees and large adult trees. Similar differences in sensitivity have also been found at the physiological level. The bases for these differences may reside in differences in stomatal behavior that alter uptake (Grulke *et al.*, 2007*a*) as well as in leaf structural differences between young and mature trees that affect internal diffusion and antioxidant capabilities (Chappelka and Samuelson, 1998; Pritsch *et al.*, 2007), both of which could result in alterations in gene expression.

Proteome analyses in rice, poplar, European beech, wheat and soybean supports the results from the above transcriptome studies. Exposure of soybean to 120 ppb O_3 for 12h/day for 3 days resulted in decreases in the quantity of proteins associated with photosynthesis, while proteins involved in carbon metabolism increased (Ahsan *et al.*, 2010). Young poplar plants exposed to 120 ppb O_3 in a growth chamber for 35 days also showed significant changes in proteins involved in carbon metabolism (Bohler *et al.*, 2007). Declines in enzymes associated with carbon fixation, the Calvin cycle and photosystem II were measured, while APX and enzymes associated with glucose catabolism increased in abundance (Bohler *et al.*, 2007). In another study to determine the impacts of O_3 on both developing and fully expanded poplar leaves, young poplars were exposed to 120 ppb O_3 for 13-h/day for up to 28 days (Bohler *et al.*, 2010). Impacts on protein quantity only occurred after the plants had been exposed to O_3 for 14 days, and at this point in time, several Calvin cycle enzymes were reduced in quantity, while the effects on the light reactions appeared later, at 21 days after beginning treatment. Some of the antioxidant enzymes increased in abundance with O_3 treatment, while others (APX) did not. O_3 did not affect protein quantity until leaves had reached full expansion, after about 7 days (Bohler *et al.*, 2010).

Valuable information about O_3 effects on plants has been provided by transcriptome and proteome studies, which allows for simultaneous analysis of changes in the expression patterns of many different genes and proteins, and which generally show between O_3-sensitive and -tolerant plants. However, earlier studies mostly focused on either an O_3-tolerant or an O_3-sensitive line but did not compare the two lines within a species. In addition, they often examined changes in gene expression at just one point in time. One study did examine the total and thiol-redox proteomes of leaf and root tissues from soybean grown under different O_3 concentrations, and that has provided a basis for choosing target antioxidant genes in my study (Galant *et al.*, 2012).

To determine if differential sensitivity of Fiskeby III and Mandarin (Ottawa) exists at the cellular or molecular level, my research included two parts:

First, I examined whether stomatal density and stomatal conductance differed between the two genotypes and whether these differences (if any exist) were associated with differential O_3 sensitivity. My hypothesis was that Mandarin (Ottawa) had greater g_s than Fiskeby III, which could contribute to its greater sensitivity to O3 because this would result in a higher does in this genotype compared to Fiskeby III.

Second, I performed a time course analysis of changes in gene expression in response to increased O_3 treatment and examined the molecular bases for resistance to O_3 among the two genotypes. In particular, I wanted to investigate the following hypotheses:

H1: That the tolerant Fiskeby III genotype upregulates defensive genes sooner and/or to a greater extent than the sensitive Mandarin (Ottawa) genotype; and,

H2: That the sensitive Mandarin (Ottawa) genotype, conversely, downregulates photosynthetic genes sooner and/or to a greater extent than the tolerant Fiskeby III genotype.

Materials and Methods

Plant growth, ozone treatment and sampling

Fiskeby III and Mandarin Ottawa were selected for this study due to their divergent responses to ozone stress. Plants were cultivated in a greenhouse with charcoal-filtered air in 6 L pots containing Fafard #2 Pro Mix (Fafard, Anderson, SC, USA), supplemented with 15 g slow release fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH, USA). Plants were maintained in a vegetative state throughout the experiment using supplemental lighting for 20 hrs/day. At 14 d after planting, seedlings were thinned to one plant per pot and treated with Marathon systemic insecticide according to the product label (OHP, Inc., Mainland, PA, USA).

At 28 days after planting, plants were moved into 12 continuously stirred tank reactors (CSTRs) (Rogers and Jeffries, 1977; Heck *et al.*, 1978) to acclimate for 2 days before exposure to ozone. Three biological replications, each in an independent CSTR, were randomly assigned to a block of 4 CSTRs, with two CSTRs in each block receiving square-wave exposures from 09:00 to 17:00 h over two days set to either 25 ppb O_3 (control plants) or 75 ppb O_3 (high O_3 plants) (Figure 1). Each CSTR contained 8 randomly placed plants (4 of Fiskeby III and 4 of Mandarin) that were sampled at four different time points (Figure 2). The fifth trifoliate was sampled from each plant at 0, 1, 2, 4, 8, 12, 24 and 48 hours after the start of treatment. Plants were permanently removed for sampling from each CSTR to prevent any volatile signals from these plants affecting the remaining plants. The leaflets were detached using a razor blade and immediately frozen in a 50 mL centrifuge tube filled with liquid nitrogen and then stored in a freezer at -80˚C. Whole leaf samples from corresponding time points were later ground and pooled for qPCR experiments (the 3

biological replicates were separated throughout the experiments but the expression data post qPCR were combined for analysis).

Foliar injury assessment and gas exchange measurement

The percentage foliar injury was assessed at 9 am for the first to sixth trifoliate leaf at 24 and 48 h plants after exposure using the protocol described by Burkey and Carter (2009). Net photosynthesis (A_n), dark respiration (r_d) and stomatal conductance (g_s) of leaves at position 3 were measured 48 hrs from the start of the ozone exposures, using a Li-6400xt portable gas exchange system (LI-COR, Inc., Lincoln, NE, USA) equipped with the 2 x 3 cm LED cuvette. Environmental conditions (mean + range) in the cuvette during gas exchange measurements for photosynthetic photon flux density (PPFD), leaf temperature, leaf-to-air vapor pressure deficit (VPD_{leaf}), RH, and CO_2 concentration were: (1500 $\pm$ 1 µmol m^{-2} s^{-1}, 35 ºC $\pm$ 1 ºC, 2.62 $\pm$ 1 kPa, 53% $\pm$ 10%, 400 $\pm$ 15 ppm).

RNA isolation and purification

Frozen leaf tissue was removed from the -80˚C freezer and immediately placed in liquid nitrogen and ground thoroughly. Total RNA was isolated from approximately 200 mg of the tissue powder using QIAGEN RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer, with minor adjustments (at step 9, 25 ul of RNase-free water was used twice to elute the RNA, instead of using 50 ul RNase-free water once). For removal of proteins and most of the free nucleotides, the RNAs were diluted with RNase free water (Thermo Fisher Scientific, Rockford, IL, USA) and treated with an equal volume of phenol: chloroform solution. After the mixture was vortexed and centrifuged at 14,000 g for 5 min, the resulting supernatant was precipitated with 1/10 volume NaOAc (3 M, pH 5.2) and 3 volumes of cold 99% ethanol and stored at -80 ˚C

overnight. The RNA precipitate was pelleted by centrifugation at full speed for 30 minutes at 4 ˚C and washed using 80% ethanol. The washed RNA pellet was solubilized in RNase free water. The purity and concentration of solubilized total RNA was assessed by measuring sample absorbance at 260/280 nm using spectroscopy (Nanodrop 2000, Thermo Fish Scientific, Rockford, IL, USA).

cDNA synthesis

First strand cDNA synthesis was performed using 2 µg of the total RNA with a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). This was performed in a 20 µl reaction system according to the manufacturer's supplied protocol.

Quantitative real-time polymerase chain reaction (qPCR)

Quantitative real-time polymerase chain reactions (qPCRs) were performed in 96-well plates using SYBR® Select Master Mix (Applied Biosystems, Foster, CA, USA) on a 7500 Real-Time PCR System (Applied Biosystems, Foster, CA) to determine the threshold cycle (Ct) for each sample. Primer sets (Table 1) (0.2 µM final concentrations for each primer) were used in a final volume of 10 µl per well. The 60s ribosomal protein L30 (Irsigler *et al.*, 2007; Le *et al.*, 2012; Tamang *et al.*, 2014) was used as an endogenous control for the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Each sample was analyzed in triplicate using 100 ng of template cDNA. The thermal profile of the qPCRs was set initially at 95˚C for 10 min, then 40 cycles at 95˚C for 15 s each and finally at 60˚C for 1 min. Amplification of the desired product was confirmed using melt curve analysis. Analysis of relative mRNA expression using the $2^{-\Delta\Delta Ct}$ method was conducted using Microsoft Excel 2010.

Statistical analysis

Gas exchange

Because both genotypes were co-located in the same chambers, they could not technically be regarded as independent samples. Therefore the differences in A_n, r_d, and g_s between genotypes within a chamber were analyzed using either a paired t-test or a one-way ANOVA of the differences between genotypes in the same chamber with ozone as the main effect. Analyses were conducted using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA), and differences were considered statistically significant if $p < 0.05$ and marginally so if $p < 0.1$. However, I also compared each genotype individually for either a time or ozone effect, using t-tests because of the problem of non-independence within the chambers and the fact that doing an analysis on differences could mask some effects due to random differences between individual plants. For gene expression over time, a repeated measures analysis was not required because individual plants were not sampled more than once.

Results

Ozone Exposures

The actual O_3 treatment levels attained during the two-day exposure period were lower than the set points of 25 ppb and 75 ppb. Over the two day exposure period, the 8 hr exposures (9 am to 5 pm) averaged 19.3 $\pm$ 0.43 ppb and 63.6 $\pm$ 0.43 ppb (mean $\pm$ se, n = 6). There were no statistical differences in exposure between day 1 and day 2 (paired t-test, p = 0.841 and p = 0.456 for the low and high O_3 treatments, respectively).

Individual chambers had some slight variations in exposure. On day one the low O_3 treatment ranged from 16.2 ppb to 23.3 ppb, and the high O_3 treatment chambers varied from 57.9 ppb to 65.7 ppb. On day 2, these same chambers ranged from 17.3 ppb to 21.7 ppb and 56.2 ppb to 63.6 ppb, respectively. Five of the six low O_3 treatment chambers and three of the six high O_3 treatment chambers had two-day means within their respective 95% confidence intervals. However, those chambers outside the confidence interval were within 0.1 to 0.4 ppb of the interval boundaries, so there was fairly good replication of the O_3 treatments across chambers over both exposure days.

Foliar injury

The injury was averaged for 3 biological replicates of the first to sixth leaves assessed. No injured or senesced leaves were found in the Fiskeby genotype in either the control or high O_3 treatment at either 24 or 48 hours after the fumigation (Table 2). However there was abundant foliar injury observed in the Mandarin genotype under high O_3 treatment at both harvest times, especially after 48 hrs (Table 2). After 24 hrs from the beginning of the O3 exposures, the fourth mature leaf (the leaf just below the one harvested for gene regulation analysis) had a mean leaf injury of 22.5 $\pm$ 7.31 % while it was only 11.7 $\pm$ 8.03%

for the slightly younger fifth leaf. However, by 48 hrs after the start of exposures, only one Mandarin plant in the low O_3 treatment showed any signs of injury (20% on one leaf), whereas for Mandarin plants in the high O_3 treatment, leaves 2 through 5 all showed substantially more injury than they did at 24 hrs. A one-way ANOVA showed that there were no differences ($p = 0.7207$) among leaf maturity levels; so all five leaves were averaged together to obtain the final leaf injury assessment, which was 38.0 $\pm$ 15.28%.

Gas exchange and stomatal conductance

Dark respiration was measured immediately following the photosynthesis measurements, but at 0 PPFD. VPD_{leaf} was significantly lower by ~0.6 kPa ($p = 0.045$) and RH higher by ~7% ($p = 0.016$) in the cuvette when illuminated at 1500 µmol m^{-2} s^{-1} for leaves of Mandarin compared to Fiskeby. In the dark, RH was still higher by 6% ($p = 0.034$), but VPD_{leaf} was no longer different ($p = 0.112$). No other environmental parameters differed between the genotypes or ozone treatments. Steady-state measurements were obtained in less than two minutes from the time of insertion into the cuvette, which would be too quick for stomata to react to the difference that was found in VPD_{leaf} between Mandarin and Fiskeby leaves, which resulted from differences in the magnitude of g_s between the two genotypes (see below for more details).

In the genotype effects analysis, a paired t-test (O_3 treatments are combined so n = 6) was used to compare Mandarin to Fiskeby across both O_3 treatments to see if there was a genotype difference in either A_n or r_d. Genotype differences for A_n or r_d within a chamber (Mandarin response – Fiskeby response) showed no ozone effect ($p = 0.109$, $p = 0.156$, respectively, Table 3). There were highly significant differences between genotypes for g_s, both in the light and in the dark ($p = 0.0002$, $p = 0.0016$, respectively, Table 3). Stomatal

conductance in Mandarin was more than double that in Fiskeby (2.4X higher) in the light, and double in the dark (2.1X higher). The A_n/g_s ratio is sometimes used to indicate the sensitivity to ozone, where higher ratios are often correlated with lowered sensitivity due to supposed production of photosynthates for antioxidant defenses and cellular repair mechanisms (Skelly *et al.*, 1996). This ratio is significantly higher (p =0.004, Table 3) for Fiskeby then for Mandarin, which suggests a greater tolerance to O_3 stress.

To analyze ozone treatment effects within each genotype, a *t*-test (n=3) was used to compare the control treatment to the high O_3 treatment for each genotype separately. There were no differences in any gas exchange parameters between the control and high ozone treatments for either genotype (Table 4).

Gene expressions

Each data point for gene expression is the average of three experimental replicates (Figure 5). A few of the data points had overly large coefficients of variance (>73%), usually due to an abnormal replicate. I adjusted these data points by taking out the aberrant replicate (for glutathione in 25 ppb at 4 h in Mandarin, and for pD1 under 75 ppb at 2,4,6 h in Mandarin, Figure 4, Figure 6, Table 5-6). Removal of these outliers did not alter the biological interpretation of the patterns, either with respect to genotypic differences or O_3 treatment effects. Rather, they simply reduced the standard errors for those data points. Therefore the discussion is mainly based on adjusted data (Figure 4, Figure 6, Table 5-6). Relative quantification (RQ) is arbitrarily determined and is the ratio of gene expression relative to four times the expression of GR2 for Fiskeby at 0 h under 25 ppb (Figure 3-6). The RQ is used because some of the original data are smaller than 1, making it hard to

differentiate from each other, especially when plotting the data. The expression of GR2 for Fiskeby at 0 h under 25 ppb times four has RQ=1.

GR2 expression in Mandarin was anomalously high ($p = 0.0164$, Table 5) for reasons unknown, and the effects was highly significant at 2h of exposure ($p = 0.0104$, Table 5). In contrast, the effects were marginally significant in Fiskeby at 2h ($p = 0.0533$, Table 5). pD1 expression was barely affected by high O_3 treatment in Mandarin across all time points and was only upregulated in Fiskeby at 2 h ($p = 0.0390$, Table 5).

There were few differences under control 25 ppb O_3 except for higher pD1 expression in Mandarin at 0 h ($p = 0.041$, Table 6) and 1 h ($p = 0.0208$, Table 6). Under high O_3, the GR2 expression at 0 h ($p = 0.0297$, Table 6) and 2 h ($p = 0.00227$, Table 6) was significantly greater in Mandarin then in Fiskeby. The temporal trends of relative expression for GR2 and pD1 are represented in Figure 3-6.

Discussion

Researchers have postulated that differential gene expression may be responsible in part for determining differences in the sensitivity of plants to ozone (Paolacci *et al.*, 2007; Puckette *et al.*, 2008; Cho *et al.*, 2013). In particular, antioxidant genes tend to be upregulated in response to O_3 exposure while metabolic genes are downregulated (Lee and Yun, 2006; Tosti *et al.*, 2006; Olbrich *et al.*, 2010; Whaley *et al.*, 2015), and higher expression of the former genes may be characteristic of ozone-tolerant plants, whereas higher expression of the latter genes may indicate higher ozone sensitivity. Those studies that examined changes in plant gene expression under O_3 stress using microarrays mostly focused on either an O_3-tolerant or O_3-sensitive genotype within a species but did not compare the two types. In addition, changes in gene expression were often examined at just one point in time and often several hours after exposure. Also, many gene expression studies have used excessively high O_3 concentrations during their exposures, which are not realistic in terms of the current ozone conditions found in most areas where soybeans are grown. I could find few studies that investigated responses at exposure times as short as just one hour of O_3 exposure except Volkov *et al*., (2006).

My study is unique in that it has addressed these short comings by measuring tolerant and sensitive genotypes within the species *Glycine max* (soybean) with exposures as short as one hour and as long as 24 hrs. In this study, there were only minor differences in gene expression of an antioxidant gene GR2 and a metabolic gene pD1 between a sensitive soybean genotype (Mandarin (Ottawa)) and a less sensitive genotype (Fiskeby III) (Tables 5 and 6). Although two genes are too small of a number to adequately represent other genes in the same category, it is also possible that some of the lack of differences in gene expression

is due to different O_3 uptake patterns, since the sensitive genotype Mandarin had a higher stomatal conductance than the tolerant genotype Fiskeby. Thus, when leaves are sampled at similar times, it would be expected that the dose would be higher in Mandarin than Fiskeby, and any differential gene expression could be due to this, rather than some inherently higher sensitivity to this pollutant.

Glutathione Reductase 2 (Glyma02g16010)

Three GR genes (GR1, GR2 and GR3) have been identified in different plant species including *Arabidopsis thaliana*, rice, kimchi cabbage and *Phragmites communis* (Marty *et al.*, 2005; Mhamdi *et al.*, 2010; Trivedi *et al.*, 2013; Kim *et al.*, 2015; Wu *et al.*, 2015; Zhang *et al.*, 2015). These genes code for the GR isoforms: GR1 targets the cytosol, while GR2 and GR3 both dually target chloroplasts and mitochondria (Chew *et al.*, 2003; Mhamdi *et al.*, 2010; Kim *et al.*, 2015; Wu *et al.*, 2015). Arabidopsis GR2 mutants are embryo-lethal (Tzafrir *et al.*, 2004), while GR1 knockout mutants show no phenotypic alterations, but a 30–60% reduction in extractable enzyme activity (Marty and Siala, 2009; Mhamdi *et al.*, 2010). GR1 and GR3 are upregulated by both biotic and abiotic stresses (Mhamdi *et al.*, 2010; Wu *et al.*, 2015). However, in a study using the microarray database Genevestigator, organellar GR2 was not up-regulated by cold, salt or osmotic stresses (Zimmermann *et al.*, 2004; Trivedi *et al.*, 2013). Ideally, GR1 would have been a better gene to investigate since it is known to be upregulated by a wide variety of stresses. However, I used the soybean GR2 gene (Glyma02g16010) due to the convenience of primer design.

Previous research has shown that glutathione (GSH) content (Schupp and Rennenberg, 1988) and GR activity (Kocsy and Owttrim, 1997) both follow a light-dependent diurnal pattern. The GSH concentration is high in midday and low during the

night, whereas GR activity increases in the dark and starts to decrease during the light period. It is possible that this pattern results from dealing with daily patterns of oxidative stress. In my study, GR2 expression gradually increased in similar proportion within each of the two genotypes and reached a peak at 2 h (11:00 h) under control O_3 (Figure 4), which is probably just the expression pattern coincident with the diurnal ryhthm for GSH and GR enzyme activity. When each genotype was exposed to the high O_3 treatment, expression peaks (at 1 h, Figure 4) were elevated when compared with the peak at 2 h for corresponding control samples, as well as shifted to an hour earlier in both genotypes. It is worth noting that the GR2 expression in Mandarin at high O_3 at 0 h was significantly higher than its control at 0 h (Table 6), and for Fiskeby in either the control or high O_3 treatment at this time. This is hard to explain because all the 0 h samples for both the Mandarin and Fiskeby genotypes should presumably have similar amounts of GR expression. Nonetheless, both genotypes were more upregulated by high O_3 at 1 hr (Figure 4), but there is no significant difference in either the extent or rate of upregulation between the two genotypes. Whaley *et al*. (2015) performed a RNA-seq analysis which showed an increased expression of glutathione genes in both genotypes after exposure to 75 ppb O_3 for 5-7 hours (Whaley *et al*., 2015). However, in another study by Chutteang *et al*., (2015) exposure to 70 ppb O_3 did not alter GR activities or leaf GSH content in the same two soybean genotypes after 4 days of exposure. They did observe a 22% higher ($p \leq 0.001$) leaf GR activity in the Mandarin genotype than in the Fiskeby genotype, which is consistent with my finding that the GR2 expression in Mandarin was greater under high O_3 at 0 h and 2 h (Figure 4).

The GR diurnal pattern described above dampened out with time in both genotypes and in both the control and high O_3 treatments and was not observed after 4 h (Figure 4). This

could explain why Chutteang *et al.* (2015) did not find any treatment effects at 4 days. It is also possible that 4 hours constitutes a metabolic cycle and since the other time points were multiples of 4 h (8 h, 12 h and 24 h), they might all be timed at the nadir of the cycle and so no daily patterns were able to be observed after 4 h. It is also possible that the first 4 hours is an adjustment period after the sudden "shock" of O_3 fumigation. The accumulating mRNAs may have reached optimal amount at which point no further transcription was needed and the intracellular redox status had approached some sort of a homeostatic equilibrium (Foyer and Noctor, 2009; Bilgin *et al.*, 2010). A third possibility is that the role GR played to reduce GSSG back to GSH was taken over by other antioxidative enzymes or GSH synthesis enzymes such as adenosine 5′-phosphosulphate (APS) reductase and γEC synthetase (Kocsy *et al.*, 2001). This process may also involve hormone regulation with feedbacks that regulate GR and other antioxidative enzymes (Overmyer *et al.*, 2003; Kovacs *et al.*, 2015).

In summary, the results from the first four hours support the idea that changes in antioxidative gene expression can happen very rapidly in response to the quick ROS burst that is elicited after sudden exposure to high O_3. As an example, a ROS peak was detected 4 hours after 300 ppb O_3 treatment in a sensitive Arabidopsis ecotype (Mahalingam, 2006). H_2O_2 was found to increase 2.3-fold within 15 min in Arabidopsis, but the return to basal levels took a few hours (Volkov *et al.*, 2006). These responses may be stress-, dose- and species- specific, however they should attract future attention to investigate earlier and potentially more robust stress responses.

Photosystem II protein D1 (Glyma13g15560.1)

There was little significant differential gene expression between the two genotypes for pD1 after exposure to O_3. Mandarin had a higher basal level of pD1 at 0 h and 1 h than

Fiskeby under control O_3 (Figure 6). The reason for this is unknown at present but could represent a higher susceptibility to light stress in the Mandarin genotype, necessitating greater synthesis and repair of the pD1 protein than in the Fiskeby genotype. pD1 expression in both genotypes responded similarly across all time points with one exception where Mandarin showed marginal treatment effects (p =0.1289) at 1 h (Table 5).

Photosystem II (PSII) is the water splitting protein-pigment complex in the chain of oxygenic photosynthesis. It is located in the thylakoid membranes of chloroplasts in plants and captures photons to extract electrons from water molecules and eventually produce ATP through the process of chemiosmosis. The reaction core of PSII is formed as a heterodimer with proteins D1 (also known as PsbA) and D2 (PsbD), which receive the excitation energy from antenna proteins CP43 (PsbC) and CP47 (PsbB) and then initiate the energy conversion process (Raymond and Blankenship, 2004). The stability of PSII is challenged by light-induced damage that especially targets its reaction center (Murata *et al.*, 2007). Each PSII complex is presumed to be damaged for every 10-100 million photons received (Eckert et al., 1991; Tyystjärvi and Aro, 1996). Therefore, photodamage to PSII occurs irrespective of light intensities but the rate and scope of damage is proportional to light intensity (Tyystjärvi, 2008). This phenomenon is generally referred to as photoinhibition (Foyer *et al.*, 1994), and it is determined by the rates of photodamage and repair of PSII (Murata *et al.*, 2007). Repair involves the degradation and re-synthesis of the damaged D1 protein (Murata *et al.*, 2007) and the constant demand for new protein copies may explain the high (10^3~10^4) RQ scale of pD1 expression compared to the much lower (~10^1) scale for GR2 expression. This may also lead to the peak of pD1 mRNA synthesis at 2 h (10:00 h) in Mandarin exposed to control O_3 because 10:00 h is when the plants start to get exposed to much higher light and therefore

could begin to experience photoinhibition (Figure 6). However, there was no significant upregulation of pD1 in Fiskeby at the same time (at 2 h) under control O_3 (Figure 6), the cause for which requires further investigation. Perhaps the D1 protein complex is more stable in Fiskeby or, due to its lower stomatal conductance, and reduced O_3 uptake, its regulation is less affected by exposure. But even at 4 h, there was still no impact of O_3 exposure in this species, and even in the more sensitive Mandarin, expression was reduced after 2 h and showed no further sensitivity due to O_3 exposure (Figure 6).

Photoinhibition can be enhanced by environmental stresses, such as cold, heat, salt and oxidative stresses (Nishiyama and Yamamoto, 2001; Yin *et al.*, 2010; Gaur and Sharma, 2014; Sui and Han, 2014). Takahashi & Murata (2008) suggested that the enhanced damage by these stressors is not due to promotion of photodamage, but rather due to inhibition of the repair of PSII by downregulating D1 protein synthesis. Such inhibition was associated with failed elongation of peptides during the D1 protein translation step in studies using cyanobacteria (Nishiyama and Yamamoto, 2001; Allakhverdiev and Murata, 2004). In my study, the expression peak of pD1 at 1h for Mandarin was marginally reduced (p =0.1289, Table 5) by exposure to elevated O_3, probably because that treatment inhibited the repair of PSII. This suggests that O_3 somehow prevented the expression of this gene at the level of transcription rather than at translation, maybe through epistatic effects (interacting effects of other genes). However, Fiskeby maintained a steady level of pD1 expression under both control and high ozone treatment across all time points (Figure 6), either due to its lower uptake, which may have resulted in minimal alterations to its redox status, and hence regulation of gene expression, or because its PSII complex is for some reason more resistant to oxidative stress. Similar to the above result, Chutteang *et al.* (2015) found that the

efficiency of energy conversion and photosynthetic electron transport was reduced in Mandarin under 70 ppb O_3 by comparing quantum yield, electron transport rate and photochemical quenching, all of which are affected by degradation of the D1 protein.

Overall, Fiskeby exhibited smaller changes in expression for either GR2 or pD1 compared to Mandarin. Similar to the trend I found, Whaley *et al.* (2015) discovered that Fiskeby is less responsive than Mandarin in expression for those genes commonly associated with oxidative stress. They found two patterns in general for the gene regulations by comparing the transcriptome of the two genotypes: the timing of differentially expressed gene as expected in the two genotypes are different, with the expression of a majority delayed in Fiskeby; a small number of genes lacked response in Fiskeby during the whole time-course when compared to Mandarin. This can be explained, in part, as due to less O_3 uptake because of lower stomatal conductance in Fiskeby than in Mandarin. Given the difference in stomatal conductance between Fiskeby and Mandarin, the same O_3 exposure would result in a lower "dose" and hence, less effect in Fiskeby than in Mandarin. However, in my study, if the expression for both GR2 and pD1 of Fiskeby at 2 h is compared with that of Mandarin at 1 h, which would somewhat equalize the dosages, there was still no expression response observed in Fiskeby. Thus, even when Fiskeby accumulates the same dose, it does not respond similarly to Mandarin, which suggests either that Fiskeby has inherently greater resistance at the molecular level than Mandarin, or, that exposure to the same dose, but applied in a chronic (low rate of uptake) manner over a longer time period, does not elicit the same molecular or physiological response as exposure to acute O_3 (high rate of uptake) for a shorter time period (Grulke *et al.*, 2007*b*; Chen *et al.*, 2009).

As discussed before, the impact of ozone on plants is determined by the O_3 concentration and duration of exposure. Chronic exposures to low levels of ozone can be found worldwide (Kangasjärvi *et al.*, 2005). In contrast, acute exposures occur less often, except in heavily polluted urban or industrial areas where the air quality is a major concern (Long and Naidu, 2002). However, initial studies at the molecular level have generally applied short acute O_3 exposures to investigate elevated O_3 effects on plants, because such experimental settings are easier to manipulate and the responses are more guaranteed under such conditions than after exposure to chronic fumigation (Kangasjärvi *et al.*, 2005). The 75 ppb O_3 chosen for the exposure concentration in my study was selected because it is near the naturally occurring elevated O_3 concentrations currently found in the Piedmont of North Carolina in recent years. If the expression of stress responsive genes was not simply delayed in Fiskeby until it accumulated a higher dose, it might be because that level of exposure is only a chronic concentration for Fiskeby but an acute concentration for Mandarin, because of its higher uptake rate and maybe greater molecular sensitivity. Since acute and chronic O_3 exposures may induce distinct response mechanisms within the leaves, the single exposure experiment I conducted may only be looking at one part of the ozone tolerance syndrome.

It was reported that while ET and methyl jasmonate are associated with signaling pathways triggered by acute O_3, they are not involved in mediating responses to chronic O_3 in Pima cotton (Grantz and Vu, 2012). Chen *et al*. (2009) found that both types of exposures could decrease leaf photosynthetic CO_2 uptake and photosystem II (PSII) efficiency (Fv/Fm) by similar percentages compared with controls, but the acute treatment induced a more spatially heterogeneous reduction, which might be due to the heterogeneous stomatal conductances across the leaf (Mott and Buckley, 2000). In my study, the response of the

whole leaf regarding gas exchange and photosynthesis was measured and therefore the actual response of the cells might have been neutralized, which means effects on highly damaged cells were obscured by other regions of the leaf that remained highly functional. Sawada and Kohno (2009) examined leaf visible damage and grain yield reduction in different rice cultivars and discovered that distinct mechanisms exist for inducing acute leaf injury and chronic yield reduction.

Based on the above knowledge, it is reasonable to further investigate whether Mandarin and Fiskeby would take up the same amount of O_3 across different time points. If not, then an experiment should be designed that provides an equivalent "dose" over a similar time period for both genotypes. This can be done by adjusting the O_3 concentration such that the fluxes ($[O_3] \times g_s$) are made equivalent. Only in this way can one be sure that the experiment is testing molecular responses that truly reflect the innate characteristics in regard to oxidative stress defense, signaling and metabolism within the tolerant and sensitive genotypes.

Table 1. Specific primers used for relative quantification of GR2 and pD1 mRNA expression by qPCR.

Primers	**Functions**	**Glyma ID**	**Tm (T°)**	**Sequence**
60s	ribosomal protein L30	Glyma17g05270	60.0	5'AAAGTGGACCAAGGCATATCGTCG3'
Glutathione Reductase 2 (GR2)	glutathione reductase	Glyma02g16010	60.1	5'GGATGTGTGCCGAAGAAGTT3'
Protein D1 (pD1)	photosystem II reaction center protein A	Glyma13g15560	60.0	5'TCCCGCTACTGCTGTTTTCT3'

Table 2. Percent leaf area with stipple 24 and 48 hours after the onset of O_3 fumigation. Values are means ± se. n=3. No statistical analysis for a genotype effect was required because of the absence of injury in all Fiskeby plants. Values for the Mandarin plants refer to the mean of the fourth and fifth leaves at 24 hrs, and the second through fifth leaves at 48 hrs.

Genotype	**[O_3]**	**Harvest time**	**% leaf injury**
Mandarin	25 ppb	24 h	0
		48 h	1.3 + 1.33
	75 ppb	24 h	17.1 ± 7.31
		48 h	38.0 ± 15.28
Fiskeby	25 ppb	24 h	0
		48 h	0
	75 ppb	24 h	0
		48 h	0

Table 3. Gas exchange comparisons between genotypes across O_3 treatments. A leaflet on the 3rd mature leaf (the leaf sampled for gene regulation analysis) was used for the gas exchange measurements. Bolded *p* values indicate significance at $p < 0.05$. *Ratio uses g_s measured in the light. Values are means ± se. n = 6.

Parameter	**Mandarin**	**Fiskeby**	***p* value**
Photosynthesis (A_n) (umol m^{-2} s^{-1})	16.2 ± 1.71	11.8 ± 1.57	0.109
Dark Respiration (r_d) (umol m^{-2} s^{-1})	-1.9 ± 0.35	-1.9 ± 0.03	0.156
Stomatal Conductance (g_s) (mol m^{-2} s^{-1}) in light	0.474 ± 0.028	0.201 ± 0.037	***<0.001***
$g_{s\text{-dark}}$ in dark (mol m^{-2} s^{-1})	0.365 ± 0.04	0.175 ± 0.03	***0.002***
A_n/g_s* (umol/mol)	34.2	58.7	***0.004***

Table 4. Gas exchange comparisons between O_3 treatments for each genotype. A leaflet on the 3rd mature leaf (the leaf sampled for gene regulation analysis) was used for the gas exchange measurements. Bolded p values indicate significance at $p < 0.05$. Values are means + se. n=3.

Genotypes	Parameters	Low O_3	High O_3	p value
Fiskeby	Photosynthesis (A_n) (umol m^{-2} s^{-1})	11.2 ± 2.24	12.4 ± 2.63	0.750
	Dark Respiration (r_d) (umol m^{-2} s^{-1})	-1.4 ± 0.41	-1.9 ± 0.04	0.359
	Stomatal Conductance(g_s) (mol m^{-2} s^{-1})	0.221 ± 0.066	0.182 ± 0.044	0.643
	$g_{s\text{-dark}}$ in Dark (mol m^{-2} s^{-1})	0.184 ± 0.059	0.165 ± 0.039	0.796
Mandarin	Photosynthesis (A_n) (umol m^{-2} s^{-1})	18.2 ± 2.99	14.3 ± 1.29	0.289
	Dark Respiration (r_d) (umol m^{-2} s^{-1})	-1.9 ± 0.03	-2.3 ± 0.28	0.340
	Stomatal Conductance(g_s) (mol m^{-2} s^{-1})	0.541 ± 0.072	0.407 ± 0.070	0.253
	$g_{s\text{-dark}}$ in Dark (mol m^{-2} s^{-1})	0.412 ± 0.045	0.295 ± 0.050	0.206

Table 5. Gene expression comparison over time between the two genotypes across the low and high O_3 treatment. Bolded p values indicate significance at $p < 0.05$. n = 3. * indicates marginal significance. For definitions of GR2 and pD1 refer to Table 1.

Gene	Genotypes	Hours	*p* value
GR2	Mandarin	0	***0.0164***
		1	0.1644
		2	***0.0104***
		4	0.5882
		8	0.2069
		12	0.6194
		24	0.8844
	Fiskeby	0	0.2760
		1	0.1320
		2	*0.0533
		4	0.4963
		8	0.1363
		12	0.7306
		24	0.2079
pD1	Mandarin	0	0.5645
		1	*0.1289
		2	0.3414
		4	0.5283
		8	0.4166
		12	0.5258
		24	0.9753
	Fiskeby	0	0.2696
		1	0.8710
		2	***0.0390***
		4	0.5448
		8	0.8927
		12	0.7447
		24	0.4183

Table 6. Gene expression comparison over time between the low and high O_3 treatment across the two genotypes. Bolded *p* values indicate significance at $p < 0.05$. n = 3. For definitions of GR2 and pD1 refer to Table 1.

Gene	O_3 Treatment	Hours	*p* valued for t-test
GR2	Low O_3	0	0.156
		1	0.235
		2	0.200
		4	0.829
		8	0.383
		12	0.178
		24	0.910
	High O_3	0	**0.030**
		1	0.484
		2	**0.002**
		4	0.946
		8	0.294
		12	0.528
		24	0.145
pD1	Low O_3	0	**0.041**
		1	**0.021**
		2	0.268
		4	0.224
		8	0.133
		12	0.448
		24	0.128
	High O_3	0	0.233
		1	0.200
		2	0.214
		4	0.112
		8	0.489
		12	0.944
		24	0.432

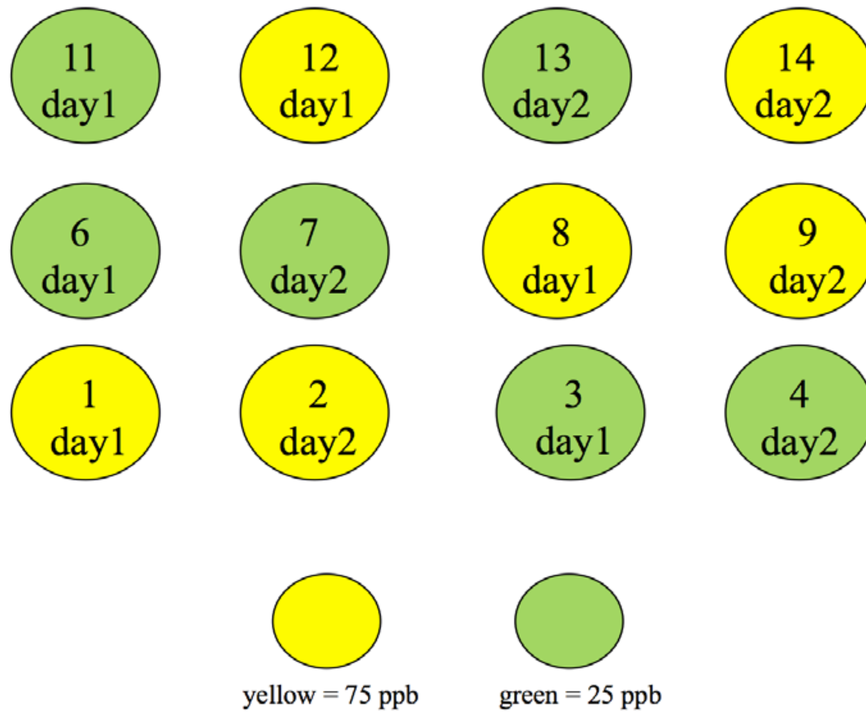

Figure 1. Greenhouse layout. Each circle represents a CSTR chamber. Day 1 and day 2 chambers contain the plants that would be harvested at 0/1/2/4 h and 8/12/24/48 h after the onset of O_3 fumigation, respectively. Yellow and green circles represent high/low O_3 chambers, respectively. Positions of the chambers within each block (rows) are randomized.

CSTR Chamber 1

CSTR 1 (75 ppb) - day 1

position	genotype	harvest time
A	Man Ottawa	1
B	Man Ottawa	0
C	Fiskeby III	2
D	Man Ottawa	4
E	Fiskeby III	1
F	Man Ottawa	2
G	Fiskeby III	0
H	Fiskeby III	4

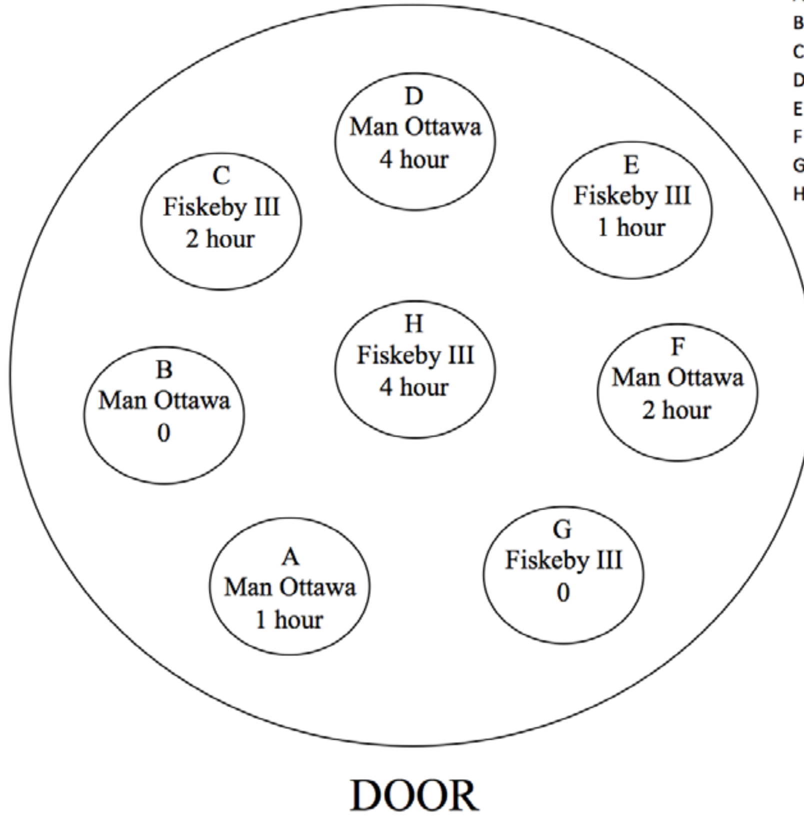

Figure 2. Example plant positions inside chamber 1. Positions were randomized for the other CSTR chambers.

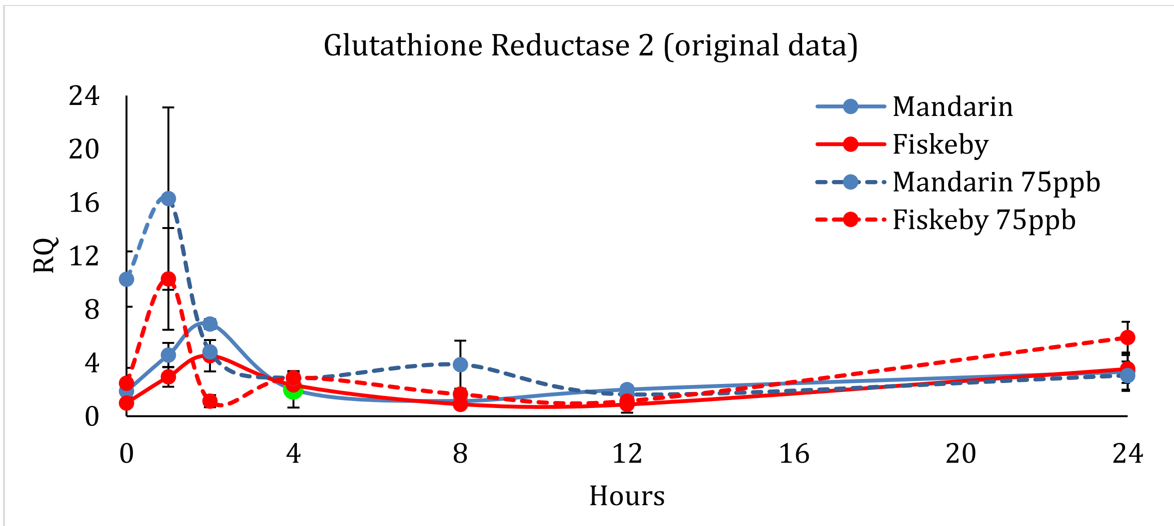

Figure 3. GR2 expression over a one-day period in the two genotypes under control and high O_3 treatments (with original data). The data points that have high coefficients of variation are indicated in green.

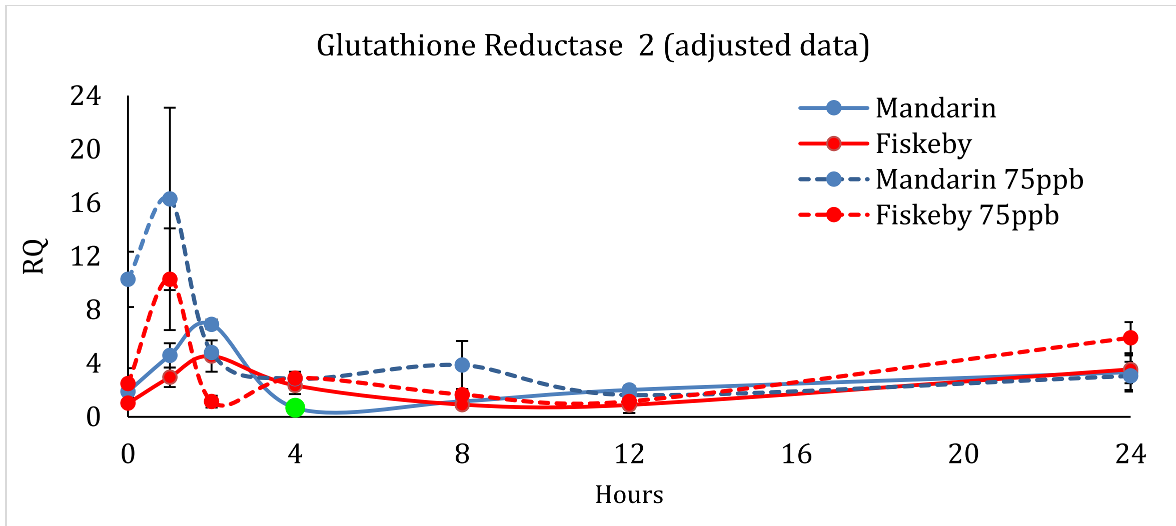

Figure 4. Adjusted GR2 expression over a one-day period in the two genotypes under control and high O_3 treatments (with adjusted data). Adjusted data points are indicated in green.

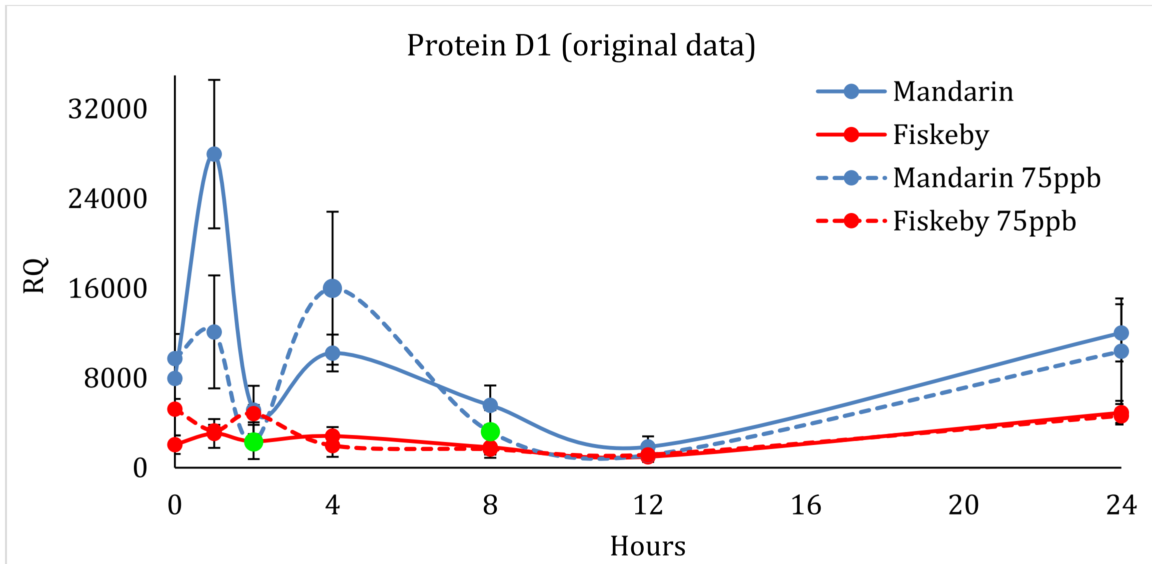

Figure 5. pD1 expression over a one-day period in the two genotypes under control and high O_3 treatments (with original data). The data points that have high coefficients of variation are indicated in green.

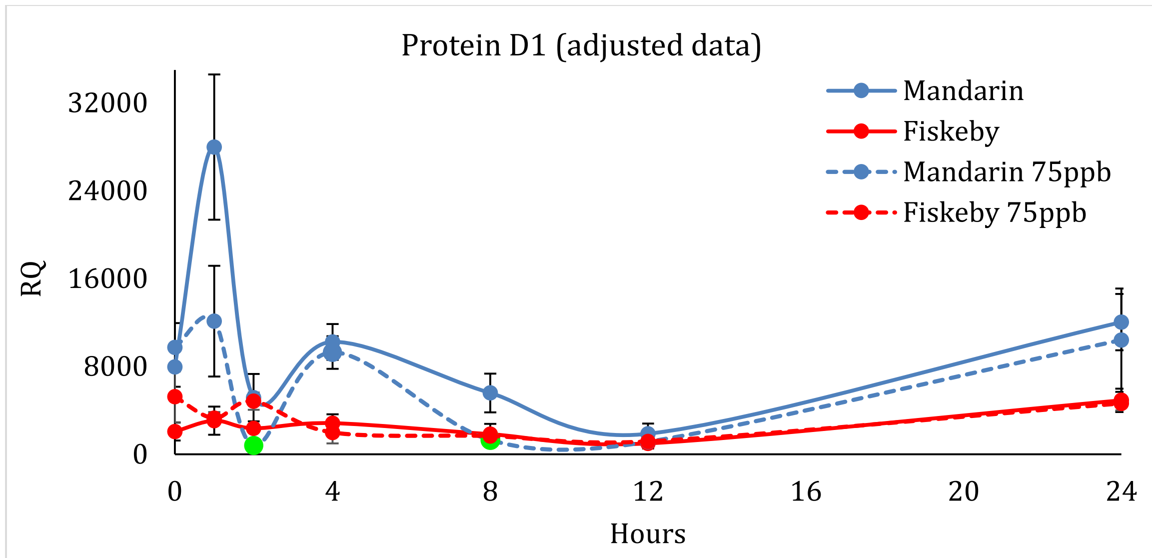

Figure 6. Adjusted pD1 expression over a one-day period in the two genotypes under control and high O_3 treatments (with adjusted data). Adjusted data points are indicated in green.

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Vita

Tongji Xing was born in May 1987 on a summer night in Taiyuan, Shanxi Province, China, as the first and only child of Jin'an Xing and Xiaofang Zhao. Growing up, Tongji helped her parents grow tomatoes, sunflowers, and bell peppers; and from this experience she learned the importance of having the correct environment in plant cultivation. Her college education consisted of her study at Shanxi University where she studied Biology. During College she spent a year abroad at Pfeiffer University where she fell in love with the natural and pristine environment of North Carolina. While at Pfeiffer, she researched the ROS scavenging effects of dietary supplements on the antioxidants system in plums. Pursuing her passion for research in plant biology, she began work on her master's in plant molecular biology at Appalachian State University in the summer of 2012. Her ongoing research efforts have led her pursue her Ph.D. at Rutgers University, where she joined the molecular biosciences program in fall of 2014. She now lives in New Brunswick, NJ, with her husband Tomas Kasza and their cat Lyla.