Arabidopsis JASMONATE-INDUCED OXYGENASES down-regulate plant immunity by hydroxylation and inactivation of the hormone jasmonic acid

Short title: Control of plant defense by inactivation of jasmonic acid

Lotte Caarls[1], Joyce Elberse[1], Mo Awwanah[1], Nora R. Ludwig[1], Michel de Vries[2], Tieme Zeilmaker[1,3], Saskia C.M. Van Wees[1], Robert C. Schuurink[2], and Guido Van den Ackerveken[1]

[1] Plant-Microbe Interactions, Department of Biology, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, The Netherlands
[2] Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
[3] Current Address: Scienza Biotechnologies, Amsterdam, The Netherlands

Corresponding author: Guido Van den Ackerveken
P.O. Box 800.56, 3508 TB Utrecht, The Netherlands
+31302533013
Email: g.vandenackerveken@uu.nl

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Author contributions:
Designed research: L.C, GvdA
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Analyzed data: L.C, N.R.L, R.S, GvdA
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ABSTRACT

The phytohormone jasmonic acid (JA) is vital in plant defense and development. Although biosynthesis of JA and activation of JA-responsive gene expression by the bioactive form JA-isoleucine (JA-Ile) have been well-studied, knowledge on JA metabolism is incomplete. In particular, the enzyme that hydroxylates JA to 12-OH-JA, an inactive form of JA that accumulates after wounding and pathogen attack, is unknown. Here, we report the identification of four paralogous 2-oxoglutarate/Fe(II)-dependent oxygenases in *Arabidopsis thaliana* as JA hydroxylases and show that they down-regulate JA-dependent responses. As they are induced by JA we named them *JASMONATE-INDUCED OXYGENASEs* (*JOXs*). Concurrent mutation of the four genes in a quadruple Arabidopsis mutant resulted in increased defense gene expression and increased resistance to the necrotrophic fungus *Botrytis cinerea* and the caterpillar *Mamestra brassicae*. In addition, root and shoot growth of the plants was inhibited. Metabolite analysis of leaves showed that loss of function of the four JOX enzymes resulted in over-accumulation of JA and in reduced turnover of JA into 12-OH-JA. Transformation of the quadruple mutant with each *JOX* gene strongly reduced JA levels, demonstrating that all four JOXs inactivate JA in plants. The *in vitro* catalysis of 12-OH-JA from JA by recombinant enzyme could be confirmed for three JOXs. The identification of the enzymes responsible for hydroxylation of JA reveals a missing step in JA metabolism, which is important for the inactivation of the hormone and subsequent down-regulation of JA-dependent defenses.

SIGNIFICANCE STATEMENT

In plants, the hormone jasmonic acid (JA) is synthesized in response to attack by pathogens or herbivores, leading to activation of defense responses. Rapidly following JA accumulation, the hormone is metabolized, presumably to prevent inhibitive effects of high JA levels on growth and development. The enzymes that directly inactivate JA were so far unknown. Here, we identify four jasmonate-induced oxygenases (JOXs) in Arabidopsis that hydroxylate jasmonic acid to form inactive 12-OH-JA. A mutant that no longer produces the four enzymes hyperaccumulates JA, exhibits reduced growth, and is highly resistant to attackers that are sensitive to JA-dependent defense. The JOX enzymes thus play an important role in determining the amplitude and duration of JA responses to balance the growth-defense tradeoff.

INTRODUCTION

The lipid-derived phytohormone jasmonic acid (JA) is an essential signaling molecule in plant defense. In response to pathogen attack or wounding, JA levels accumulate resulting in activation of a subset of immune genes and the production of defensive secondary metabolites (1). Multiple negative feedback mechanisms control JA levels and JA-responsive gene expression, presumably to minimize inhibition of plant growth that is associated with JA-mediated defense responses (2). In healthy plants, under non-stressed conditions, low levels of JA are present, and the activation of JA-responsive genes is prevented by JAZ repressor proteins that bind transcriptional activators of the JA pathway (3–7). The conjugate of JA with isoleucine(JA-Ile) strongly promotes binding of JAZ repressors to the F-box protein COI1 (4, 8, 9), resulting in the degradation of JAZ proteins and subsequent activation of JA-responsive gene expression (10, 11). At the same time, *JAZ* gene expression is activated by JA resulting in the subsequent repression of JA-responsive gene expression (4, 5, 12). Excess JA and JA-Ile are also inactivated by hydroxylation, forming 12-hydroxy-JA (12-OH-JA) and 12-OH-JA-Ile (13–16).

The hydroxylated form of JA is thought to be inactive as it does not trigger degradation of JAZ repressors, and treatment with 12-OH-JA accordingly does not induce JA-responsive gene expression, nor does it inhibit root growth or seed germination (17, 18). 12-OH-JA has been identified in several plant species, including Arabidopsis, maize, potato, tomato and rice, and accumulates in Arabidopsis after wounding and *Botrytis cinerea* infection (13, 17, 19, 20). Strikingly, hydroxylation of JA to 12-OH-JA by a monooxygenase produced by the blast fungus *Magnaporthe oryzae* attenuates plant immune responses to this pathogen (18). So far, no plant enzyme that hydroxylates JA has been identified (21). Hydroxylation of JA-Ile, on the other hand, has been described in Arabidopsis and is mediated by three cytochrome P450 enzymes, CYP94B3, CYP94B1 and CYP94C1 (14, 22–24). 12-OH-JA can be formed from 12-OH-JA-Ile by cleavage of the isoleucine group by two amidohydrolases (25). However, an Arabidopsis double mutant that no longer produces these enzymes still accumulates 12-OH-JA, implying that other enzymes catalyze direct hydroxylation of JA to 12-OH-JA.

Apart from cytochrome P450 enzymes, members of the 2-oxoglutarate (2OG) Fe(II)-dependent oxygenase family are involved in oxygenation/hydroxylation reactions in plants (26). Interestingly, several 2OG oxygenases were shown to hydroxylate and inactivate plant hormones; e.g. two different groups of 2OG oxygenases inactivate gibberellic acid (GA) by hydroxylating either bioactive 19-GA or an inactive precursor of GA (27, 28). More recently, the active form of auxin was reported to be hydroxylated and inactivated by the 2OG oxygenase DAO in rice (29) and Arabidopsis (30, 31). Finally, the defense hormone salicylic acid (SA) is hydroxylated by the 2OG oxygenase SA 3-HYDROXYLASE (S3H) (32). Since inactivation of hormones via hydroxylation by 2OG oxygenases is common in plants, we hypothesized that 2OG oxygenases could function as JA-hydroxylases as well. Here, we describe the identification of a clade of four 2OG oxygenases that are transcriptionally induced by JA, which we named JASMONATE-INDUCED OXYGENASES (JOX). We provide metabolic and biochemical evidence that these enzymes are responsible for hydroxylation of JA to 12-OH-JA. Furthermore, phenotypic studies of mutant and overexpression lines show that the JOXs are involved in down-regulation of JA-dependent responses, thereby affecting plant defense and

growth. These results identify a new class of enzymes in JA metabolism that perform an essential role in in controlling defense responses to necrotrophs and herbivorous insects.

RESULTS

Four JASMONATE-INDUCED OXYGENASES group in a distinct clade in Arabidopsis

To identify JA-induced 2OG oxygenases, we first constructed a phylogenetic tree of 93 Arabidopsis proteins that each contain two conserved 2OG oxygenase Pfam domains; the C-terminal PF03171 (2OG-Fe(II) oxygenase superfamily), and the N-terminal PF14226 (non-haem dioxygenase in morphine synthesis N-terminal). Phylogenetic clustering of the 93 proteins revealed distinct families (Fig. S1), that largely overlapped with previously described clades (26) which were based on 2OG oxygenases of six plant species ranging from unicellular alga to the flowering plants Arabidopsis and rice. Projection of transcriptome data showed that 2OG oxygenases that are induced in Arabidopsis seedlings by treatment with the defense-related hormones methyl jasmonate (MeJA) or SA were only present in a cluster of 50 proteins that encompass 14 clades as defined by Kawai *et al.* (26) (Fig. 1). Clade 38 (in red) contains the *S3H/DLO1* and *DMR6* genes that are induced by SA (32, 33). The expression of six genes encoding 2OG oxygenases was induced more than 2-fold by MeJA treatment (indicated in bold; Fig. 1). Of two weakly induced genes, At5g20550 is a gene of unknown function, whereas LDOX (At4g22880) encodes an enzyme involved in anthocyanin biosynthesis (34). A third MeJA-induced gene is *DIN11* (At3g49620), which was described as a senescence-associated gene involved in the response to viral infection (35). Strikingly, clade 46 (in blue) contains four 2OG-oxygenases of which 3 were clearly induced at 3 h after treatment with MeJA (Fig. 1).

The JA-responsiveness of the four 2OG oxygenase genes of clade 46 was experimentally verified in five-week-old plants treated with MeJA. This treatment increased transcript levels of all four genes (Fig. 2) that were, therefore, named *JASMONATE-INDUCED OXYGENASEs* (*JOXs*). The expression of *JOX1* (At3g11180), which was not MeJA-induced in seedlings according to publically available microarray data (Fig 1, 36), was highly induced in adults plants at 1h after MeJA treatment, and was slightly higher than in mock-treated plants at 2-6 h after MeJA treatment (Fig. 2). The expression patterns of *JOX2* (At5g05600) and *JOX3* (At3g55970) were similar: induction was low at 1 h and 2 h after treatment, but high at 6 h after treatment. Expression of *JOX4* (At2g38240) was induced at all time points, but showed a different temporal behavior: it was highly induced at 1 h, lower at 2 h and high again at 6 h after treatment (Fig. 2). In the *coi1* mutant, that does not have a functional JA receptor and is impaired in MeJA-responsive gene expression (37), none of the *JOX* genes were induced by MeJA (Fig. 2). Interestingly, expression of the four *JOX* genes was strongly induced in plants infected by *B. cinerea* or infested by the caterpillar *Mamestra brassicae*, which both induce JA accumulation (Fig. S2). The fact that the four related *JOX* genes are all activated through the general COI1-dependent JA-response pathway make them prime candidates to be involved in JA metabolism.

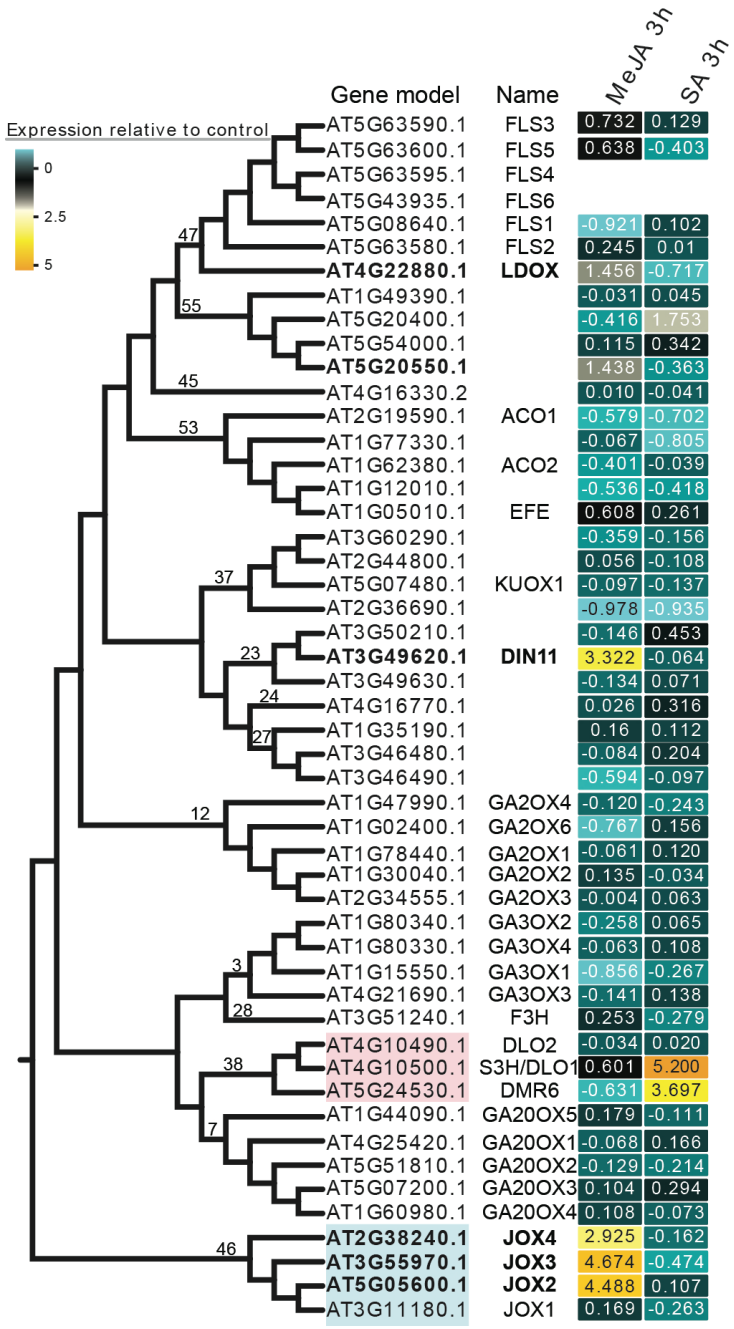

Figure 1. Phylogenetic tree of SA- and MeJA-induced and related 2OG oxygenases of Arabidopsis. The cladogram shows the relatedness of 50 2OG oxygenases selected from the phylogram in Figure S1. For each protein model, the name (when available) is supplied. For each clade, the number which was assigned to it by Kawai et al. (2014) is indicated. The heat map indicates the $\log_2$ fold change of the corresponding genes in Arabidopsis seedlings 3 h after MeJA or SA treatment. Indicated in pink is clade 38, which contains SA-induced S3H/DLO1. 2OG oxygenases induced by MeJA are indicated in bold. Clade 46 containing the JOX genes induced by MeJA is indicated in blue.

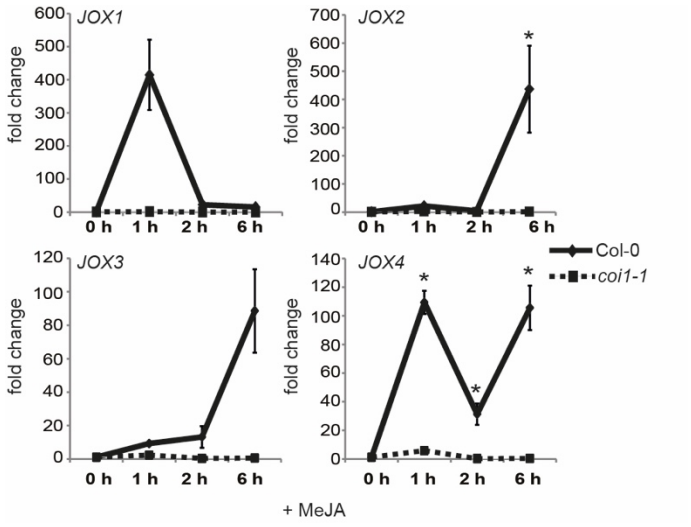

Figure 2. Expression of *JOX* genes after MeJA treatment. qRT-PCR analysis of expression of *JOX1, JOX2, JOX3* and *JOX4* in response to MeJA treatment in 5-week-old Col-0 and *coi1-1.* Shown is the expression (fold change) at 0 h, 1 h, 2 h, or 6 h after MeJA treatment, relative to that in mock-treated plants at the same time. Error bars indicate standard error. An asterisk indicates a significant higher expression compared to mock-treated plants ($P \leq 0.05$; two-way ANOVA).

JOX enzymes are negative regulators of JA responses

The presumed function of the JOX enzymes in JA hydroxylation is expected to influence JA-related biological processes. We therefore set out to test phenotypes affected by JA in mutant plants. Since the four *JOX* genes could act redundantly, a quadruple mutant *jox1 jox2 jox3 jox4*, hereafter referred to as *joxQ*, was generated by crossing four T-DNA insertion lines in each of which one of the four JOX genes was disrupted. JA-related phenotypes, e.g. resistance to necrotrophic pathogens and herbivorous insects, were then analyzed in the *joxQ* mutant. Lesions caused by infection with the necrotrophic fungus *B. cinerea* were significantly smaller in the *joxQ* mutant compared to those on wild-type Col-0 leaves (Fig. 3A&B). In addition, larvae of the generalist caterpillar *M. brassicae* were smaller and weighed significantly less when fed on *joxQ* compared to those fed on Col-0 (Fig. 3C&D). This suggests that defense against both necrotrophic pathogens and herbivorous caterpillars is upregulated in *joxQ* plants. To understand the molecular basis of the increased resistance in the *joxQ* mutant, we measured expression of JA-responsive defense genes before and after infection or infestation. Already before *B. cinerea* infection, expression of the JA/ET-responsive *PDF1.2* and *ORA59* genes was higher in the *joxQ* mutant compared to Col-0; the transcription factor gene *ORA59* was expressed 15-fold higher, while expression of *PDF1.2* was increased 9000-fold (Fig. 3E). During infection with *B. cinerea, ORA59* and *PDF1.2* levels strongly increased and were significantly higher in the *joxQ* mutant than in Col-0 (Fig. 3E). Similarly, expression of the JA-responsive transcription factor gene *MYC2* was higher in the *joxQ* mutant than in Col-0 under control conditions. Levels of this gene increased after *M. brassicae* feeding, and stayed slightly higher in the *joxQ* mutant (Fig. 3F).

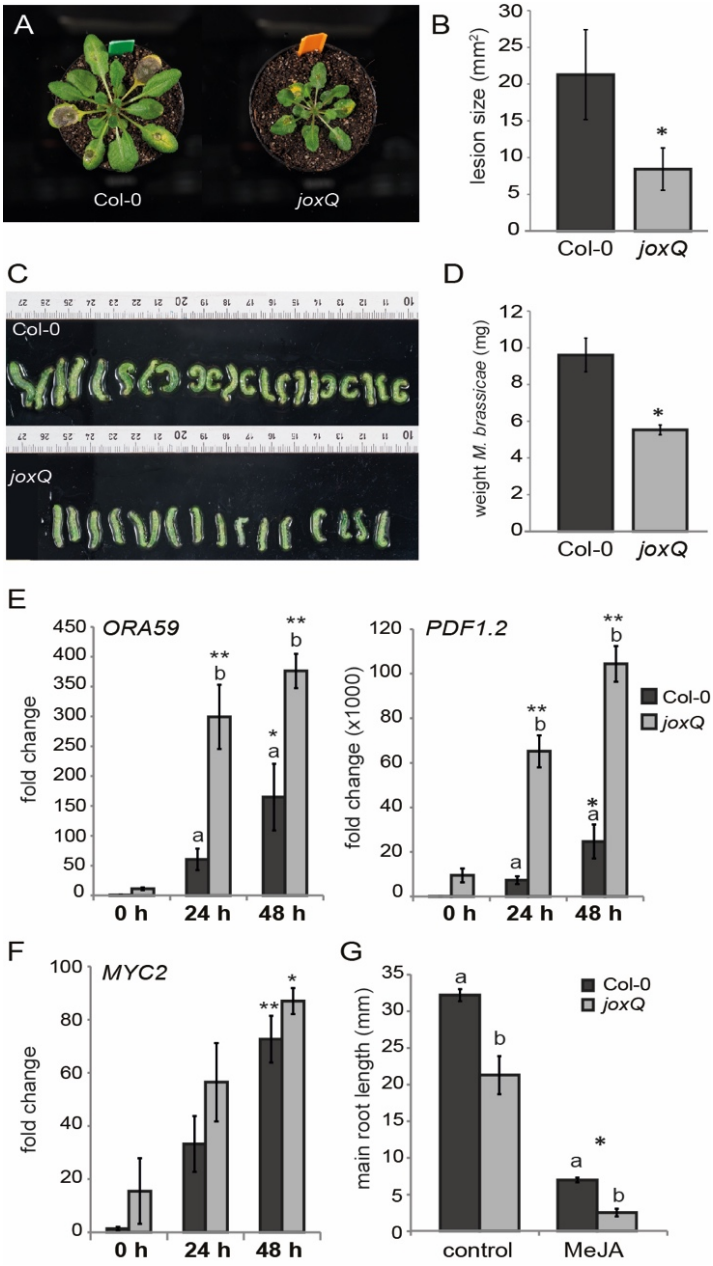

Figure 3. The *joxQ* quadruple mutant displays phenotypes reminiscent activated JA signaling (A) Representative pictures of reduced disease symptoms caused by *B. cinerea* infection on *joxQ* compared to Col-0. (**B)** Area size of necrotic lesions caused by *B. cinerea* in Col-0 and *joxQ*. **(C)** Growth of *M. brassicae* caterpillars on Col-0 and *joxQ* plants. Caterpillars were photographed after 8 days of feeding. **(D)** Caterpillar fresh weight after eight days of feeding. An asterisk denotes a significant difference between caterpillar weight on Col-0 and *joxQ* (*t* test, $P \leq 0.001$). **(E)** Expression of the JA-responsive genes *ORA59* and *PDF1.2* before infection (0 h) and after 24 or 48 h of *B. cinerea* infection of Col-0 or *joxQ*, relative to Col-0 at 0 h. **(F)** Expression of the JA-responsive gene *MYC2* before (0 h) and after 24 or 48 h of *M. brassicae* feeding on Col-0 or *joxQ*, relative to Col-0 plants at 0 h. (E,F) Different letters

indicate significant differences between genotypes. An asterisk indicates a significant higher expression compared to Col-0 plants at 0 h (two-way ANOVA, Tukey post-hoc test; * $P \leq 0.05$; ** $P \leq 0.001$). **G)** Root growth of seedlings grown for 15 days on ½ MS medium without (control) or with 50 µM MeJA. Different letters indicate significant differences between genotypes per treatment. An asterisk indicates a significant growth inhibition by MeJA (two-way ANOVA, $P \leq 0.05$).

JA is also known to inhibit plant growth and delay flowering time (38, 39). In accordance with this, the *joxQ* quadruple mutant was consistently smaller than wild-type plants (Fig. 3A) and exhibited reduced root growth on ½ MS medium (Fig. 3G). In addition, in the presence of 50 µM MeJA, the reduction in main root length of *joxQ* was significantly stronger than that of Col-0 (Fig. 3G). These phenotypes support the idea that in wild-type plants JOX proteins suppress JA-mediated growth inhibition. Moreover, flowering time was delayed by 6 days in the *joxQ* mutant compared to Col-0 under short-day conditions and the mutant produced fewer seeds than wild-type Col-0 (Fig. S3). The disease resistance and other JA-related phenotypes of the quadruple *joxQ* mutant are reminiscent of plants with activated JA responses, and are possibly caused by high JA levels.

The *joxQ* mutant hyperaccumulates JA

The accumulation of JA and its bio-active form JA-Ile could explain the phenotypes of the *joxQ* quadruple mutant and was therefore measured by LC-MS/MS. We found that JA levels were about 5x higher in the *joxQ* mutant than in Col-0 under non-treated conditions (Fig. 4A). Three hours after wounding, which is known to trigger JA accumulation, JA levels tripled to 11 ng/g FW in wild-type plants. In the *joxQ* mutant, JA rose to 170 ng/g FW (Fig. 4A). JA-Ile was not detected in plants that had not been wounded. After wounding, JA-Ile accumulated to low but detectable levels in Col-0. In the *joxQ* mutant, JA-Ile levels were significantly higher than in Col-0 (Fig. 4A). The increased accumulation of JA and JA-Ile in the *joxQ* mutant suggests that in wild-type plants the JOX proteins negatively affect the accumulation of JA and JA-Ile, possibly via hydroxylation of JA. To further study this, *JOX1* (At3g11180) was overexpressed in the Col-0 background (*JOX1* OX). In accordance with our hypothesis, JA levels were slightly lower than in Col-0 under basal conditions when *JOX1* was overexpressed and more importantly, JA and JA-Ile levels did not increase after wounding in this line (Fig. 4A). The results of both the mutant and overexpression line suggest that after wounding, the JOX enzymes act to reduce amounts of JA, possibly via hydroxylation.

If JOXs indeed hydroxylate JA, we would expect low levels of 12-OH-JA in the *joxQ* mutant. However, 12-OH-JA was not detected in plants before wounding, and was about 3-fold higher in the *joxQ* mutant in response to wounding compared to Col-0 (Fig. S4A). This was lower than the 14-fold increase in JA levels in *joxQ* compared to Col-0. Possibly, 12-OH-JA detected in *joxQ* is generated by cleavage of the conjugated isoleucine of 12-OH-JA-Ile by aminohydrolases ILL6 and IAR3 (25). In *JOX1* OX we expected increased 12-OH-JA to accumulate, however, 12-OH-JA was not detected in this line (Fig. S4A). We hypothesize that hydroxylated JA cannot be

detected because JA levels in this OX line are so low, or that the produced 12-OH-JA is further metabolized and therefore not detectable. To reduce the difference in JA levels between the different lines, leaves of wild-type, *joxQ* and *JOX1* OX plants were immersed in a 100 µM JA solution. Three hours after treatment, jasmonates were measured. In JA-treated leaves, JA levels in *joxQ* were similar to Col-0 in *joxQ*, and lower in the *JOX1* OX line (Fig. 4B). Strikingly, the level of 12-OH-JA was much lower in the *joxQ* mutant than in Col-0. In contrast, in *JOX1* OX, the levels of 12-OH-JA were higher than in Col-0 (Fig. 4B). This indicates that the conversion of JA into 12-OH-JA is reduced in the *joxQ* mutant, and enhanced in the *JOX1* OX line. We conclude that the *joxQ* mutant hyperaccumulates JA and JA-Ile under basal and biotic stress conditions, and accumulates reduced 12-OH-JA after treatment with JA. In contrast, a line overexpressing *JOX1* builds up less JA, and accumulates more of the hydroxylated form when treated with JA.

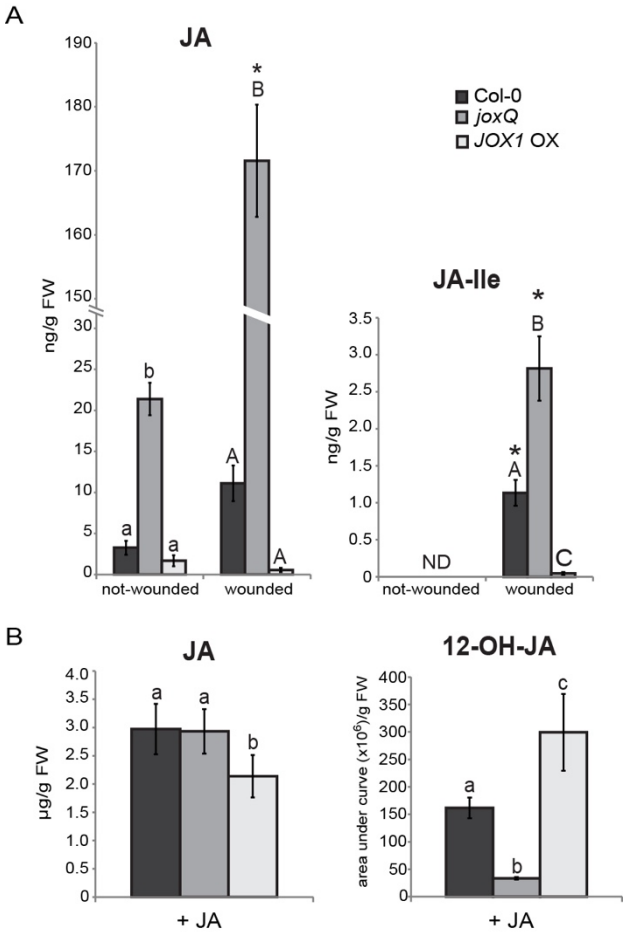

Figure 4. The *joxQ* mutant accumulates JA and is impaired in 12-OH-JA production after JA treatment. (A) JA and JA-Ile levels in leaves with or without wounding (3 h after mechanical damage) of Col-0, *joxQ* and *JOX1* OX plants. Each data point represents the mean of four biological replicates. Error bars indicate standard error. JA levels were calculated by correcting for the internal standard of JA and for leaf weight. JA-Ile levels were calculated by using a standard curve and corrected for leaf weight. Different letters indicate statistically significant differences between genotypes at the same treatment. An

asterisk indicates that wounding significantly induced the compound (two-way ANOVA; Tukey post-hoc test; $P \leq 0.05$). **(B)** Accumulation of JA and 12-OH-JA in plant exposed for 3 h to 100 µM JA. Each data point represents the mean of four biological replicates. Error bars indicate standard error. JA levels were calculated by correcting for the internal standard of JA and for leaf weight. For 12-OH-JA, the area under the curve (parent ion/daughter ion: 225/59) was recorded and corrected for recovery of JA and leaf weight. Different letters indicate statistically significant differences between genotypes (two-way ANOVA; Tukey post-hoc test; $P \leq 0.05$).

Overexpression of each individual *JOX* complements *joxQ* phenotypes

The presumed metabolic function of the JOX enzymes is to hydroxylate JA. To determine if all JOX enzymes have the capability to reduce JA levels and responses, we constitutively expressed each *JOX* gene from the 35S promoter in the quadruple *joxQ* mutant. For each *JOX*, at least two independent homozygous transformants were selected. First, we observed that overexpression of each *JOX* rescued the growth phenotype of the quadruple *joxQ* mutant. While five-week old *joxQ* plants were significantly smaller than Col-0, all *JOX*-transformed plants resembled Col-0 plants (Fig. 5A). Secondly, the high expression of *PDF1.2* that was observed in *joxQ* plants (Fig. 3E), was reverted to wild-type levels in all overexpression lines (Fig. 5B). Finally, we measured levels of JA and JA-derivatives in all transformed lines. In untreated adult plants of *joxQ*, JA levels were about 3 times higher than in Col-0. Overexpression of *JOX1*, *JOX2* and *JOX3* resulted in depletion of JA. Overexpression of *JOX4* resulted in JA levels similar to those in Col-0 (Fig. 5C). Taken together, these results show that each individual JOX can reduce JA levels to those in wild-type plants or lower, resulting in complementation of JA-related phenotypes (Fig. 5).

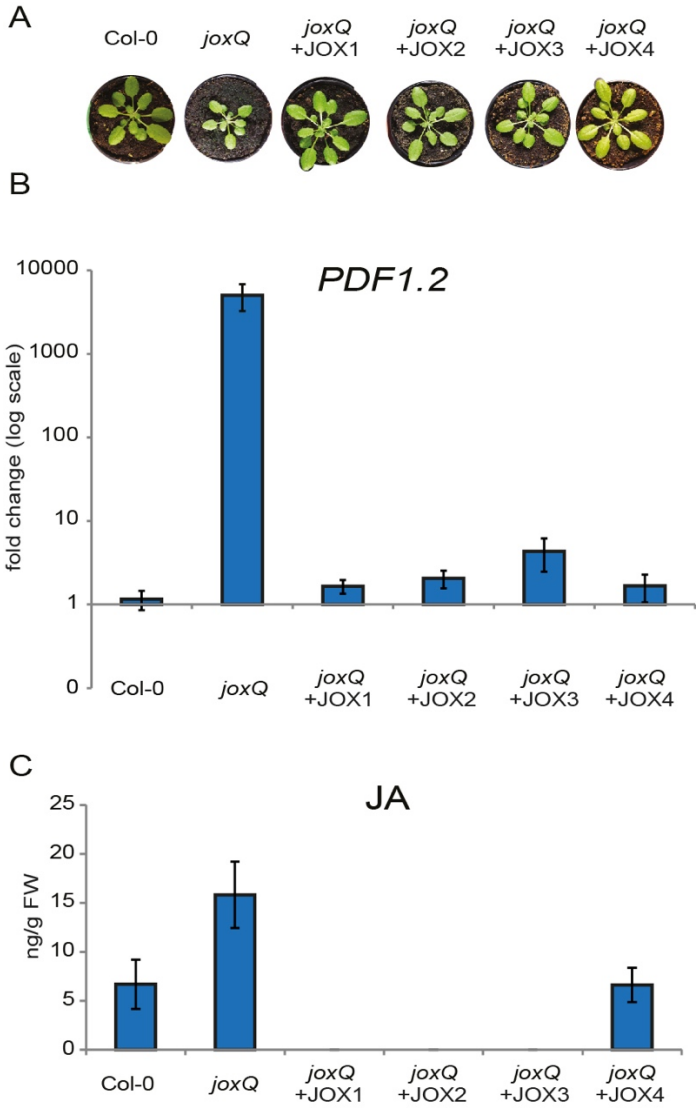

Figure 5. Overexpression of each individual JOX complements *joxQ* phenotypes. (A) Growth phenotype of representative plants of Col-0, *joxQ* and overexpression lines of JOX1, JOX2, JOX3, JOX4 in the *joxQ* background. **(B)** Expression of *PDF1.2* in untreated Col-0, *joxQ* and overexpression lines of *JOX1*, *JOX2*, *JOX3*, *JOX4* in the *joxQ* background. Expression is normalized to the reference gene At1g13320, and relative to expression in Col-0 plants. Data points represent the mean of four to twelve plants, error bars indicate standard error. For *joxQ JOX* overexpression lines, the mean of two or three (JOX2) independent homozygous transformant lines is shown. **(C)** Amount of JA in untreated leaves of Col-0, *joxQ* and overexpression lines of JOX1, JOX2, JOX3, JOX4 in the *joxQ* background. JA levels were calculated by correcting for the internal standard of JA, and by dividing by leaf weight. Each data point represents the mean of four to eight biological replicates. Error bars indicate standard error. For JOX overexpression lines, average is shown of two or three (JOX2) independent homozygous transformant lines.

JOX1, JOX2 and JOX4 hydroxylate JA *in vitro*

To determine whether the JOX proteins can catalyze the hydroxylation of JA to 12-OH-JA without other plant proteins present, assays were conducted with JA as substrate and recombinant JOX enzymes produced in *E. coli*. The reaction products were analyzed by LC-MS/MS. Lysates of *E. coli* expressing JOX1, JOX2 and JOX4 effectively converted JA to 12-OH-JA, resulting in almost complete conversion of the JA substrate, and high levels of 12-OH-JA (Fig. 6). The lysate of *E. coli* expressing JOX3 produced low amounts of 12-OH-JA, which were higher than that of the negative control (reaction mixture without lysate) but not higher than the amount of 12-OH-JA produced in the enzymatic assay with *E. coli* expressing the SA-hydroxylase S3H/DLO1. We thus conclude that recombinant JOX1, JOX2 and JOX4 have JA-12-hydroxylase activity *in vitro* in the absence of other plant-derived enzymes and compounds.

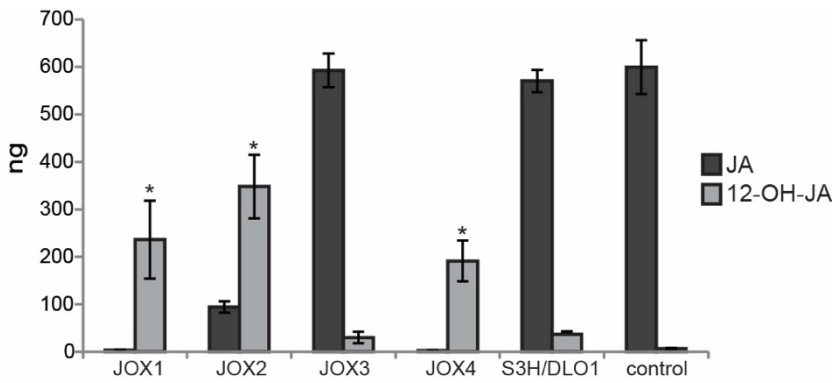

Figure 6. JOX1, JOX2 and JOX4 are JA-hydroxylases *in vitro*. Enzyme assays with JOX1, JOX2, JOX3, JOX4 and S3H/DLO1 and JA as substrate. Shown are the amounts of JA and 12-OH-JA at the end of enzyme assay. Each data point represents the mean of three biological replicates. Error bars indicate standard deviation. An asterisk denotes a significant difference between the JOX enzyme and the SA-hydroxylase S3H/DLO1 (*t* test, $P \leq 0.05$). Amounts of JA and 12-OH-JA were calculated using standard curves. Control: Enzymatic reaction mixture without lysate. The experiment was repeated with similar results.

Discussion

Four JA-induced 2OG oxygenases (JOX) from a single paralogous family in Arabidopsis were identified that strongly contribute to negative regulation of JA-dependent responses. Plants in which the four genes were mutated (*joxQ*) accumulated high levels of JA, exhibited smaller growth, enhanced expression of defense genes, and resistance to both *B. cinerea* and *M. brassicae*. We provide evidence from metabolic measurement and enzymatic assays that the four JOX enzymes control JA levels by hydroxylation of JA to the inactive 12-OH-JA. By keeping

JA levels low the JOX enzymes are important in the determining the amplitude and duration of JA responses and balance the growth-defense tradeoff.

Inactivation of plant hormones by 2OG oxygenase-mediated hydroxylation appears to be a common theme in plants to control their hormone levels. The hormones GA and SA are hydroxylated by 2OG oxygenases that are closely related to the JOXs, i.e. GA2OXs that hydroxylate GA (27) (clade 12 in Fig. 1), and S3H (clade 38) that hydroxylates SA (32). Two more distantly related 2OG oxygenases were recently shown to inactivate the plant hormone auxin (30, 31). Clade 46 of the 2OG oxygenases as defined by Kawai *et al.* (26) consists of four Arabidopsis members, encoded by the genes At3g11180, At5g05600, At3g55970 and At2g38240, which we named *JOX1*, *JOX2*, *JOX3,* and *JOX4*, respectively. We show that the members of this clade act by oxidative inactivation of JA, and prevent overaccumulation of JA and indirectly its bio-active form JA-Ile.

The observation that JA-levels are high in the *joxQ* mutant already in untreated conditions (Fig. 4A), suggests that JOXs contribute to reduction of JA levels in these non-induced conditions. After wounding, JA levels increased dramatically in the *joxQ* mutant, supporting a function of these enzymes in reducing accumulation of JA in response to activation of JA responses such as those triggered by wounding. That this mechanism is controlled by JA itself is evidenced by the induction of the *JOX* genes by MeJA treatment, by *M. brassicae* feeding and by *B. cinerea* infection, the first of which was shown to be dependent on JA-coreceptor COI1. This is reminiscent of mechanisms of oxidative inactivation of hormones as discussed above and in particular of the mechanism of JA-Ile hydroxylation (14, 23). Enzymes from the cytochrome P450 family inactivate JA-Ile: CYP94B1 and CYP94B3 hydroxylate JA-Ile to 12-OH-JA-Ile (22–24) and CYP94C1 further oxygenates this compound to 12-COOH-JA-Ile (14). Our identification of four 2OG oxygenases that convert JA into the inactive 12-OH-JA, further elucidates JA metabolism. From 12-OH-JA-Ile, 12-OH-JA can be produced by the amidohydrolases IAR3 and ILL6, which cleave the isoleucine group of JA-Ile and 12-OH-JA-Ile (25). However, the JOX enzymes can hydroxylate JA directly, and majorly contribute to the removal of JA in plants in undisturbed growth conditions and in response to wounding or pathogen attack. The quadruple mutant of these enzymes shows similar phenotypes as the JA-Ile hydroxylase mutants, e.g. enhanced expression of JA-responsive genes and increased sensitivity to JA-dependent inhibition of root growth (Fig 3, 14). This suggests that hydroxylation of JA by the JOX enzymes contributes to inactivation of the active JA signal to a similar extent as hydroxylation of JA-Ile. The levels of the bioactive JA-Ile are likely directly influenced by the amount of JA, as we show that the levels of JA and JA-Ile are in equilibrium after wounding (Fig. 4A).

The importance of hydroxylation of JA is emphasized by the apparent evolution of four different enzymes with the same function. It is possible that each enzyme functions in different processes in the plant or in distinct plant tissues. Preliminary evidence for this comes from the timing and amplitude of the expression induced by MeJA, *B. cinerea* and *M. brassicae,* which was different between the *JOX* genes (Fig. 2, Fig. S2). In addition, we have so far only tested expression in leaf tissue, while spatial expression of each *JOX* gene within the leaf or within the plant could be different. In a similar way, the SA-hydroxylase gene *DLO1* and its paralog *DMR6* have similar

but distinct activities due to their pathogen-induced expression in different parts of downy mildew-infected leaves (33). Experiments to localize the expression of *JOXs* and complementation assays under their own promoter could further elucidate the different functionalities of the four JOX enzymes. In an enzyme assay, recombinant JOX1, JOX2 and JOX4 were shown to be able to produce 12-OH-JA from JA in the absence of other plant proteins. JOX3 was unable to hydroxylate JA in this *in vitro* assay. However, in our complementation studies, all four enzymes, including JOX3, were shown to be able to complement all phenotypes of the *joxq* mutant, indicating that each JOX enzyme can lower JA levels *in vivo.* Possibly, the JOX3 enzyme requires activation by another plant protein or compound before it is active as an JA-hydroxylase, or folding of the recombinant JOX3 protein is not correct.

In plants, JA is converted to derivatives that are biologically active, or reduced active or inactive (16). 12-OH-JA has been characterized as an inactive form of JA, as it is not capable of degrading JAZ9 and does not induce expression of JA-responsive genes or inhibition of root growth (15, 17, 18). The compound has been suggested to have tuber-inducing capabilities (40), but what the role of this compound is in non-tuber forming plants such as Arabidopsis is not clear. It is likely that hydroxylation of JA is a quick mechanism to inactivate JA,. Following production of JA, expression of *JOX* genes would quickly be induced, after which the accumulation of JA, and subsequently the expression JA-responsive genes expression, is dampened. This is yet another negative feedback system that would be active in the JA pathway, in addition to, e.g. the activation of JAZ repressor genes by JA (4, 5, 12). Moreover, similar hydroxylation mechanisms control levels of other plant hormones (23, 30, 32). The identification of JOX enzymes elucidates another major step in the metabolic pathway of plants. Our data shows that it is imperative that plants balance JA levels by controlling JA metabolism, as inhibitive effects on growth are evident in the *joxQ* mutant. As expression of the JOX genes is induced by JA, plants can quickly shut off JA-dependent responses after their activation to prevent negative effect of high JA levels on growth and development. The JOX enzymes thus contribute to balance the growth/defense tradeoff.

Material and Methods

All material and methods are described in SI Material and Methods..

Acknowledgments

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