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Exploring the impact of wounding and jasmonates on ascorbate metabolism

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Abstract

Vitamin C (ascorbate, AsA) is the most abundant water-soluble antioxidant in plants. Ascorbate provides the first line of defense against damaging reactive oxygen species (ROS), and helps protect plant cells from many factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attack. Plant defenses against these stresses are also dependent upon jasmonates (JAs), a class of plant hormones that promote ROS accumulation. Here, we review evidence showing that wounding and JAs influence AsA accumulation in various plant species, and we report new data from *Arabidopsis* and tomato testing the influence of JAs on AsA levels in wounded and unwounded plants. In both species, certain mutations that impair JA metabolism and signaling influence foliar AsA levels, suggesting that endogenous JAs may regulate steady-state AsA. However, the impact of wounding on AsA accumulation was similar in JA mutants and wild type controls, indicating that this wound response does not require JAs. Our findings also indicate that the effects of wounding and JAs on AsA accumulation differ between species; these factors both enhanced AsA accumulation in *Arabidopsis*, but depressed AsA levels in tomato. These results underscore the importance of obtaining data from more than one model species, and demonstrate the complexity of AsA regulation.

Keywords

Ascorbate; vitamin C; jasmonate; ROS; wounding; *Arabidopsis*; tomato

Introduction

Ascorbic acid (vitamin C, AsA) is essential for human health, and in order to survive humans must obtain this nutrient from their diet, primarily from fresh fruits and vegetables [60]. AsA is also critical to plant health, as evidenced by the fact that no plant mutant completely devoid of AsA has ever been described. Despite the importance of AsA, however, the factors regulating its metabolism in plants are not well-understood. AsA content in plants varies in response to abiotic and biotic stresses, including wounding (Table 1), and several studies (Table 2) suggest that jasmonates (JAs), a class of plant hormones that regulate many plant stress responses, may

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influence AsA metabolism [62,90,125]. Whereas the majority of studies on wounding (Table 1) reported a decrease in AsA, the majority of prior studies on exogenous JAs (Table 2) reported an increase in AsA. However, none of these prior studies compared the effects of these two treatments in the same plant species and tissue type. The goal of this study was to examine the effects of wounding and methyl jasmonate (MeJA) treatment on intact plants in the same species, and we also explored potential diversity among species by comparing wounding and MeJA influence on AsA in two different models. In this paper, we present an overview of what is currently known about the influence of wounding and JAs on AsA metabolism in plant cells and tissues, and we also report our current findings on AsA metabolism in response to mechanical injury and treatment with exogenous JAs in *Arabidopsis* and tomato (*Solanum lycopersicum*).

Ascorbate metabolism and function in plants

A biosynthetic pathway for AsA was not defined until 1998 [121]. Subsequent discoveries revealed that AsA is synthesized via multiple pathways [2,11,61,121,123]. Major routes to AsA formation in plants (Fig. 1) include: the D-mannose/L-galactose (Man/Gal) [121], D-galacturonate (GalU) [2], L-gulose (Gul) [123] and *myo*-inositol (MI) [61] pathways. These routes appear to be highly interrelated, as seen in the case of VTC4 (Fig 1). This enzyme has been shown to use L-*myo*-inositol-1 phosphate as a substrate in addition to L-galactose-1-phosphate, and to contribute to both MI and AsA metabolisms [115]. The factors regulating these metabolic pathways are just beginning to be understood. Recently, screening of an activation-tagged *Arabidopsis* collection allowed the discovery of *AMR1* (for ascorbic acid mannose pathway regulator 1), a gene that coordinately and negatively regulates multiple genes in the Man/Gal route to AsA [133]. A purple acid phosphatase with phytase activity has also been shown to channel phytate to the MI pathway to AsA, and may modulate AsA levels by controlling the availability of MI as a precursor for AsA biosynthesis [132]. Recycling may also play a significant role in AsA accumulation in plant tissues since AsA content can be increased by enhanced expression of AsA recycling enzymes [21].

In plants, AsA participates in enzymatic and non-enzymatic detoxification of reactive oxygen species (ROS) generated by aerobic cellular metabolism and by abiotic stresses such as wounding, ozone, and UV-B damage [29,97,98]. Ascorbate may also contribute to responses to herbivory through its role in metabolism of glucosinolates [18,25,122]. In addition, as a cofactor to some iron-and copper-containing enzymes, AsA plays an important role in the biosynthesis of certain plant hormones, including ethylene and gibberellic acid, and cell wall glycoproteins and secondary metabolites with antimicrobial properties [81].

Ascorbate metabolism is also closely intertwined with cell wall biosynthesis and remodeling. As illustrated in Fig. 1, multiple intermediates that participate in the Man/Gal and MI pathways are also used as precursors of cell wall components. Furthermore, a “VTC2 cycle” that links AsA biosynthesis with photosynthesis and cell formation was recently proposed based on the findings that the VTC2 enzyme can use glucose-1 phosphate and GDP-D-glucose as substrates in addition to GDP-L-galactose, and that a GDP-D-mannose-2'-epimerase activity exists in plants [124]. In addition, since extracellular H₂O₂ and hydroxyl radicals contribute to the cross-linking of cell wall polymers and proteins [5,94], the direct effect of AsA on ROS might influence cell wall expansion [95]. Also, as a substrate for ascorbate oxidase, AsA is implicated in the control of cell expansion and cell division through the control of the apoplastic redox status [49,51,79]. Furthermore, production of extensins involved in cell wall modification is controlled by the enzyme prolyl hydroxylase which uses AsA as a substrate [30,114].

The numerous roles of AsA [11,79,80] and the emerging role of ROS in many plant physiological processes emphasize the need to understand regulation of AsA metabolism in plants [67,68,116]. Moreover, the pathways for AsA synthesis and recycling appear to be

influenced by numerous stresses including mechanical wounding [46] and by jasmonates, a family of stress-responsive signaling compounds [70,85]. Therefore, regulation of AsA metabolism in response to injury and other stresses represents a particularly rich area for study.

Impact of wounding on ascorbate metabolism

Wounding has been shown to alter the total AsA content of many plant tissues (Table 1). The effects of injury on AsA vary among plant species, and possibly also among different plant organs, and the nature of the mechanical injury can also influence the outcome; for example, bruising potato tubers decreases AsA, whereas cutting them increases AsA content [69]. However, for the majority of species that have been examined, wounding results in a decrease in total AsA content. Another effect of wounding on AsA content is that in some plants, injury decreases the ratio of reduced to oxidized AsA [110,120,126]. It is worth noting that the experiments reported in Table 1 were performed with detached tissues, and so additional experiments are needed to determine if similar phenomena occur in intact plants. Further work is also required to identify the factors regulating total AsA content and redox status in wounded tissue, because regulation of this wound response is not yet well understood.

Numerous studies in a variety of plant species have shown that wounding influences the transcriptional regulation of various genes that govern AsA biosynthesis and recycling (e.g. [10,19,32,40,46,55,89]). However, few generalizations can be made based on these studies, and the observed gene expression patterns are not necessarily predictive of actual AsA levels; for example, in acerola, genes encoding AsA biosynthetic enzymes were up-regulated 24 h after wounding and down-regulated at 48 h, although total AsA content in wounded plants was depressed relative to controls at both time points [10]. Furthermore, the factors regulating the expression and activity of AsA synthesis and recycling enzymes in response to wounding has not been extensively investigated. Because JAs mediate many wound responses and are also known to influence expression of certain genes involved in AsA metabolism [89,90] they could potentially contribute to wound-response changes in AsA pools.

Jasmonate metabolism and function

Jasmonic acid, MeJA, their isomers, biosynthetic precursors, and several derivatives including jasmonoyl-amino acid conjugates are collectively referred to as jasmonates (JAs) [119]. The ground breaking work of Vick and Zimmerman [117] facilitated the elucidation of the pathway for JA biosynthesis (Fig. 2). The initial step involves the release of α -linolenic acid (LA), a C₁₈ fatty acid with three double bonds (18:3), from membranes or storage lipids in response to developmental or stress signals by the action of a lipase [47]. LA is subsequently converted to 12-oxo-phytodienoic acid (OPDA) by sequential activities of three plastidial enzymes, lipoxygenase, allene oxide synthase, and allene oxide cyclase [56,75,91]. The remaining steps are localized to the peroxisome and involve the reduction of OPDA by OPDA reductase to yield 3-oxo-2-(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) [57,117]. OPC-8 is activated by OPC-8:CoA ligase to produce OPC-8-CoA [53]. Finally, JA is derived from OPC-8-CoA following three cycles of β -oxidation involving three peroxisomal enzymatic functions (acyl-CoA oxidase, multi-functional-protein, and L-3-ketoacyl-CoA thiolase), which are encoded by a small gene family in *Arabidopsis* [28,57,91]. The volatile JA metabolite, JA methyl ester (MeJA), is formed by methylation of the C-1 carboxyl group by a JA carboxy methyltransferase [96]. Also, MeJA can be de-esterified to JA in a reversible reaction by MeJA esterase [107,127]. Activation of JA to jasmonoyl-isoleucine (JA-Ile) is done by the JA:amino synthetase (jasmonate resistant 1, or JAR1) [41,103,109].

In general, jasmonate signaling in plants is dependent upon coronatine insensitive 1 (COI1), although the JA precursor OPDA is reported to regulate a distinct set of COI1-independent responses [58,106,111,128]. The F-box protein COI1 is a component of a multi-protein E3-

ubiquitin ligase that mediates degradation of JA ZIM domain (JAZ) proteins that repress transcription of JA-responsive genes [22,31,113]. Although multiple JAs are present in plant tissues, only the JA conjugate JA-Ile and to a lesser extent JA-leucine (JA-Leu) and JA-valine (JA-Val) promote the COI1-JAZ interaction [16,22,50,101,113]. Recently it was also shown that (+)-7-*iso*-JA-Ile is the bioactive form which accumulates predominately in wounded tissues, while the (–)-JA-Ile form is inactive [36,109].

Exogenous application of JAs is one of the methods used to investigate the effects of these compounds on various plant physiological processes. This method has allowed investigators to identify JA-responsive genes [13,44,54]. Exogenously applied MeJA influences the expression of many genes [100] and the plant responses associated with JA signaling tend to correlate with changes in gene expression [83]. Another commonly used approach to studying JA function is the use of mutant lines in *Arabidopsis* and tomato that are defective in JA signaling. Some of the *Arabidopsis* mutants with defective JA biosynthesis (Fig. 2) include *fad7-2* [64] and *acx1/5* (*acyl-CoA oxidase1/5*) [53,93]. The *Arabidopsis* *FAD7* gene is responsive to wounding and encodes a plastidial ω -3-fatty acid desaturase that catalyzes the desaturation of 18:2 fatty acids in plastid membranes [63,64,72]. The *fad7-2* mutant has less than 30% of wild type levels of the JA precursor linolenic acid(18:3) and 16:3 fatty acids when grown at temperatures ranging from 18 and 28°C [64]. Loss of function of *FAD7* also results in diminished linolenic acid (less than 10% of wild type) and JA signaling in the mutant tomato line *spr2* [56]. JA accumulation can also be inhibited by defects in acyl-CoA oxidase (ACX) family members which catalyze the first β -oxidation step in JA synthesis (Fig. 2).

Arabidopsis plants defective in *ACX1* show reduced activity on long-chain fatty acyl-CoA substrates and JA production in response to wounding [1,93]. However, disruption of *ACX5* has no obvious phenotypic or JA synthesis consequences [1,93]. Interestingly, wound-induced JA production is abolished in the *Arabidopsis* *acx1/5* (*acx1/5*) double-mutant suggesting *ACX5* might inhibit *ACX1* activity in wild type plants [93]. The role of *ACX1* in JA synthesis is further supported by the findings from the *acx1* tomato mutant which showed that this line produces only 5% of wild type levels of JA in wounded tissues [57]. Nonetheless, residual JA in both *Arabidopsis* and tomato *acx1* mutants suggests potential for compensation in JA synthesis by other *ACX* isoforms. Others have shown that the *Arabidopsis* *acx1acx5* is also resistant to indole-3-butyric acid and endogenous auxin [1,57,93], stressing the importance of β -oxidation in multiple hormone pathways in plants.

Several *Arabidopsis* mutants impaired in their ability to perceive JA have been isolated including *jar1* and *coi1* [35,104]. Both *jar1* and *coi1* show reduced expression of the vegetative storage protein (VSP) after exposure to JA or wounding [13,14,104]. The JA insensitivity of *jar1* is apparently due to its inability to form bioactive amino acid conjugates of JA [103], whereas mutants with defects in *COI1* cannot release JA-responsive genes from negative regulation by JAZ proteins [16,101]. The *jar1* mutant is fertile but *coi1* is male-sterile and exogenous application of MeJA does not restore fertility in *coi1*, consistent with *COI1* function as a JA-Ile receptor [129]. The tomato *jai1* (*jasmonate-insensitive1*) mutant is defective in JA signaling, including inability to express JA-responsive genes. *JAI1* encodes a tomato homologue of the *Arabidopsis* *COI1* [58]. Surprisingly and in contrast with the *Arabidopsis* *coi1*, *jai1* is male fertile but impaired in female fertility [58].

Studies utilizing JA mutants and/or exogenous application of JAs have demonstrated that JAs are involved in many diverse aspects of plant biology [27]. JAs also regulate plant responses to a variety of abiotic and biotic stress factors, such as wounding, water-deficit, UV and ozone exposure, pathogen attack, and herbivory, as well as induction of volatile signals in plant-plant and plant-insect interactions [59]. A unifying feature of the diverse stresses that activate JA signaling is that they all generate ROS, and mutants deficient in JA signaling have enhanced susceptibility to oxidative stress [33]. In addition, JAs are known to induce ROS production

when applied exogenously [131] and when produced *de novo* in response to leaf wounding [74,99]. Plants with compromised ability to produce ROS are also impaired in their responsiveness to leaf damage by wounding [88]. Thus, ROS production and containment may be essential components of a functional wound response process [99], and JAs likely play a role in regulating the redox balance of stressed or wounded plant tissues. Given that JAs and AsA both contribute to plant defenses against oxidative stresses, the potential interaction between these two factors merits further investigation.

Interaction between JA and AsA

The impact of endogenous JA signaling on AsA metabolism in plants is currently not well-understood. Several studies have shown that AsA increases in response to exogenous JAs in several plant species (Table 2), which suggests that JAs may regulate AsA metabolism in plants. However, these reports do not provide information about AsA metabolism in mutants deficient in JA synthesis or perception. Such mutants may help discern the influence of endogenous JAs on base-line AsA levels, as well as the role of JAs in regulating AsA metabolism in response to stresses such as wounding. Other signaling molecules such as salicylic acid (SA) also induce AsA [4], and the relative importance of JAs in AsA regulation is unknown. Furthermore, it is not yet clear whether induction of AsA by exogenous JAs (Table 2) is a direct JA signaling response that requires all components of JA synthesis and signaling, or is triggered by an effect of JA treatment such as ROS generation, which is caused by but not specific to JA. It has been reported that the *opr3* mutation in *Arabidopsis*, which inhibits JA synthesis [105], also causes a modest impairment in ozone-responsive induction of certain transcripts involved in AsA synthesis via the Man/Gal pathway [90]. Because exogenous MeJA also increased expression of *VTC1* and *VTC2* transcripts and enhanced AsA accumulation after 48 h, the authors concluded that JAs may regulate AsA metabolism under oxidative stress [90]. However, further work is needed to determine whether exogenous JAs and other stress conditions (e.g. mechanical wounding) influence AsA metabolism in a JA-signaled manner.

In addition to the potential effects of JA signaling on AsA accumulation, conversely, the quantity and redox state of AsA may also influence JA signaling. For example, in tomato, silencing of the terminal enzyme in the Man/Gal pathway resulted in a decrease in the ratio of AsA to DHA and also increased several JA-responsive transcripts such as proteinase inhibitors and arginine decarboxylase [3]. Recent global gene expression studies suggest that exogenous MeJA promotes up-regulation of several genes in the Man/Gal pathway to AsA biosynthesis and also of *DHA reductase (DHAR)*, a gene encoding an enzyme involved in AsA recycling [76,90,92]. Another study found that in tobacco (*Nicotiana tabacum*) BY2 cells, MeJA treatment stimulated the transcription of *GDP-mannose 3',5'-epimerase (GME)* and a putative *Arabidopsis L-gulonolactone oxidase (GLOase)* [125].

This gap in our understanding of potential links between JAs and AsA led us to apply physiological and genetic approaches to assess the influence of exogenous MeJA and mechanical wounding on AsA metabolism. *Arabidopsis* and tomato were chosen for this study because they are widely-used models to study JA signaling, wounding, and responses to biotic and abiotic stress; furthermore, mutants deficient in JA signaling are available in both species.

Results

Ascorbic acid content in JA mutants

To assess the influence of JA synthesis and signaling on AsA metabolism (Fig. 1) in *Arabidopsis* and tomato we measured AsA in wild type (WT) plants and in mutant lines impaired in JA synthesis or downstream signaling (Fig. 2). In *Arabidopsis*, there were highly significant differences in AsA content among the four genotypes tested (Fig. 3A; one-way

ANOVA, $P < 0.0001$). Total AsA levels were significantly lower in the *Arabidopsis* double mutant *acx1/5* [93], which is strongly blocked in JA production, than in WT plants (Student's *t* test, $P = 0.0001$) or in the other JA mutants, *fad7-2* (Student's *t* test, $P = 0.0008$) and *jar1-1* (Student's *t* test, $P = 0.0002$) (Fig. 3A). The *fad7-2* mutant also had lower AsA content than WT plants, and this difference was statistically significant at $\alpha = 0.1$ but not at $\alpha = 0.05$ (Student's *t* test, $P = 0.0579$). In contrast to *acx1/5* and *fad7-2* [64], the JA activation mutant *jar1-1* [104] produced similar AsA levels as WT (Student's *t* test, $P = 0.1679$) (Fig. 3A).

Whereas defects in JA synthesis decreased AsA accumulation in *Arabidopsis* (Fig. 3A), the trend in tomato was for JA synthesis mutants to have higher AsA content than WT plants (Fig. 3B). Differences in AsA content among the three tomato genotypes tested were statistically significant at $\alpha = 0.1$ but not at $\alpha = 0.05$ (Fig. 3B; one-way ANOVA, $P = 0.0547$). The tomato *spr2* mutant, which lacks a functional fatty acid desaturase 7 and has dramatically reduced JA levels [56], produced approximately 19% more AsA than the Castlemart WT (Fig. 3B, Student's *t* test, $P = 0.0194$). AsA levels in the *acx1* mutant [53] were similar to levels in *spr2* (Fig. 3B, Student's *t* test, $P = 0.7858$), and were ~15% higher than AsA levels in WT, although this difference was not statistically significant (Student's *t* test, $P = 0.1894$). However, it should be noted that poor germination led to a small replicate number for *acx1* ($n = 5$ in *acx1* versus $n = 18$ in the rest of the genotypes), which reduced the power of our statistical analysis, and may have compromised our ability to detect significant differences between *acx1* and the other genotypes.

Impact of exogenous MeJA and wounding on AsA content in WT plants

Next we tested the effect of exogenous JA on AsA in foliar tissues of *Arabidopsis* and tomato. From 1 to 5 h after MeJA application to *Arabidopsis*, rosette leaves contained on average $0.2 \mu\text{mol g}^{-1}$ FW more AsA than mock-treated leaves, representing a 7% increase in AsA (Fig. 4A; effect of MeJA treatment according to a two-way ANOVA: $P = 0.0002$). AsA content in MeJA-treated plants remained elevated relative to mock-treated controls even after 24 h (not shown). This is consistent with previous reports that observed induction of AsA in *Arabidopsis* after exogenous application of MeJA to cell suspension cultures or in plant tissue cultures grown in bioreactors (Table 2). AsA levels in both mock- and MeJA-treated *Arabidopsis* also increased significantly with time over the course of the 5 h sampling period (Fig. 4A; effect of time according to a two-way ANOVA: $P < 0.0001$), although there was no significant interaction between the effects of time and MeJA treatment (two-way ANOVA: $P = 0.3620$). The experiment had begun shortly (1.5 h) after the lights turned on in the growth chamber, and so the gradual increase in AsA content observed in both treatment groups over the time course may represent a response to light [12]. Although others have reported enhanced AsA oxidation in response to MeJA [62], we did not detect oxidized AsA exceeding 5% of total AsA, nor did we see any dramatic changes in the abundance of oxidized AsA in response to MeJA. This might be due to a shorter period of exposure to MeJA (maximum 24 h), to differences in the assays used to measure DHA, and our method of growing the plants (i.e. in soil rather than hydroponically).

We also measured the AsA content of WT *Arabidopsis* foliage following mechanical wounding in order to assess how AsA levels respond to wounding in intact plants. Mechanical wounding is a common assay used to interrogate gene and metabolite responsiveness to tissue damage and the process of defense induction by wounding [85,86]. Earlier reports indicated that some of the genes encoding AsA recycling enzymes are induced by mechanical wounding [46] which may lead to enhanced AsA; however, other studies demonstrated that wounding decreases the AsA content of detached tissues (Table 1). Our results show that in intact WT *Arabidopsis*, AsA increased significantly by about 8.0% in wounded samples (ANOVA: $P = 0.0065$) and remained elevated throughout the course of the experiment (Fig. 4B).

In contrast, wounding caused an average decrease of ~26% in the total AsA content of tomato foliage (Fig. 5B; effect of wounding according to a two-way ANOVA: $P=0.0240$). AsA content in this experiment was not influenced by time ($P=0.6287$), instead, the wound-induced decrease in AsA was consistent at all time points, and persisted 24 h after treatment. We also performed a side-by-side comparison of the effects of exogenous MeJA and wounding on the AsA content of WT tomato at 24 h (Fig. 5A). There were significant differences among the treatment groups (ANOVA: $P=0.0235$), and total AsA was ~23% lower in wounded foliage than in untreated controls (Student's *t* test, $P=0.0150$). AsA content in MeJA-treated plants was similar to levels in wounded foliage (Student's *t* test, $P=0.8032$), and was ~20% lower than in plants that were mock-treated with water. The difference between MeJA- and water-treated plants was statistically significant at $\alpha=0.1$ but not at $\alpha=0.05$ (Student's *t* test, $P=0.0829$). Interestingly, the AsA content of water-treated plants was intermediate between that of untreated controls (Student's *t* test, $P=0.4623$) and wounded plants (Student's *t* test, $P=0.1258$). This suggests that foliar sprays may contribute to the effects of exogenous hormone treatments.

Impact of wounding on AsA content in JA mutants

To test whether wound-induced JAs are required for the wound-responsive increase in AsA observed in *Arabidopsis*, we measured AsA in wounded leaves of the *acx1/5* double mutant. Consistent with the results in Fig. 3A, leaf samples from *acx1/5* plants contained significantly less AsA than samples from WT plants (about 26% less AsA) (Fig. 4C; effect of genotype according to a three-way ANOVA: $P<0.0001$). Wounding also caused a significant increase in AsA content (effect of wounding according to a three-way ANOVA: $P=0.0004$), and there was no significant interaction between plant genotype and wound treatment ($P=0.2458$), suggesting that AsA levels in both WT and *acx1/5* plants responded similarly to wounding. In fact, wounding steadily increased AsA over time in both *acx1/5* and WT genotypes by approximately $0.2 \mu\text{mol g}^{-1} \text{FW}$, an equivalent to about 6% increase (Fig. 4C). Similar to our results in Fig. 4A, AsA content in both control and wounded plants varied over time ($P=0.0150$), but time did not show any significant interactions with other variables in this experiment ($P>0.10$); thus, these differences in AsA content at different points in the photoperiod did not influence our conclusions about the effects of plant genotype and wounding on AsA content.

Next, the effect of wounding on the AsA content of tomato was assessed in the JA synthesis mutant *spr2* and the signaling mutant *jail* [58]. Mechanical wounding resulted in a significant decrease in AsA in WT, *spr2* and *jail* backgrounds (Fig. 5C; effect of wounding according to a two-way ANOVA: $P=0.0012$). Similar to our results in *Arabidopsis*, there was no significant interaction between genotype and treatment ($P=0.3503$), indicating that wounding had similar effects on AsA levels in mutant and WT plants. Genotype did, however, significantly influence AsA content (overall effect of genotype according to a two-way ANOVA: $P<0.0001$), and total AsA was on average ~30% higher in *spr2* than in WT plants (Student's *t* test, $P<0.0001$). However, we were unable to compare baseline AsA content in *jail* with levels in WT or *spr2* plants because all of the *jail* plants were wounded 5-days prior to the experiment for the purpose of applying PCR to confirm the *jail* mutation [58]. This wounding may have depressed AsA levels in the *jail* mutant relative to the other genotypes.

Influence of wounding and MeJA on expression of AsA biosynthetic genes

The enhanced AsA in response to wounding and MeJA in *Arabidopsis* may have been a result of enhanced expression of AsA biosynthetic genes. Others have reported induction of AsA biosynthetic genes by MeJA in cell cultures and intact plants [90,125]. Transcript profiling data obtained from the Genevestigator v3 database [43] indicate that several AsA biosynthetic genes are differentially regulated in response to MeJA and wounding (Fig. 6). Genevestigator data indicate that when *Arabidopsis* leaves were treated with MeJA, several genes from the Man/Gal pathway [121], notably *PMI*, *VTC1* and *VTC5* were induced, but not genes in the MI

pathway [61]. However, MeJA also appears to suppress other AsA biosynthetic genes in these two pathways as well (Fig. 6). Similarly, data in Fig. 6B indicate induction of Man/Gal genes within 3 h of wounding, but *MIOX2*, *MIOX4* and *MIOX5* appear to be suppressed by this treatment.

For comparison to the Genevestigator gene expression data, we used quantitative RT-PCR to monitor the expression of *VTC1*, *GME* and *MIOX4*, genes known to contribute to AsA production in *Arabidopsis* [60] 3 and 6 h after wounding and exogenous MeJA. Relative expression was normalized to *actin2* expression and then to the respective WT control for each time point using pair-wise fixed reallocation randomization test [78] with 2000 randomizations. As an additional control, the co-expression of wound and JA- responsive genes *VSP1* and *VSP2* were used to monitor the effectiveness of MeJA treatment in WT and wounding in WT and *acx1/5* genotypes. As expected, *VSP* was significantly induced by MeJA ($P=0.001$) (Fig. 7D) and wounding ($P=0.021$) (Fig. 7H) in WT leaves, but not in the JA-deficient mutant *acx1/5* ($P>0.10$), and the level of *VSP* mRNA was extremely low in *acx1/5* relative to wounded and unwounded WT controls ($P<0.05$) (Fig. 7H).

Three hours after wounding, *VTC1* and *MIOX4* were significantly induced in WT plants by a factor of 1.73 ($P=0.041$) and 13.80 ($P=0.001$), respectively (Figs. 7E and 7G), although the expression of these genes returned to roughly base-line levels by 6 h after wounding (Figs. 7E and 7G). In contrast, neither *VTC1* ($P=0.2850$) nor *MIOX4* ($P=0.4420$) were significantly induced in wounded leaf tissue from *acx1/5* plants (Figs. 7E and 7G). Also, we found no statistical significance in *GME* expression in response to wounding after 3 and 6 h for any genotype (Fig. 7F; $P>0.50$).

Results presented in Fig. 7A show that, similar to wounding, MeJA treatment resulted in a significant increase ($P=0.001$) in the expression of *VTC1* (by a factor of 2.24) 3 h after treatment. The *VTC1* mRNA returned to levels similar to that of untreated control plants 6 h after MeJA treatment (Fig. 7A, $P=0.498$), which is similar to the kinetics we observed for wounding (Fig. 7E). MeJA appeared to have no effect on the expression of *GME* and *MIOX4* after 3 and 6 h (Figs. 7B and 7C, $P>0.50$).

Discussion

Impact of exogenous and endogenous JAs on AsA

Exogenous MeJA enhanced AsA in *Arabidopsis* rosette leaves (Fig. 4A). This result is in agreement with other findings about the influence of MeJA on oxidative processes in *Arabidopsis* [62]. To evaluate the role of endogenously produced JAs in AsA metabolism we also measured AsA in mutants of *Arabidopsis* and tomato that are impaired in JA synthesis or signaling. Compared to WT plants, we observed a significant decrease in AsA in the *Arabidopsis acx1/5* mutant that is severely compromised in its ability to produce JA (Fig. 3A). We chose to work with this double mutant instead of the single mutants available for the ACX gene due to redundancy and possible compensation of the multiple isoforms of this enzyme in *Arabidopsis* [1,28]. AsA levels were also lower in the *fad7-2* mutant, although this difference was on the border of statistical significance. Together, our experiments with exogenous JA treatment and with JA mutants suggest that JAs are regulators of AsA metabolism in *Arabidopsis*. However, several points in JA biosynthesis and signaling are controlled by multiple genes [119]; for example, *fad7* in *Arabidopsis* shows considerable functional redundancy with *fad8* [64]. Furthermore, multiple JAs (e.g. OPDA) have been shown to have biological activity [106]. Therefore, single mutations such as *fad7-2* and *jar1-1* are not sufficient to block JA signaling completely, and this may limit their impact on AsA metabolism. Consequently, *jar1-1*, which is partially blocked in production of the bioactive JA conjugate JA-Ile [23,64,108] has AsA content similar to WT (Fig. 3A).

Genevestigator transcript data revealed that several *Arabidopsis* AsA biosynthetic genes are induced by exogenous MeJA with their induction peaking within 3 h (Fig. 6A), suggesting a role for these genes in AsA biosynthesis in response to MeJA. The differential response to MeJA of Man/Gal and MI genes (Figs. 6 and 7) suggests that JAs may up-regulate some but not all pathways to AsA. Moreover, our qRT-PCR data (Fig. 7) suggest in leaf tissue the Man/Gal pathway might be the major contributor to AsA synthesis in response to MeJA (Figs. 7A-G). Also, induction of *MIOX5* in cell cultures by MeJA (Fig. 6A) suggests that other pathways to AsA may be responsive to MeJA during different stages of development or over longer periods of exposure to MeJA.

Results obtained from tomato presented a very different picture from those obtained in *Arabidopsis*. Whereas MeJA-treatment increased total AsA content in *Arabidopsis* (Fig. 4A), MeJA treated plants had ~20% less AsA than water-treated controls (Fig. 5A). Moreover, the *spr2* mutant in tomato (comparable to the *fad7* mutant in *Arabidopsis*) had ~19% less total AsA than WT tomato plants (Fig. 3B). These results suggest that JAs influence AsA accumulation in tomato as well as in *Arabidopsis*, but that they may have opposite effects in the two species.

Impact of wounding on AsA, and the role of JAs in this wound response

Mechanical wounding is a common assay used to interrogate plant responses to stresses that cause cellular disruption, such as herbivory. Earlier reports suggested that some of the genes encoding AsA recycling enzymes are induced by mechanical wounding [40] which may lead to enhanced AsA and stress tolerance [34]. Here we demonstrate that wounding enhances AsA accumulation in *Arabidopsis* foliage (Figs. 4B and C), and also influences expression of genes involved in multiple pathways for AsA biosynthesis (Figs. 6 and 7). Analysis of data deposited at Genevestigator suggest that wounding up-regulates several genes required for AsA biosynthesis via the Man/Gal pathway (*PMI*, *VTC1*, 2, and 5), but may suppress expression of genes that contribute to AsA biosynthesis via the MI pathway (*MIOX* 2, 4, and 5) (Fig. 6B). In contrast, our qRT-PCR results showed a dramatic induction of *Arabidopsis MIOX4* and modest induction of *VTC1* by wounding in WT leaf tissue (Figs. 7E and 7G). This suggests that, unlike exogenous MeJA, mechanical wounding stimulates multiple pathways to AsA. Interestingly, other authors have also detected induction of a *MIOX*-like gene in apple after wounding [130].

Because wounding activates JA signaling, it is also widely used in the study of JA-mediated defense. The observed increase in AsA after treating *Arabidopsis* leaves with MeJA and wounding are in agreement with earlier observations suggesting a role for JAs in AsA metabolism [90,125]. To explore the potential role of JAs in mediating the effects of wounding on AsA metabolism, we compared this wound response in WT plants and in JA biosynthesis and signaling mutants. Interestingly, mechanical wounding failed to induce *VTC1* and *MIOX4* transcripts in leaves of the *Arabidopsis* JA synthesis mutant *acx1/5*, suggesting that *de novo* JAs are required for the wound responsiveness of these genes. It should be noted however, that both *VTC1* and *MIOX4* enzymes are involved in cell wall biosynthesis [26,48] therefore the gene induction we observed after wounding may be indicative of changes in cell wall formation, rather than in AsA metabolism. As shown in Fig. 4C mechanical wounding also increased AsA in the *acx1/5* mutant. This suggests that although JAs are necessary for the transcriptional induction of certain genes involved in AsA biosynthesis (Fig. 7E-G), they are not required to affect AsA production in response to wounding (Fig. 4C). Therefore, other factors in addition to JA may be involved in AsA regulation in response to wounding.

Interestingly, in WT tomato, mechanical wounding resulted in suppression of AsA production, similar to the effects of exogenous MeJA (Figs. 5A-C). This wound response was also observed in tomato mutants deficient in JA synthesis (*spr2*) and signaling (*jai1*) (Fig. 5C), suggesting

that JA signaling is not required for wound-induced AsA suppression. A decline in AsA in response to mechanical wounding has been observed in many cases (Table 2). For example, in pelargonium (*Pelargonium peltatum*), AsA was diminished by almost 50% 8 h after wounding [7]. Since mechanical wounding and JA promote a rapid increase in H₂O₂ in tomato [74], metabolism of H₂O₂ may deplete AsA in wounded tissues [7]. Plants detoxify H₂O₂ through the action of AsA peroxidases (APXs), which use AsA as a reductant to scavenge ROS. Therefore, the influence of exogenous MeJA and wounding on AsA in tomato may be due to enhanced ROS production and utilization of AsA in ROS control through the activity of APXs [38]. Consistent with this idea, induction of *APX2* is independent of JAs but requires H₂O₂ [20]. The fact that wounding depletes AsA content in tomato but not in *Arabidopsis* may also suggest that in *Arabidopsis*, other antioxidants such as glutathione [73] may play a more central role in ROS containment than AsA. It is also reported that anthocyanins participate in controlling ROS levels in *Arabidopsis* over extended periods of exposure to oxidative stress [70], but this seems unlikely to occur over the short assay periods used in our study.

Our data suggest that other factors in addition to JA may mediate the impact of wounding on AsA in both tomato and *Arabidopsis*. One of these additional factors might be ethylene, which is induced by wounding and is known to interact with JA in certain defense responses [77]. There is precedence for ethylene involvement in regulation of APX activity in response to ROS and ROS-inducing stress factors [66]. APXs are prominent membrane proteins in cellular compartments housing β -oxidation reactions [17] such as those involved in JA synthesis [57]. Also, JAs and ethylene are thought to act together in conferring resistance to toxic elements, such as selenium, which are known to induce ROS [112]. However, we cannot rule out that other wound-induced signals which also promote ROS independent of JA [6], including abscisic acid (ABA) [42] may also have an impact on AsA metabolism. Therefore, additional studies on ethylene and ABA mutants would help clarify the role of these hormones in AsA metabolism.

Conclusions

While the effects of JAs and wounding differed between *Arabidopsis* and tomato, within each species these different factors had similar effects on AsA accumulation. In *Arabidopsis*, certain defects in JA synthesis led to decreased base-line AsA levels, and exogenous MeJA and wounding both enhanced AsA accumulation. Conversely, in tomato, certain JA mutants displayed enhanced AsA levels, and MeJA and wounding both suppressed AsA accumulation. The convergent effects of JAs and wounding within species could be due to the fact that both JAs and wounding induce ROS, rather than to a direct role of JAs in wound responses. Thus, wounding can modify AsA content even in mutants with deficiencies in JA synthesis, such as *acx1/5* in *Arabidopsis* and *spr2* in tomato. However, the fact that *acx1/5* negates the influence of wounding on expression of the AsA biosynthetic genes *VTC1* and *MIOX4* suggests that endogenous JAs contribute to at least certain aspects of wound-responsive changes in AsA metabolism. Additional experiments are needed to investigate whether the *VTC1* and *MIOX4* induction we measured after wounding is required for cell wall formation or AsA synthesis. As illustrated in Fig. 1 there is some overlap between the genes in the Man/Gal pathways that are known to be induced by JAs and the ones that are under the control of the AMR1 regulatory protein. It would be interesting to test if the AMR1 itself is also regulated by JAs. These additional work is needed to fully understand the role of JAs in regulating base-line AsA levels in plants, and to explore how JAs may interact with other signals in the plant that govern the effects of wounding on AsA accumulation.

Methods

Plant materials and growth conditions

Arabidopsis thaliana seed for wild type (var. Columbia, Col-0, CS60000), and JA mutants were surface-sterilized and plated on solid Murashige and Skoog (MS) medium plus 3% sucrose. Plates were stratified for 3 days at 4°C and then transferred to a growth chamber kept at 23°C, 65% relative humidity and a 12/12 h light/dark regime under $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation. After 7 days, seedlings were potted in 2 × 2 in pots containing *Arabidopsis* Growth Medium (Lehle Seeds, Round Rock, TX) and maintained in the same growth chamber until reaching growth stage 5.10 [15] when tissue was either collected for steady-state foliar AsA analysis, wounded or treated with 50 μM MeJA.

Tomato seeds for wild type (variety Castlemart) and JA mutants, *jai1* [58] and *spr2* [56] were surfaced-sterilized and pre-germinated on Whatman paper soaked with sterile water on petri dishes. When the radicles had emerged and were about 2 cm in length, seedlings were transferred to soil in 3.5 × 3.5 in pots containing Sunshine LC1 potting mix (Sun Gro, Bellevue, WA). Screening of *jai1* plants was done by placing pre-germinated seedlings in Whatmann paper soaked with 1 mM MeJA as described in [58] and selecting seedlings that did not show stunting of root growth. For additional confirmation that we had selected plants homozygous for the mutant allele, a PCR screen for the *jai1* allele was also performed [58]. Greenhouse-maintained plants were periodically sprayed with Orthene 75S (EPA Reg# 59639-26-AA) and Kelthane (EPA Reg# 7401-TX1) to control thrips infestation.

Wounding and MeJA treatments

Leaf wounding in *Arabidopsis* was done as previously described [108]. Essentially the two basal rosette leaves of plants in 5.10 growth stage [15] were wounded by crushing the leaf across the mid-vein once with a hemostat. After wounding plants were maintained under the same conditions in the growth chamber. For MeJA treatment a 50 μM solution was prepared from a stock solution of 1 mM MeJA in sterilized double-distilled water [58]. Plants were sprayed with MeJA solution or water alone until drip and maintained under the same conditions in the growth chamber until the end of the experiment. In tomato, leaf wounding was done by crushing a leaflet on the second fully expanded leaf from the top of each plant at two points across the mid-vein with a hemostat. After wounding, *Arabidopsis* and tomato plants were maintained under the same conditions in the growth chamber or greenhouse, respectively. For MeJA treatment, 50 or 75 μM solutions were prepared from a stock solution of 1 mM MeJA in sterilized double-distilled water [58]. Tomato plants were sprayed with MeJA solution or water alone until drip and maintained under the same conditions in the growth chamber or greenhouse until the end of the experiment.

Ascorbate measurements

Foliar ascorbate content was determined by the ascorbate oxidase assay [60,82]. Tissue samples were harvested between 9 and 10 am (1–2 h after lights go on) and immediately frozen in liquid nitrogen and stored at –80°C till analysis. Samples were ground in 6% (w/v) *meta*-phosphoric acid, and centrifuged at 15,000 g for 5 min. Reduced AsA was determined by measuring the decline in A_{265} (extinction coefficient of $14.3 \text{ cm}^{-1} \text{ mM}^{-1}$) after addition of 1 unit of ascorbate oxidase (Sigma, St. Louis, MO) to 1 mL of the reaction mix including tissue extract and 100 mM potassium phosphate (pH 6.9). Oxidized AsA was determined in a 1-mL reaction mixture including 1 μL of 2 mM dithiothreitol after incubating at room temperature for 20 min. Total AsA was the sum of reduced and oxidized AsA.

Gene expression analysis

The web-based Genevestigator v3 microarray database and analysis tool [43] was used to generate gene expression comparisons for MeJA and wound response of select AsA genes as per developer's instructions at <https://www.genevestigator.com/gv/index.jsp>. Data from Genevestigator consisting of the log₂-transformed values of the ratio of gene expression between control and treated (plus MeJA or wound) samples were subsequently plotted. *Arabidopsis* AsA biosynthetic genes analyzed include *phosphomannose isomerase 1* (*PMI1*, At3g02570), *phosphomannose mutase* (*PMM*, At2g45790), *GDP-D-mannose pyrophosphorylase* (*VTC1*, At2g39770), *GDP-D-mannose -3',5'-epimerase* (*GME*, At5g28840), *GDP-L-galactose phosphorylase* (*VTC2*, At4g26850; *VTC5*, At5g55120), *L-galactose-1-phosphate phosphatase* (*VTC4*, At3g02870), *L-galactose dehydrogenase* (*GaldH*, At4g33670), *L-galactono-1,4-lactone dehydrogenase* (*GLDH*, At4g47930) and *myo-inositol oxygenase 1* (*MIOX1*, At1g14520), *MIOX2* (At2g19800), *MIOX4* (At4g26260), and *MIOX5* (At5g56640).

Real-time reverse-transcription PCR

Arabidopsis wild type (Col-0, CS60000) and *acx1/5* were grown, wounded, and treated with MeJA as described previously. For wounding experiment, WT-control, WT-wounded, *acx1/5*-control, and *acx1/5*-wounded treatments were tested 3- and 6-h after wounding. For MeJA experiment, WT-mock (just water) and WT-MeJA treatments were tested 3- and 6-h after MeJA application. Four biological replications per treatment were performed in both experiments.

RNA was extracted using TRI reagent® (Molecular Research Center, Cincinnati, OH) using manufacturer's instructions with some modifications. Briefly, 1 ml of TRI reagent® was added to ~100 mg of tissue grinded in liquid nitrogen and centrifuged at 7,500 rpm for 15 min at 4°C. Supernatant was collected and 0.2 ml of chloroform was added and incubated for 5 min at room temperature. After centrifugation at 7,500 rpm for 20 min at 4°C, 0.5 ml of clear aqueous phase was transferred to a fresh tube and 0.25 ml of 0.8 M NaCitrate/1.2 M NaCl and isopropanol were added. RNA was precipitated overnight at -20°C. Next day RNA was washed twice with 70% ethanol and resuspended in 35 µl of nuclease-free water. Total RNAs were treated with DNase I (New England Biolabs, Ipswich, MA) following manufacturer's instructions. For reverse transcriptase-mediated PCR, cDNA was synthesized from 0.5 µg of DNA-free RNA using oligo dT (18) primer with M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA) following manufacturer's instructions. For PCR amplification, each sample was run in duplicate using 25 ng of cDNA, 12.5 µl of Quantitec SYBR green (QIAGEN, Valencia, CA), and 200 nM gene specific primers in a total volume of 25 µl in a Mx3000P QPCR system (Stratagene, La Jolla, CA) using the following thermal profile: enzyme activation 95°C/15-min and 40 amplification cycles at 94°C/15-sec, 58°C/30-sec, and 72°C/30-sec. *VTC1* (At2g39770, Forward 5'-TGTTGACGAAACCGCTACAA-3', reverse 5'-CCACCCGATGATACTGCTC-3') and the *VSP1* (At5g24780.1)-*VSP2* (At5g24770) co-amplifying *VSP1-2* (Forward 5'-CTCCCAAATCCACTCTACTACG-3', reverse 5'-GTGCCAAAACGGCTACAAAG-3') primers were designed using Primer3 v0.4.0 [87]. *GME* (At5g28840, Forward 5'-CGATGAGTGTGTTGAAGG-3', reverse 5'-AGATTGTTGTCTGAGTTACG-3') and *actin2* (At3g18780, Forward 5'-GTATCGCTGACCGTATGAG-3', reverse 5'-CTGCTGGAATGTGCTGAG-3') were taken from Zhang and co-workers [133] *MIOX4* (At4g26260, Forward 5'-GAGATGAATGCATTTGGCCG-3', reverse 5'-TTTATCTAATTTTCATATTCAGCCC-3') was taken from [48]. Ct values were calculated from ROX baseline corrected fluorescence in the MxPro-Mx300P v.4 software (Stratagene, La Jolla, CA). Amplification specificity was verified at the end of PCR amplification by using Mx300P dissociation curve analysis. Relative gene expression was normalized to *actin2*

expression and then to the respective wild type control for each time point using the calculation software for the relative expression in real-time PCR (REST) using pair wise fixed reallocation randomization test [78].

Statistical analysis

Analysis of variances (ANOVA) and mean separation using Student's t tests were performed in JMP® Statistical Discovery Software v.8.0 using standard linear models (SAS Institute, Cary, NC).

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Abbreviations

ABA	abscisic acid
ACX	acyl-CoA oxidase
AOC	allene oxide cyclase
AOS	allene oxide synthase
AMR1	ascorbic acid mannose pathway regulator 1
AsA	ascorbate
APX	ascorbate peroxidase
COI1	coronatine insensitive 1
DHA	dehydroascorbate
DHAR	DHA reductase
FAD7	ω -3-fatty acid desaturase 7
Gal	L-galactose
GalDH	Gal dehydrogenase
GalU	D-galacturonate
GalUR	GalU reductase
GlcU	glucuronate
GlcUR	GlcU reductase
GLDH	L-galactono-1,4-lactone dehydrogenase
GLOase	L-gulonono-1,4-lactone oxidase
GME	GDP-mannose 3',5'-epimerase
Gul	L-gulose
JA	jasmonic acid
JAs	jasmonates

JACs	JA-amino acid conjugates
JAI1	jasmonate-insensitive 1
JA-Ile	jasmonoyl-L-isoleucine
JA-Leu	jasmonoyl-L-leucine
JMT	jasmonic acid carboxy methyl transferase
JAR1	jasmonate resistant 1
JA-Trp	jasmonoyl-L-tryptophan
JA-Val	jasmonoyl-L-valine
KAT	L-3-ketoacyl-CoA thiolase
LA	α -linolenic acid
LOX	lipoxygenase
Man/Gal	D-mannose/L-galactose
MeJA	JA methyl ester
MJE	MeJA esterase
MI	<i>myo</i> -inositol
MIOX	MI oxygenase
MFP	multi functional protein
OPC-8	3-oxo-2-(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-oxo-phytodienoic acid
PMI	phosphomannose isomerase
PMM	phosphomannose mutase
ROS	reactive oxygen species
SA	salicylic acid
VSP	vegetative storage protein
VTC	vitamin C deficient mutant

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Figure 1.

Pathways involved in ascorbic acid biosynthesis and regeneration in plants and their interaction with other metabolic networks. The pathways shown are: the mannose/galactose (Man/Gal) route, the gulose (Gul) shunt, the galacturonase (GalU) pathway, and the *myo*-inositol (MI) route [reviewed in 60]. The proposed “VTC2 cycle” [124] is also shown. This cycle that links photosynthesis, ascorbate and cell wall biosynthesis was proposed based on the fact that VTC2 is able to use glucose-1 phosphate and GDP-D-glucose as substrates and also on the existence of a GDP-D-mannose-2'-epimerase activity in plants. A purple acid phosphatase with phytase activity has been shown to channel phytate to the MI pathway to ascorbate [132], while VTC4 has been shown to also use L-*myo*-inositol-1 phosphate and contribute to both *myo*-inositol and ascorbate metabolisms[115]. Genes encoding enzymes highlighted in red are induced in response to MeJA or wounding according to data from Genevestigator and results herein and [125]. As indicated by the gray minus symbol, multiple genes in the Man/Gal route are negatively regulated by AMRI (ascorbic acid mannose pathway regulator 1), an F-box containing protein [133]. Intermediates highlighted in green that participate in the Man/Gal and MI pathways are also involved in the biosynthesis of cell wall components, while dehydroascorbate is the precursor of tartaric, oxalic and threonic acids. Enzymes: 1 phosphoglucose isomerase (EC 5.3.1.9); 2 phosphomannose isomerase (PMI, EC 5.1.3.1.8); 3 phosphomannose mutase (PMM, EC 5.4.2.8); 4 GDP-mannose pyrophosphorylase (VTC1, EC 2.7.7.13); 5 GDP-mannose,3',5'-epimerase (GME, EC 5.1.3.18); 6 GDP-galactose phosphorylase (VTC2, EC 2.7.7. B2); 7 L-galactose-1-P phosphatase (VTC4); 8 L-galactose dehydrogenase (GalDH, EC 1.1.1.48); 9 L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3); 10 D-galacturonate reductase (GalUR); 11 aldono-1,5-lactonase (EC 3.1.1.17); 12 GDP-mannose-2'-epimerase; 13 inositol phosphate phosphatase (EC 3.1.3.25); 14 *myo*-inositol oxygenase (MIOX, EC 1.13.99.1); 15 glucuronate reductase (GlcUR, EC 1.1.1.19); 16 glucuronolactonase (GNL, EC 3.1.1.19); 17 L-gulonono-1,4-lactone oxidase (GLOase, EC

1.1.3.8); 18 dehydroascorbate reductase (DHAR, EC 1.8.5.1). Where omitted EC number have not been assigned.

Figure 2.

The jasmonate biosynthetic pathway and signaling. *Arabidopsis* and tomato mutants used in this study are indicated. The JA precursor α -linolenic acid (α -LA), an 18:3 fatty acid (18:3 FA) is produced from linoleic acid, an 18:2 fatty acid (18:2 FA) by fatty acid desaturase (FAD) enzymes [56,64,65]. JA biosynthesis begins with the release of α -LA from chloroplast membranes by the action of a lipase [47]. JA biosynthesis is inhibited in the *Arabidopsis* mutant *fad7-2* (fatty acid desaturase 7-2) and the tomato mutant *spr2* (suppressor of prosystemin-mediated responses 2) because loss of function of fatty acid desaturase 7 (FAD7) in these mutants limiting availability of linolenic acid [56,64]. α -LA is subsequently transformed to 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) by sequential activities of lipoxygenase (LOX, EC 1.13.11.12), allene oxide synthase (AOS), allene oxide cyclase (AOC, EC 5.3.99.6) and 12-oxo-phytodienoic acid (OPDA) reductase 3 (OPR3, EC 1.3.1.42) enzymes

[56,75,91]. OPC-8 is activated by OPC-8 ligase 1(OPCL1) to produce OPC-8-CoA [53]. OPC-8-CoA undergoes three cycles of β -oxidation in the peroxisome to form JA, and these steps are catalyzed by acyl-CoA oxidase (ACX, EC 1.3.3.6), multi functional protein (MFP), and L-3-ketoacyl-CoA thiolase (KAT, EC 2.3.1.16). The *acx1/5* (*acyl-CoA oxidase 1/5*) *Arabidopsis* mutant and the *acx1*(*acyl-CoA oxidase 1*) tomato mutant are compromised in the first β -oxidation step, leading to deficiency in JA [1,28,53,93]. JA is metabolized to the volatile derivative MeJA by a JA carboxy methyl transferase (JMT, EC 2.1.1.141) [96]. The reversible reaction catalyzed by a MeJA esterase (MJE) converts MeJA to JA [107]. JA is activated by conjugation to Ile by jasmonate resistant 1 (JAR1) to form jasmonoyl-isoleucine (JA-Ile) [103]. The *Arabidopsis jar1-1*(*jasmonate resistant 1-1*) mutant is impaired in JA-Ile production in response to mechanical wounding [108]. Other jasmonoyl-amino acid conjugates (JACs) are present in plants [103] and some of these e.g. jasmonoyl-tryptophan (JA-Trp) modulate other hormone pathways [102]. The *Arabidopsis coi1* (*coronatine insensitive 1*) and tomato *jail* (*jasmonate insensitive 1*) mutants are insensitive to JA due to a compromised JA-Ile receptor coronatine insensitive 1 (COI1) [35,58,128,129]. Binding of JA-Ile to the COI1 receptor leads to degradation of jasmonate ZIM domain (JAZ) proteins that repress JA-responsive genes resulting in various JA-signaled responses [22,50,113,129].

Figure 3.

Foliar AsA content of *Arabidopsis* and tomato wild type (WT) and JA mutant plants. **(A)** Leaf samples from *Arabidopsis* WT (Col-0, CS60000) and JA mutant plants were harvested at developmental stage 5.10 [15]. The JA mutant *fad7-2* [64] is partially blocked in JA production, the *acx1/5* mutant [53] is strongly impaired in its ability to produce JA [93] and the *jar1-1* mutant [104] is partially blocked in production of the bioactive JA conjugate, jasmonoyl-isoleucine in response to leaf wounding [23,103,108]. **(B)** Leaf samples were collected from 3-week-old tomato WT (variety Castlemart) and JA mutant plants. The JA mutant *spr2* is blocked in desaturation of 18:2 FA to 18:3 FA due to loss of function of a plastidial ω -fatty acid desaturase [56] and *acx1* is impaired in a JA synthesis step involving β -oxidation [57].

Values annotated with different letters are significantly different (LSD Student's *t* at $\alpha=0.05$). The content of oxidized AsA was less than 5% for all genotypes in **A** and **B**. Error bars indicate standard errors of the means ($n>10$ for all genotypes with the exception of $n=5$ for tomato *acx1*). Fresh weight (FW).

Figure 4.

AsA production in response to exogenous MeJA and mechanical wounding in *Arabidopsis*. (A) WT (Col-0, CS60000) plants were at the developmental stage 5.10 [15] when rosette leaves were sprayed with water (mock) or solution containing 50 μM MeJA and AsA content was determined following a time course. (B) Two fully-expanded (base) rosette leaves from plants at similar developmental stage as in A were wounded with a hemostat across the mid-vein and AsA determined following a time course. (C) Comparison of AsA production in response to mechanical wounding in WT and *acx1/5* plants. Two rosette leaves from wounded (WT + W and *acx1/5* + W) and non-wounded control (WT C and *acx1/5* C) plants at stage 5.10 [15] were sampled through a 6 h time-course. The content of oxidized AsA in both MeJA- and wound-treated samples was less than 5% for all time points. Error bars indicate standard deviation of the means from two independent experiments (n>10). Fresh weight (FW).

Figure 5.

AsA production in response to exogenous MeJA and mechanical wounding in tomato WT (variety Castlemart) and JA mutant plants. **(A)** Effect of leaf wounding and exogenous MeJA (75 μ M) on AsA content of WT plants after 24 h. Values annotated with different letters are significantly different (LSD Student's t at $\alpha=0.05$) **(B)** Time-course analysis of effect of mechanical wounding on foliar AsA content of WT. The content of oxidized AsA for all time points was less than 5% **(C)** Effect of mechanical wounding on foliar AsA content of WT, *spr2*, and *jai1* plants after 24 h. Wounding significantly decreased total AsA in all three genotypes. AsA levels in *spr2* were significantly ($P<0.05$) higher than WT and *jai1*. Error bars

indicate standard errors of the means from two independent biological experiments ($n>10$).
Fresh weight (FW).

Figure 6.

AsA biosynthetic gene transcript meta-profile analysis for JA and wound responses in *Arabidopsis*. **(A)** AsA biosynthetic gene transcript expression dynamics in *Arabidopsis* cell suspensions treated with 50 μ M MeJA after 30 min to 6 h and **(B)** rosette leaves subjected to mechanical wounding for 1 to 3 h. Data are from Genevestigator v3 [43] and consist of the $\log_2$ -transformed values of the ratio of gene expression between control and treatment (plus MeJA or wound).

Figure 7.

Analysis of MeJA and wound response of *Arabidopsis* AsA and JA genes by quantitative RT-PCR. (A-D) Induction of *VTC1* (A) *GME* (B) *MIOX4* (C) and *VSP* (D) by MeJA in WT (Col-0, CS60000) plants. Mock-3 or -6 are samples from plants sprayed with water alone 3 and 6 h post-treatment. MeJA-3 or -6 are samples from plants sprayed with a solution containing 50 μ M MeJA 3 and 6 h post-treatment. (E-H) Induction of *VTC1* (E) *GME* (F) *MIOX4* (G) and *VSP* (H) by mechanical wounding in WT (Col-0, CS60000) and *acx1/5* plants. C-3 or -6, are samples from control plants 3 and 6 h post-wounding. W-3 and -6 are samples from wounded plants 3 and 6 h post-wounding. Error bars indicate standard errors of the means. Relative gene expression was normalized to *actin2* expression and then to the respective WT control/mock for each time point (pair wise fixed reallocation randomization test * $P < 0.01$; ** $P < 0.05$).

Table 1

Summary of the effect of mechanical wounding on plant ascorbate metabolism

Species (common name)	Tissue	Wounding	Response	Reference
<i>Apium graveolens</i> (celery)	Stem	Shredding	Total AsA decreased 53%	[84]
<i>Arabidopsis thaliana</i> (mouse-ear cress)	Leaf	Crushing	Total AsA increased	This study
<i>Brassica napus</i> var. Bronowski (rapeseed)	Leaf	Cutting	Total AsA decreased 21% AsA/DHA ratio decreased from 11 to 5	[120]
<i>Brassica oleracea</i> convar. <i>capitata</i> var. <i>alba</i> (white cabbage)	Leaf	Shredding	Total AsA decreased 11%	[84]
<i>B. oleracea</i> convar. <i>capitata</i> var. <i>rubra</i> (red cabbage)	Leaf	Shredding	No significant changes after wounding	[84]
<i>Cucurbita pepo</i> (zucchini)	Root	Shredding	Total AsA decreased 53%	[84]
<i>Daucus carota</i> (carrot)	Root	Shredding	Total AsA decreased 82%	[84]
<i>Dioscorea rotundata</i> (white yam)	Tuber	Cutting	Total AsA increased 300%	[45]
<i>Diospyros kaki</i> var. Fuyu (persimmon)	Fruit	Cutting	Total AsA decreased	[126]
<i>Fragaria ananassa</i> var. Selva (strawberry)	Fruit	Cutting	Significant oxidation of AsA, but no changes in total AsA	[126]
<i>Kalanchoë daigremontiana</i> (alligator plant)	Stem	Cutting	Ratio reduced AsA/total AsA in apoplast decreased to 33% of the original, while total AsA remained largely unaffected	[110]
<i>Malpighia glabra</i> (acerola)	Leaf	Cutting	Total AsA decreased 50%	[10]
<i>Pastinaca sativa</i> (parsnip)	Root	Shredding	Total AsA decreased 76%	[84]
<i>Raphanus sativus</i> (red radish)	Root	Shredding	No significant changes after wounding	[84]
<i>Solanum lycopersicum</i> var. Castlemart (tomato)	Leaf	Crushing	Total AsA decreased	This study
<i>Solanum tuberosum</i> var. Katahdin (potato)	Tuber	Cutting	Total AsA of potatoes stored for 2 d increased 100 to 300%	[8]
<i>S. tuberosum</i> var. Mayqueen	Tuber	Cutting	Total AsA of potatoes stored for 7 w increased 200%	[37]
<i>S. tuberosum</i> var. Belrus	Tuber	Cutting Bruising	Total AsA increased 400% Total AsA decreased 350%	[69]
<i>S. tuberosum</i> var. Russet Nortkotah	Tuber	Shredding	Total AsA decreased 32%	[84]
<i>S. tuberosum</i> var. Binje	Tuber	Cutting	Threonic acid, a product of AsA oxidation increased	[39]

Table 2

Summary of the effect of jasmonates on plant ascorbate metabolism

Species (common name)	Tissue/cell/Organ	Jasmonate	Concentration (μ M)	Treatment duration	Response	Reference
<i>Arabidopsis thaliana</i> (mouse-ear cress)	Rosette leaves	MeJA	50	0–5 h	Elevated AsA from 1–5 h	This study
<i>A. thaliana</i>	Seedlings	JA	30 and 200	48 h	Elevated AsA, 300%	[90]
<i>A. thaliana</i>	Rosette leaves	MeJA	1, 10 and 100	6 d	Elevated AsA	[62]
<i>A. thaliana</i>	Cell cultures	MeJA	50	21 h	Increase in <i>de novo</i> AsA synthesis	[125]
<i>Brassica napus</i> (rapeseed)	Roots and shoots	MeJA	1	5 d	AsA unchanged in roots but elevated in shoots	[24]
<i>B. oleracea</i> (wild mustard)	Florets	MeJA	1000	1 h	Enhanced AsA degradation	[71]
<i>Fragaria vesca</i> (woodland strawberry)	Leaf	MeJA	100	8 d	Slow decrease in AsA in response to dehydration stress	[118]
<i>Nicotiana tabacum</i> (tobacco)	Cell cultures	MeJA	50	0–48 h	Increase in <i>de novo</i> AsA synthesis	[125]
<i>Panax ginseng</i> (ginseng)	Root cultures	MeJA	200	0–9 d in bioreactor	Elevated AsA	[4]
<i>Prunus triloba</i> (plum)	Whole fruit	MeJA	0, 10 and 1000	12 h	Elevated AsA, 140%	[52]
<i>Solanum lycopersicum</i> (tomato)	Cut fruit	MeJA	~ 100	0–15 d at 4°C	Elevated AsA from 1–15 d	[9]
<i>S. lycopersicum</i>	Leaves	MeJA	75	0–24 h	Decline in AsA	This study