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THE ROLE OF ASCORBIC ACID IN THE PLANT – HERBIVORE INTERACTION

THE ROLE OF ASCORBIC ACID IN THE PLANT – HERBIVORE INTERACTION

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Entomology

By

Kelly Ann Carruthers
University of Arkansas
Bachelor of Science in Biology, 2008

May 2012
University of Arkansas

ABSTRACT

Ascorbic acid (AsA), vitamin C, is an important molecule that is vital for both human and plant health, because it aids in the detoxification of reactive oxygen species (ROS) caused by various abiotic and biotic stresses including UV, salinity, ozone, and attack from pathogens. In order to understand the role of AsA in the plant-herbivore interaction, we looked at the effects of the wound-responsive hormones, jasmonate, wounding, and herbivory on AsA content. Our study is the first to directly compare the effects of wounding and jasmonates on AsA levels in plants. We looked at the effects of wounding and methyl jasmonate (MeJA) on JA-deficient and JA-insensitive genotypes of tomato to further determine if JA mediates the effects of wounding on AsA in plants, because JA is involved in many other wound responses. Wounding in two cultivars of tomato lowered AsA content as did exogenous MeJA application, suggesting a link between these two. Wounding of mutant lines impaired in JA signaling showed a decrease in AsA content similar to wild type, indicating AsA response to wounding does not require JAs. These results show a complicated relationship between AsA, JA, and wounding, in which both JA and wounding appear to but may act independently. Few studies explore ascorbate's role in actual plant systems with associated herbivores. We evaluated the effects of multiple types of herbivores on AsA of both tomato and Arabidopsis, providing the first comparison of different types of herbivore damage on AsA content among plant types. Tomato and Arabidopsis responded differently to herbivory. Ascorbate decreased significantly in response to caterpillar feeding in tomato foliar tissue with no significant difference in systemic or root tissue. Whereas, Arabidopsis saw no significant difference in treated tissue but saw significant decrease in AsA of systemic tissue of aphid and caterpillar treated plants. We were, also, able to determine high AsA content of a *MIOX4* overexpression line and use it in determining the effects of AsA on

caterpillars in conjunction with WT, moderate AsA, and vtc1 mutant, low AsA. The *MIOX4* overexpression line supported caterpillars of a greater weight than either WT or *vtc1*.

This thesis is approved for
recommendation to the
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DEDICATION

To my family and friends:
For listening, understanding, making me laugh,
and for all the love and support you give
I would not be where I am or who I am without you.
For mom, dad, and Lea, above all else

To Tobi,
For loving me
and for believing in me
at times when I forgot how to

To Col. Lott,
Semper Fi and
live long and prosper!

To my students at Liberty High school
You can accomplish anything with
hard work and respect.

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CHAPTER I

Effects of Wounding and Jasmonates on Ascorbic Acid Content of Tomato Foliage

ABSTRACT

Ascorbic acid (AsA), vitamin C, is vital for both human and plant health, because it aids in the detoxification of reactive oxygen species (ROS) caused by various abiotic and biotic stresses including UV, salinity, ozone, and attack from pathogens. The plants' ability to defend against these stresses is also dependent upon a class of wound-responsive hormones called jasmonates (JAs). In order to understand the role of AsA in the plant-herbivore interaction, changes in AsA content due to physical damage by wounding have been observed in plants. Similarly, previous studies have investigated the effects of JAs on AsA. Typically, wounding and wound-responsive JAs have been studied independently of one another. Our study is the first to directly compare the effects of wounding and JAs on AsA levels in plants. We looked at the effects of wounding and exogenous application of methyl jasmonate (MeJA) on JA-deficient and JA-insensitive genotypes of tomato (*Solanum lycopersicum*) to further determine if JA mediates the effects of wounding on AsA in plants. Wounding in two cultivars of tomato lowered AsA content as did exogenous MeJA application, suggesting a link between these two. Ascorbate content was higher in JA-deficient cultivars as compared to wild type tomato for one experiment, suggesting that endogenous JA may play a role in AsA regulation. We exogenously applied MeJA to *spr2*, a JA-deficient mutant, and wild-type (WT) (cv. Castlemart) and found that AsA decreased in all cases. This suggested that exogenous MeJA application restores the WT levels of AsA. Wounding of *spr2* and *jai1*, mutant lines impaired in JA signaling, showed a decrease in AsA content similar to WT, indicating AsA response to wounding does not require JAs. These results suggest a complicated relationship between AsA, JA, and wounding. Both JA and wounding appear to cooperate but may act independently. Further experiments are necessary to fully understand the interactions between AsA, JA, and wounding.

INTRODUCTION

L-Ascorbic acid or ascorbate (AsA), also known as, vitamin C, is most noted for its importance in human health; it is an essential dietary requirement, obtained through fruits and vegetables for humans in the prevention of scurvy (Lorence and Nessler 2007). Ascorbate is also essential for plant health. This is evident by the fact that no plant mutant totally devoid of AsA has ever been described (Suza et al. 2010). Still, despite the critical importance of AsA for human and plant health there is relatively little known about what influences AsA metabolism. There is evidence that mechanical wounding and exogenous application of methyl jasmonate, (MeJA) can affect AsA metabolism (Tables 1 and 2). Wounding is an important component of herbivore damage, and jasmonates (JAs) are wound-inducible signals that play a major role in induced resistance to insects and pathogens as well as to certain abiotic stresses. Therefore, we are interested in the impact of wounding and JAs on AsA accumulation, because they could potentially influence AsA content in insect-challenged plants. Previous studies have investigated the role of mechanical wounding on AsA content on a variety of plants and tissues, and a majority of these studies have shown a decrease in AsA content in response to wounding (Table 1). A majority of other studies have investigated the role of MeJA application on AsA content and report an increase in AsA content in response to exogenous application of MeJA (Table 2). This suggests that wounding and MeJA cause opposite responses in AsA content, but this may be because the two treatments were not compared in the same species and tissue types. The goals of this study were to compare the effects of wounding and MeJA treatment and to determine if JAs mediate the effects of wounding on AsA content by examining wound responses in mutant lines impaired in JA signaling. Because JA and wounding are both involved in plant responses to

herbivory, this study provides a starting point to explore components the responses in the absence of influence from insect saliva or other oral secretions.

Function of AsA and Metabolism

Ascorbic acid is important for a diverse array of functions, including a number of physiological processes in the plant. Its primary functions are as an antioxidant, an enzyme cofactor, and modulator of cell signaling (Foyer and Noctor 2011). As an antioxidant, AsA is involved in the detoxification of reactive oxygen species (ROS) from both by normal, aerobic, cellular metabolism. ROS may be produced by photosynthesis, and by abiotic stresses from ozone, wounding and UV damage, by means of both enzymatic and non-enzymatic reactions (Davey et al. 1999; Smirnoff 2001; Smirnoff et al. 2001). When L-ascorbic acid decreases from interaction with an ROS, an increase in L-dehydroascorbate occurs. In healthy tissue, L-dehydroascorbate, or oxidized AsA, is approximately 5% of total AsA.

Ascorbate is an important enzyme cofactor. For example, AsA is a cofactor for several dioxygenases, non-heme and iron-containing enzymes which aid in the formation of plant hormones and secondary metabolites (Prescott and John 1996). Ascorbate can be a cofactor for myrosinases, as well, which are imperative for the formation of more toxic byproducts of the plant defenses against herbivores, such as glucosinolates in the Brassica family (Burmeister et al. 2000; Conklin et al. 1997; Wittstock and Halkier 2002). Ascorbate can also serve as a substrate for enzymes, including ascorbate oxidase (AO) which aids in the control of cell expansion and division. Ascorbate modulates cell signaling by affecting AO within the apoplast (Pignocchi and Foyer 2003).

Ascorbate is synthesized via multiple biosynthetic pathways, although none were described for AsA until 1998 (Wheeler et al. 1998). Since then, 3 additional alternative routes

have been described: D-mannose/L-galactose (Wheeler et al. 1998), L-galacturonate (Agius et al. 2003), L-gulose (Wolucka and Van Montagu 2003), and *myo*-inositol (Lorence et al. 2004). An understanding of the pathways involved and the mechanisms regulating them has only recently begun to emerge. For example, a protein named AMR1 (ascorbic acid mannose pathway regulator 1) was found to regulate several genes in the D-mannose/L-galactose pathway (Zhang et al. 2009). Wounding and exogenous MeJA have also been shown to influence AsA metabolism (Table 1 and 2), but the mechanisms are still unclear.

Wounding

Wounding due to herbivore damage is a common stress faced by plants. Wounding may be caused by insects and other invertebrates and vertebrates and may be characterized by tissue damage and oxidative stress. Wounding gives insight as to how insect herbivores may affect AsA metabolism without the influence of saliva. Wounding studies have been done on a wide variety of plants, on a variety of tissue types, with a variety of wounding styles, including shredding, crushing, and cutting (Table 1). Total AsA content had been reported to decrease due to tissue wounding in 11 of 19 studies. Total AsA content increased in response to wounding in 5 of 19 studies. In 4 of 19 studies, total AsA did not change significantly. While no significant change in AsA content occurs from wounding in strawberries, there is a significant oxidation of AsA, which shows a change in redox status (Wright and Kader 1997). Wounding may influence the redox status of AsA; for example, in the alligator plant, *Kalanchoe daigremontiana*, the ratio of reduced AsA decreased by one third in the apoplast (Takahama 1993). Ascorbate in its reduced state is both useful for ROS scavenging and for the plant. Reduced AsA can neutralize ROS by providing a stable reaction as an electron and proton donor, which converts AsA into L-dehydroascorbic acid (DHA), the oxidized form.

A potato variety, Bintje, showed an increase in threonic acid, an oxidation product of AsA, by tuber cutting (Galindo et al. 2009) (Table 1). Though trends of decreased amounts of AsA induced by wounding are common, there are still multiple examples in which the opposite is true. Because most studies used detached tissues to assess wounding, experiments need to be done with intact tissue to determine if the effects are the same. Ascorbate has been reported to vary according to tissue type (Lorence et al. 2004), developmental stage of the plant (Zhang et al, 2009), time of day (Burkey et. al. 2003) and light intensity (Hamner et al. 1945). Only a few studies of the ones listed in Tables 1 and 2 have reported these variables in their studies, therefore it is likely that some of the variations seen are due to differences in the experimental conditions used by these research groups.

JA function and biosynthesis

Jasmonates (JAs) also influence AsA content for many plants. The collective term "jasmonates" refers to jasmonic acid (JA), its derivatives, including methyl jasmonate (MeJA), JA-isoleucine and other conjugates (Wasternack 2007). Jasmonates are important for plant health by participation in wounding and systemic acquired resistance (Creelman and Mullet 1997). Similar to AsA, JAs play a vital role in plant growth, development, and response to stress (Creelman and Mullet 1997). In several studies, JA has been shown to affect AsA response. In response to ozone, several genes involved in AsA recycling were induced in WT *Arabidopsis thaliana* but were not induced in JA-deficient mutants of Arabidopsis (Sesaki-Sekimoto et al. 2005). JA has been observed to correlate with antioxidant levels in a study that showed that differing concentrations of MeJA was linked with an increase in several antioxidants (Li and Staden 1998). MeJA induction begins the cascade leading to AsA content changes, as well as many other metabolic changes (Wolucka et al. 2005), and MeJA is induced by herbivory, as well

(Reinboth et al. 1994; Creelman et al. 1992). In 1984, the JA biosynthetic pathway was elucidated (Vick and Zimmerman 1984). The process begins with the release of linolenic acid from storage lipids or membranes, which is converted to 12-oxo-phytodienoic acid. Through further conversion and reduction in the peroxisome, JA is synthesized (Ishiguro et al. 2001). JA can be converted to the volatile, MeJA an isomer of JA, by methylation of the C-1 carboxyl group with help of the enzyme, JA carboxy methyl transferase (Seo et al. 2001). Methylation can be reversed by MeJA esterase, converting MeJA back into JA (Stuhlfelder et al 2004, Wu et al. 2008).

Exogenous application of MeJA is a common technique for studying the effects of JAs on plant physiological processes along with AsA metabolism. Studies consistently have shown a link between JAs and AsA by treating different plants with either MeJA or JA (Table 2). Total AsA content was increased due to exogenous MeJA in 7 of 8 studies, and total AsA content remained unchanged in the remaining study. Fewer conclusions can be drawn from tomato exogenously applied with JA. One study reports increased total AsA, one decreased, and the other remained unchanged. It is important to note that different cultivars of tomato and different tissues were used among the 3 studies that examined the effects of JA on total AsA content. Overall, a majority of the studies showed an increase in AsA content with exogenous application of MeJA (Table 2).

Although all previous studies have relied on exogenous JA treatments to study the impact of JAs on AsA, several mutant tomato lines that are deficient in JA signaling are also available to address this topic. The *spr2* mutant is defective in the synthesis of linolenic acid which leads to JA formation (Lee and Howe 2003). Similarly, *acx1* plants contain a point mutation in Acx1 which causes the plant to be defective in the biosynthesis of JA. The mutation affects the beta-

oxidation, necessary to synthesize JA (Powers 2006). Finally, *jai1*, jasmonic acid insensitive-1, does synthesize JA, unlike the other two mutants, but is unable to sense it (Chen 2006).

In this study, we investigated the role of wounding on AsA and of JA in the plant-insect interaction, because both have a strong relationship with herbivory. Though there are many studies comparing wounding and JA, individually, none test them within the same system. Consequently, little is known about the effects of endogenous JA on AsA metabolism. We utilized JA signaling mutants, wounding and exogenous MeJA application to examine the relationships between JA, wounding, and AsA, all of which may influence plant-insect interaction.

MATERIALS AND METHODS

Surface sterilization of seeds. In experiments involving JA-deficient and JA-insensitive genotypes of tomato, all genotypes, including control var. Castlemart (CM), were surface sterilized prior to planting on wet filter paper in petri dishes. Seeds were rinsed for 2 min with 70% EtOH, followed by a solution of 50% bleach with 0.05% Tween 20 (ICN Biochemicals Cat#103168). After 6 minutes in the 50% bleach solution, seeds were then rinsed 6 – 8 times with sterile ddH_2O, and then plated on filter paper soaked with ddH_2O.

Plant materials. Five tomato genotypes were used for this study: Two WT tomato cultivars, UC82B and Castlemart, and three mutant lines that were developed in a Castlemart background, and that are deficient in jasmonate signaling: *spr2*, *jai1*, and *acx1* (obtained from G. Howe, Michigan State University). In experiments that utilized only WT cultivars, seeds were directly planted into soil, whereas in experiments involving the *jai1* mutant plants, seeds from all genotypes were surface sterilized and germinated on filter paper soaked but not pooled with

water in petri dishes, and *jai1* mutant plants were then exposed to MeJA for detection of homozygous recessive traits. After sowing or transplanting from petri dishes, all plants were grown in 3" pots with soil (Sun Gro Sunshine Mix #1 Professional Growing Mix) supplemented with 15-9-12 Osmocote Plus slow-release fertilizer (Scotts). Plants either planted from seed or transplanted from petri dishes were grown under greenhouse conditions (ca. 24 to 27˚C, L16:D:8 photoperiod). Plants were watered 3 times a day, 6 days a week with a dilute nutrient solution containing 1000mg/L $CaNO_3$ (Hydro Agri North America, Tampa FL), 500 mg/L $MgSO_4$ (Giles Chemical Corp, Waynesville, NC), and 500 mg/L Hydroponic 4-18-38 Growmore fertilizer (Growmore, Gardena, CA), and the 7^{th} day were watered three times with water only.

Jai1 trait detection. Selection of *jai1-1* mutants was performed in F2 segregating populations. JA-insensitive, *jai1* mutants were surface sterilized and germinated in the presence of light on filter paper in petri dishes containing only autoclaved ddH_2O until radicals were approximately 1 cm in length. Water soaked but did not pool on the filter paper; filter paper was rehydrated when necessary. At 4 or 5 days after planting, depending on germination rate, seedlings were then transferred to petri dishes with filter paper soaked but not pooled with 1 mM MeJA for detection of homozygous recessive genes (1 mM MeJA made by adding 2 µL MeJA to 75 µL EtOH and adding the mixture to 10 mL water). Purple coloration due to anthocyanin accumulation in the hypocotyls and stunted root growth indicated the presence of a dominant gene, whereas green coloration due to lack of anthocyanin accumulation and normal root growth indicated presence of the homozygous recessive trait. Petri dishes were maintained in the presence of light (80 – 90 µmol $m^{-}2\ s^{-1}$) to insure the development of strong symptoms in wild-type plants. Plants were ranked for the amount of purple observed, after treatment, from 0, no purple, to 4, strong purple coloring and stunting of growth. Plants marked with a 0 were checked for homozygous recessive

trait by DNA extraction of leaf discs collected from the cotyledons, prior to experimentation. Extraction and dilution were done using REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich, St. Louis, MO), and Green GoTaq Polymerase (Promega, Madison, WI) was used for amplification along with a forward primer to test presence of dominant and recessive traits and reverse primers for both traits (jai Forward: 5'- GTG GAG ACG ATA TGT TGA GAC TAA-3' jai wt Reverse: 5'- CCA TGG AGT CCA TCA CCT AAC AGT-3' jai mutant Reverse: 5'- GTG GTC AGA TCA GAG CCC TCT ATT -3'). These were compared to negative and positive controls. Initial and denaturation steps were at 94˚C for 3min followed by 35 cycles of denaturation at 94˚C for 1min, annealing at 50˚C for 1min and extension 72˚C for 2 min. Final extension was set to 72˚C for 10min, and samples were stored at 4˚C.

Wounding treatment. Wounds to tomato foliar tissue were made using a hemostat to crush the leaves twice along the midvein: Once approximately halfway between stem and tip of the leaf and once closer to the tip of the leaf. Typically, the primary leaflet to the right of the terminal leaflet was wounded. However, if more tissue needed to be collected, the first leaflet to the left of the terminal leaflet was also wounded and collected. Leaflets were chosen from healthy, fully expanded leaves. The experiment testing the affect of wounding of 6 week old WT Castlemart cultivar over several time points used the 4^{th} fully expanded leaf from the bottom; the experiment involving 5 week old UC82 used the 3^{rd} fully expanded leaf from the bottom; and the experiments using 5 week old JA-deficient and JA-insensitive genotypes used the 2^{nd} fully expanded leaf from the top.

Methyl jasmonate application. Foliar application of synthetic 75 µM MeJA solution (Sigma-Aldrich Cat#392707) was accomplished by using a spray bottle (Sprayco 32 oz adjustable nozzle bottle). The upper surface of treated leaves was sprayed at a distance of approximately 1' away

with MeJA on the 2nd fully expanded leaf from the top, until water dripped from the tip of the leaf.

Tissue collection for AsA measurement. Plants were between 5 and 6 weeks old at the time of tissue collections, and tissue was collected between 9 and 10 am. We attempted to standardize leaf sample size, and leaves were cut and weighed (leaf between 100 and 160 mg, optimally 150mg), placed in a Whirl-Pak plastic bag (VWR Cat#11216373), and flash frozen by placing the bag immediately into liquid nitrogen. Samples were stored at -80˚C until total, reduced, and oxidized AsA measurements were taken.

Ascorbate measurement. Measurements of total, reduced, and oxidized AsA content in foliar tissue were obtained by ascorbate oxidase assay as previously reported (Rao and Ormrod 1995; Lorence et al. 2004). Samples were ground in ice-cold 6% meta-phosphoric acid (MPA), divided into 2 – 3 subsamples to reduce technical variation into 0.75 mL aliquots and centrifuged at 13,000 rpm for 5 min to remove cellular debri. Supernatant from each subsample was then divided into 2 cuvettes (50 µL of supernatant/cuvette; UltraVette UV-disposable cuvettes, VWR West Chester, PA) containing 950 µL of K-phosphate buffer for separate analysis of oxidized and reduced AsA content. Oxidized AsA was determined by comparing sample absorbance before and after incubation with 1 µL of 1 mM DL-dithiothreitol (DTT, FW = 154.25) (IBI Scientific Cat#IB21040). DTT containing cuvettes were incubated in the dark for 20 min at room temperature. Reduced AsA was determined by comparing sample absorbance before and after adding 1 unit of enzyme, AO (MP Biomedicals Cat#190075) to each cuvette and leaving at room temperature for 1 min. A Perkin Elmer UV/VIS spectrometer Lambda Bio 20 connected to a Gateway 2000 desktop computer using Perkin Elmer UVWinLab software was used to read absorbance at a wavelength of 265 nm. Absorbance units were converted to concentration of

AsA using the extinction coefficient for pure AsA, 14.3 mM^{-1}. Total AsA was determined by adding oxidized and reduced AsA.

Ascorbate oxidase efficiency test. When new aliquots of AO were made, prior to sample testing, an enzyme efficiency test was performed for AO using 69.3 mg of pure AsA added to 10 mL 6% meta-phosphoric acid. A 1 µL aliquot of this solution was added to a cuvette containing 1 mL of K-phosphate buffer (0.1M, pH 6.9). Absorbance was read at approximately 0.5. A 1 unit AO aliquot was then added to the cuvette, and absorbance declined for approximately 1 min until it reached nearly zero.

Statistical analysis. One way and two way analysis of variance (ANOVA), Welch's Weighted ANOVA, and means separation by Student's *t* test, were performed in JMP Statistical Discovery Software v.7.0 (SAS Institute, Cary, NC). Experiments with unequal variances used Welch's Weighted ANOVA.

RESULTS

Impact of wounding on foliar AsA over time in WT plants. Total AsA levels were significantly lower in wounded plants than in untreated controls (two way ANOVA; $F=5.12$; $df=1,37$; $p=0.03$) but did not vary significantly among time points ($F=0.08$; $df=3,37$; $p=0.97$), and there was no significant interaction between treatment and time ($F=1.20$; $df=3,37$; $p=0.32$) (Figure 1). Similarly, reduced AsA levels were significantly lowered due to wounding as compared to untreated control plants (two way ANOVA; $F=7.38$; $df=1,37$; $p=0.01$) but were not significantly different between time points (two way ANOVA; $F=0.11$; $df=3,37$; $p=0.96$) or between the interaction of treatment and time ($F=0.59$; $df=3,37$; $p=0.63$). A marginally significant ($p<0.1$) difference in oxidized AsA was observed due to time (Welch's Weighted

ANOVA; F=2.32; df=7,13.02; p=0.08). This difference appears to exist between untreated plants at 24 hours and other untreated plants at 3, 6 and 12 hours (Student's *t* test; 3 h: p=0.01; 6 h: p=0.02; 12 h: p=0.03). No significant differences were observed in the ratio of oxidized to reduced due to treatment (two way ANOVA; F=0.15; df=1,37; p=0.70), time (F=0.79; df=3,37; p=0.51), or interaction between treatment and time (F=1.29; df=3,37; p=0.29) (Figure 1).

Effects of MeJA and wounding on WT tomato cultivar. In the Castlemart cultivar, a significant difference in total AsA content among wounded, MeJA and water treatments and untreated controls was observed (one way ANOVA; F=3.39; df=3,62; p=0.02) (Figure 2). In comparison to control, wounding (Student's *t* test; p=0.02) and MeJA application (Student's *t* test; p=0.01) caused a significant decrease in AsA content, and AsA content in the water treatment was the intermediate. Reduced AsA content was also significantly different among treatment groups (one way ANOVA; F=3.33; df=3,62; p=0.03) and decreased in response to wounding (Student's *t* test; p=0.02) and MeJA treatment (Student's t test; p=0.01) but not water (Student's t test; p=0.47). No significant differences were observed in oxidized AsA among treatment groups (one way ANOVA; F=0.79; df=3,62; p=0.50) or in the ratio between oxidized and reduced AsA (one way ANOVA; F=0.78; df=3,62; p=0.51).

In the WT UC82 tomato cultivar, total AsA content decreased significantly when treatment was applied to leaves as compared with tomato foliage sprayed with water alone (two way ANOVA; F=3.04; df=2,65; p=0.05) (Figure 3). No significant differences were observed between the 6 and 24 h after treatment (F=2.49; df=1,65; p=0.12) or due to interaction between time and treatment (F=0.14; df=2;65; p=0.87). Reduced AsA decreased in response to treatment (F=3.05; df=2,65; p=0.05) but did not respond to time (F=2.35; df=1,65; p=0.13) or interaction between time and treatment. (F=0.12; df=2;65; p=0.89). Oxidized AsA did not differ

significantly in response to treatment (F=1.30; df=2,65; p=0.28) but did not respond to time (F=1.84; df=1,65; p=0.18) or interaction between time and treatment (F=0.49; df=2;65; p=0.62).Similarly, the ratio between oxidized and reduced did not differ significantly in response to treatment (F=1.32; df=2,65; p=0.27) but did not respond to time (F=1.92; df=1,65; p=0.17) or interaction between time and treatment. (F=0.33; df=2;65; p=0.72).

Reference levels of AsA in Castlemart, *spr2*, *acx1*, and *jai1* tomato genotypes. Our experiment was conducted to compare baseline AsA levels in *jai1*, *spr2*, *acx1*, and WT. Total AsA was not significantly different among WT, *spr2*, *acx1* and *jai1* genotypes of tomato (one way ANOVA; F=1.29; df=3,47; p=0.29), and no significant differences were observed in reduced AsA (F=1.28; df=3,47; p=0.29), oxidized AsA, (F=0.86; df=3,47; p=0.47) or the ratio between oxidized and reduced (F=0.52; df=3,47; p=0.67) (Figure 5).

Effects of wounding and MeJA on tomato mutants defective in JA synthesis.

An experiment was conducted to determine the reference levels of AsA and the effects of wounding and MeJA on mutants impaired in JA signaling. We were able to test baseline levels of AsA in *spr2*, *acx1*, and WT Castlemart plants but were unable to include *jai1* because of wounding prior to treatment and tissue collection. Also, due to limited numbers of viable mutant plants, we were unable to form a complete, randomized block design. Wounding and MeJA applications were limited, and these treatments were separated in analysis. Both Castlemart and *spr2* received MeJA application and wounding treatments, and *jai1* was only wounded.

Reference levels of AsA in Castlemart, *spr2*, and *acx1* tomato genotypes. We wanted to determine the baseline levels of JA signaling-impaired mutants as compared with WT. A significant difference was observed among genotypes (one way ANOVA; F=3.14; df=2,38; p=0.05). Total AsA content was significantly higher in *spr2* plants than WT, and total AsA

content of *acx1* fell between *spr2* and WT (LSMeans Student's *t* at α=0.05). There was no significant differences were observed in reduced AsA (one way ANOVA; F=2.11; df=2,38; p=0.14), oxidized AsA (F=1.24; df=2,38; p=0.30), or the ratio between oxidized and reduced AsA (F=1.01; df=2,38; p=0.38) (Figure 4).

Effect of wounding on WT and JA Mutant Tomato Plants. JA-signaling impaired mutants were wounded in comparison with WT plants in order to determine whether wounding would have a different effect on plants deficient or insensitive to JA. Total AsA content was significantly reduced by wounding (two way ANOVA; F=11.23; df=1,84; p=0.001) and was significantly different between the genotypes (F=14.06; df=2,84; p<0.0001), but there was no significant interaction among the genotypes and the wounding treatment (F=1.06; df=2,84; p=0.35), suggesting a similar response to wounding in all genotypes (Figure 6). By genotype, total AsA content was significantly higher in *spr2* is than in WT or *jai1* (LSD student's *t* α=0.05), and by treatment, control plants had higher levels of total AsA than wounded plants (LSD student's *t* α=0.05). As with total AsA, reduced AsA was significantly affected by both treatment (two way ANOVA; F=9.34; df=1,84; p=0.003) and genotype (F=11.68; df=2,84; p<0.0001), and no interaction was observed between the two independent variables (F=1.61; df=2,84; p=0.21). Again, *spr2* was significantly higher within genotype and unwounded treatments were significantly higher (LSD student's *t* α=0.05). Oxidized AsA was not significantly different among genotypes (two way ANOVA; F=0.69; df=2,84; p=0.51), treatment (F=0.31; df=1,84; p=0.58), or the interaction between the two (F=0.41; df=2,84; p=0.67). Similarly, the ratio between oxidized and reduced AsA was not significantly different among genotypes (two way ANOVA; F=1.41; df=2,84; p=0.25), treatment (F=0.87; df=1,84; p=0.35), or the interaction between the two (F=0.67; df=2,84; p=0.51). (Figure 6).

Effect of MeJA Application on WT and JA Mutant Tomato Plants. JA-signaling impaired mutants and WT Castlemart plants were sprayed with MeJA to determine if reapplying JA to JA-deficient plants would cause a different outcome than the decrease observed in WT plants. Total AsA content was significantly reduced by MeJA application overall for both WT and *spr2* tomato genotypes (two way ANOVA; F=8.37; df=1,64; p=0.01) (Figure 7). Total AsA was also significantly different between genotypes (F=11.25; df=1,64; p=0.001) but not significant difference due to interaction between genotype and treatment (F=0.51; df=1,64; p=0.48). Reduced AsA was also significantly different due to MeJA application (two way ANOVA; F=6.70; df=1,64; p=0.01) and significantly different between genotypes (F=9.26; df=1,64; p=0.003) but not significantly different in the interaction of genotype and treatment (F=0.89; df=1,64; p=0.35). Both total AsA and reduced AsA were higher in spr2 than WT and higher in untreated plants as opposed to MeJA treated plants (Student's *t* at α=0.05). Oxidized AsA was significantly different between genotypes (two way ANOVA; F=9.26; df=1,64; p=0.003) and treatments (F=6.70; df=1,.64; p=0.01) but not significantly different in the interaction of genotype and treatment (F=0.89; df=1,64; p=0.35). The ratio between oxidized and reduced AsA was not significantly different between genotypes (two way ANOVA; F=0.41; df=1,64; p=0.53), treatment (F=0.74; df=1,64; p=0.39), or the interaction between genotype and treatment (F=0.88; df=1,64; p=0.35) (Figure 7).

DISCUSSION

Effect of wounding on AsA content. In both WT tomato cultivars (Castlemart and UC82), wounding caused a decrease in AsA content. This is consistent with the majority of the previous data reported in the literature (Table 1). Most of the deviation from the pattern of decreased AsA witnessed occurs in Arabidopsis, which are known to have increased AsA upon mechanical

wounding. It is unclear as to why AsA behaves differently in response to wounding in Arabidopsis than many other plants. This could be due to the unique chemistry of Arabidopsis, and other Brassicas. AsA plays a significant role in anti-herbivore defenses by aiding in the production of more toxic byproducts of glucosinolates (Krumbein et al. 2005). Because herbivory is a form of tissue wounding, this could account for why this species may behave differently. For the majority of other plants studied, it is possible that different plant tissues and even different types of wounding can cause differences in AsA metabolism (Reyes et al. 2007). Another theory may be that the effect of wounding on AsA content may be influenced by the balance between AsA and other antioxidants in the plant. For example, because oxidative stress occurs from wounding another antioxidant such as nitric oxide (NO) may respond more quickly, decreasing the plant's dependence on AsA as the primary antioxidant in the system (Arasimowicz et al. 2008). Perhaps, if a plant uses AsA as its primary antioxidant, it may upregulate AsA synthesis or recycling in response to wounding resulting in an increase in total AsA. Alternatively, if AsA is the primary antioxidant for a particular plant, it might be used up in response to wounding, whereas another plant that depends more heavily on other antioxidants might be able to better preserve the AsA pool. The mechanisms of why AsA decreases in some plants and not others are still unknown and merit further study.

We also sought to determine at what time after wounding that AsA decreases. We found that AsA tended to decrease over all sampled time within 24 h of wounding, although not significantly for all sampled times. These data are consistent with a study that suggests that AsA content changes rapidly, within the first 2 h after wounding (Suza et al. 2010). This may explain why we saw the trend of decreased AsA content over times sampled between 3 and 24 h after wounding. Although AsA is studied in terms of human health and function, relatively little is

known about AsA in the plant-insect interaction. The mechanisms that are involved in AsA metabolism due to wounding are especially unclear, but there is evidence to suggest that JA may be one mechanism of the process.

Effect of exogenous MeJA on AsA. MeJA application to both WT tomato cultivars (Castlemart and UC82) decreased AsA significantly, which was similar to the response to wounding for both cultivars. Furthermore, subsequent work in Arabidopsis has shown that the effects of wounding and MeJA treatment on AsA are similar to one another, even though these effects differ from those observed in tomato (Suza et al. 2010). Similarly, in tomato, *S. lycopersicum*, exogenous JA and MeJA application elicited the same responses as beet armyworm feeding (Thaler et al. 1996). This raises the possibility that JAs may contribute to the effect of wounding on AsA accumulation, since JAs are wound-inducible and mediate many other wound responses (Creelman and Mullet 1997). Alternatively, it is possible that both wounding and JAs respond independently to some common variable, for example ROS, that may impact AsA.

Role of endogenous JAs on AsA content. We investigated the role of endogenous JAs on the impact of wounding on AsA content. Wounding to both WT and JA-deficient and JA-insensitive genotypes caused a significant decrease in AsA content. Previous work is consistent with our data; AsA was decreased in caterpillar-fed foliar tissue of WT and *spr2* plants (M. Arevalo and F. Goggin, personal communication). Though the experiment used plants fed by caterpillars as opposed to strictly wounding, it aids in the understanding for the relationship between wounding, JA, and AsA. Although these mutants are either deficient in JA synthesis or unable to recognize JA, it does not affect changes in AsA due to wounding. Because the impact of wounding on AsA in the JA-deficient and JA-insensitive genotypes was similar to the effects in WT plants, this strongly suggested that JA was not required for the decrease in AsA observed in response to

wounding. This could have been due to the possibility that AsA, as the primary antioxidant, may be used up in response to wounding. Conversely, it may be possible that wounding and JA respond independently to a common variable, like ROS production upon wounding. There is little information available about the response of AsA to wounding of JA-deficient or JA-insensitive genotypes of tomato. Our data indicated that JAs were not required for the impact of wounding on AsA content, but that JAs may have possibly regulated constitutive AsA levels in unwounded tissues.

We chose *spr2*, *acx1*, and *jai1* genotypes of tomato, because they all have some deficiencies in either JA biosynthesis or recognition, each stemming from a unique mutation. Using these different genotypes allow us to determine what role JA plays, assuming the responses were similar across all genotypes. We found that AsA content of *acx1* and *jai1* mutants were not significantly different but observed a trend of higher AsA content in *acx1*. Although not for all analyses, *spr2* often exhibited higher AsA content than WT. More replications will be needed to detect significant differences because we often were unable to get data from the 18 replicates required to reach significance, as revealed by a power analysis. Previous studies have also shown elevated AsA content of *spr2* mutant tomato as compared with the WT control (Arevalo 2010). When applied with exogenous MeJA, AsA content of WT and JA mutant, *spr2*, tomato foliage was significantly reduced. Treating *spr2* with MeJA restored it to WT levels of AsA. In *jai1* mutants, however, we did not observe an increase in AsA content, which may be due to the nature of the mutant. The two genotypes (*spr2* and *acx1*), have mutations that cause a defect the synthesis of JA (Lee and Howe 2003; Powers et al. 2006). Whereas, *jai1* produces a normal level of JA but is unable to react to particular wound signals (Li et al 2002, Chen et al. 2006). All mutants should be retested for AsA levels with more replicates

in order to determine if a significant difference between JA-deficient and JA-insensitive genotypes and wild type exists. Data is also limited on MeJA application and wounding of JA mutants and their connection with AsA. However, these data are important in the investigation of the overall involvement of JA and MeJA in the process of AsA change, because JA and wounding are so interconnected.

Further experimentation needs to be done with other JA-deficient or JA-insensitive genotypes and wild type genotypes treated with mechanical wounding and exogenous MeJA application. More replication could provide a better picture of the impact of JAs on AsA by comparing the outcomes of a variety of mutants marked by the same deficiency. Moreover, analysis of gene expression and enzyme activities related to AsA synthesis and recycling may help to identify key components that alter ascorbate metabolism in response to wounding and/or JA. In this way, we may be able to identify similarities in how AsA biosynthetic pathways respond to wounding and MeJA treatment, and we can determine if wounding induces these responses in JA-deficient mutants. Further comparison could also help us evaluate other factors outside of wounding and JA that affect AsA metabolism. In addition, future research should include other plants, including Arabidopsis, in which wounding and JAs are known to enhance AsA accumulation (Suza et al. 2010). These studies should shed light on the regulation of an important antioxidant, which may alter plant tolerance of many biotic and abiotic stresses.

FIGURES AND FIGURE LEGENDS

Table 1. Summary of literature on the effects of wounding on various plants and tissues.

Species (common name)	Tissue	Wounding	Response	Reference
Apium graveolens **(celery)**	Stem	Shredding	Total AsA - 53% decrease	(Reyes et al. 2007)
Arabidopsis thaliana **(mouse-ear cress)**	Leaf	Crushing	Total AsA - increased	(Suza et al. 2010)
Brassica napus **var. Bronowski (rapeseed)**	Leaf	Cutting	Total AsA - 21% decrease; AsA/DHA ratio – 11 to 5 decrease	(Watanabe et al. 2007)
Brassica oleracea **convar. *capitata* var. *alba* (white cabbage)**	Leaf	Shredding	Total AsA - 11% decrease	(Reyes et al. 2007)
B. oleracea **convar. *capitata* var. *rubra* (red cabbage)**	Leaf	Shredding	No significant change	(Reyes et al. 2007)
Cucurbita pepo **(zucchini)**	Root	Shredding	Total AsA - 53% decrease	(Reyes et al. 2007)
Daucus carota **(carrot)**	Root	Shredding	Total AsA - 82% decrease	(Reyes et al. 2007)
Dioscorea rotundata **(white yam)**	Tuber	Cutting	Total AsA - 300% increase	(Ikediobi et al. 1989)
Diospyros kaki **var. Fuyu (persimmon)**	Fruit	Cutting	Total AsA - decrease	(Wright and Kader 1997)
Fragraria ananassa **var. Selva (strawberry)**	Fruit	Cutting	Significant oxidation of AsA, but no changes in total AsA	(Wright and Kader 1997)
Kalanchoë daigremontiana **(alligator plant)**	Stem	Cutting	Ratio reduced AsA/total AsA in apoplast - 33% decrease; total AsA largely unaffected	(Takahama 1993)
Malpighia glabra **(acerola)**	Leaf	Cutting	Total AsA – 50% decrease	(Badejo et al. 2009)
Pastinaca sativa **(parsnip)**	Root	Shredding	Total AsA - 76% decrease	(Reyes et al. 2007)
Raphanus sativus **(red radish)**	Root	Shredding	No significant changes	(Reyes et al. 2007)
Solanum lycopersicum **var. Castlemart (tomato)**	Leaf	Crushing	Total AsA - decrease	(Suza et al. 2010)
Solanum tuberosum **var. Katahdin (potato)**	Tuber	Cutting	Total AsA of 2 d stored potatoes - 100–300% increase	(Asselbergs and Francis 1952)
S. tuberosum **var. Mayqueen**	Tuber	Cutting	Total AsA of 7 w stored potatoes - 200% increase	(Fukuda et al. 1995)
S. tuberosum **var. Belrus**	Tuber	Cutting/Bruising	Total AsA – 400% increase/Total AsA –350% decrease	(Mondy and Leja 1986)
S. tuberosum **var. Russet Nortkotah**	Tuber	Shredding	Total AsA – 32% decrease	(Reyes et al. 2007)
S. tuberosum **var. Bintje**	Tuber	Cutting	Threonic acid, a product of AsA oxidation, increased	(Galindo et al. 2009)

Table 2. Summary of literature on the effects of MeJA application to various plants and their tissue.

Plant	Tissue Type	Solution	Concentration	Outcome	Citation
Arabidopsis	Cell suspensions	MeJA	50µM Solution	Total AsA increased	(Wolucka et al. 2005)
Panax ginseng	Roots	MeJA	200µM Solution	Total AsA higher at 5, 7, and 9 days	(Ali et al. 2007)
Tomato	Fruits – cut up	MeJA	22.4µL/L	Total AsA higher at all time points	(Ayala-Zavala 2008)
3 Plum Varieties	Fruits	MeJA	0, 10^{-5}, or 10^{-3} M vapor	Total AsA increased at fruit ripening stage for all 3 varieties	(Khan and Singh 2007)
Strawberry	leaves	MeJA	0.1 mM Solution	Water stressed plants treated with MeJA increased in total AsA	(Wang 1999)
Arabidopsis	Leaves	MeJA	10^{-6}, 10^{-5} and 10^{-4} M	Total AsA increased	(Maksymiec and Krupa 2002)
Dark and light grown canola	Roots and Shoots	MeJA	1 mM Solution	Total AsA in roots not effected Total AsA increased in shoots of both light and dark canola	(Comparot et al. 2002)
Tomato cv. UC82	Leaves	JA	1.5 mM	Total AsA increased	(M. Arevalo and F. Goggin, personal Communication)
Tomato cv. Castlemart	Fruits	JA	1.5 mM	Total AsA decreased	(M. Arevalo and F. Goggin, personal Communciation)
Tomato cv. Castlerock, Motelle, Castlemart	Leaves	JA	1.5 mM	No change in total AsA	(Arevalo 2010)

Figure 1. Impact of wounding on foliar ascorbic acid content over time. Total and reduced AsA content in tomato foliage (cv. Castlemart) decreased significantly in response to mechanical wounding but did not vary among time points tested (3, 6, 12, and 24h), and there was no significant interaction between wounding and time (two way ANOVA; $\alpha = 0.05$). There were no significant differences observed due to either independent variable in both oxidized AsA and the ratio of oxidized and reduced AsA. Error bars represent the standard error of the means (SEM) for total AsA.

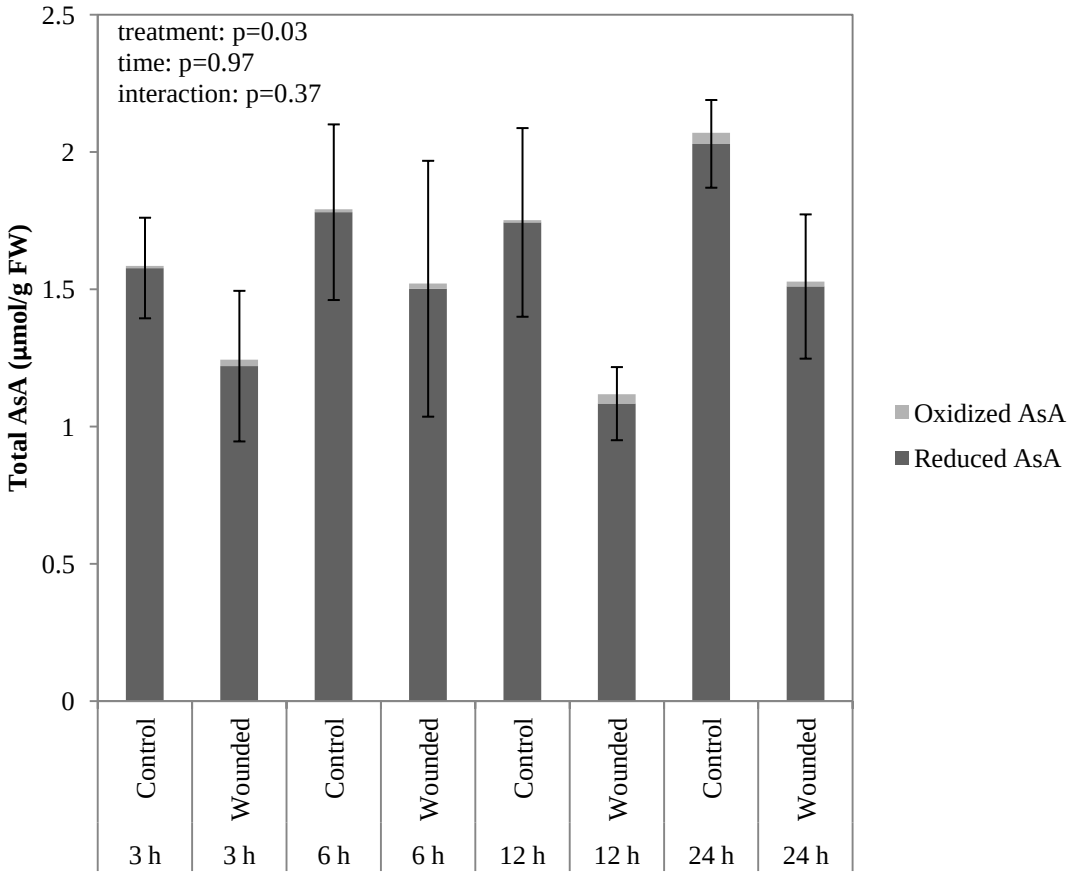


Figure 2. Effect of MeJA and wounding on AsA content in tomato foliage (cv. Castlemart). At 24h after wounding or treatment with MeJA (75 μM), there were significant differences in total and reduced AsA in treated foliage, but no changes were observed in oxidized AsA content or the ratio between oxidized and reduced AsA (one-way ANOVA; $\alpha = 0.05$). Different letters indicate values that are significantly different (Student's *t* test; $\alpha = 0.05$). Error bars represent the SEM for total AsA.

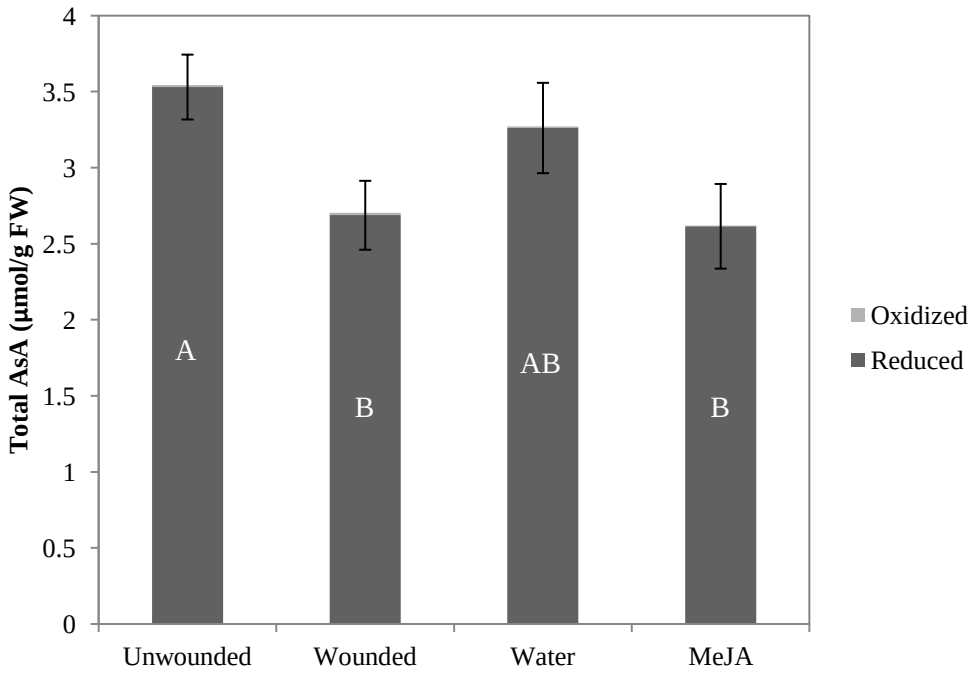


Figure 3. Effect of MeJA and wounding on AsA content in tomato foliage (cv. UC82). Total and reduced AsA content in tomato foliage (cv. UC82) showed a significant decrease in response to treatment (mechanical wounding and 75 μM MeJA) but did not vary among time points tested (6 and 24h), and there was no significant interaction between treatment and time (two way ANOVA; $\alpha = 0.05$). There were no significant differences observed due to either independent variable in both oxidized AsA and the ratio of oxidized and reduced AsA. Error bars represent the SEM for total AsA.

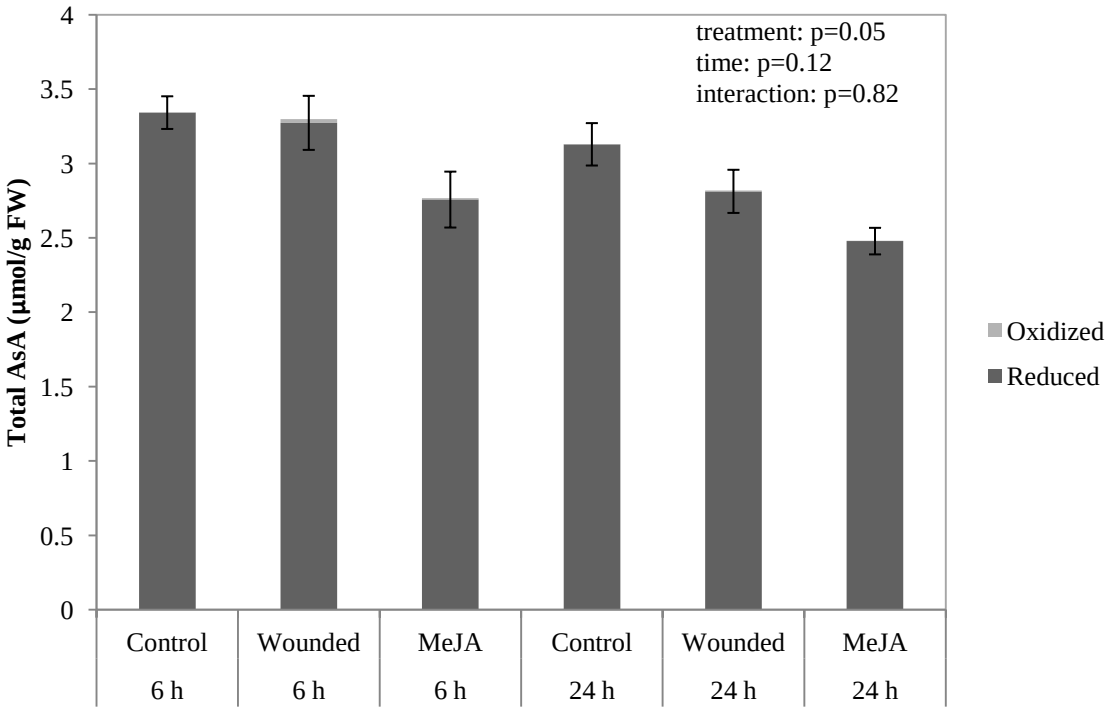


Figure 4. Baseline Levels of AsA in Castlemart, *spr2*, and *acx1* tomato genotypes. Total AsA was significantly different among WT and two JA-deficient mutants, *spr2* and *acx1*. There were no significant differences observed in reduced AsA, oxidized AsA, or the ratio between oxidized and reduced AsA (one way ANOVA; $\alpha = 0.05$). Different letters indicate values that are significantly different (Student's *t* test; $\alpha = 0.05$). Error bars represent the SEM of total AsA.

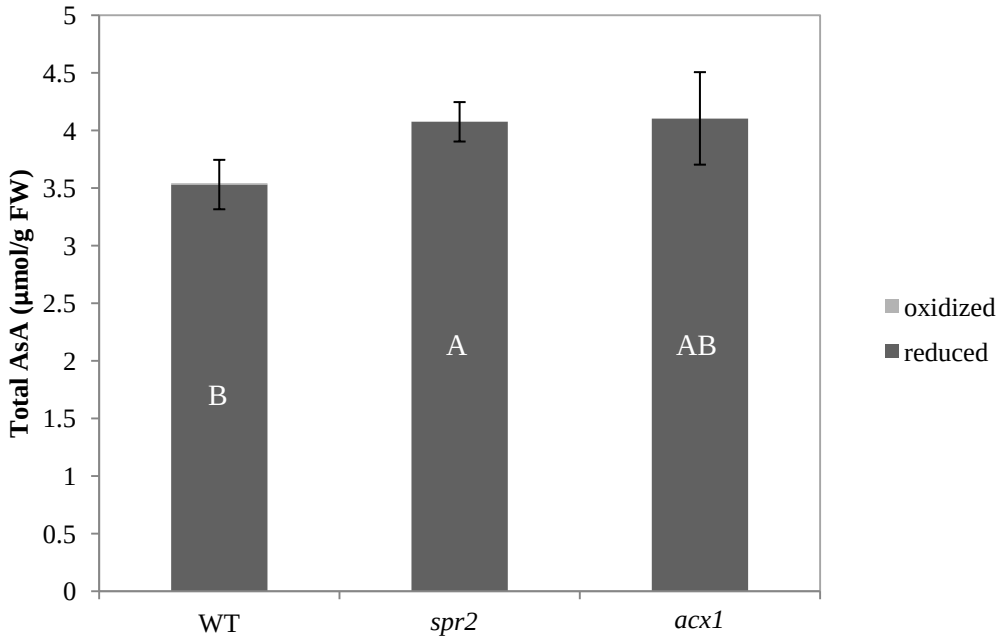


Figure 5. Baseline Levels of AsA in Castlemart, *spr2*, *acx1*, and *jai1* tomato genotypes. No significant difference was observed in total, reduced, oxidized AsA and the ratio between oxidize and reduced AsA of foliar tissue collected from WT and JA-signaling impaired mutants, *spr2*, *acx1*, and *jai1* (one way ANOVA; $\alpha = 0.05$). Error bars represent the SEM of total AsA.

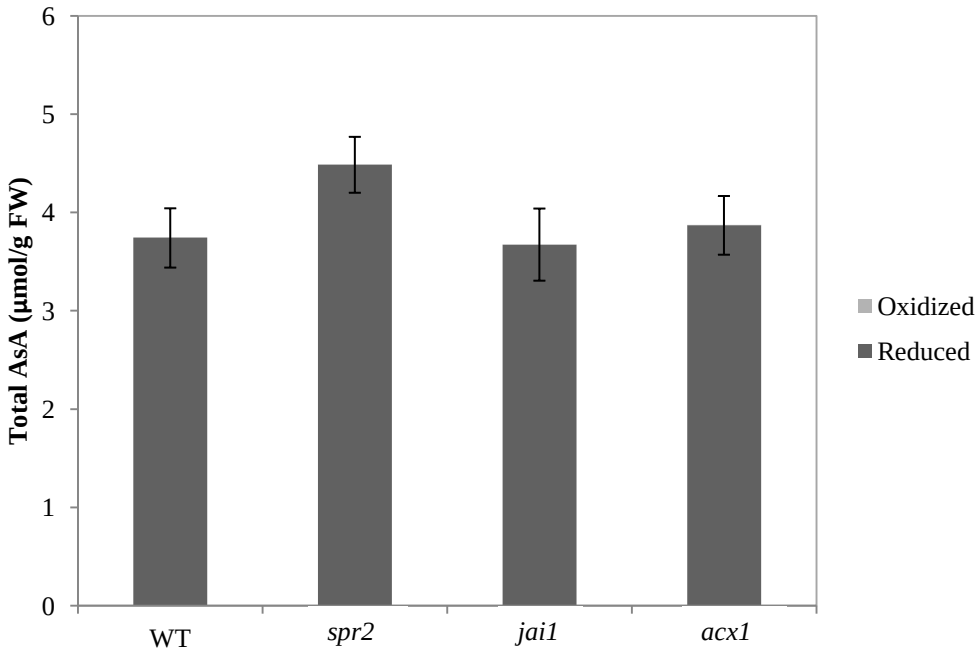


Figure 6. Effect of wounding on AsA content in WT tomato (cv. Castlemart) and JA-signaling impaired mutants. Total and reduced AsA content significantly decreased with mechanical wounding and was significant among the genotypes. There was no significant interaction between the effects of genotype and wounding treatment (two way ANOVA; $\alpha = 0.05$). There were no significant differences in oxidized AsA or the ratio between oxidized and reduced AsA. Error bars represent the SEM of total AsA.

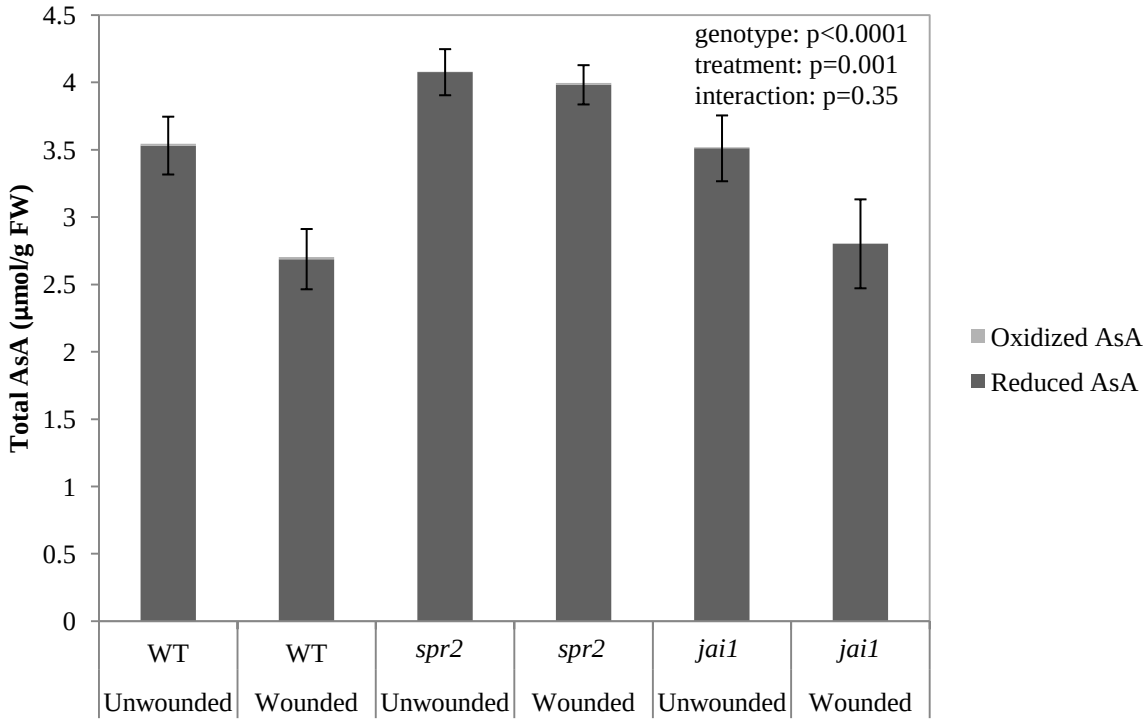
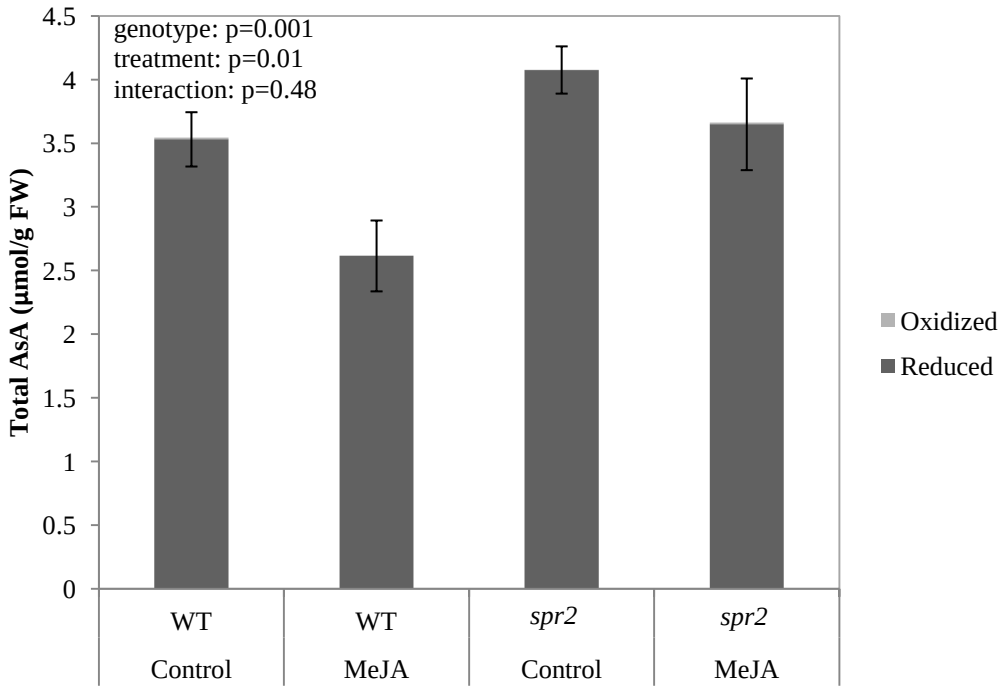


Figure 7. Effect of MeJA Application on AsA content in WT and the JA-deficient *spr2* mutant. At 24 h after treatment with 75 μM MeJA, total and reduced AsA content decreased significantly by MeJA application overall for both genotypes of tomato and was significantly different between genotypes with no interaction between treatment and genotype (two way ANOVA; $\alpha = 0.05$). There was no significant difference in oxidized AsA or the ratio between oxidized AsA and reduced AsA. Error bars represent the SEM of total AsA.



CHAPTER II

AsA in Plant-Herbivore Interactions

ABSTRACT

Most if not all herbivorous insects require a dietary source of L-ascorbic acid (AsA, vitamin C), which is important as an antioxidant and enzyme cofactor. In plants, AsA also minimizes oxidative damage and can facilitate resistance to certain abiotic and biotic stresses. Because AsA can enhance plant health, but is also a beneficial dietary component for herbivores, it is important to evaluate the role of AsA in the intimate plant-herbivore interaction. The goals of this study were to evaluate how herbivory influences AsA metabolism in plants, and also how AsA abundance in plants could influence host suitability for herbivores. We measured the effects of three species of herbivores (the potato aphid, *Macrosiphum euphorbiae*, the beet armyworm, *Spodoptera exigua*, and a root-knot nematode, *Meloidgyne javanica*) on AsA content in tomato (*Solanum lycopersicum*), providing the first direct comparison of different types of herbivore damage (piercing-sucking, chewing, and root-galling) on AsA content. Furthermore, we compared the effects of caterpillar and aphid herbivory in two different plant species, tomato and *Arabidopsis thaliana*. In tomato, AsA content decreased significantly in response to caterpillar feeding in locally damaged foliage but showed no significant systemic effects in undamaged leaves or root tissue. Aphids and nematodes had no statistically significant effect on AsA content in leaves or roots. On Arabidopsis, caterpillars (*S. exigua*) and aphids (the green peach aphid, *Myzus persicae*) had no significant effects on AsA levels in locally-infested leaves but caused a significant systemic decrease in the AsA content of uninfested foliage. Thus, the effects of herbivory on AsA content in plants appeared to vary depending upon the plant and herbivore species. To assess how AsA levels in Arabidopsis might influence susceptibility to insect herbivores, we compared beet armyworm feeding and growth on wild-type plants and two genotypes with altered AsA accumulation: *vtc1*, a mutant impaired in AsA biosynthesis, and

MIOX4 L33-4, a transgenic line that has enhanced AsA content as a result of overexpression of the biosynthetic enzyme, myo-inositol oxygenase 4 (MIOX4). Weight gain and feeding damage by the beet armyworm larvae was greater on the MIOX4 overexpression line that on WT or *vtc1* plants, suggesting the transgene increased plant susceptibility to caterpillars. In summary, these results indicate that herbivores modify AsA content in plants, and suggest that AsA levels in plants may also modify herbivore performance.

INTRODUCTION

Increasing L-ascorbic acid (AsA, also known as ascorbate or vitamin C) in food crops could benefit humans because it is a dietary requirement in human health. It could potentially also enhance plant vigor and yield because AsA is vital for plant health as an antioxidant and enzyme cofactor and by aiding in cell division, growth, senescence, and stress resistance (Conklin 2001). However, before crops with enhanced AsA are released commercially, we must also consider how this trait could affect plant susceptibility to biotic stresses. One important stress to plants is damage by herbivores, including by insects and nematodes. Some studies suggest that herbivores may alter AsA levels and that AsA content may also influence plant susceptibility to herbivory (Goggin et al. 2010). Therefore, we must study the role of AsA in the plant-herbivore interaction to understand how herbivores may alter the accumulation of this important nutrient, and how altering AsA levels in crops might influence herbivory.

The Effects of Herbivores on Ascorbic Acid

Herbivores are diverse and have broad impact on plants. Although AsA is found in every plant and most plants are predated on, relatively few studies have directly measured the effects of insect herbivores on plant-derived AsA. These studies have shown that certain foliar-feeding herbivores cause a decrease in AsA content (Bi et al. 1995 and 1997; Jiang 1996), whereas root-knot nematode infection on tomato cause an increase in AsA content of resistant cultivars and no change in AsA content of susceptible cultivars (Arrigoni et al. 1979). A decrease in L-ascorbic acid, the reduced form of AsA, from caterpillar feeding corresponded with an increase in L-dehydroascorbate, the oxidized form (Bi 1995; Bi 1997). Ascorbate functions as an antioxidant by detoxifying reactive oxygen species (ROS) produced both by aerobic, cellular metabolism from normal processes, such as photosynthesis and by stresses, such as wounding (Smirnoff 2001; Davey et al. 1999; Smirnoff et al. 2001). This has implications for herbivory, as

mastication by a variety of herbivores creates ROS. It is unclear, however, whether the effects would be the same across many herbivorous insects due to the different modes of feeding.

Though there are few studies that have directly measured AsA in plants challenged by herbivores, many studies have shown an effect of insects on components of AsA metabolism, including ascorbate oxidase (AO) and ascorbate peroxidase (APX). AO and APX catalyze the oxidation of AsA, and the unstable radical and derivates produced quickly degrade if they are not recycled back to the bioactive, reduced form, L-AsA. Several studies suggest that herbivory increases AO and APX activity (An et al. 2009; Bi and Felton 1995; Felton et al. 1997; Moloi et al. 2008; Chakraborty 2005). If these increases in AO and APX activities are not paired with concomitant increases in recycling enzymes, AO and APX could mediate AsA losses resulting from herbivory (Goggin et al. 2010). Herbivores can also alter the transcript abundances of genes encoding APX and AO, although patterns of AO and APX gene expression can vary widely depending upon the plant species, the herbivore, and the AO and APX isoform (Goggin et al. 2010). The diversity of responses in APX and AO regulation suggest the complexity of AsA regulation and the variety of factors that may influence AsA abundance in herbivore challenged plants.

The Effects of Dietary Ascorbic Acid on Herbivores

Since it is observed that insects can affect AsA content of the plant, it is also equally important to know how the insect may be affected by a change in AsA content. Originally, it was thought that insects could synthesize AsA (Vanderzant and Richardson 1963), and that therefore played little role in the nutrition of the insect (House 1962). However, that idea has since changed (Chippendale 1975; Dadd 1957) based on evidence that at least some insects are not able to synthesize AsA (Gupta et al. 1972), and that dietary AsA in artificial diets has

beneficial effects on numerous herbivorous insects (El-Karaksy and Idriss 1990; Kramer and Seib 1982).

To understand why AsA could be beneficial to the insect, it is important to determine where AsA might be located or possibly stored within the insect. Only a few studies on this topic exist and mainly explore this as it relates to Lepidopterans and not other orders. For example, *Trichoplusia ni, Depressaria pasinacella,* and *Manduca sexta* seem to have higher AsA content in the gut than in the fat body, which could suggest an antioxidant role in detoxifying ROS consumed by the insect or generated within the gut (Timmerman et al. 1999; Kramer and Seib 1982). However, the pH of the Lepidopteran midgut is fairly high compared to the lower pH of AsA, suggesting that AsA may not be an effective antioxidant in the gut (Felton and Duffey 1991; Smirnoff 1996). Because AsA is water soluble, it would be less likely that AsA is stored in the fat body of caterpillars. Evidence also suggests that AsA enzyme recycling activities occur in *H. zea,* which demonstrates the importance of AsA within the species (Felton and Summers 1995). Ascorbate-recycling has been shown in *Orgyia leucostigma,* where ascorbate in may suppress peroxide formation in the midgut lumen by reducing hydrogen peroxide to water. The dehydroascorbate produced is reduced back into ascorbate with the help of glutathione (Barbehenn et al. 2001).

Ascorbate storage in the insect gives an indication of the significance of AsA within an insect. The importance of AsA in the diet is well established by studies that have investigated the role of AsA in an insect's diet through optimization of artificial diets. Complete depletion of AsA in diets had severe negative consequences for the insects that fed on them (Vanderzant et al. 1962; Lindroth and Weiss 1994). Typically, there is an optimal range of AsA content in a diet for a particular insect species' development. For example, *Bombyx mori* exhibits enhanced silk

production and an increase in fecundity when within the optimum range of AsA (El-Karaksy and Idriss 1990). However, there are negative outcomes associated with AsA content both above and below that ideal range (Pristavko and Dovzhenot 1974; Garg and Mahajan 1994, Garg and Mahajan 1993; Levinson and Navon 1969; Chippendale et al. 1965; Khlistovskiy and Alfimov 1979; Mittler et al. 1970; Dadd et al. 1967; Bridges and Norris 1977; Davis 1966; Chang and Kurashima 1999; Popham and Shelby 2009; Coudron et al. 2008). AsA provides a benefit for many insects reared on artificial diets, suggesting an importance in insect health.

Impact of AsA Content in Plants on Herbivores

Although artificial diet studies demonstrate that dietary AsA influences the growth and survival of many herbivorous insects, few have examined how AsA content in plants influences plant susceptibility to insects or other herbivores. One approach has been to look at insect performance on host plants that show natural variation in AsA content. In a study of seven different cultivars of tomato, no significant correlation was detected among the relative amount of AsA and the amount of feeding damage inflicted by the cotton leafworm, *Spodoptera littoralis*, or the Colorado potato beetle, *Leptinotarsa decemlineata*, but mortality of these insects was lower cultivars with higher AsA content (Antonious et al. 1999). AsA content variations among different host plants of the gypsy moth, *Lymantria dispar*, did not correlate with changes in caterpillar health (Roth et al. 1994). It is difficult to test differences in the effects of AsA alone among different plant species, because each species has a unique chemical make-up. A second approach has been to manipulate AsA content in plants by direct application of AsA, its biosynthetic precursors, or of inhibitors of AsA synthesis. AsA supplemented to leaves did not seem to affect *L. dispar* health significantly (Roth et al. 1994). In another study, susceptible cultivars of tomato supplemented with AsA were more resistant to root-knot

nematode, *M. incognita.* When resistant cultivars were supplemented with lycorine, an inhibitor of AsA synthesis, resistance decreased (Arrigoni et al. 1979). This, however, does not present a clear picture as to the effect of AsA on herbivores. Another more preferable approach to address this question is to compare insect performance on isogenic plant lines with enhanced or suppressed AsA accumulation due to alteration in the function or expression of single genes.

AsA mutant plants usable for testing AsA effects on insects

Ascorbate can be synthesized by at least 4 biosynthetic pathways in plants, and AsA content in the model plant *Arabidopsis thaliana* can be modified through manipulation of biosynthetic enzymes in these pathways (Figure 1). Arabidopsis with modified AsA content could be used to assess the effect of AsA of plants on insects. A point mutation in the *vtc1* mutant line causes a single amino acid substitution in the enzyme, GDP-mannose pyrophosphorylase, which catalyzes the conversion of D-mannose-1-P to GDP-D-mannose (Lukowitz et al. 2001; Wheeler et al. 1998) (Figure 1). This mutation reduces GDP-mannose pyrophosphorylase activity and causes AsA content to be 25% of that found in WT plants (Conklin 2001). Overexpression of *myo*-inositol oxygenase, MIOX, an enzyme which catalyzes the conversion of *myo*-inositol to D-glucoronate causes an increase in AsA content (Lorence et al. 2004). Only one of these mutants has ever been tested with insects previously. The larvae of *S. littoralis* fed on *vtc1* and gained more weight than on WT Arabidopsis (Schlaeppi et al. 2008). This suggests that a change in AsA can influence insect health.

The goal of this study was to determine the effects of herbivores with different feeding types on tomato and Arabidopsis. This was the first study to compare multiple herbivores on a single plant species. Tomato plants were infested with caterpillars, aphids, nematodes or left uninfested as controls. Similarly, Arabidopsis plants were infested with caterpillars, aphids or

left uninfested as controls. In addition, we needed to determine the effects of AsA on insect herbivores to investigate the role of AsA in the plant-herbivore interaction. We received seed of four independent plants of segregating population of MIOX4 overexpressers. We first determined which of these individual plants displayed the highest AsA content and we called this *MIOX4* L33-4. These *MIOX4* L33-4 (high AsA), WT, and *vtc1* (low AsA) were infested with caterpillars to determine differences in weights of caterpillars and differences in amount of plant damage.

MATERIALS AND METHODS

Plant Materials.

Genotypes. Experiments were performed with tomato (*Solanum lycopersicum* cv. Castlemart), *Arabidopsis thaliana* (Nessler) ecotype Columbia (WT; seeds kindly provided by Dr. Craig L. Nessler, Virginia Tech University, hereafter referred to as WT), and with a transgenic Arabidopsis line (*MIOX4* L33) with strong constitutive expression of MIOX4 (kindly provided by Dr. Argelia Lorence, Arkansas State University). The MIOX4 over-expression line was developed by the Lorence Laboratory as follows (Yactayo-Chang J, Suza W, Lorence A, unpublished). The *MIOX4* cDNA (Lorence et al. 2004) was subcloned into the *Kpn*I/*Sac*I sites of the binary vector pBIB-Kan (Becker 1990) placing it under the control of the CaMV 35S promoter with duplicated enhancer between the 5' tobacco etch virus leader and the NOS terminator. This construct was transformed into *Agrobacterium tumefaciens* strain GV3101. WT *A. thaliana* plants were transformed with the *MIOX4:pBIB-Kan* construct via the floral dip method (Clough and Bent 1998), and primary transformants (T0 generation) were selected on Murashige and Skoog (Murashige and Skoog 1962) plates containing 500 mg L^{-1} carbenecillin

and 100 mg L^{-1} kanamycin. Southern blot analysis performed with L33 have shown that this line has 4 copies of the *MIOX4:pBIB-Kan* insert (Lorence, unpublished data). Assays for this project (described further below) were conducted with the T3 generation.

Arabidopsis culture. Seeds were surface sterilized by rinsing for 5 minutes with 70% EtOH, followed by 10 minutes in 3.075% sodium hypochlorite (from commercial bleach) with 0.05% Tween 20 (ICN Biochemicals). Seeds were then rinsed with sterile ddH_2O 6-8 times and then plated on Murashige and Skoog (MS) (Murashige and Skoog 1962) medium plus 1% sucrose (JT Baker) and 0.7% phytagar (Gibco), with pH adjusted to between 5.7 and 6.0 by KOH. For experiments that involved insect performance on Arabidopsis, plants were grown in growth chambers (150 $\mu molm^{-2}s^{-1}$; L16:D8 photoperiod; 65% humidity) and vernalized at 4˚C for 3 days prior to germination. Arabidopsis were transplanted 12 to 14 after planting. Plants were watered every 1 to 2 days and fertilized weekly. Herbivore challenged Arabidopsis were grown the same way but humidity could not be controlled due to the type of growth chamber used. Arabidopsis MIOX4 overexpression line 33 (Lorence et al. 2004) was tested for AsA levels to determine whether MIOX4 overexpression would exhibit high levels AsA to be used in subsequent experiments. The MIOX4 overexpressers were grown in growth chambers (150 $\mu molm^{-2}s^{-1}$; L16:D8 photoperiod) but began with lowered light intensity due to an error, until it was corrected approximately 4 days after being transplanted. The growth chamber was unable to control humidity. Arabidopsis MIOX4 overexpressers were germinated in selection 1X MS media containing 1% sucrose, 0.7% phytagar, and 100mg/L kanamycin (Invitrogen Corporation, CA). MIOX4 overexpressers were transplanted to soil 21 to 22 days after initial planting, because selection media caused slowed growth in plants and light levels were lowered. Plants were watered every 2 to 3 days and fertilized weekly, with the exception of the first 2 weeks in which

these Arabidopsis plants only received water.

Tomato culture. *S. lycopersicum* cv. Castlemart was germinated in vermiculite (Sun Gro, WA), and after 13 days, seedlings were transplanted into 10 oz Styrofoam cups filled with sand supplemented with 15 mL of 15-9-12 Osmocote plus slow-release fertilizer (Scotts, Ohio) sprinkled on the top of the sand. Plants were maintained at (ca. 24 to 27˚C, L16:D8 photoperiod) in the greenhouse and received fertilizer 3 times a day, 6 days a week with a dilute nutrient solution containing 1000mg/L $CaNO_3$ (Hydro Agri North America, Tampa FL), 500 mg/L $MgSO_4$ (Giles Chemical Corp, Waynesville, NC), and 500 mg/L Hydroponic 4-18-38 Growmore fertilizer (Growmore, Gardena, CA). On the 7^{th} day, plants were watered 3 times with water only. Some of the plants became infected with thrips, but all plants that showed signs of infestation were excluded from the experiment.

Insect and Nematode Materials. The potato aphid, *Macrosiphum euphorbiae* (clone WU11), and the root-knot nematodes, *Meloidogyne javanica* (isolate VW4), were reared as previously described (Hebert et al. 2007; Lambert et al. 1992). Infective juveniles of the root-knot nematode were collected from a hydroponic culture reared on tomato (Lambert et al. 1992). The green peach aphid, *Myzus persicae*, was reared on cabbage, *Brassica oleracea* (cv. Flat Dutch), at ~24˚C (L16h: D8 photoperiod). Larvae of the beet armyworm, *Spodoptera exigua*, were obtained from Dr. Ken Korth's lab, Department of Plant Pathology, U. of Ark, Fayetteville, AR, or purchased from Benzon, where they were reared on general purpose Lepidoptera diet (Benzon, Carlisle, PA).

Impact of herbivores on AsA content of tomato. Four and a half week old tomato plants were challenged with beet armyworm larvae, potato aphids, or root-knot nematodes grown under

greenhouse conditions (ca. 24 to 27˚C) to compare the effects of different types of feeding damage on AsA content in foliage and roots of the host plant.

For plants assigned to the caterpillar treatment group or the aphid treatment group, insects (1 second-instar larva/cage or 5 adult aphids/cage) were confined to individual leaflets using mesh clip cages (4 cm diameter cages) placed on the lateral leaflet closest to the terminal leaflet of the fourth and fifth fully expanded leaves from the bottom of the plant (2 cages/plant; 8-14 plants/treatment group) and allowed to feed for 48 h prior to tissue collection. Empty clip cages were also placed at the same leaflet positions on all other plants in the experiment, including uninfested controls and nematode-inoculated plants, to ensure consistency throughout the experiment. Wire supports were used to support the weight of the cages. Plants assigned to the nematode treatment group were inoculated with approximately 3,000 root-knot nematodes per plant by pipetting nematodes suspended in water into the sand surrounding the root system. To facilitate the inoculation process, plastic straws had been placed vertically in the sand of all pots at the time of transplanting to allow the roots to grow around them, and these were removed at the time of inoculation to create small vertical channels in the sand (~4 cm deep) into which nematodes could be pipetted without mechanically damaging the roots. For consistency, straws were also removed from the uninfested control and the plants used for the caterpillar and aphid treatments. Water without nematodes was pipetted into the sand. Uninfested leaflets from a different leaf were collected from tomato challenged with a variety of herbivores that were caged on a different leaf. Uninfested leaflets in the same leaf position of the leaf directly above and to the right of the treated leaf were collected to determine the systemic effect of herbivory in AsA content in tomato (Figure 2A). When the data was analyzed, each plant was treated as a replicate and each cage was treated as a subreplicate.

Impact of herbivores on AsA content of Arabidopsis. Five and a half week old Arabidopsis plants were challenged with caterpillars and aphids to compare the effects of feeding types of herbivores on AsA content. Plants were inoculated at growth stage 6.5, 5.5 weeks after planting and were kept in growth chamber (23˚C). The 6.5 stage is characterized by inflorescence growth and 50% of flowers open (Boyes et al. 2001). For herbivore challenged Arabidopsis, one second-instar beet armyworm or 5 green peach aphids were confined to clip cages (3 cm diameter) on each of two large, opposing basal leaves (2 cages/plant, 12-13 plants/treatment group). Empty cages were placed on untreated control plants. When the data was analyzed, each plant was treated as a replicate and each cage was treated as a subreplicate.

Uninfested foliage on a different leaf was collected from Arabidopsis challenged with a variety of herbivores, caged on a different leaf. Uninfested leaves on the inside of the plant on a separate level of growth than the treatment were chosen in Arabidopsis (Figure 2B). Roots were not collected because the growth media used for Arabidopsis did not allow for roots to be cleanly separated from the soil.

Tissue Collection. All tissue collected was cut, weighed, placed in a Whirl-Pak plastic bag (VWR Cat#11216373), and flash frozen, immediately, in liquid nitrogen. Tissue typically weighed between 100 and 160 mg, optimally 150 mg. Some samples, however, were between 50 and 100 mg and had a smaller number of replicates. Samples were stored at -80˚C until ascorbate measurements were taken.

Impact of MIOX4 on AsA content in Arabidopsis. Because the transgenic Arabidopsis line (L33) that overexpresses *MIOX4* was segregating, AsA content was highly variable in individual plants of this line. Therefore, in order to identify transgenic materials with high AsA that could be used for subsequent experiments, foliar AsA content was compared in plants of the T3

generation derived from four different hemizygous T2 parents (*MIOX4* L33-1 = seeds derived from L33 T1P1/T2P2; L33-2 = seeds derived from L33 T1P1/T2P3; L33-3 = seeds derived from L33 T1P1/T2P4; L33-4 = seeds derived from L33 T1P1/T2P7).

*F*oliage for determination of AsA levels tissue was done at the 6.5 stage in Arabidopsis, similar to plants that were used for all other experiments. Due to retarded growth, these plants were approximately 8 weeks old when they reached the 6.5 stage.

Ascorbate Measurement. Measurement of AsA in foliar tissue was performed using the ascorbate oxidase assay, as previously described (Rao and Ormrod 1995; Lorence et al. 2004). Samples (50-150 mg ea) were ground in ice-cold 6% meta-phosphoric acid (MPA) at a ratio 150mg/1.5mL. Immediately after grinding the tissue in MPA, samples were split into 2 subsamples and centrifuged at 13,000 rpm for 5 minutes. Each subsample was then divided into 2 cuvettes (50 µL of supernatant/cuvette; UltraVette UV-disposable cuvettes, VWR West Chester, PA) containing 950 µL of K-phosphate buffer for analysis of oxidized and reduced AsA content. Oxidized AsA was determined by adding 1µL of 1mM DL-Dithiothreitol (DTT, FW = 154.25) (IBI Scientific, Iowa) under dim lighting, due to DTT's light sensitivity and incubated in dark for 20 min at room temperature. Reduced AsA was determined by adding 1µL of enzyme, ascorbate oxidase (MP Biomedicals Cat#190075) and incubated for 1min at room temperature. A Perkin Elmer UV/VIS spectrometer Lambda Bio 20 connected to a Gateway 2000 desktop commuter using Perkin Elmer UVWinLab software was used to read absorbance at a wavelength of 265nm before and after adding enzyme or DTT. Total AsA was determined by summing oxidized and reduced AsA.

Prior to testing samples, a confirmation of enzyme activity test was performed for AO, when new aliquots of AO (MP Biomedicals, Ohio) were made. 69.3 mg of pure AsA was added

to 10ml 6% MPA. Then, 1µL of this solution was added to a cuvette containing 1mL of K-phosphate buffer. Absorbance was read at approximately 0.5. A 1µL AO was then added to the cuvette, and absorbance declined for approximately 1min until it reached nearly zero.

Impact of altered AsA content in Arabidopsis on herbivores. Caterpillars feeding and weight gain, as well as aphid population growth, were assessed on WT plants, *vtc1*, and *MIOX4* L33-4. Aphid performance was assessed once, whereas the caterpillar experiment was performed twice; however, only two genotypes (WT and *MIOX4* L33-4) were included in the first assay with caterpillars because of insufficient growth of *vtc1* plants. In the caterpillar performance experiment that included *vtc1*, MIOX4 overexpressers were planted 2 days prior to WT and *vtc1* to account for retardation in growth from the antibiotic selection media in attempt to synchronize plants to same developmental stage at time of bioassay. All plants were plated at the same time in the experiment that did not include *vtc1*. Plants differed in sizes and were grouped to obtain a comparable distribution of sizes within each genotype, so that size did not vary with genotype. Two neonate beet armyworm larvae were confined on each 5 to 6.5 week old plant at the 6.5 stage by sleeves of transparency film taped to fit into 500 CC pots topped with breathable mesh and anchored by plastic tags. Weights of the neonates were too small to be measured at the start of the experiment. The caterpillars were allowed to feed for 7 days in a growth chamber (23˚C). It should be noted that these sleeves if not properly secured, could allow enough space for caterpillars to leave the plant. Consequently, some caterpillars were lost. For assay 1, the total area of damage was measured (mm^2) 3 days after infestation by measuring length and width of every hole created by the caterpillar and adding those areas together. For assays 1 and 2, damage was also measured day 7 after infestation, either by measuring feeding holes where possible, or by rating damage visually on a scale from 1-4 (Figure 3).

In the aphid performance experiment, *vtc1* genotypes were planted 8 days after WT because of an increased growth rate experienced in experiments prior to this study. Two adult green peach aphids were confined to each 5 to 6.5 week old plant at the 6.5 stage by transparency film sleeves adjusted and taped to fit into 500 CC pots with breathable mesh on top and secured by plastic tags. Aphid numbers were counted after 7 days.

Statistical analysis. Analysis of variance (ANOVA), both one way and two way, Welch's Weighted ANOVA, likelihood ratio, means separation by Student's *t* test, and log transformations were performed in JMP Statistical Discovery Software v.7.0 (SAS Institute, Cary, NC). When needed, variances were stabilized by log transformation of the data, and Welch's Weighted ANOVA was used when data did not meet requirements for ANOVA.

RESULTS

Impact of herbivores on AsA content of tomato. Tomato was infested with the beet armyworm or the potato aphid to compare the effect of herbivores with different feeding behavior on AsA content in local (infested) and systemic (uninfested) plant tissues. For locally infested tissues and the corresponding untreated controls, there were significant differences in total AsA content (oxidized and reduced) (Oneway ANOVA; $F=59.45$; $df=2,29$; $p<0.0001$) and reduced AsA ($F=51.19$; $df=2,29$; $p<0.0001$), but no significant differences in oxidized AsA ($F=1.13$; $df=2,29$; $p=0.34$) or in the ratio of oxidized to reduced AsA ($F=0.83$; $df=2,29$; $p=0.45$) (Figure 4A). Compared to the untreated control, foliage locally infested by caterpillars had 63% less total AsA (Student's *t* test; $t=2.05$; $p<0.0001$) and 65% less reduced AsA content ($t=2.05$; $p<0.0001$). AsA content was not significantly different between foliage infested with aphids and untreated controls ($t=2.05$; $p=0.27$).

In uninfested foliage collected from plants challenged with aphids or caterpillars, no significant systemic effect was observed in total AsA (Oneway ANOVA; $F=1.79$; $df=2,36$; $p=0.18$) (Figure 4B), reduced AsA ($F=1.56$; $df=2, 36$; $p=0.19$), oxidized AsA ($F=0.04$; $df=2,36$; $p=0.96$), or the ratio between oxidized and reduced ($F=0.88$; $df=2,36$; $p=0.43$).

Root tissue was also collected to determine if there was a systemic effect in roots due caterpillar and aphid infested foliage. No significant difference was found among treatments in total AsA (Oneway ANOVA; $F=0.72$; $df=2,28$; $p=0.50$), reduced AsA ($F=0.46$; $df=2,28$; $p=0.63$), oxidized AsA ($F=1.18$; $df=2,28$; $p=0.32$) or the ratio between oxidized and reduced AsA ($F=1.47$; $df=2,28$; $p=0.25$) (Figure 4C).

Nematodes were inoculated into the roots to determine if there is a local effect of nematodes on AsA content of roots. Total AsA content (Oneway ANOVA; $F=1.48$; $df=1,17$; $p=0.24$), reduced AsA ($F=0.33$; $df=1,17$; $p=0.57$), oxidized AsA ($F=0.94$; $df=1,17$; $p=0.34$), and the ratio of oxidized to reduced AsA ($F=1.41$; $df=1,17$; $p=0.25$) of tomato root did not differ significantly due to root-knot nematodes (Figure 5A). Foliage was also collected to determine if there is a systemic effect due to nematode infested roots on AsA content in foliage. Total AsA content ($F=.05$; $df=1,27$; $p=0.83$), reduced AsA ($F=0.06$; $df=1,26$; $p=0.81$), oxidized AsA ($F=0.11$; $df=1,26$; $p=0.74$), and the ratio of oxidized to reduced AsA ($F=0.12$; $df=1,26$; $p=0.79$) of tomato foliage did not differ significantly due to root-knot nematodes (Figure 5B).

Nematode treatment was done side by side with the caterpillar and aphid treatments, with empty insect cages on the same leaf positions that were used to measure the local and systemic effects of these foliar herbivores. Therefore, we can also directly compare the effects of all 3 herbivores. In infested leaves, significant differences among treatments were served for total and reduced AsA (total: Oneway ANOVA; $F=43.48$; $df=3,35$; $p<0.0001$, reduced: $F=39.14$; $df=3,35$; $p<0.0001$), but not for oxidized AsA ($F=1.09$; $df=3,35$; $p=0.37$) or the ratio of oxidized to reduced AsA ($F=0.91$; $df=3,35$; $p=0.44$). Only caterpillars caused a decrease in AsA content (total and reduced AsA) (Student's *t* test: total: $t=2.03$; $p<0.0001$; reduced: $t=1.99$; $p<0.0001$), as AsA levels were not significantly different in aphid infested leaves (total: $t=1.74$ $p= 0.10$; reduced: $t= 1.34$; $p= 0.19$) or nematode treated (total: $t=1.40$ $p= 0.18$; reduced: $t=0.29$; $p= 0.77$) compared to control plants.

In systemic foliage, no significant differences were observed among the four treatment groups in total AsA (Oneway ANOVA; $F=1.3628$; $df=3,45$; $p=0.267$), reduced AsA ($F=1.346$; $df=3, 45$; $p=0.272$), oxidized AsA ($F=0.031$; $df=3,45$; $p=0.993$), or the ratio between oxidized and reduced ($F=0.749$; $df=3,45$; $p=0.529$).

In comparing the effects of all herbivores on the AsA content of roots, no significant difference in total (Oneway ANOVA; $F=0.83$; $df=3,34$; $p=0.49$), reduced ($F=0.48$; $df=3,34$; $p=0.70$), oxidized ($F=1.08$; $df=3,34$; $p=0.37$), or the ratio between oxidized and reduced AsA ($F=1.42$; $df=3,33$; $p=0.25$) was observed.

Impact of herbivores on AsA content of Arabidopsis. To assess changes in AsA in Arabidopsis due to herbivores of different types, Arabidopsis were infected with caterpillar and aphids. Locally infested foliage was collected to determine the effect of herbivores on AsA content in Arabidopsis. No significant difference was observed in total AsA content of foliage infested with either aphids or caterpillars (Oneway ANOVA; F=1.01; df=2,32; p=0.37) (Figure 6A). Similarly, no difference was found between treated and untreated foliar tissue for reduced AsA (F-1.23; df=2,32; p=0.31), oxidized AsA (F=0.37; df=2,32; p=0.69) and the ratio between oxidized and reduced AsA (F=0.49; df=2,32; p=0.62).

Uninfested foliage was collected to determine the systemic effect of herbivores on AsA content in Arabidopsis. Herbivory caused a significant systemic effect on total AsA content (Oneway ANOVA; F=4.76; df=2,34; p=0.02). Caterpillar feeding decreased total AsA content by approximately 32% in systemic, untreated tissue (Student's *t* test; t=2.03; p=0.01), and aphid feeding decreased total AsA content by approximately 32%, as well (Student's *t* test; t=2.03; p=0.01) (Figure 6B). For both insects, the observed decline in total AsA was a result of a drop off in reduced AsA (Oneway ANOVA; F=4.91; df=2,34; p=0.01), while oxidized AsA remained comparable to levels in untreated control plants (F=0.73; df=2,34; p=0.49). The ratio of oxidized AsA to reduced AsA showed no significant difference among treatment groups (F=1.89; df=2,34; p=0.17).

Impact of MIOX4 on AsA content in Arabidopsis. The MIOX4 overexpression line used for this study had 4 copies of the transgene (A. Lorence, personal communication) and the T3 generation plants used in this study were segregating for the presence of the transgene.

Ascorbate levels were compared in T3 progeny from 4 different T2 parents (all derived from the same T1 parent) grown on selection media in order to select for the lineage with the highest AsA content. These were compared against WT and *vtc1*, a mutant with low AsA. Total AsA content differed between plant genotypes (Log transformed; Oneway ANOVA; $F=29.45$; $df=5,43$; $p<0.0001$) (Figure 7). In comparison to WT, *vtc1* showed an approximately 69% decrease in total ascorbate content (Student's *t* test; $t=2.02$; $p<0.0001$). With the exception of *MIOX4* L33-4, all other overexpression lines, *MIOX4* L33-1, *MIOX4* L33-2, and *MIOX4* L33-3, showed no difference in AsA content from WT (Student's *t* tests; $t=2.02$; $p=0.42$, $p=0.63$, $p=0.49$, respectively). However, *MIOX4* L33-4 showed an approximately 45% increase in AsA content over WT (Student's *t* test; $t=2.02$; $p=0.04$). Changes in total AsA content were due to a difference in reduced AsA (Log transformed; Oneway ANOVA; $F=28.65$; $df=5,43$; $p<0.0001$). Reduced AsA of *MIOX4 L33-4* was significantly higher than WT (Student's *t* test; $t=2.02$; $p=0.04$), and WT was significantly higher than *vtc1* (Student's *t* test; $t=2.11$; $p<0.0001$). No significant difference was observed in oxidized AsA among genotypes (Oneway ANOVA; $F=0.12$; $df=5,43$; $p=1.00$) or the ratio between oxidized and reduced AsA ($F=1.70$; $df=5,43$; $p=0.16$).

Impact of altered AsA content in Arabidopsis on herbivores. The effects of altered AsA content in plant on beet armyworm performance were tested in two independent bioassays. Caterpillar weight and amount of feeding was measured on Arabidopsis genotypes with low (*vtc1*), high (*MIOX4* overexpression line), and wild-type levels of AsA (WT). The lineage *MIOX4* L33-4 was used for testing because it contained the highest level of AsA.

Bioassay 1: WT and *MIOX4* L33-4 were treated with 2 beet armyworms. At 3 days after inoculation (DAI), no significant difference in consumed leaf area was observed between WT and *MIOX4 L33-4* (Oneway ANOVA; F=0.02; df=1,22; p=0.90) (Figure 8A). Damage was reevaluated at 7 DAI by ranking the overall damage of the whole plant on a scale of 1 (low damage) to 4 (high damage). A categorical ranking was used to measure damage on day 7 because too much damage had occurred to the leaf margins to allow the precise measurement of feeding holes. Damage ranks were not significantly different between WT and *MIOX4 L33-4* (Likelihood Ratio; χ^2=3.28; p=0.35) (Figure 8B).

At 7 DAI, caterpillars did not have significantly different weights when feeding on WT or *MIOX4 L33-4* genotypes (Welch's ANOVA; F=1.58; df=1,30.36; p=0.22) (Figure 8C).

Bioassay 2: This experiment included *vtc1* along with WT and *MIOX4* L33-4. For this assay we did not measure damage at 3 DAI, because no significance was found from bioassay 1. Measurement was taken of total area of consumed plant tissue after 7 days of infestation. A significant difference in damage was observed among genotypes (Oneway ANOVA; F=4.86; df=2,44; p=0.01) (Figure 9A). The *MIOX4* L33-4 over-expression line sustained the most damage with approximately 74% more damage than WT (Student's *t* test; t=2.02; p=0.01) and approximately 105% more damage than *vtc1* (t=2.02; p=0.01). WT and *vtc1* genotypes were not significantly different (t=2.02; p=0.59). At 7 days, there were also significant differences among genotypes in caterpillar weight (Oneway ANOVA; F=3.76; df=2,58; p=0.03) (Figure 9B). Caterpillars weighed more on *MIOX4* L33-4 than on both WT (Student's *t* test; t=2.00; p=0.02) and *vtc1* (t=2.00; p=0.03).

DISCUSSION

Our study revealed differences in how three different herbivores (caterpillars, aphids, and root-knot nematodes) impact AsA content in tomato. Caterpillars caused a significant decrease in AsA content in locally infested foliage, similar to prior observations that AsA levels declined in response to beet armyworm challenge in two additional tomato genotypes, cv. Castlemart and the *spr2* mutant (M. Arevalo, personal communication). The decrease in total AsA content due to caterpillar feeding was similar in magnitude to the effect of mechanical wounding of tomato (Chapter 1). In contrast to caterpillars, neither aphids nor root-knot nematodes appeared to affect AsA content in their host plants. Our findings with nematodes are consistent with those of Arrigoni et al. For consistency, straws were also removed from the uninfested control and the plants used for the caterpillar and aphid treatments, water without nematodes was pipetted into the sand (1979), who reported that nematode infestation did not influence AsA content in the roots of another susceptible tomato cultivar. It is possible that the decrease in AsA content observed in leaflets fed upon by caterpillars was due to wounding, and that feeding by aphids and nematodes did not alter AsA content because these herbivores did not cause as extensive mechanical disruption.

No systemic effects of herbivory on AsA content were observed in uninfested foliage of plants challenged with caterpillars, aphids or nematodes. Total AsA of roots appeared to be lower in caterpillar infested plants, but this was not statistically significant, possibly because of the low number of replications and the low relative AsA content observed in root tissue.

Our study also revealed differences in how two different plant species, tomato and Arabidopsis, responded to caterpillars and aphids. In Arabidopsis, no significant differences were found in AsA content of foliage locally infested with aphids and caterpillars, which is

surprising, considering that mechanical wounding of Arabidopsis is known to cause an increase in AsA content (Suza et al. 2010). Perhaps there is some aspect of caterpillar feeding that counteracts the mechanism that causes an increase in AsA in response to wounding in Arabidopsis. It is possible that glucosinolates, the anti-herbivore defense system in Arabidopsis, may play a role in the difference observed between tomato and Arabidopsis by influencing the way the insect feeds. Ascorbate serves as an enzyme cofactor for myrosinases, and this is imperative for the hydrolyzation of the glucosinolates found within the order Bassicales (Burmeister et al. 2000; Conklin et al. 1996; Wittstock and Halkier 2002). These data suggest that wounds imparted by insect feeding do not have as large an effect on AsA content in Arabidopsis as they do in tomato. However, more replicates are necessary to determine if there are significant changes in local AsA content due to caterpillar and aphid feeding.

In contrast to our results for leaves on which insects were caged, unchallenged leaves from the same plants showed a systemic decreased in AsA content in response to caterpillar feeding and aphid infestation. This suggests that, unlike tomato, the effects of aphid and caterpillar damage are not restricted to only local tissue. It is peculiar that we would see more marked in changes in AsA in uninfested foliage than in local, infested leaves. Perhaps, some degradation in sample occurred as observed by unusually high levels of oxidized AsA content in these leaves. However, these data give us tantalizing evidence that herbivores may have systemic effects on AsA content in their hosts, even in undamaged, uncaged leaves. More work is needed to determine the effects that herbivores have on AsA independent of wounding, because many prior studies of insect oral secretions have shown that feeding damage by insects has different effects on the plant for mechanical wounding (Felton and Korth 2000).

Our study revealed differences in plant damage or the weights of the caterpillars in bioassay 2. The most damage occurred to *MIOX4* L33-4 plants, and the heaviest caterpillars were found on *MIOX4* L33-4 plants as compared to both WT and *vtc1*. Damage of WT and *vtc1* were not significantly different from one another, but the lightest insects fed on WT plants, which is similar to previous results (C. Avila personal communication). Previous experiments have showed a difference between *vtc1* and WT, in which caterpillars weighed more after feeding on *vtc1*, but the experiment used fewer replicates. *MIOX4* L33-4 plants showed similarities in both bioassays by having an increased amount of damage and supporting greater larval weights than WT. However, bioassay 1 revealed no significant differences in plant damage or insect weight. The difference in these two bioassays is likely due to the fewer number of replicates used in bioassay 1 and growth issues from insufficient lighting for plants used in bioassay 1. Regardless, these data suggest that increased AsA in these plants may contribute to a benefit to insect health in terms of weight and serve as a detriment to the plant in terms of damage.

The results of our bioassays with MIOX4 overexpressers differ from prior results with another transgenic line in which AsA accumulation was enhanced through transgenic expression of the enzyme L-gulono-1,4-lactone oxidase (GLOase). These plants have a strong constitutive expression of a rat gene encoding for the enzyme GLOase and have two-fold greater AsA than control plants (Lorence and Nessler 2007). A preliminary experiment indicated that beet armyworm feeding and growth was reduced on GLOase as compared to WT (C. Avila personal communication). This suggests that, although high AsA is common to both GLOase and *MIOX4* L33-4 mutants, there could be a key component that is different between these mutants that cause their effects to be different on the caterpillar growth and feeding. Perhaps overexpression of

MIOX4 also affects factors other than AsA that may influence plant susceptibility to insects. For example, *myo*-inositol is involved in synthesis of several isomeric inositols, which are involved in resistance to a variety of stresses (Loewus and Murthy 2000).

Future analysis of the expression of genes encoding for MIOX and the enzyme GLOase could be used to determine where differences lie and why we observed different outcomes. It is possible the altering the genes encoding for these two genotypes differ in expression of other enzymes related to AsA synthesis and the ability for AsA to be reformed or recycled in the system. This may help us to identify key differences in how AsA biosynthesis alters proteins and enzymes that play roles in anti-herbivory. Future studies should also examine the potential multi-generational effects of AsA content in the plant on the herbivore, such as hatch rate, because the effect of anti-herbivore defenses may be more evident in future generations of the herbivore. It would be necessary to lengthen this experiment to determine the effects of increased AsA due to *MIOX4* L33-4 plants on insect longevity and to study these effects over a larger part of the insect's life. There is also a need for more plants with altered ascorbate levels to understand the effect of AsA on insects. This could have important implications for agriculture. In the future it would be interesting to determine if aphid population growth is affected by MIOX4 overexpression, because no significant change in population growth of aphids occurred between *vtc1*, WT and GLOase plants. (Appendix).

Conclusions. Our data suggests that different types of herbivores can have different affects on AsA content in their host plants, and that the effects of a single herbivore such as the beet armyworm may also vary between different host species. Caterpillar feeding caused a decrease in AsA content in tomato, with no systemic effect on AsA content in uninfested leaves or root tissue. In Arabidopsis, AsA levels were unaffected by herbivory in locally-infested tissue but

decreased significantly in the uninfested foliage, which may have been due to insufficient lighting conditions. Future experimentation is needed to tease apart what effects herbivores have on tissue of a variety of plants. Development of AsA mutants of different plant species is essential to further our understanding of the effects of AsA on herbivores, since our study suggests that the role of AsA in plant-insect interactions may vary in different plant species.

FIGURES AND FIGURE LEGENDS

Figure 1. AsA Biosynthesis in Arabidopsis. AsA can be synthesized by 4 different pathways in the plant: the *myo*-inositol (MI) pathway, the L-gulose pathway, the D-mannose/L-Galactose pathway, and the D-galacturonate pathway. An exchange of a sole amino acid translated from the sequence at the VTC1 locus causes AsA content to be reduced by 70% of that found in WT plants. Overexpression of *myo*-inositol oxygenase, MIOX, causes an increase in AsA content. Transformation with the enzyme, L-gulono-1,4-lactone oxidase (GLOase) doubles AsA content. This figure is based on (Suza et al. 2010).

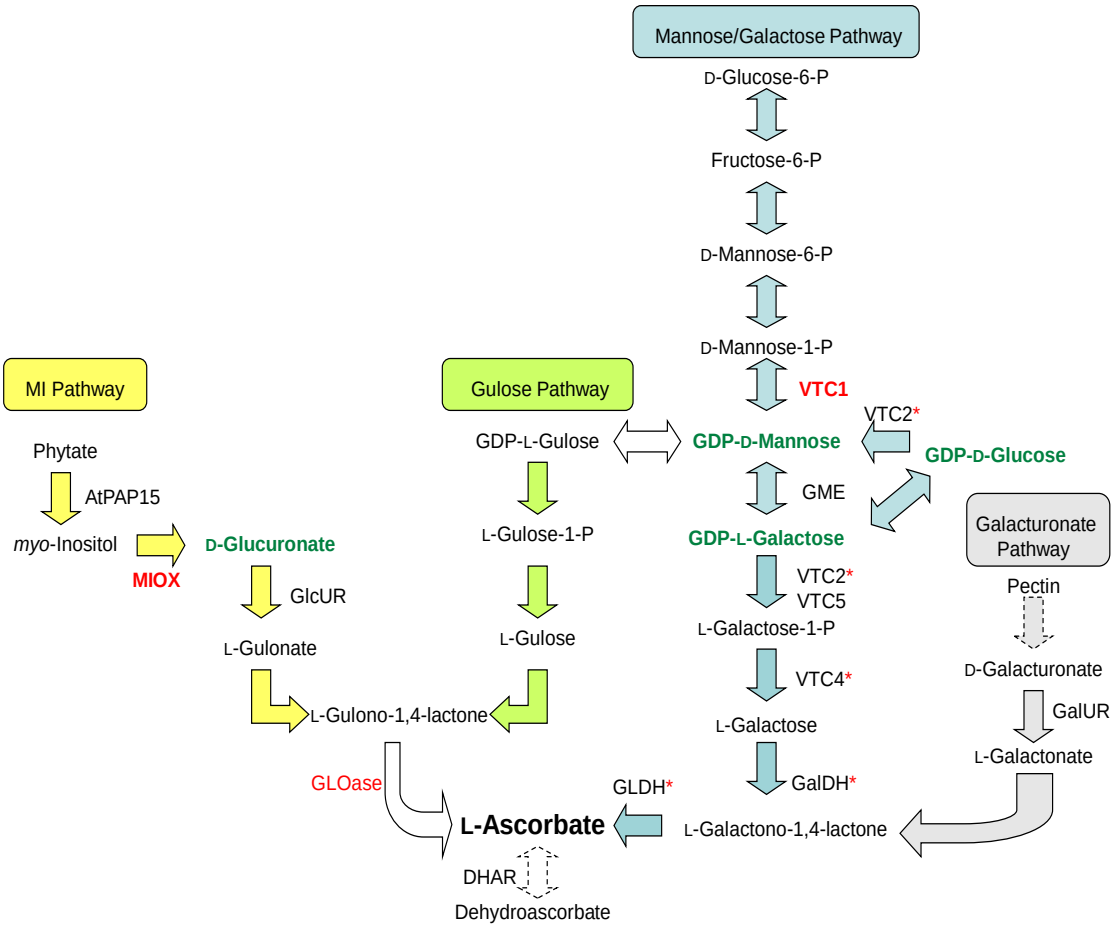
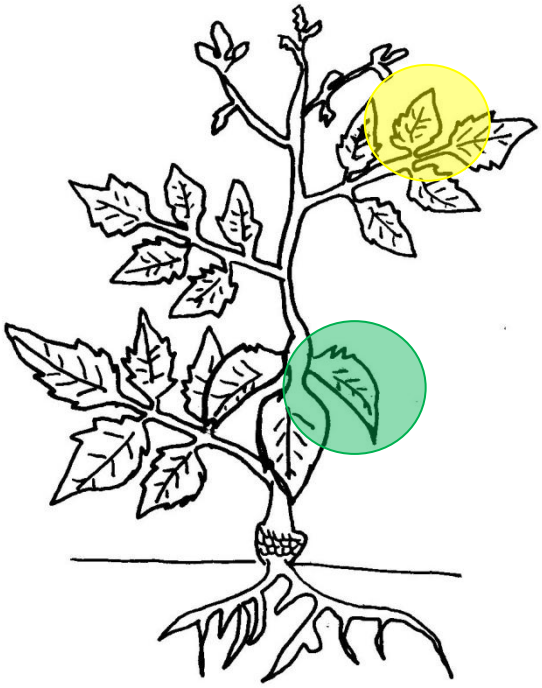


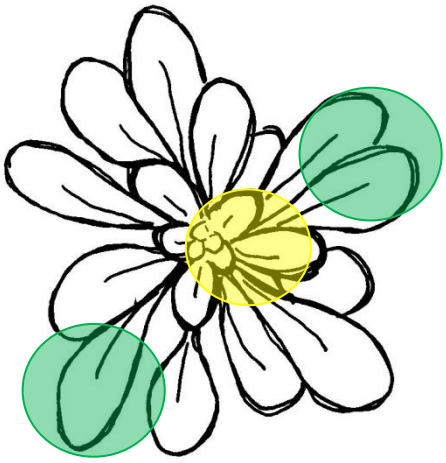
Figure 2. Uninfested leaves of tomato and Arabidopsis for testing systemic response to herbivores. Uninfested leaflets at the same leaflet position of the leaf directly above and to the right of the infested leaf were collected to determine the systemic effect of herbivory in AsA content in tomato (A). Uninfested leaves on the inside of the plant on a separate level of growth than infested leaves were chosen in Arabidopsis (B).

A



Infested leaves

B



Uninfested leaves

Figure 3. Arabidopsis damage rating from 1 (low damage) to 4 (high damage).

1

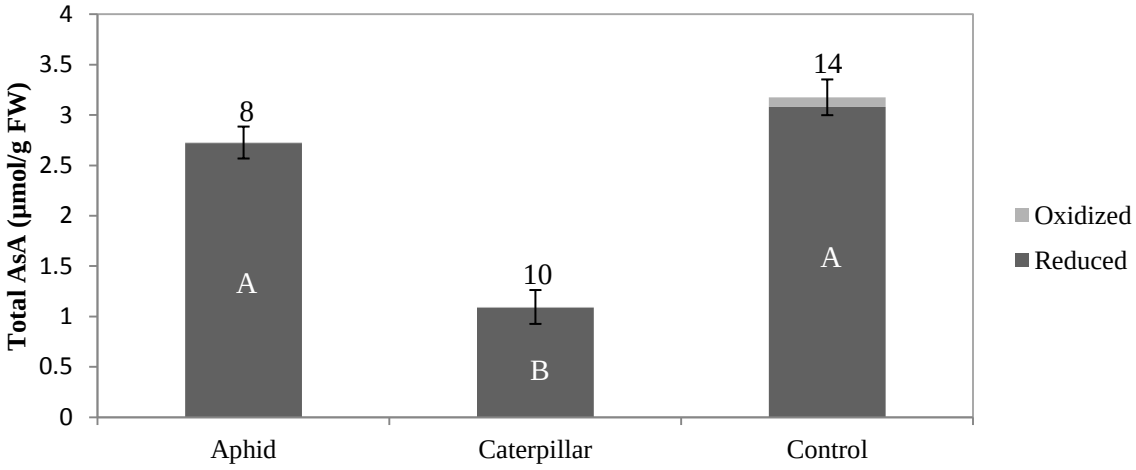
2

3

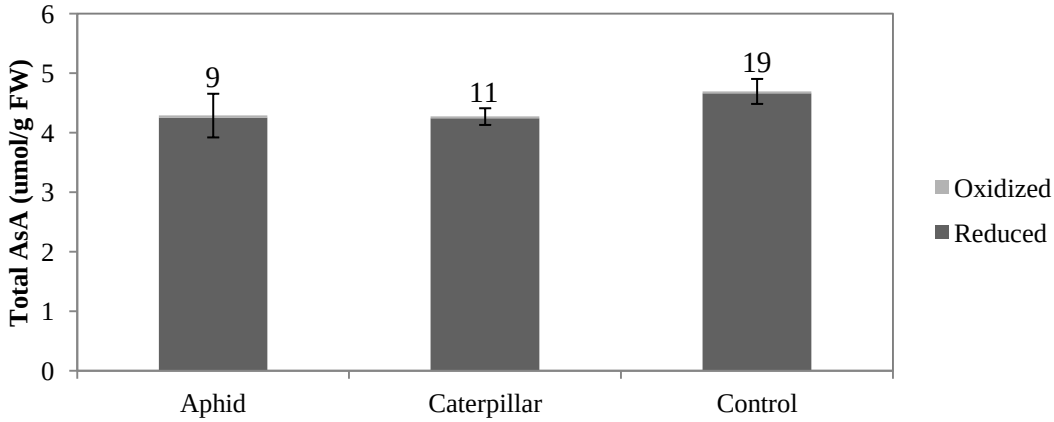
4

Figure 4. Impact of aphid and caterpillar infestation on AsA content in tomato. Reduced and oxidized AsA were measured in locally infested leaves (A), systemic leaves unexposed to herbivores (B), or roots (C) each at 48h after plants were challenged with caterpillars or aphids, or were mock-infested with empty cages (control). Different letters represent significantly different means for total (oxidized + reduced) AsA according to a Student's *t* test. Error bars represent the standard error of the means (SEM) of total AsA. Numbers represent number of replicates for each treatment.

A


Total AsA (µmol/g FW)
4
3.5
3
2.5
2
1.5
1
0.5
0
8
10
14
A
B
A
Aphid
Caterpillar
Control
Oxidized
Reduced

B


Total AsA (umol/g FW)
6
5
4
3
2
1
0
9
11
19
Aphid
Caterpillar
Control
Oxidized
Reduced

C

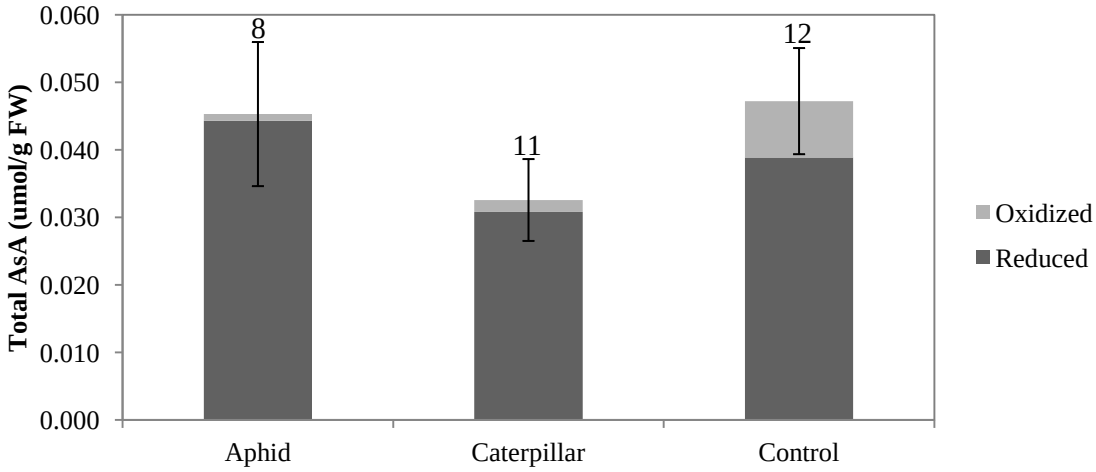
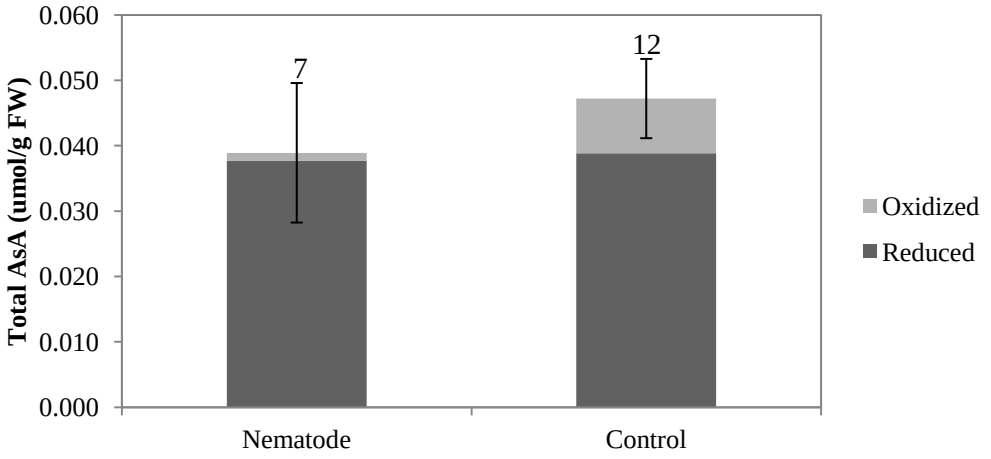

Total AsA (umol/g FW)
0.060
0.050
0.040
0.030
0.020
0.010
0.000
8
11
12
Aphid
Caterpillar
Control
Oxidized
Reduced

Figure 5. Effect of nematode infestation on AsA content in tomato. Reduced and oxidized AsA was measured in locally infested root tissues (A) and foliage (B) 48h after plants were inoculated with nematodes, or were mock-inoculated with water (control). Foliar and root tissue were collected. Error bars represent the SEM of total AsA. Numbers represent number of replicates for each treatment.

A



B

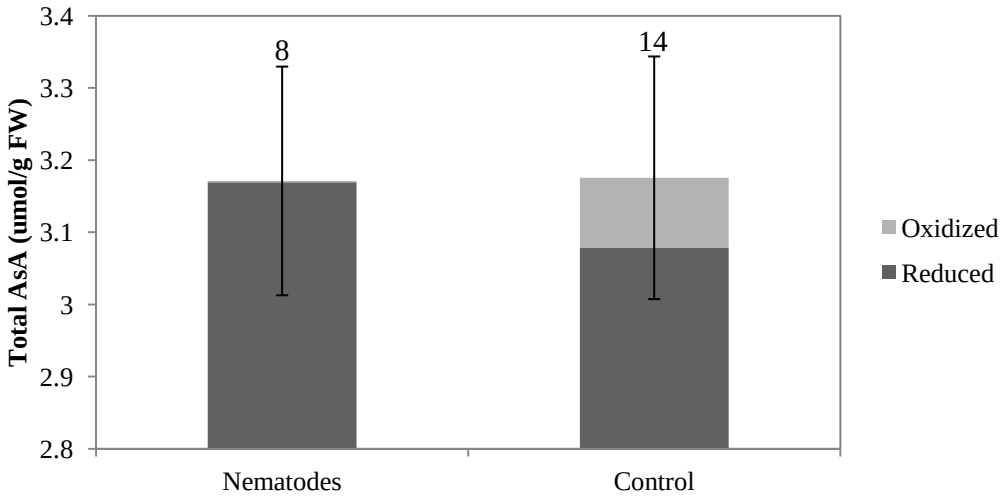
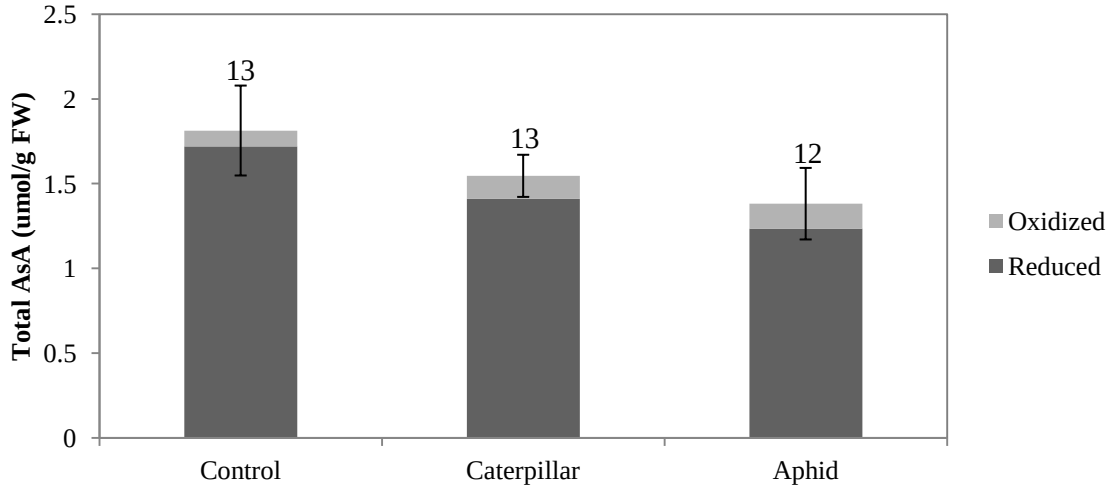


Figure 6. Impact of aphid and caterpillar infestation on AsA content in Arabidopsis. Reduced and oxidized AsA were measured in Arabidopsis that were locally infested leaves (A) and systemic leaves unexposed to herbivores (B) 48h after plants were challenged with caterpillars or aphids, or were mock-infested with empty cages (control). Different letters represent significantly different means for total (oxidized + reduced) AsA according to a Student's *t* test. Error bars represent the SEM of total AsA. Numbers represent number of replicates for each treatment.

A



B

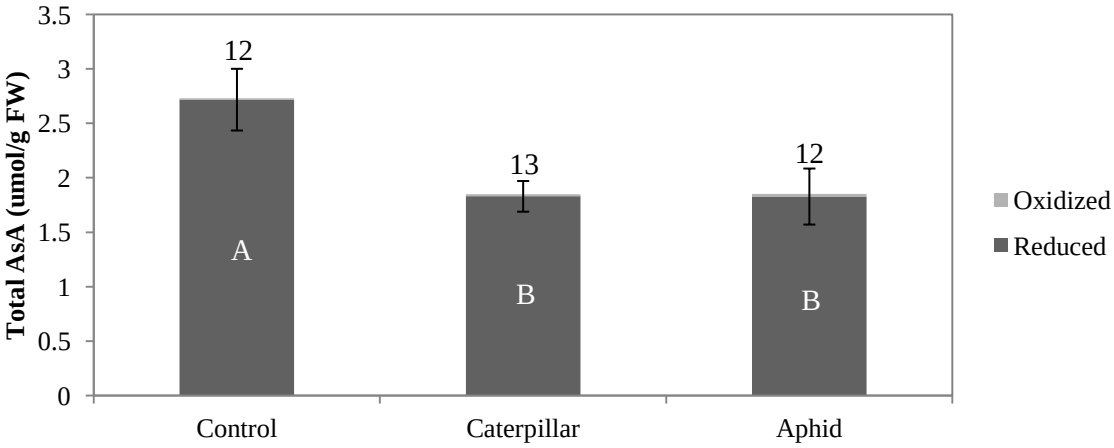


Figure 7. The effect of the *vtc1* mutation and overexpression of *MIOX4* on AsA content in *Arabidopsis*. AsA levels were compared in *vtc1*, WT, and the 4 copies of the transgenic line 33 of MIOX4 overexpressers (*MIOX4* L33-1, *MIOX4* L33-2, *MIOX4* L33-3, and *MIOX4* L33-4). Different letters represent significantly different means for total (oxidized + reduced) AsA according to a Student's *t* test. Error bars represent the SEM of total AsA. Numbers represent number of replicates for each treatment.

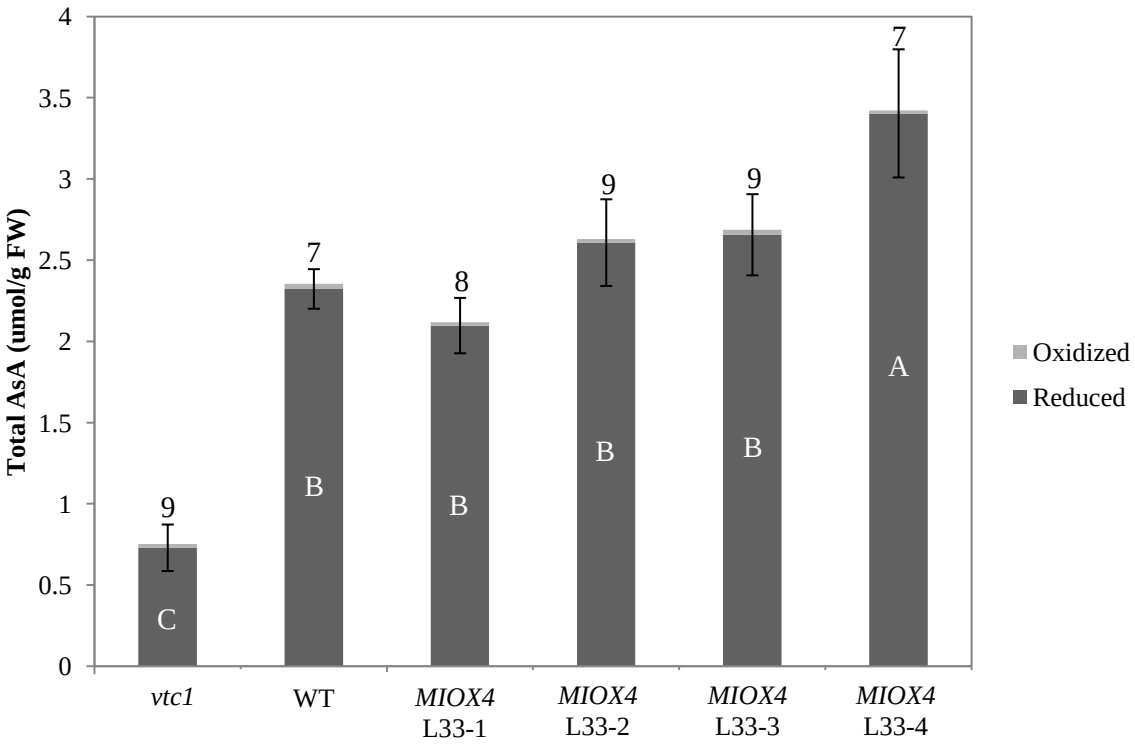
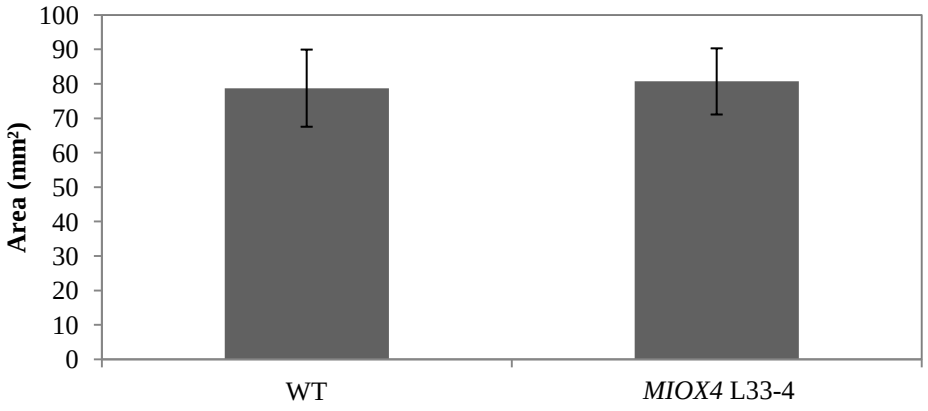
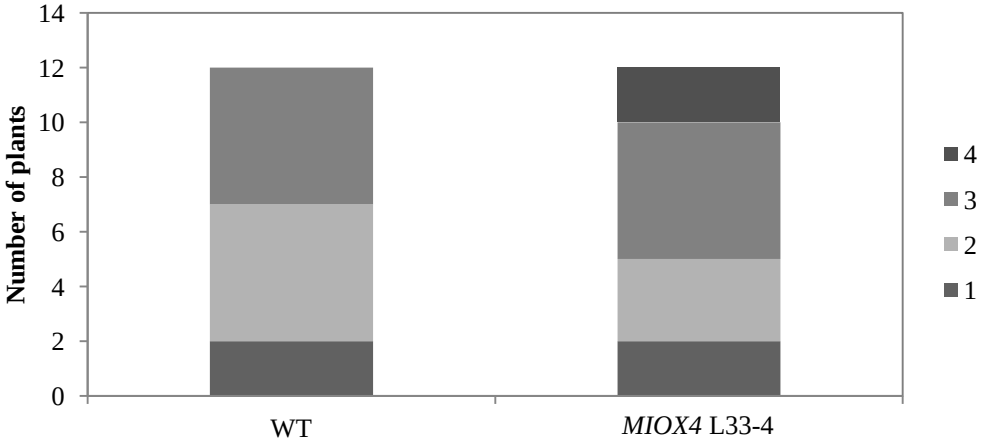


Figure 8. Caterpillar feeding damage and performance on WT and MIOX4 overexpression line (L33–4) of Arabidopsis. At 3 days after inoculation (DAI) with 2 neonate beet armyworm larvae, no significant difference was observed in the total area of feeding damaged (mm^2) between the WT cultivar and the *MIOX4* L33-4 overexpression line of Arabidopsis. Error bars represent the SEM of total area (A). At 7 DAI, Damage was assessed again using a ranking from 1 (low damage) to 4 (high damage) and WT and *MIOX4* L33-4 plants again showed no significant difference (B). No significant differences were observed between weights (mg) of caterpillars (*S. exigua*) that fed on WT and *MIOX4* L33-4 overexpression line of Arabidopsis at 7 DAI (C). Error bars represent the SEM of total AsA.

A


100
90
80
70
60
50
40
30
20
10
0
Area (mm²)
WT
MIOX4 L33-4

B


14
12
10
8
6
4
2
0
Number of plants
WT
MIOX4 L33-4
4
3
2
1

C

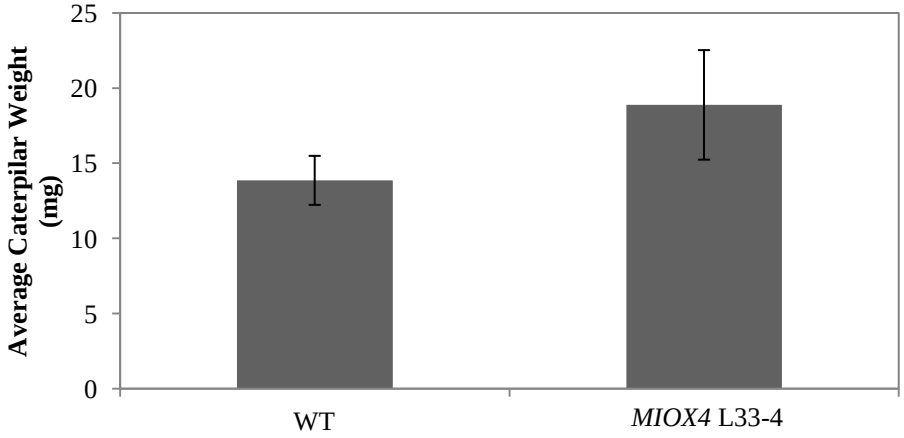
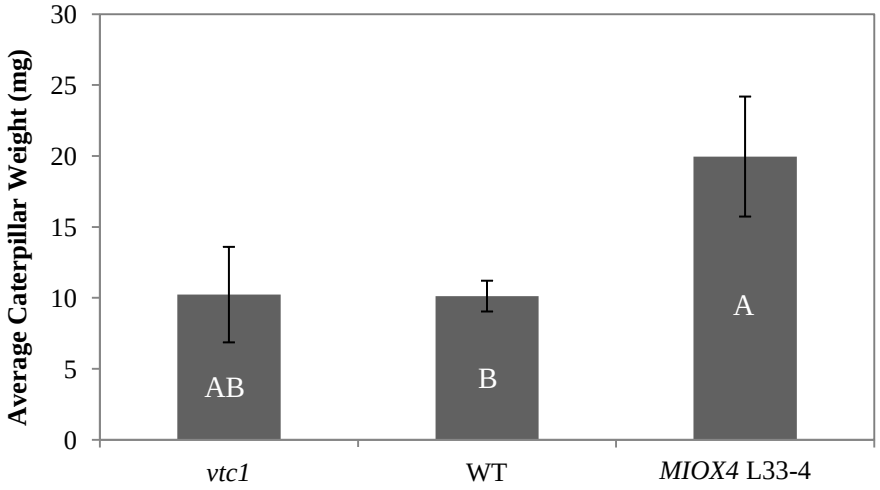
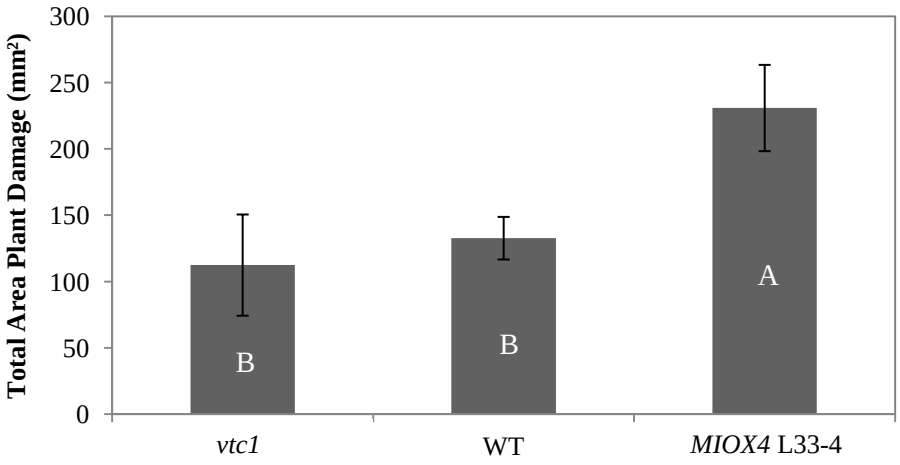

25
20
15
10
5
0
Average Caterpilar Weight (mg)
WT
MIOX4 L33-4

Figure 9. Caterpillar performance and total damage area due to caterpillar feeding on *vtc1*, wild type, and MIOX4 overexpression line of Arabidopsis. At 7DAI, a significant difference was observed in total area of plant damage (mm^2) among *vtc1*, WT, and *MIOX4* L33-4. Error bars represent SEM of total area of plant damage (A). A significant difference was also observed in caterpillar weights (mg) among *vtc1* , WT, and *MIOX4* L33-4. Error bars represent SEM of average caterpillar weight (B). Different letters represent significantly different means by Student's *t* test.

A

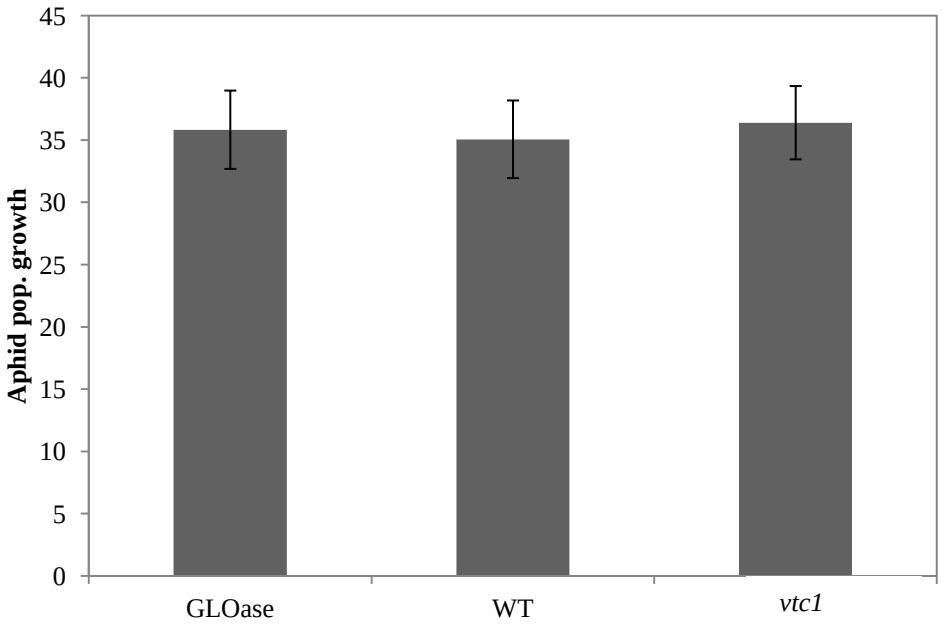


B



APPENDIX

Aphid performance on *vtc1*, WT and GLOase of Arabidopsis. Arabidopsis transformed with gene encoding for the enzyme, L-gulono-1,4-lactone oxidase (GLOase) have twice the levels of AsA as WT plants (Lorence and Nessler 2007). This enzyme catalyzes the conversion of L-gulono-1, 4-lactone to ascorbate (Lorence et al. 2004; Smirnoff 2001; Wolucka and Van Montagu 2003). To test the effects of AsA on aphid performance, population growth of aphids was measured on 3 genotypes of Arabidopsis: GLOase (high AsA), WT (normal AsA), and *vtc1* (low AsA). Green peach aphids (*Myzus persicae*) were added to Arabidopsis at the 6.5 stage. Five aphids were confined by a sleeve and allowed to feed and reproduce over 7 days. No significant difference in population growth was observed among genotypes. (Oneway ANOVA; $F=0.158$; $df= 2$; $p=0.858$). Error bars represent SEM of aphid population growth.



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