Jasmonate Precursor Biosynthetic Enzymes LOX3 and LOX4 Control Wound-Response Growth Restriction[1][OPEN]

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Wound-response plant growth restriction requires the synthesis of potent mediators called jasmonates (JAs). Four 13-lipoxygenases (13-LOXs) produce JA precursors in Arabidopsis (*Arabidopsis thaliana*) leaves, but the 13-LOXs responsible for growth restriction have not yet been identified. Through loss-of-function genetic analyses, we identified LOX3 and LOX4 as the principal 13-LOXs responsible for vegetative growth restriction after repetitive wounding. Additional genetic studies were carried out in the gain-of-function *fatty acid oxygenation 2* (*fou2*) mutant that, even when undamaged, shows JA-dependent leaf growth restriction. The *fou2 lox3 lox4* triple mutant suppressed the *fou2* JA-dependent growth phenotype, confirming that LOX3 and LOX4 function in leaf growth restriction. The *fou2* mutation affects the TWO PORE CHANNEL1 (TPC1) ion channel. Additional genetic approaches based on this gene were used to further investigate LOX3 function in relation to leaf growth. To activate LOX3-dependent JA production in unwounded plants, we employed hyperactive TPC1 variants. Expression of the *TPC1ΔCa$_i$* variant in phloem companion cells caused strongly reduced rosette growth in the absence of wounding. Summarizing, in parallel to their established roles in male reproductive development in Arabidopsis, LOX3 and LOX4 control leaf growth rates after wounding. The process of wound-response growth restriction can be recapitulated in unwounded plants when the LOX3 pathway is activated genetically using a hyperactive vacuolar cation channel.

Herbivore damage can lead to spectacular changes in the growth of long-lived plants. Similarly, long-term plant growth can be modified strikingly as a result of repetitive leaf wounding inflicted by skilled bonsai gardeners. These effects are characterized by altered vegetative growth often leading to stunted plants. In the laboratory, and over briefer timescales, comparable effects on plant growth can be observed in short-lived species including Arabidopsis (*Arabidopsis thaliana*). For example, when the rosettes of this species are subject to repetitive wounds over a period of ~3 weeks, the newly formed leaves they produce are smaller with shortened petioles compared to those on unwounded plants (Yan et al., 2007). These effects on growth were found to depend on the production of the lipidic regulator jasmonate (JA). Specifically, leaf and petiole growth restriction was strongly attenuated in the JA-synthesis mutant *allene oxide synthase* (*aos*; Yan et al., 2007) and in a JA-signaling mutant (Zhang and Turner, 2008). Therefore, a key function of JA pathway-activating signals produced in damaged organs is to travel to apical tissues to reprogram future growth, allowing plants to optimize their defense strategies (Huot et al., 2014; Guo et al., 2018; Ballaré and Austin, 2019; Fernández-Milmanda et al., 2020). Importantly, the activation of JA signaling after wounding requires the de novo synthesis of JA (Browse, 2009; Chini et al., 2016; Howe et al., 2018). Specifically, JA biosynthesis in the aerial tissues of Arabidopsis depends on four distinct 13-lipoxygenases (13-LOXs), namely LOX2, LOX3, LOX4, and LOX6. Each of these 13-LOXs can produce JA precursors in leaves and each LOX appears to contribute in a different way to defense gene expression (Chauvin et al., 2013, 2016; Grebner et al., 2013). However, unlike for reproductive development, the contributions of individual 13-LOXs to vegetative growth modulation in Arabidopsis remains poorly understood.

Like wound-response leaf growth restriction (Zhang and Turner, 2008), Arabidopsis reproductive development requires the *CORONATINE-INSENSITIVE1* gene (Xie et al., 1998). *CORONATINE-INSENSITIVE1* encodes the receptor for jasmonoyl-Ile (JA-Ile; Sheard et al., 2010; Howe et al., 2018) and 12-hydroxy-JA-Ile (Jimenez-Aleman et al., 2019; Poudel et al., 2019), a compound implicated, together with JA-Ile, in the control of wound-induced growth restriction (Poudel et al., 2019). In terms of JA biosynthetic enzymes, a LOX was found to be essential

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for male flower formation in maize (Acosta et al., 2009), Subsequently, LOX3 and LOX4 were identified as the 13-LOXs that produce the JA precursors necessary for normal reproductive development in Arabidopsis (Caldelari et al., 2011). That is, although all four Arabidopsis 13-LOXs produce precursors for JA synthesis, LOX2 and LOX6 cannot replace LOX3 and LOX4 to maintain male fertility. The study of the roles of JA in reproductive development has been extended to the cellular level, and the cells in floral organs that are necessary for JA signaling leading to fertility have been identified (Jewell and Browse, 2016). By contrast, the roles of 13-LOXs in vegetative development in Arabidopsis remain poorly understood. To our knowledge, no specific 13-LOXs have an attributed role in any aspect of leaf growth. We therefore set out to address this gap in our knowledge. We used a collection of reduced-function *lox* single and multiple mutants. In each case, two alleles for each LOX were previously characterized biochemically for their ability to make JAs in wounded leaves (Caldelari et al., 2011; Chauvin et al., 2013). One of each allele was used in this study, allowing the individual inputs of each LOX on rosette growth to be assessed genetically.

To complement loss-of-function studies and to further investigate the activity of 13-LOXs in rosette growth restriction, we also took an opposite, gain-of-function approach. Here, we attempted to activate growth-regulating 13-LOXs in Arabidopsis in the absence of wounding. Our strategy was based on the *fatty acid oxygenation upregulated2* (*fou2*) mutant (Bonaventure et al., 2007a). At the molecular level, the *fou2* mutation affects TWO-PORE CHANNEL1 (Peiter et al., 2005; Hedrich et al., 2018; Pottosin and Dobrovinskaya, 2018), increasing the open-probability of this vacuolar cation channel (Bonaventure et al., 2007a; Beyhl et al., 2009; Lenglet et al., 2017). In the undamaged state, adult-phase *fou2* leaves have elevated levels of JA compared to the wild type, and the *fou2* transcriptome indicates high JA pathway activity (Bonaventure et al., 2007b). Relevant to that study is the fact that *fou2* has small, epinastic leaves with short petioles. Generation of *fou2 aos* double mutants that cannot produce JA is known to suppress much of the effect of *fou2* on leaf growth, although this double mutant does not completely rescue the leaf epinasty that is typical of *fou2* (Bonaventure et al., 2007b). We investigated whether *fou2* complementary DNA (cDNA; $TPC1^{D454N}$) could be used to activate JA signaling leading to rosette growth restriction. The results reveal roles for specific 13-LOXs in leaf growth and also identify a cell population likely to play critical roles in wound-response vegetative growth plasticity.

RESULTS

Damaged Plants Are Smaller, with Greater Defense Capabilities than Their Undamaged Counterparts

Wild-type plants that had been fed on by *Spodoptera littoralis* larvae for 11 d under controlled conditions showed obvious signs of damage and their rosettes appeared to be smaller than the undamaged wild type (Fig. 1A). However, each leaf showed variable signs of damage, and a more controlled wounding protocol was therefore employed. We used serial mechanical wounding to better control for the effects of leaf damage and to test whether plants that had been damaged were more resistant to herbivores. Starting with 2-week-old plants, developing leaves were wounded at 3-d intervals in such a way that leaf 1 was damaged first by crushing 50% of the lamina, then leaf 2, and so forth. In total, seven leaves were wounded. As a control, plants were handled identically but not wounded. At the end of the treatment, and 3 d after the last of seven wounds (or handling, as was the case of the control unwounded plants), wounded wild-type rosettes were smaller than

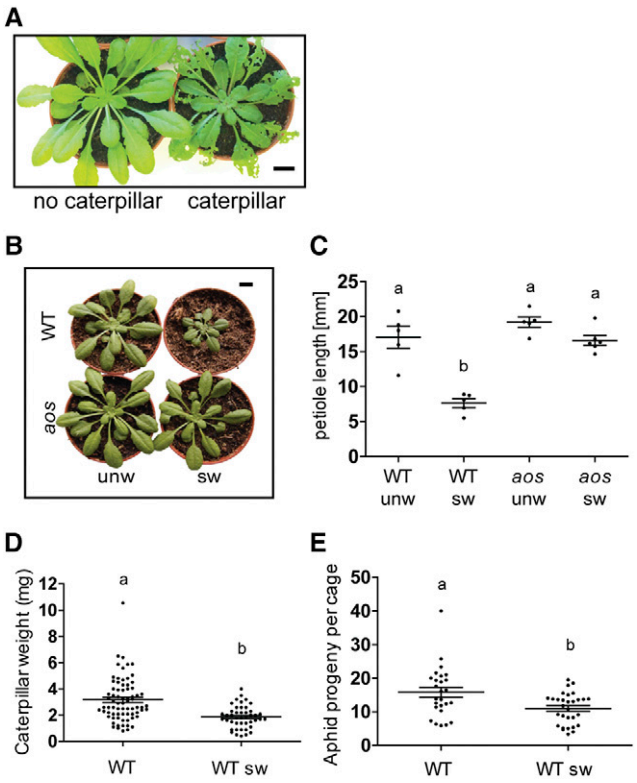

Figure 1. Enhanced defense in serially wounded plants. A, Rosette morphology of undamaged wild-type (WT) plant and plant after 11 d of *S. littoralis* feeding. Scale bar = 1 cm. B, Rosette morphology of wild type and *aos* after serial wounding. The background was digitally rendered in white in the image. unw, Unwounded; sw, serially wounded. Scale bar = 1 cm. C, Petiole length after serial wounding in wild type and *aos* mutant. The mean petiole length of leaves number 6, 7, and 8 in each plant is counted as one biological replicate. Bars represent the means (±SEM), n = 5 to 6 D, Biomass of *S. littoralis* after 11 d feeding on unwounded wild type or serially wounded wild type (WT sw). Bars are means (±SEM) of three combined experiments. Wild type, n = 73; wild type sw, n = 44. E, Reproductive success of cabbage aphids (*B. brassicae*) on unwounded or serially wounded plants. Aphid success was monitored 14 d after placing nymphs on plants. Bars are means (±SEM) of three combined experiments; wild type, n = 26; wild type sw, n = 30. Lowercase letters indicate significant difference as determined by Tukey's HSD test ($P < 0.001$).

those of unwounded plants (Fig. 1B) and showed reduced petiole extension (Fig. 1C). The effects of wounding on petiole growth were reduced in the *aos* mutant relative to the wild type (Fig. 1, B and C). Reduced growth due to JA pathway activity has been linked to increased levels of defense (Huot et al., 2014; Guo et al., 2018). We compared herbivore performance on unwounded plants and plants that had been mechanically wounded in bioassays using the lepidopteran herbivore *S. littoralis* and the cabbage aphid *Brevicoryne brassicae*. Weight gain by *S. littoralis* was reduced on the prewounded plants compared to undamaged plants (Fig. 1D). Similarly, aphid fecundity, as judged by the production of adult aphids, was reduced on the prewounded plants relative to the undamaged plants (Fig. 1E).

Identification of 13-LOXs that Operate in Wound-Activated Rosette Growth Responses

Because wound-response growth restriction is JA-dependent and is associated with decreased defense against herbivores, we sought to identify the branch of the JA synthesis pathway necessary for this process. Four 13-LOXs contribute to JA production in wounded leaves (Chauvin et al., 2013; 2016). The effects of serial wounding on growth restriction in single *lox* mutants for each of these genes was tested. Figure 2, A and B, shows that the wild type and all single *lox* mutants displayed wound-response growth restriction. Next, *lox* triple mutants (Chauvin et al., 2013) were wounded repetitively. Each of these lines retains a single functional JA precursor-producing LOX. When subjected to serial wounding, the *lox2 lox4 lox6* mutant displayed growth restriction similar to the wild type (Fig. 2, C and D), whereas the other triple mutants showed less wound-response growth restriction relative to the wild type. In the triple mutants, mass reduction in response to wounding was lowest in *lox2 lox3 lox4*, followed by *lox3 lox4 lox6*, then *lox2 lox3 lox6*. The *lox* quadruple mutant that cannot produce JA (Chauvin et al., 2013) showed relatively little wound-response growth restriction. A final series of experiments compared the

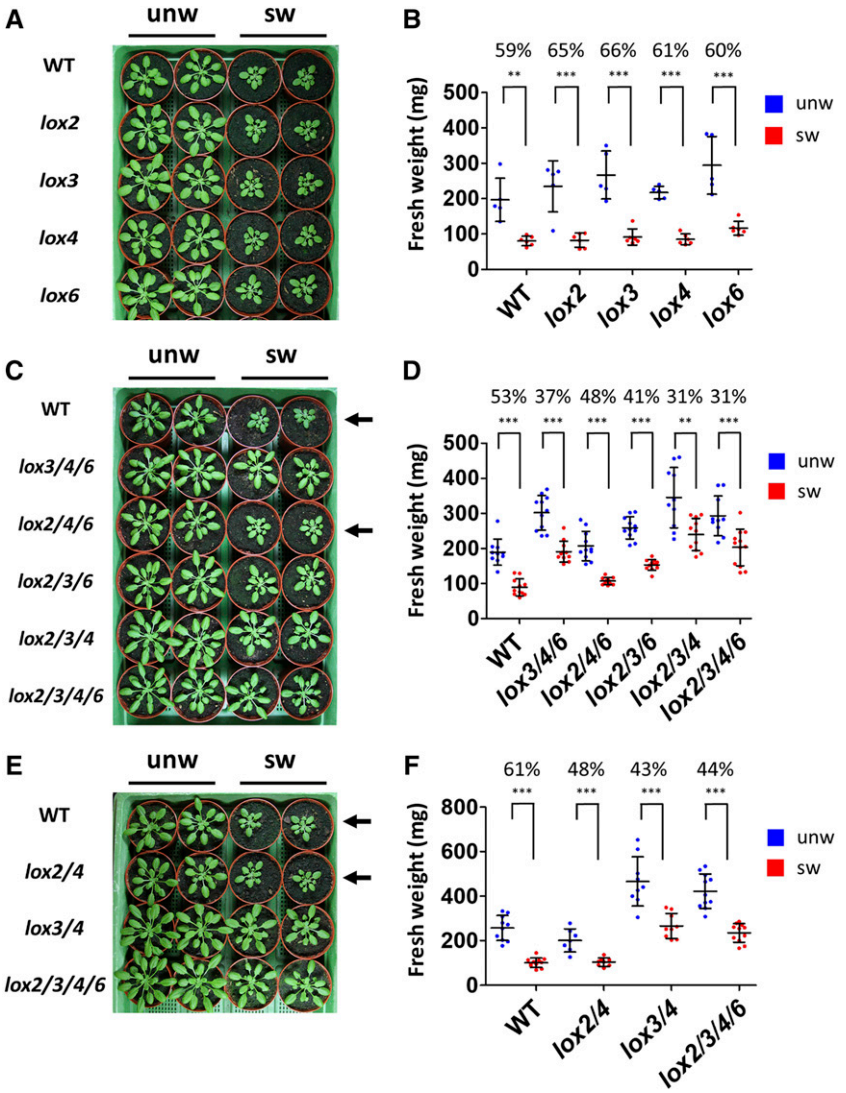

Figure 2. Effect of serial leaf wounding on rosette growth of *lox* mutants. Rosette morphology of unwounded and serially wounded wild type (WT) and *lox* mutants. unw, Unwounded; sw, serially wounded. In each case, two representative plants are shown. The ratios (percent) represent fresh weight reduction resulted by wounding. A, Rosettes of wild type and *lox* single mutants. B, Fresh weights of unwounded and serially wounded wild type and *lox* single mutants. $n = 5$ to 6. C, Rosette morphology of wild type, *lox* triple mutants, and the *lox* quadruple mutant. D, Fresh weights of unwounded and serially wounded plants. $n \geq 10$. E, Rosette morphology of wild type, *lox* double mutants, and the *lox* quadruple mutant. F, Fresh weights of unwounded and serially wounded plants. $n \geq 7$. Arrows in C and E indicate genotypes showing strong growth restriction after wounding. Bars represent the mean of fresh weight (±SD). Asterisks indicate significant difference as determined by Student *t* test (**$P < 0.01$ and ***$P < 0.001$).

growth of the *lox2 lox4* and *lox3 lox4* double mutants in response to serial wounding. In these assays, the *lox3 lox4* double mutant behaved like the quadruple *lox* mutant. The *lox2 lox4* double mutant showed slightly stronger growth restriction than *lox3 lox4* (Fig. 2, E and F). Comparison of fresh weights of unwounded plants from the same dataset (Supplemental Fig. S1) showed that the fresh masses of the quadruple and triple *lox* mutants and the *lox3 lox4* double mutant were greater than the wild type. In summary, these results based on biochemically and genetically verified loss-of-function *lox* alleles show that LOX3, and, to a lesser extent, LOX4, produce the majority of JA precursors necessary for wound-response rosette growth restriction.

The *fou2* Phenotype Is Suppressed When *LOX3* and *LOX4* Are Mutated

A mutant that affects petiole length is *fou2*, a plant gene that overproduces JA in the adult phase (Bonaventure et al., 2007a). JA production in *fou2* is necessary for the shorter-than-wild-type petioles typical of this mutant, and petiole length was restored in *fou2 aos* double mutants that cannot produce JA (Bonaventure et al., 2007b). Based on the results of the serial wounding assays shown in Figure 2, we therefore generated *fou2 lox3 lox4* triple mutants. We found that the strong JA-dependent phenotype of *fou2* was suppressed in the *fou2 lox3 lox4* triple mutant (Fig. 3). The level of suppression of the *fou2* phenotype was similar to that reported for the *fou2 aos* double mutant (Bonaventure et al., 2007a), which was used as a control in these experiments. These results further confirm the roles for LOX3 and LOX4 in JA-dependent growth control and raise the intriguing possibility that stunted, bonsai-like wound phenotypes could be recapitulated through the genetic activation of 13-LOXs in the absence of wounding. To do this, we chose LOX3 because it had the strongest effect among the four 13-LOXs on wound-response growth restriction. The xylem and phloem were chosen as target tissues because (1) the *LOX3* promoter activity domain spans both these regions (Chauvin et al., 2016), and (2) both the phloem and xylem are known to play critical roles in leaf-to-leaf wound signaling (Nguyen et al., 2018). Methods to activate JA production via LOX3, and therefore mimic wound-response growth restriction, were developed taking advantage of the gain-of-function nature of the *fou2* mutation.

Hyperactive TPC1 Stimulates LOX3-Dependent Growth Restriction

In *fou2*, the Asp 454 to Asn (D454N) mutation in TWO-PORE CHANNEL1 ($TPC1^{D454N}$) increases the open probability of this channel (Bonaventure et al., 2007a; Beyhl et al., 2009). This is because Asp 454, along with other acidic residues in TPC1, binds inhibitory Ca^{2+} in the vacuolar lumen, changing the voltage sensitivity of this voltage-gated cation channel (Guo et al., 2017). However, the *fou2* mutation is semidominant, and wild-type *TPC1* alleles inhibit the effects of $TPC1^{D454N}$ (Bonaventure et al., 2007a). In preliminary experiments to activate JA-dependent growth phenotypes with $TPC1^{D454N}$, and to overcome its semidominance, $TPC1^{D454N}$ was expressed in the *tpc1-2* null background (Peiter et al., 2005), with expression in the phloem driven by the *SUC TRANSPORTER2* (*SUC2*) promoter (Truernit and Sauer, 1995), with expression in xylem contact cells driven by the *LIPOXYGENASE6* (*LOX6*) promoter (Chauvin et al., 2013), or with expression in the bundle sheath driven by the *SCARECROW* (*SCR*) promoter (Wysocka-Diller et al., 2000). The 5-week-old *SCRpro*::$TPC1^{D454N}$ plants (Supplemental Fig. S2A) did not display a *fou2*-like phenotype, and the *LOX6pro*::$TPC1^{D454N}$ plants (Supplemental Fig. S2B) displayed only a slight *fou2*-like phenotype at this stage. By contrast, *SUC2pro*::$TPC1^{D454N}$ expressed in *tpc1-2* phenocopied *fou2* (Supplemental Fig. S2C), including causing elevated expression of the JA-signaling marker *JAZ10* (Supplemental Fig. S2D). As expected, when the wild-type *TPC1* sequence was expressed under the *SUC2* promoter in the *fou2* background, the *TPC1* cDNA partially reverted the *fou2* phenotype toward that of the wild type (Supplemental Fig. S2E) and suppressed the elevated levels of *JAZ10* transcript detected in *fou2* (Supplemental Fig. S2F).

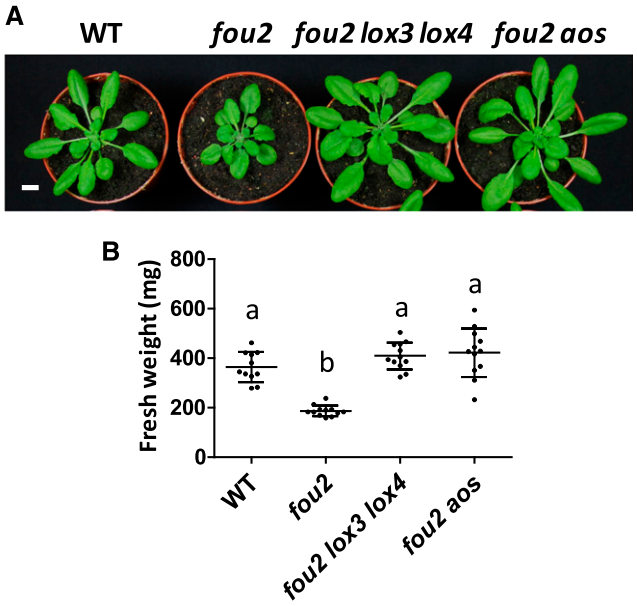

Figure 3. Mutations in *LOX3* and *LOX4* genes largely suppress the *fou2* phenotype. Plants were grown for 5 weeks in short-day conditions. A, Rosette morphology of wild type (WT), *fou2*, *fou2 lox3 lox4*, and *fou2 aos*. Scale bar = 1 cm. The phenotype of the *fou2 aos* mutant was as reported in Bonaventure et al. (2007b). B, Fresh weights of wild type, *fou2*, *fou2 lox3 lox4*, and *fou2 aos*. Data are means (±SD), $n = 11$ to 12. Lowercase letters indicate statistically significant differences as determined by Tukey's HSD test ($P < 0.05$).

These initial findings raised the possibility that JA precursor production by LOX3 could be activated through the expression of hyperactive TPC1 variants, provided that these variants had sufficient genetic penetrance to overcome the presence of wild-type *TPC1* alleles. The single D454N mutation in TPC1^{D454N} reduces Ca^{2+} binding to a vacuole lumen-located inhibitory sensor (Beyhl et al., 2009). More recently, and together with Asp 454, the residues Asp 240 and Glu 528 were also found to participate in channel-inhibiting Ca^{2+} coordination. The TPC1 ^{D240A,D454A,E528A} variant, termed "TPC1ΔCa$_i$," largely removes luminal calcium inhibition without affecting the cation permeation specificity observed in the wild-type channel (Guo et al., 2017). We reasoned that hyperactive TPC1ΔCa$_i$ might display sufficient genetic penetrance to be employed in backgrounds harboring wild-type *TPC1* alleles to activate growth restriction through LOX3. Experiments were thus designed to test this.

Based on preliminary results (Supplemental Fig. S2), *TPC1ΔCa$_i$* was expressed in the phloem. For this, *SUC2*pro::*TPC1ΔCa$_i$* was transformed into the wild

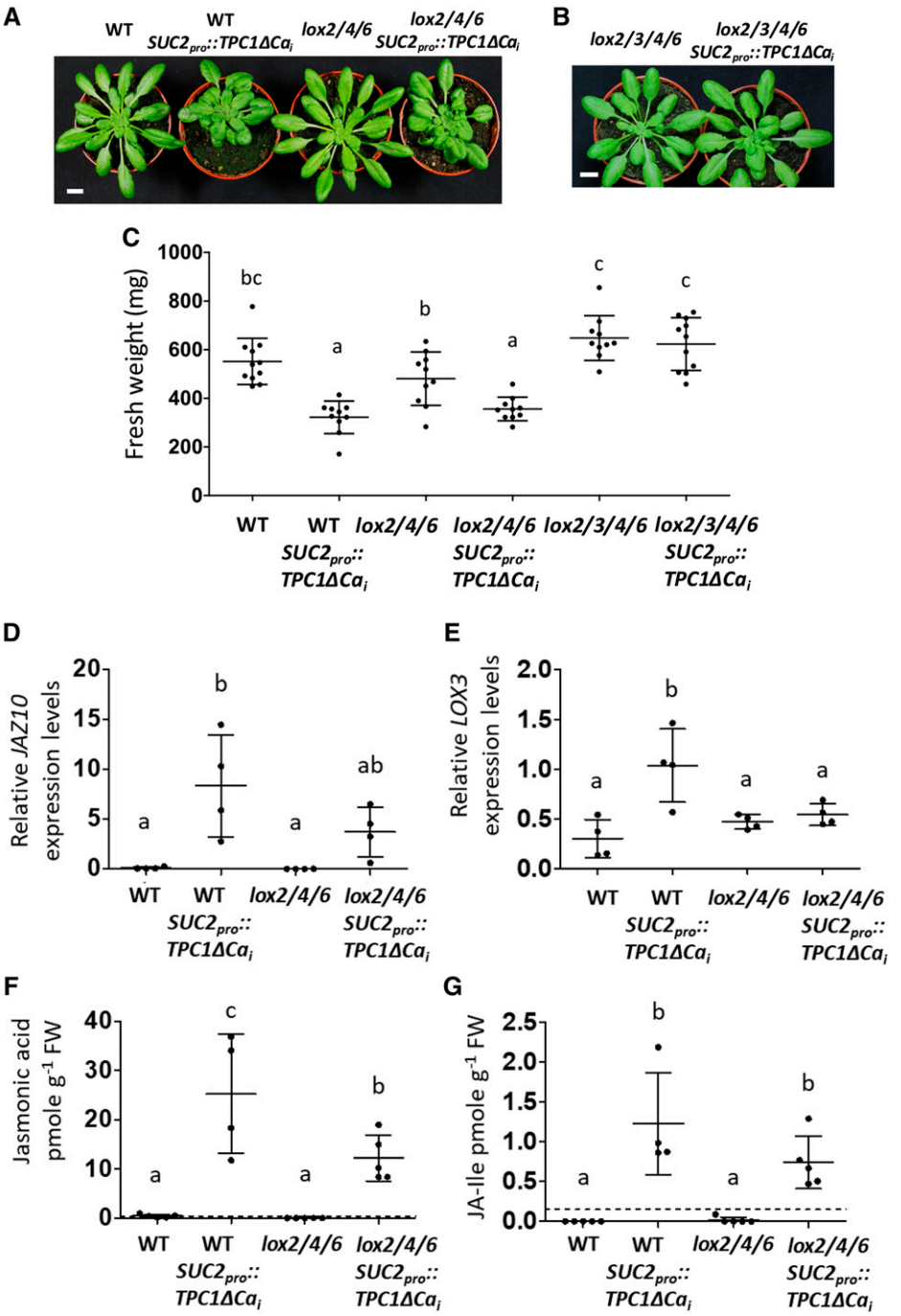

Figure 4. Expressing *TPC1ΔCa$_i$* in phloem companion cells stimulates LOX3-dependent growth restriction. All plants were 5 weeks old. A, Rosette morphology of wild type (WT), the wild type expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*, *lox2/4/6*, and *lox2/4/6*-expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*. B, Rosette morphologies of the *lox* quadruple mutant and the same plant transformed with *SUC2$_{pro}$::TPC1ΔCa$_i$*. Scale bars = 1 cm. C, Fresh weights of wild type, the wild type expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*, *lox2/4/6*, *lox2/4/6* expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*, *lox* quadruple mutant, and *lox* quadruple mutant expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*. D, *JAZ10* transcript levels relative to reference gene *UBC21*. E, *LOX3* transcript levels relative to *UBC21*. F, JA levels in the leaves of the wild type, the wild type expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*, *lox2/4/6*, and *lox2/4/6* expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*. The dashed horizontal line shows the limit of quantification. G, JA-Ile levels in the leaves of the wild type, the wild type expressing *SUC2$_{pro}$::TPC1ΔCa$_i$*, *lox2/4/6*, and *lox2/4/6* expressing *SUC2$_{pro}$::TPC1ΔCa$_i$* The dashed horizontal line shows the limit of quantification. Data are means (±SD); $n = 10$ to 11 (C), $n = 4$ (D and E), and $n = 4$ to 5 (F and G). Lowercase letters indicate statistically significant differences as determined by Tukey's HSD test ($P < 0.05$).

type and into the *lox2 lox4 lox6* triple mutant that retains *LOX3* as the only functional 13-LOX gene. Both the wild type and the *lox2 lox4 lox6* lines expressing *SUC2*-pro::*TPC1ΔCa$_i$* displayed *fou2*-like phenotypes (Fig. 4A; Supplemental Fig. S3). As a control, *SUC2*pro::*TPC1ΔCa$_i$* was transformed into the *lox2 lox3 lox4 lox6* quadruple mutant that lacks the ability to make JA. The transformed quadruple mutant displayed a weak phenotype at the rosette center (Fig. 4B; Supplemental Fig. S3), consistent with the fact that part of the effect of *fou2* on leaf growth is JA-independent (Bonaventure et al., 2007a, 2007b). Further analyses of the transformants revealed that *TPC1ΔCa$_i$* expression in the wild type and in *lox2 lox 4 lox6* reduced rosette fresh weight, but this was not significantly affected in the *lox* quadruple mutant that does not produce JA (Fig. 4C). *JAZ10* levels were found to be elevated in wild type and in *lox2 lox4 lox6* triple mutant plants expressing *SUC2*pro::*TPC1ΔCa$_i$* (Fig. 4D). The relative levels of the LOX3 mRNA were also measured in the wild type, the wild type expressing *TPC1ΔCa$_i$*, and in the *lox2 lox4 lox6* triple mutant without and with the *TPC1ΔCa$_i$* transgene. Whereas expression of *TPC1ΔCa$_i$* in the wild type increased *LOX3* mRNA levels ~3-fold, there was no corresponding increase in *LOX3* mRNA level in the triple-mutant background (Fig. 4E).

Finally, to investigate whether expression of *SUC2*-pro::*TPC1ΔCa$_i$* in the wild type and in the *lox2 lox4 lox6* triple mutant altered the levels of jasmonic acid and JA-Ile in leaves, the levels of these molecules were measured. Increases in both jasmonic acid (Fig. 4F) and JA-Ile (Fig. 4G) were detected in both genetic backgrounds expressing *TPC1ΔCa$_i$* relative to the control genetic backgrounds.

DISCUSSION

JA-controlled leaf growth restriction is likely to be widespread in nature and can be activated by repetitive wounding (Yan et al., 2007) or by repeated mechanostimulation (Chehab et al., 2012). This process is, at least in part, a consequence of repression of cell division (Noir et al., 2013) and is controlled by MYC transcription factors (Major et al., 2017). Although growth and defense can be at least partially uncoupled (Campos et al., 2016), the ability of plants to channel resources from growth to defense is likely to have adaptive value (Huot et al., 2014; Guo et al., 2018). Consistent with this, we found that repetitively wounded plants slowed weight gain in a lepidopteran, and also limited reproduction in a hemipteran, herbivore. The fact that repetitive wounding affected plant growth and that this correlated with resistance to insects prompted us to investigate which branch of the JA biosynthesis pathway operates to control post-wounding leaf growth.

A Role for LOX3 and LOX4 in Wound-Response Growth

Here, we were able to identify LOX3 and LOX4 as the key 13-LOXs that act, through the production of JA precursors, in wound-response leaf growth restriction. We also noted that multiple *lox* mutations generally caused an increase in the fresh mass of unwounded plants. This was expected because *aos* mutants in JA biosynthesis have higher dry masses than the wild type (Yan et al., 2007). LOX3 and LOX4 are known to contribute to JA production in response to osmotic stress (Grebner et al., 2013) and after leaf wounding (Chauvin et al., 2013). This same 13-LOX pair in Arabidopsis is essential for male reproductive development (Caldelari et al., 2011) and the two 13-LOXs also play roles in root defense against nematodes (Ozalvo et al., 2014).

With this work, all four JA precursor-producing 13-LOXs in Arabidopsis now have attributed roles in wounded rosettes (Fig. 5). In addition to the functions of LOX3 and LOX4 described herein, LOX2 produces JA precursors as well as defense-related metabolites called "arabidopsides," which accumulate rapidly in and near wounds (Glauser et al., 2009). Concerning *LOX6*, corresponding loss-of-function mutants are compromised in rapid JA synthesis in leaves distal to damage sites (Chauvin et al., 2013; Grebner et al., 2013; Gasperini et al., 2015). LOX2 and LOX6 also function together to upregulate LOX3 and LOX4 (Chauvin et al., 2016). In this work, expression of *TPC1ΔCa$_i$* in the wild-type background caused increases in *LOX3* mRNA levels. However, and consistent with the model for *LOX3*/*LOX4* regulation by LOX2 and LOX6 proposed in Chauvin et al. (2016), no increases in *LOX3* mRNA were detectable in the *lox2 lox4 lox6* triple mutant. The result suggests that hyperactive TPC1 variants exert their activating effect on LOX3-dependent JA precursor synthesis at the post-transcriptional level.

A question emerging from this study is raised by the fact that all four 13-LOXs produce JAs after leaf wounding (Chauvin et al., 2013). LOX3, LOX4, and

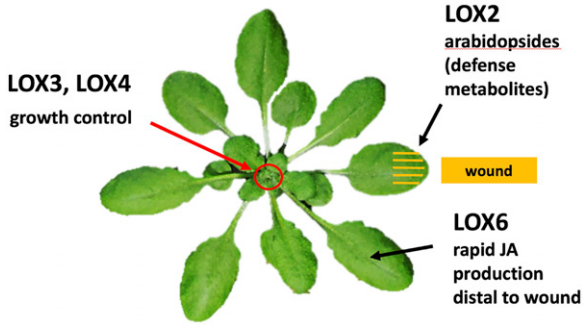

Figure 5. Specific 13-LOX functions in wounded Arabidopsis rosettes. All four 13-LOXs produce JA precursors in wounded leaves and all four play roles in defense against lepidopteran herbivores. LOX2 is expressed throughout soft tissues (including the mesophyll) but not (or at very low levels) in the mature leaf vasculature. LOX3, LOX4, and LOX6 are primarily expressed in phloem- and xylem-associated cells in both developing and mature leaf veins. Only the functions that are largely specific to distinct LOXs in Arabidopsis leaves are emphasized. From Chauvin et al. (2013, 2016), Glauser et al. (2009), Grebner et al. (2013), and this work.

LOX6 are expressed principally in phloem- and xylem-associated cells in adult-phase primary leaf veins (Chauvin et al., 2013). Moreover, JA precursors made by LOX6 in xylem contact cells can move out of these cell populations (Gasperini et al., 2015). Why, then, do LOX3 and LOX4 and not LOX2 and/or LOX6 operate in the control of wound-response leaf growth restriction? It is possible that JA precursors or JAs produced by LOX6 and LOX2 in response to wounding do not reach the stem cell populations that determine the rate of leaf primordia development. In a potentially interesting parallel, we note that transcripts for all four Arabidopsis 13-LOXs are found in developing flowers (Klepikova et al., 2016), and yet only LOX3 and LOX4 produce JAs essential for reproductive development in this plant (Caldelari et al., 2011).

Phloem-Derived Signals Can Activate the JA Pathway

Which cell types produce the signals that lead to wound-response rosette growth restriction? To investigate this, we sought to control this process genetically in the absence of wounding. The *LOX3* promoter activity domain in primary leaf veins spans both the regions of xylem and phloem (Chauvin et al., 2016). Cells in one or both of these tissues were candidates that might produce the signals that stimulate environmentally linked growth restriction. In this respect, the phloem was of particular interest because this tissue plays central roles in growth plasticity (López-Salmerón et al., 2019). Additionally, key roles of the phloem in the induction of antiherbivore defense mechanisms in leaves have been discovered (Nguyen et al., 2018). Remarkably, expressing $TPC1^{D454N}$ in the null background *tpc1-2* under the phloem-companion-cell–specific *SUC2* promoter phenocopied *fou2*. To increase the dominance of *fou2*, we exploited *TPC1ΔCa*$_i$ ($TPC1^{D240A,D454A,E528A}$), which strongly increases the open probability of the channel (Guo et al., 2017). The first key test with the *TPC1ΔCa*$_i$ cDNA was to express it in a restricted cellular domain under the *SUC2* promoter in the *lox2 lox3 lox4 lox6* quadruple mutant background that does not produce biologically active JAs in response to wounding (Chauvin et al., 2013). This test (Fig. 4B; Supplemental Fig. S3) revealed that the rosette diameters and leaf shapes of the *lox* quadruple mutants with or without *TPC1ΔCa*$_i$ were similar. In 5-week-old plants, only a few of the younger leaves in the rosette of the quadruple mutant transformed with *TPC1ΔCa*$_i$, displayed some epinasty. This experiment served as the basis for transforming the *lox2 lox4 lox6* triple mutant with *TPC1ΔCa*$_i$ to try to activate LOX3, and thereby control rosette growth. A further control was expression of *TPC1ΔCa*$_i$ in the wild-type background. The wild-type plants expressing *SUC2::TPC1ΔCa*$_i$ displayed a *fou2*-like phenotype.

By extending this approach to a *lox2 lox4 lox6* background that carries the functional *LOX3* gene, we found that *TPC1ΔCa*$_i$-dependent ion fluxes generated in the phloem could activate the LOX3 branch of the JA synthesis pathway. The mechanism underlying this phenomenon is unknown, but membrane depolarization after wounding appears to play a role in JA pathway activation in Arabidopsis leaves (e.g. Lenglet et al., 2017; Nguyen et al., 2018). Phloem cells are highly excitable (Sibaoka, 1962; Hafke and van Bel, 2013; Hedrich et al., 2016), and wounding may therefore activate JA synthesis by altering membrane potentials and Ca^{2+} fluxes in these cells. Such a scenario may be widespread in plants subjected to repetitive wounding. The art of bonsai may rest on related mechanisms.

CONCLUSION

Loss-of-function genetic studies show that LOX3 and LOX4 together control wound-response leaf growth in Arabidopsis. These two 13-LOXs operate in both vegetative growth and reproductive development, highlighting that their functions are distinct from those of LOX2 and LOX6. Further demonstrating a role of LOX3 in controlling leaf growth, we were also able to activate LOX3-dependent JA production in unwounded leaves. This was accomplished using a novel approach based on gain-of-function mutations in a vacuolar ion channel. In terms of future work, hyperactive ion channel variants could find broader use in investigating mechanisms of cell-to-cell signaling within the vasculature, a phenomenon that underlies much of plant physiology.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transfer-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/). Arabidopsis (*Arabidopsis thaliana*) Columbia (Col-0) was the wild type and the background for *lox* mutants. The *lox* alleles used were *lox2-1* (Glauser et al., 2009), *lox3b* (SALK_147830), *lox4a* (SALK_ 071732), and *lox6a* (SALK_138907). Other mutants used were *tpc1-2* (*SALK_145413*), *fou2* (At4g03560, $TPC1^{D454N}$; Bonaventure et al., 2007a), and *aos* (At5g42650; Park et al., 2002), which were all in the wild-type background. Plants were grown individually in 7-cm–diameter pots on soil at 70% humidity and 100 μE m^{-2} s^{-1} photosynthetically available radiation under short-day conditions (10-h light at 22°C).

Serial Wounding

For serial wounding, a series of wounds was inflicted from d 14 of growth when the first two leaves were slightly bigger than the cotyledons. Fifty percent of the tip of leaf 1 was wounded with forceps. Control plants were handled identically but not wounded. Plants were then incubated for 3 d before 50% wounding of leaf 2. The same schema was repeated with 3 d of resting between each wound event and the series of wounds was stopped 3 d after leaf 7 was wounded. Five to six plants of each genotype (wild type or *aos*) were used for petiole measurements. For each plant, petiole lengths of the leaves 6, 7, and 8 were measured from photographs using the software ImageJ (https://imagej.nih.gov/ij/index.html) and their combined lengths were averaged.

Insect Bioassays

No-choice bioassays were performed with neonate *Spodoptera littoralis* placed on 5-week-old plants grown under short-day conditions identical to those described above. Six larvae were placed on each individual plant (11 plants per genotype) and allowed to feed for 11 d. Larvae were then weighted

on a precision balance XP205 DeltaRang (Mettler-Toledo). Aphid bioassays performed with *Brevicoryne brassicae*. Unwounded or serially wounded plants were placed in cages (11 plants per cage) and five adult aphids were deposed on each plant. After 48 h (day0), the adults were removed. After 72 h, nymphs were removed, leaving only five nymphs on each plant. Nymphs were counted at day 11 and the nymphs and adult aphids were counted at day 14. The aphid performance was calculated as follows: ([number of nymphs on day 11 + number of nymphs on day 14]/ number of adults on day 14).

Gene Expression Analysis

At the time of tissue collection, the leaves (unwounded or wounded) were collected and immediately frozen in liquid N_2. RNA isolation and reverse transcription quantitative PCR was as described by Gfeller et al. (2011) using the following primer pairs: *UBC21* (At5g25760) transcripts were quantified using the primers 5′-CAGTCTGTGTGTAGAGCTATCATAGCAT-3′ (ubc21F) and 5′-AGAAGATTCCCTGAGTCGCAGTT-3′ (ubc21R). *JAZ10* (At5g13220) transcripts were quantified using primers 5′-ATCCCGATTTCTCCGGTCCA-3′ (jaz10F) and 5′-ACTTTCTCCTTGCGATGGGAAGA-3′ (jaz10R). *LOX3* (At1g17420) transcripts were quantified using primers 5′-AACACAACCACA TGGTCTTAAACTC-3′ (lox3F) and 5′-GGAGCTCAGAGTCTGTTTTGATAA G-3′ (lox3R).

Plasmid Construction and Transformation

Vectors were based on MultiSite Gateway Technology (www.invitrogen.com). Promoters were amplified from wild-type genomic DNA with indicated oligonucleotides for: the 2-kb upstream region directly preceding the first ATG of SCR (At3g54220; Malamy and Benfey, 1997), LOX6 (At1g67560; 5′-CGGGGT ACCGGTTGTTGAAATTTCTGATGCT-3′ and 5′-TTCCCCCCCCGGGTTTTGT TTGGAGTTTGGCAGT-3′), and the 4-kb upstream region directly preceding the first ATG of SUC2 (At1g22710; 5′-CGGGGTACCCTGCTAAAACTATTC CATTTCAAAATG-3′ and 5′-TTCCCCCCCCGGGATTTGACAAACCAAGAAA GTAAG-3′). After amplification, these sequences were verified and cloned via restriction digestion with *Xma*I and *Kpn*I into a modified pUC57 (Chauvin et al., 2013) to create pEN-L4-promoter-R1 clones. The open reading frame of TPC1 (At4g03560) and TPC1D454N was amplified from cDNA from wild-type and fou2 plants (5′-ACAAAAAGCAGGCTTAATGGAAGACCC-3′ and 5′-AGAAA GCTGGGTTGTGTCAGAAGTGGAACACT-3′). Amplification products were recombined into pDONR221 (Invitrogen) to produce pEN-L1-gene-L2 clones. To generate promoter fusion with proteins under the control of endogenous promoters, pEN-L4-promoter-R1 plasmids were recombined with pEN-L1-CDS-L2 into pEDO097pFR7m24GW by double Gateway Technology to obtain SUC2pro-TPC1D454N, SCRpro-TPC1D454N, LOX6pro-TPC1D454N, and SUC2pro-TPC1 clones. All constructs were introduced into Arabidopsis backgrounds by floral dip *Agrobacterium*-mediated transformation. For promoter fusions, transformed seeds expressing red fluorescence protein in T1, T2, and T3 lines were selected by fluorescence microscopy. TPC1ΔCai (Guo et al., 2017) was synthesized by GenScript. The following mutations were introduced into the TPC1 coding sequence: Asp (GAC) 240-Ala (GCC), Asp (GAT) 454-Ala (GCT), and Glu (GAA) 528-Ala (GCA). The synthetic gene was cloned into the Entry vector pDONR/Zeo and recombined with pUC57 carrying the SUC2 promoter for plant transformation. From multiple transformants with similar phenotypes, a minimum of two independent transgenic lines for individual each construct was used in experiments.

JA and JA-Ile Analyses

JA and JA-Ile measurements were performed as described by Glauser et al. (2014). Plants were 5 weeks old and had not been wounded. The internal standards used were JA-d5 for JA and JA-Ile-$^{13}C_6$ for JA-Ile. Limits of quantifications for JA and JA-Ile were 0.4 and 0.15 pmol g^{-1} fresh weight, respectively.

Statistical Analysis

Statistical significance in pairwise comparisons was evaluated by Student's *t* test. Multiple comparisons using ANOVA followed by Tukey's honestly significant difference (HSD) test were performed in the software R 3.2.2 (www.r-project.org).

Accession Numbers

Sequence data TPC1 can be found in the GenBank/EMBL data libraries under accession numbers 825655/ NP_567258.1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of serial leaf wounding on rosette growth of *lox* mutants.

Supplemental Figure S2. Expressing *TPC1*^{D454N} in phloem companion cells of *tpc1-2* phenocopies *fou2*.

Supplemental Figure S3. Independent transformants show similar phenotypes and fresh weights to those used for Figure 4 in the main text.

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