Short title: Initiation of JA biosynthesis in wounded plants

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Footnotes

List of author contribution: A.J.K. conceived the original research plans. A.N.K. and A.J.K. designed experiments. A.J.K., A.N.K., R.E.H., T.M. and T.Z. performed experiments. T.Z. made the construct and generated transgenic lines. R.E.H. conducted substrate feeding experiments. A.N.K. conducted most of the experiments. All contributed to analyzing the data. A.N.K. and A.J.K. wrote the article with contributions by all listed authors.

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Abstract

The basal level of plant defense hormone jasmonate (JA) in unstressed leaves is low, but wounding causes its near instantaneous increase. How JA biosynthesis is initiated is uncertain but the lipolysis step that generates fatty acid precursors is generally considered to be the first step. We used a series of physiological, pharmacological, genetic, and kinetic analysis of gene expression and hormone profiling to demonstrate that the early spiking of JA upon wounding does not depend on the expression of JA biosynthetic genes. By using a transgenic system, we showed how decoupling the responses to wounding and JA prevents the perpetual synthesis of JA in wounded leaves. We then used DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) as a model wound-responsive lipase to demonstrate that although its transient expression in leaves can elicit JA biosynthesis to a low level, an additional level of activation is triggered by wounding which causes massive accumulation of JA. This wound-triggered boosting effect of DAD1-mediated JA synthesis can happen directly in damaged leaves or indirectly in undamaged remote leaves by the systemically transmitted wound signal. Finally, protein stability of DAD1 was influenced by wounding, α-linolenic acid, and mutation in its catalytic site. Together, the data support mechanisms that are independent of gene transcription and translation at work to initiate the rapid JA burst in wounded leaves and demonstrate how transient expression of the lipase can be used to reveal changes occurring at the level of activity and stability of the key lipolytic step.

INTRODUCTION

Terrestrial plants are subject to attacks by plant-feeding insects. The lipid-derived hormone jasmonate (JA), a collective term used to describe jasmonic acid and its precursors and derivatives, is a key phytohormone that orchestrates many of the defense responses against insects (Wasternack and Hause, 2013, Howe *et al.*, 2018). Rapid induction of the JA-dependent signaling pathway is critical for a timely response to fast moving aggressors like insects. Indeed, judging from the speed of JA-responsive marker gene expression, the JA signaling pathway is induced within several minutes of insect herbivory or mechanical tissue injury (Mousavi *et al.*, 2013, Toyota *et al.*, 2018).

The molecular details of transcriptional regulation in the JA signaling pathway have been revealed (Chini *et al.*, 2007, Thines *et al.*, 2007, Yan *et al.*, 2007). The centerpiece for this mechanism is a nuclear residing co-receptor complex consisting of CORONATINE INSENSITIVE 1 (COI1) and a JASMONATE ZIM-domain (JAZ) protein (Xie *et al.*, 1998). COI1 is the F-box protein part of the E3 ubiquitin ligase complex, Skp1-Cul1-F-box protein (SCF^{COI1}) and JAZs are transcriptional repressors of transcription factors (TFs) that control JA-responsive gene expression. The complex formation between COI1 and JAZ facilitated by JA results in the polyubiquitination and subsequent proteolytic degradation of JAZs which then leads to a transcriptional activation of JA-regulated genes. Since the physical interaction between COI1 and JAZ requires the bioactive form of JA, most prominently, (+)-*7-iso*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.*, 2009), it implies that JA must first be present for this transcriptional system to work.

The core JA biosynthetic pathway begins in the plastid and proceeds through the peroxisome before finally being converted to JA-Ile in the cytosol (Vick and Zimmerman, 1983, Schaller and Stintzi, 2009, Koo, 2018, Wasternack and Feussner, 2018). The generally accepted first step of JA biosynthesis is the liberation of 18-carbon fatty acids (FAs) containing three double bonds (C18:3 $\Delta^{9,12,15}$) called α-linolenic acid (α-LA) from phospholipids or galactolipids in the plastid membrane by phospholipase A-type 1 (PLA1) lipases (Conconi *et al.*, 1996, Ryu, 2004, Bonaventure *et al.*, 2011). DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) (At2g44810; PLA-Iβ1) is the first established lipase to be involved in JA biosynthesis (Ishiguro

et al., 2001). In addition, there are seven PLA1s that group closely with DAD1 in phylogenic trees that also have predicted plastid transit peptides named DAD1-like PLA1s (Rudus *et al.*, 2014). Of these, DONGLE (DGL) was proposed to be the primary lipase involved in wound-induced JA biosynthesis in leaves (Hyun *et al.*, 2008) but a subsequent study disputed the claim and instead identified PLA-Iγ1 as another contributor to JA biosynthesis in wounded leaves (Ellinger *et al.*, 2010). Other recent studies have identified PLASTID LIPASE2 (PLIP2) and PLIP3 to be involved with ABA-induced JA biosynthesis (Wang *et al.*, 2018).

Upon release from the membrane lipids by phospholipases, α-LA is converted into *cis*-(+)-12-oxophytodienoic acid (OPDA) by 13-LYPOXYGENASE (LOX), ALLENE OXIDE SYNTHASE (AOS) and ALLENE OXIDE CYCLASE (AOC) in the plastid. OPDA is further metabolized in the peroxisome by a series of enzymes, including OPDA REDUCTASE 3 (OPR3), OPC-8:0 CoA LIGASE1 (OPCL1), ACYL COA OXIDASE1/5 (ACX1/5) and other β-oxidation cycle enzymes to produce jasmonic acid. Jasmonic acid is finally conjugated to an amino acid, most prominently, isoleucine by JASMONATE RESISTANT 1 (JAR1) in the cytosol (Staswick and Tiryaki, 2004).

Although the biosynthetic pathway is relatively well characterized, the regulatory aspects of the pathway and how JA biosynthesis is initiated upon wounding remain unclear (Koo and Howe, 2009, Bonaventure and Baldwin, 2010, Scholz *et al.*, 2015, Mielke *et al.*, 2021). The amount of JA in unwounded leaves can vary widely depending on developmental stage and environmental conditions but it is generally very low and only detectable by sensitive modern mass spectrometers (Creelman and Mullet, 1995, Schmelz *et al.*, 2003, Glauser *et al.*, 2009). Wounding activates rapid *de novo* synthesis of JA within 2-5 min both locally and systemically (Chung *et al.*, 2008, Glauser *et al.*, 2008, Koo *et al.*, 2009). The fast timing suggests that the biosynthetic capacity (e.g., enzymes) may be already present in untreated leaves before wounding (Maffei *et al.*, 2007).

In this study, we first used a series of physiological, pharmacological, genetic, and kinetic analyses of gene expression and hormone profiling to demonstrate that the early spiking of JA upon wounding does not depend on transcriptional or translational induction of JA biosynthetic genes. We then confirm and add to earlier findings that JA biosynthesis is only limited by substrate availability. Next, by using a transgenic system, we demonstrate how a decoupling between responses to wounding and JA prevents perpetual synthesis of JA in wounded leaves,

and identify *DAD1-like PLA1* and *JAR1* genes to the selective responders to wounding. We then use DAD1 as a wound-inducible model lipase to demonstrate that transient activation of *DAD1* transcription can trigger JA biosynthesis to a small extent but that additional wound-activated post-transcriptional steps boost DAD1-mediated JA synthesis. We show that this boosting effect does not require direct tissue damage by showing that it occurs over long distance in undamaged leaves. Finally, we report the findings about DAD1 protein stability under normal and stress conditions, as an example of post-transcriptional mode of regulation.

RESULTS

Wound-triggered JA biosynthesis precedes transcription of JA biosynthetic genes and is not stopped by inhibition of gene transcription or translation

When rosette leaves (leaf 3,4; local) of 24-d-old Arabidopsis were mechanically wounded using a pair of hemostats jasmonic acid and JA-Ile levels rose linearly for about 30 min (Supplemental Fig. S1A and B), consistent with what have been reported before (Chung *et al.*, 2008). Clear increases can be detected within 5 min. The level of JA before 5 min can be inferred through extrapolating a straight line through the origin (0 min), indicating that JA is made well before 5 min. There was also a rapid synthesis of jasmonic acid and JA-Ile in the systemic undamaged leaves (leaf 6,7) of wounded plants within 5 min of local leaf wounding (Supplemental Fig. S1C and D), as reported before (Glauser *et al.*, 2009, Koo *et al.*, 2009). A time course gene expression analysis was carried out on tissue samples collected together with the above JA profiling samples to see how transcription of JA biosynthetic genes and other JA responsive genes respond to wounding compared with the speed of hormone accumulation. All early (*OPR3*, *OPCL1, JAZ7*) and late (*JAR1*) gene markers were induced by wounding (Supplemental Fig. S1E). The earliest significant increases were detected at around 10 min for *OPCL1* and *JAZ7*, and 20 min for *OPR3* (Fig. 1E), and 30 min for *JAR1* (Supplemental Fig. S1E). Similar kinetic behavior of transcription was observed in the systemic leaves with increases of *OPCL1sys* detected the earliest among the four markers at 10 min (Supplemental Fig S1F). Thus, all marker genes were observed to lag behind the increases of JA in both the local and systemic tissues.

Next, we tested whether inhibition of gene expression could affect wound-induced JA biosynthesis. Plants were pretreated with 1 mM cordycepin, a potent transcriptional inhibitor (Sorenson *et al.*, 2018) for 1 h. Such treatment resulted in complete inhibition of *JAZ7* and *OPR3* transcription by wounding, demonstrating the efficacy of the treatment (Fig. 1A). Hormone measurements in those plants showed that cordycepin treatment did not cause JA levels to change compared to the mock treatment (Fig. 1B). When both the mock and cordycepin pretreated plants were wounded (1 h), there were no measurable differences in jasmonic acid or JA-Ile levels between the two groups (Fig. 1B), showing that transcriptional inhibition of JA biosynthetic genes had minimal impacts on wound-induced JA accumulation.

Building onto a similar idea, we then tested whether inhibition of protein translation could have any impact on wound-induced JA levels. Plants were pre-incubated in buffers containing 0.2 mM cycloheximide (CHX) which is a potent translational inhibitor (Chung *et al.*, 2008). Presence of CHX eliminated synthesis of DAD1-*Myc* protein in a transgenic plant (to be described more in later sections), both short term (15, 30, 60 min) and long term (6 h), demonstrating the efficacy of the treatment (Fig. 1C). However, wounding in the presence or absence of CHX had no impact on JA levels (Fig. 1D). CHX by itself did not change JA levels compared to mock as well (Fig. 1D). These results show that blocking transcription or translation has no major effect on wound-induced JA accumulation and that enzymes needed for initial JA biosynthesis are already present in these tissues before wounding.

Expression of JA biosynthetic genes alone does not trigger *de novo* JA biosynthesis

To test whether JA biosynthesis can be initiated by the induction of several JA biosynthetic genes, Arabidopsis leaves were sprayed with 5 μM coronatine (COR), a bacterial toxin and a potent mimic of JA-Ile which is known to induce JA responses (Katsir *et al.*, 2008). COR induced most, if not all, JA biosynthetic genes, including *LOX2*, *AOS*, *AOC1*, *OPR3*, *OPCL1*, and *ACX1* as shown by RNA-Seq experiment (Attaran *et al.*, 2014) (Supplemental Fig. S2A,), as well as by time course qRT-PCR analysis of two marker genes, *OPR3* and *JAZ8* (Supplemental Fig. S2B). However, the same treatment did not cause endogenous jasmonic acid nor JA-Ile to increase during the course of 12 h treatment (Supplemental Fig. S2C), consistent with earlier studies probing similar questions using structural variants of JA or isotope-labeled JA precursors

(Koch *et al.*, 1999, Miersch and Wasternack, 2000, Pluskota *et al.*, 2007, Scholz *et al.*, 2015) and our earlier experiment using COR (Koo *et al.*, 2009). Additionally, to see if pretreatment with COR has any impact on subsequent wound-induced JA biosynthesis, plants were first sprayed with COR for 1 h and then wounded for 30 min. Wounding increased jasmonic acid in both mock and COR-treated plants but there was no additional increase of jasmonic acid levels in the COR pretreated group (Supplemental Fig. S2C). For JA-Ile, there was even a strong reduction by COR pretreatment (Supplemental Fig. S2C), which may be attributed to the increased turnover (Caarls *et al.*, 2017, Heitz *et al.*, 2019, Poudel *et al.*, 2019) since many genes involved in JA-Ile catabolism (e.g., *CYP94C1*, *ILL6*) are also induced by COR (Supplemental Fig. S2A).

Precursor applications result in OPDA and JA biosynthesis without wounding

If induction of biosynthetic gene expression doesn't trigger JA biosynthesis, what will trigger JA biosynthesis? Here, we revisited some of the earlier studies where JA responses were elicited by exogenous supply of JA precursors, such as α-LA (Vick and Zimmerman, 1983, Farmer and Ryan, 1992, McConn and Browse, 1996, Christeller and Galis, 2014). In our experiment, we used intact Arabidopsis seedlings and a semi-*in vitro* system using isolated pea chloroplasts to study the kinetics of the α-LA conversion to JA metabolites. When Arabidopsis seedlings (12-d-old) were incubated in a liquid media containing 50 or 100 μM α-LA, there was a dose- and time-dependent increase of jasmonic acid (Supplemental Fig. S3A and B). The time-dependent was clear within 5 min of incubation which lasted until the end of the assay period of 1 h. We additionally treated *fad3fad7fad8* mutant with exogenous α-LA. The *fad3fad7fad8* mutant is deficient in endogenous α-LA and C16:3 FAs and consequently cannot produce JA even by wounding (Supplemental Fig. S3). However, when incubated with α-LA, *fad3fad7fad8* was able to accumulate jasmonic acid to levels equivalent to that produced by WT. This shows that even though *fad3fad7fad8* lacks endogenous α-LA or JA, it still possesses biosynthetic capacity to convert α-LA to JA. The fact that both WT and *fad3fad7fad8* produced similar levels of JA from exogenous α-LA substrate shows that the bulk of the JA in the WT was also made from the exogenously supplied precursor (α-LA) and not by secondary elicitation of more endogenous JA biosynthesis. Another important implication of these results is that the

preexisting enzymes of JA biosynthesis in these seedlings are constitutively active and does not require additional activation steps to be able to catalyze the biosynthetic steps.

Next, intact chloroplasts isolated from pea (*Pisum sativum*) were tested for semi-*in vitro* synthesis of OPDA by exogenous α-LA. Pea has been extensively used for isolating large quantities of intact chloroplasts (Koo *et al.*, 2004). In the absence of exogenous α-LA, there was minimal change of OPDA level over a course of 90 min incubation period. However, when supplied with α-LA (100 μM), a dramatic increase of OPDA (>1,200 pmol/mg chlorophyll (mgChl)) was observed within 5 min that saturated after 5 min (Supplemental Fig. S3E). This is a relatively large amount of OPDA compared to OPDA produced by wounded pea leaves (<50 pmol/mgChl) (Supplemental Fig. S4A). A trace amount of jasmonic acid (<5 pmol/mgChl) and no JA-Ile was detected in the incubation mixture as expected (Supplemental Fig. S4B). The precursor-product relationship was established by feeding these chloroplasts with a stable-isotope labeled α-LA ([$^{13}C_1$]-α-LA) which was converted to [$^{13}C_1$]-OPDA (Supplemental Fig. S3F) with no significant increase in unlabeled OPDA. These results demonstrate that similar to the whole seedlings, isolated chloroplasts which rely on all of their OPDA biosynthetic enzymes on the imports from the cytosol (since all are encoded by nuclear-genome) already possess the full biosynthetic capacity and that no other elicitation steps are needed to be able to convert exogenous α-LA to OPDA.

Most wound-inducible plastidial *PLA1* genes are not induced by JA in leaves

Membrane lipid hydrolysis that generates free FAs including α-LA is generally considered as the first step of JA biosynthesis. The substrate feeding experiments showing that the entire biosynthetic steps beyond the precursor generation step can run freely which strongly implies that such lipolysis step is the rate limiting step for the initiation of JA biosynthesis. Thus, we examined expression of seven plastid-localized *DAD1-like PLA1*s, including *DAD1*, *DGL* and *PLA-1γ1* that have previously been published for their role in JA biosynthesis (Yang *et al.*, 2007, Rudus *et al.*, 2014, Kelly and Feussner, 2016). Of these, five showed varying degree of transcript increases upon wounding (Fig. 2A). Induction was most prominent with *DAD1* followed by *DGL*, *PLA1-Iα2*, *PLA1-Iβ2*, and *PLA1-Iγ1*, although statistical significance was weak (p>0.05) for all except *DAD1*. Even though absolute comparison of expression levels between genes are

not accurate for this type of semi-quantitative qRT-PCR analysis, *DAD1* consistently gave the highest relative expression level changes followed by *DGL* and *PLA1-Iβ2*. Although they are expressed in response to wounding, none of them were induced until 20 min after wounding which is significantly slower than the increase of JA, casting doubts that their transcriptional induction elicits JA biosynthesis. Expression of *DAD1-like PLA1*s in the systemic leaves of wounded plants also resembled their local expression pattern (Supplemental Fig. S5), showing that the systemic wound signal can trigger their gene expression although, again, their induction needed at least 20 min (Supplemental Fig. S1C and D).

We then looked at the expression of these seven *DAD1-like PLA1*s in the COR-treated RNAseq data (Fig. 2B) (Attaran *et al.*, 2014). Interestingly, none of them were induced by COR. This is a clear deviation from the other JA biosynthetic genes that are induced by both wounding and COR (Supplemental Figs. S1 and S2). This pattern of expression was further verified with the *DAD1* gene by qRT-PCR in a side-by-side wounding vs. jasmonic acid treatment comparisons (Fig. 2C). Expression of marker genes *JAZ7* and *OPR3* showed that the jasmonic acid treatment was effective. In addition to *DAD1*, *JAR1*, another key enzyme in the production of JA-Ile, was also found to be exclusively induced by wounding but not by COR or jasmonic acid (Fig. 2B and C) (Suza and Staswick, 2008).

Transgenic plants expressing *DAD1* under JA-inducible promoter display symptoms of chronic exposure to JA

The observation that expression of *DAD1-like PLA1* genes can be turned on preferentially by wounding and not by JA/COR unlike other JA biosynthetic genes may be an important regulatory mechanism for preventing perpetual synthesis of JA. This is because if the entire JA pathway was under a positive feedback regulation, even a minor tissue damage or any other kind of stress or developmental program that causes JA to accumulate will be caught in a never-ending loop of JA synthesis. To disprove such auto-amplification theory, we simulated the free-running "lipase-JA-lipase-JA" circuit *in planta*. We generated a transgenic plant with a gene construct carrying the recombinant *DAD1* gene controlled by a JA-inducible *OPR3* promoter. Function of DAD1 for wound-induced JA biosynthesis in leaves is not proven through loss-of-function studies most likely due to functional redundancies among plastidial PLA1s (Ellinger *et*

al., 2010). However, *DAD1* was chosen here based on its proven biochemical activity (Ishiguro *et al.*, 2001) in JA-biosynthesis and it being the strongest wound-inducible *PLA1* in leaves (Fig. 2). For the JA-inducible promoter, a 1.5-kb upstream region of *OPR3* gene was used that had been shown to be effective in driving the expression of reporter genes in response to JA, wounding or insect herbivory (Body *et al.*, 2019). The resulting transgenic Arabidopsis lines carrying *OPR3promoter:DAD1* construct (*OPR3pro:DAD1*) were severely stunted and constitutively accumulated anthocyanin even when grown under standard growth conditions (Fig. 3A, B and D). These phenotypes were reminiscent of plants grown on JA-containing media. Introduction of the *OPR3pro:DAD1* construct in a JA-deficient *aos* mutant background suppressed the phenotypes and reverted back to the WT phenotype (Fig. 3A and D), showing that the stressed phenotypes of *OPR3pro:DAD1* were due to the JA pathway. RT-PCR analysis of *DAD1* transcripts showed that *DAD1* was expressed at high levels even in mock-treated *OPR3pro:DAD1* plants (Fig. 3C). Additive effects of exogenous JA on *DAD1* expression in the *OPR3pro:DAD1* plants were not obvious due to the already high basal levels. However, the induction was clearer in the *OPR3pro:DAD1*/*aos* plants where the basal level of *DAD1* transcripts was low (Fig. 3C). Exogenous JA treatment resulted in a substantial increase of anthocyanin in *OPR3pro:DAD1* compared to equally treated WT or *OPR3pro:DAD1*/*aos* (Fig. 3D). Hormone measurements showed that *OPR3pro:DAD1* plants constitutively accumulated high levels of jasmonic acid (~800 pmol/gFW) compared to WT or *OPR3pro:DAD1*/*aos* (<10 pmol/gFW) (Fig. 3E). JA-Ile levels were also higher in the *OPR3pro:DAD1* plants except that its relative content compared to jasmonic acid (~0.5%) was lower than those normally observed in wounded tissues (~10%) (Fig. 3E). This is likely contributed by low expression of *JAR1* in *OPR3pro:DAD1* plants even with increased jasmonic acid as shown earlier (Fig. 2C). These results illustrate the detrimental impacts of having *DAD1* expression controlled by JA, and thereby explain why it is necessary to have its promoter not respond to JA.

Transient induction of *DAD1* expression in leaves without wounding can trigger low levels of JA but cotreatment with wounding boosts JA synthesis several fold

We next wanted to know whether expression of *DAD1* is sufficient to trigger JA biosynthesis or there are other layers of regulation besides gene expression. Although, the

chronic JA phenotypes of *OPR3pro:DAD1* plants imply that *DAD1* expression without wounding can cause JA production, that could be due to *OPR3* promoter activity in various cell types at diverse developmental stages beginning from the embryos. In addition, as mentioned before, what was puzzling was that wound induction of *DAD1* expression (and other *PLA1*s) lags far behind JA biosynthesis (Fig. 2 and Supplemental Fig. S5) implying that gene expression is not the primary driver of initial JA burst. However, if preexisting DAD1 (and other PLA1) enzymes are responsible for JA biosynthesis upon wounding, then question rises as to why then they would not cause JA to accumulate in higher levels in resting plants. In order to further probe these questions, we generated a chemical-inducible transgenic system (Supplemental Fig. S6) where *DAD1* transcription can be induced by exogenous application of dexamethasone (dex) that does not occur in plants. Full-length *DAD1* fused to a C-terminus *Myc* epitope tag was cloned into a dex-inducible vector (Aoyama and Chua, 1997), and the resulting construct was transformed into Arabidopsis (*Pdex:DAD1-Myc*). Out of the 16 T1 plants that survived antibiotic marker selection, six lines displayed significant induction of *DAD1* transcript when their leaves were treated with 30 μM of dex (Supplemental Fig. S6A). Those six lines also contained more JA (Supplemental Fig. S6B). A homozygous line was selected and used for more detailed analyses.

A time series experiment showed that the increase of *DAD1* transcripts can be detected within 4 h of dex application and the levels continued to rise (8 h) (Fig. 4A). Immunodetection of DAD1-*Myc* using an antibody against the *Myc* epitope showed DAD1-*Myc* protein to be also induced by 4 h and continue to increase (8 h) (Fig. 4B). No DAD1-*Myc* protein was detected prior to the treatment with dex (0 h), showing absolute dependence of its expression on dex. Two of the three detected bands increased upon dex treatment over time and thus appeared to be specific to the DAD1-*Myc*. The upper weaker band (P) is likely to be the precursor form of DAD1-*Myc* before the cleavage of the chloroplast transit peptide and the stronger lower band (M) the mature form, judging from their sizes and preferential partitioning to the supernatant and the pellet fractions, respectively, upon centrifugation of chloroplasts (Supplemental Fig. S7A). Apart from the two, one which appears to be a nonspecific band was detected in all samples. This band appears only when certain batches of commercial *Myc* antibodies are used (e.g., Fig. 4B, 6A, 7A and Supplemental Fig. S7A) and does not appear when other batches are used (Figs. 5C, 7B-C and 7F). Importantly, correlated with the increases of DAD1-*Myc* transcripts and

proteins, there were increases of jasmonic acid and JA-Ile (Fig. 4C and Supplemental Fig. S7B). This shows that expression of DAD1 can trigger JA production without wounding. However, the levels were quite low. Compared to ca. 7 nmol/gFW of jasmonic acid induced by wounding (2 h), dex triggered only ca. 0.5 nmol/gFW (Fig. 4C). JA-Ile to jasmonic acid ratio was also low (< 2%) when induced by dex compared to that by wounding (~ 10 %) (Fig. 4C and Supplemental Fig. S7B). This low level of JA-Ile was similar to what was observed in *OPR3pro:DAD1* plants (Fig. 3E) and likely to be related to the unresponsiveness of *JAR1* expression to JA (Fig. 2C and Supplemental Fig. S7C and D).

We then tested whether wounding has any additional effect on DAD1-induced JA biosynthesis. For this, *Pdex:DAD1-Myc* plants that had already been treated with dex were subsequently wounded and were compared with those that received only dex or only wounding treatments. Singular treatment with dex had very little effect on the increase of jasmonic acid (~ 50 pmol/gFW) (Fig. 4E) as seen before (Fig. 4C). Wounding raised the level to ~ 8 nmol/gFW which is equivalent to the level reached by wounded WT leaves (Fig. 4E). However, when both dex and wounding were applied together in the *Pdex:DAD1-Myc* plants, the jasmonic acid level rose to as much as 50 nmol/gFW, which is about 6-fold compared to wounding alone and 1000-fold compared to dex treatment alone (Fig. 4E). *DAD1* transcript levels remained similar between dex and dex+wound treated plants during these treatments (Fig. 4D). These results show that although DAD1 expression alone (by dex) can elicit some JA synthesis additional wounding significantly enhances this DAD1-mediated JA biosynthesis.

While carrying out these experiments, we also noticed that there were variations in the levels of JA induced by dex between experiments with the jasmonic acid levels ranging from ca. 50 pmol/gFW (Fig. 4E) to ca. 500 pmol/gFW (Fig. 4C). We eventually found that these variations were strongly influenced by the age of the plants (Fig. 5A). JA content was especially very low (< 2 pmol/gFW) in plants younger than 15-d-old but increased slowly until 25-d, and by 30-d, the levels jumped to 7 nmol/gFW. This was not due to variabilities in the inducibility of *DAD1* transcripts nor DAD1 proteins by dex in these plants (Fig. 5B and C). It was also not due to the variability in their abilities to synthesize JA by wounding in these different-aged plants (Fig. 5D). The reason for this developmental variation is still unclear but the observations from earlier stage (<15-d) plants are especially telling because they represent cases where presence of abundant DAD1 proteins is failing to trigger JA biosynthesis, implying additional layer(s) of

regulation for JA biosynthesis upon wounding. Wounding can unlock this limitation imposed upon DAD1 regardless of their ages, because the cotreatment with dex and wounding resulted in large boosts of JA synthesis in both stages (Fig. 4E and Fig. 5E).

The wound signal that amplifies JA production by *DAD1-Myc* can be transmitted systemically over a long distance

This putative regulatory element that is activated by wounding to boost DAD1-*Myc*-mediated JA synthesis may be physicochemical by nature, happening as a result of random cell breakage rather than by a controlled signaling mechanism. Examples of such uncontrolled events may include random mixing of DAD1-*Myc* enzymes with broken membrane debris out of cellular context. This possibility can be tested by looking at the systemic wound responses in undamaged intact tissues. If DAD1-Myc proteins that had been pre-induced (by dex) in these systemic leaves can be activated to boost JA biosynthesis as seen earlier by systemic wound signal coming from remote damaged leaves, this would discount the artifactual uncontrolled enzyme theory and favor a regulated posttranslational activation model. To pre-induce systemic DAD-Myc expression, the systemic leaves (leaf 6 and 7) were treated with dex for 6 h prior to any wounding (Fig. 6). Then, the untreated local leaves (leaf 3 and 4) were wounded. JA was measured in the systemic undamaged leaves 15 min after the local leaf wounding. As a control, the same experiment was carried out on *Pdex:DAD1-Myc* that had not been treated with any dex. As expected, DAD1-*Myc* protein was induced by the dex treatment in the systemic leaves but not in the local untreated leaves (Fig. 6A). Wounding of the control plants (no dex) increased systemic jasmonic acid levels to ~70 pmol/gFW (Fig. 6B inset). The dex-alone in the systemic leaves induced jasmonic acid to ~7 nmol/gFW (UW+dex in Fig. 6B) as seen before (Fig. 5). However, when the local leaves were wounded this systemic jasmonic acid level rose to ~35 nmol/gFW (W+dex in Fig. 6B), a 5-fold increase from dex alone and a 500-fold increase from wounding alone.

DAD1 protein is unstable and can be stabilized by wounding but degrades more quickly in the presence of α-LA

One of our hypotheses was that the molecular target for the signaling event that boosted DAD1-*Myc*-mediated JA biosynthesis may be the lipase itself. We began exploring this possibility by monitoring the dynamics of DAD1-*Myc* proteins levels over time (Fig. 7A). DAD1-*Myc* was first induced by treating *Pdex:DAD1-Myc* plants with dex for 12 h, and then 0.2 mM CHX was added to inhibit protein translation. Protein extracts at various time points were then probed with antibodies against *Myc* (for DAD1-*Myc*) and compared with other JA-biosynthetic marker proteins, LOX, AOC and JAR1 (Fig. 7A). Interestingly, in contrast to LOX, AOC, or JAR1 that remained largely unchanged over the monitored period of 3 h, DAD1-*Myc* levels were markedly reduced by 1 h indicative of faster protein turnover. Using similar assay, we then tested whether DAD1-*Myc* protein stability is affected by the presence of α-LA which is the product of DAD1 catalysis and the primary precursor of JA biosynthesis (Fig. 7B). Inclusion of different concentrations of α-LA in the incubation media was found to promote the degradation of DAD1-*Myc* protein, resulting in a clear reduction of signal by 40 min with 50 μM α-LA and 10-20 min with 100 μM α-LA. Encouraged by the results, we then tested whether wounding has any effect (Fig. 7C). In contrast to α-LA, wounding delayed degradation of DAD1-*Myc*, maintaining signals after 90 min when most of them in the no wounding samples disappeared. Next, we created a catalytically inactive version of DAD1-*Myc* enzyme, DAD1^{mut}-*Myc*, by introducing Ala substitutions to the amino acid residues that constitute the catalytic triad (S295A, D352A, and H416A) (Ishiguro *et al.*, 2001) which is critical for the catalytic activity of lipases (Fig. 7D). Consequently, stably transformed Arabidopsis lines expressing this mutated version of gene (*Pdex:DAD1^{mut}-Myc*) were not able to induce JA biosynthesis by dex nor could boost its accumulation by dex+wound treatment (Fig. 7E). Interestingly, the mutated DAD1^{mut}-*Myc* protein persisted longer than DAD1-*Myc* (Fig. 7F). Together, these results show that DAD1 protein stability may be one of the targets of regulation, perhaps contributing to elicitation of JA biosynthesis in wounded leaves.

DISCUSSION

Although JA biosynthetic pathway has been elucidated (Vick and Zimmerman, 1983, Wasternack and Hause, 2013) and transcription of biosynthetic genes has been extensively studied (Reymond *et al.*, 2004, Devoto and Turner, 2005, Pauwels *et al.*, 2009, Howe *et al.*,

2018), how JA can be produced so quickly by wounding remains unclear. The fast synthesis of JA by wounding cannot be easily explained by a model that depends on transcriptional activation of JA biosynthetic enzymes. The biosynthetic gene transcripts lagged far behind JA accumulation both in the local and systemic leaves (Fig. 2, Supplemental Figs. S1 and S5). Induction of JA biosynthetic gene expression by COR or JA could not elicit *de novo* synthesis of JA, consistent with earlier reports (Koch *et al.*, 1999, Miersch and Wasternack, 2000, Pluskota *et al.*, 2007, Koo *et al.*, 2009, Scholz *et al.*, 2015) nor did it result in more synthesis of JA when wounded subsequently (Supplemental Fig. S2). This is consistent with failed previous attempts to elevate basal JA levels by simply overexpressing a few biosynthetic enzymes in transgenic plants even though subsequent wounding of these plants may have raised JA levels over WT (Laudert *et al.*, 2000, Bachmann *et al.*, 2002, Stenzel *et al.*, 2003, Sharma *et al.*, 2006, Wasternack, 2007, Rudus *et al.*, 2014) despite claims of success by some others (Harms *et al.*, 1995). More direct evidence against the transcriptional activation model came from our inhibitor studies where blockage of either transcription or translation had no major impact on wound induced JA biosynthesis (Fig. 1).

In fact, the positive feedback model of JA synthesis by JA-responsive gene expression can be problematic. This was demonstrated by our transgenic system (*OPR3pro:DAD1*) that was designed to produce more JA by JA-responsive gene expression (Fig. 3). We found that there may be at least two steps of JA biosynthesis that prevent this run-on feedforward mechanism. One is the lipid hydrolysis step at the beginning of JA biosynthesis and the second is the final conjugation step that joins nascent jasmonic acid with Ile. All seven plastidial *DAD1-like PLA1*s and *JAR1* selectively responded to wounding but not to JA or COR (Fig. 2). In addition, on multiple occasions (Fig. 3E and Supplemental Fig. S7), JAR1 acted as a limiting factor for JA-Ile increases despite high jasmonic acid levels. For examples, JA-Ile levels were ~ 0.5 % of jasmonic acid levels both in *OPR3pro:DAD1* and dex-induced *Pdex:DAD1-Myc.* These levels are much lower compared to the wound response where JA-Ile levels typically reach 5–20% of the jasmonic acid levels (Supplemental Fig. S1A and B) (Suza and Staswick, 2008) or even higher (> 20%) in the systemic leaves (Supplemental Fig. S1C and D). In this way, plants seem to have been selecting to exclude *PLA1*s and *JAR1* from the positive feedforward mechanism which is prevalent among other JA metabolic and signaling genes.

With regards to the major limiting factor for JA biosynthesis, there is a wide consensus among scientists that it is the substrate or the precursor availability (Vick and Zimmerman, 1983, Farmer and Ryan, 1992, McConn and Browse, 1996, Wasternack, 2007, Christeller and Galis, 2014). Our substrate feeding assays using whole Arabidopsis seedling and isolated pea chloroplasts (Supplemental Fig. S3A-B) once again made it clear that exogenously supplied α-LA can be converted to JA without wounding. It is important to point out the implications of this observation, that is, all enzymes in the pathway are present and active in leaves prior to wounding. This is not to say that any other additional regulatory step(s) may not exist, for example, to fine tune the catalytic potential of various enzymes in the pathway, but to emphasize the fact that the biosynthesis will run its full course without any need for an intervention as long as the substrate is available. The fact that *fad3fad7fad8* could metabolize exogenous α-LA to JA with the same rigor as the WT showed that the basal level of biosynthetic enzymes is maintained even in the absence of JA (Supplemental Fig. S3D). The *fad3fad7fad8* results also discount the possibility of α-LA acting as a signaling molecule to initiate JA biosynthesis (e.g., by triggering more lipid hydrolysis) rather than being a mere substrate for these feeding experiments. This is further supported by the stable isotope feeding experiment where [^{13}C]-labelled α-LA was converted to [^{13}C]-OPDA without any increases in endogenous OPDA (Supplemental Fig. S3F). The chloroplast feeding experiment also shows that the chloroplasts even when isolated from the cytosol have enough enzymes to sustain initial synthesis of OPDA. The reaction, however, plateaued within 5 min. It is not clear whether this is due to the complete exhaustion of α-LA substrates or cessation of enzyme catalysis (either by turnover or inactivation). Immunoblots seem to indicate that at least LOX and AOC proteins seem to be present for hours (Fig. 7A) although this may be little different in isolated chloroplasts where fresh supply of enzymes from the cytosol is cut off. In addition, we routinely observed that the isolated chloroplasts are active hours after preparation. The substrate exhaustion theory can be explained by other metabolic sinks besides OPDA synthesis, such as incorporation into glycerol lipids (Koo *et al.*, 2005). Alternatively, we cannot rule out the possibility of feedback inhibition by OPDA. More biochemical assays are needed to explore this avenue of research.

Several lipases involved in JA biosynthesis have been described from multiple species (Ishiguro *et al.*, 2001, Hyun *et al.*, 2008, Kallenbach *et al.*, 2010, Cai *et al.*, 2014, Wang *et al.*, 2018). However, those involved in wound-induced JA biosynthesis in Arabidopsis have

remained elusive due to problems attributed to gene redundancy (Ellinger *et al.*, 2010). Our gene expression study identified several of these plastidial *DAD1-like PLA1*s to be induced by wounding both in the local and systemic leaves. Of these, we chose DAD1 as a model lipase in our study because of its proven biochemical function in JA biosynthesis. Although contribution of DAD1 to wound-induced JA biosynthesis in leaves is not demonstrated through loss-of-function studies (Ellinger *et al.*, 2010) and DAD1 is considered mainly as a flower lipase, its strong induction by wounding in leaves combined with its enzymatic activity makes it likely to contribute to JA biosynthesis in the wounded leaves. JA accumulation in both the *OPR3pro:DAD1* and *Pdex:DAD1-Myc* plants support this prediction. However, there is more to it than its mere presence that elicits JA biosynthesis.

First, the fast speed of wound-elicited JA biosynthesis (Supplemental Fig. S1) would suggest action of pre-existing DAD1 (and other PLA1s). But the low basal levels of JA in untreated leaves would indicate their limited activity. Second, the chronically stressed phenotypes of *OPR3pro:DAD1* plants would suggest its enzymatic action in the absence of wounding (Fig. 3). It must however be taken into account that *OPR3* promoter driven *DAD1* can potentially be expressed in all cell types throughout all developmental stages. In addition, since *OPR3pro:DAD1* is under the positive feedforward regulation by JA, all these cells at all developmental stages are expected to contain higher levels of JA than those in the WT plants. Nevertheless, it does show that when DAD1 is present (in high levels) it will cause JA to be made. Third, transient expression of DAD1-*Myc* in *Pdex:DAD1-Myc* plants induced JA accumulation (Fig. 4). However, the JA levels varied depending on the developmental stage of the leaves with the younger than 15-d old plants showing minimal accumulation. In fact, the JA levels stayed relatively low in most developmental stages until the plants reached 30 d (Fig. 5). The fact that the induced DAD1-*Myc* protein levels remained similar throughout these developmental stages and that there was essentially no difference in their abilities to synthesize JA upon wounding clearly show that there is more to it than the mere presence of DAD1 proteins for the full elicitation of JA biosynthesis. This is consistent with previous reports showing minimal increases in JA when DAD1 or other PLA1s were expressed under cauliflower mosaic virus 35S promoter (Ellinger *et al.*, 2010, Rudus *et al.*, 2014). These studies were mostly focused on wound-induced JA levels but their unwounded data show little change from the controls. It may be worthwhile to note that sudden increase in JA at 30 d coincide with the vegetative-to-

reproductive transition. The significance of this is currently unknown. Fourth, regardless of plant ages, wounding boosted the production of JA to several fold higher than that by *DAD1-Myc* expression alone (by dex) or by wounding alone (Figs. 4 and 5). This boosting effect cannot be explained by differences in *DAD1-Myc* expression levels since they were not different whether the plants were wounded or not whether the plants were young or old. It is also unlikely scenario that DAD1-Myc has somehow impacted other lipase activity. This boosting effect can be recapitulated in remote undamaged leaves indicating that the effect is based on a signaling event rather than by misregulation, for example, by random mixing of enzymes and substrates in broken cells. Thus, it seems like DAD1 when expressed can induce JA biosynthesis. However, the degree to which it can elicit JA is heavily influenced by the cell type and developmental stage contexts. Importantly, wound signal can lift sanctions imposed upon DAD1, allowing it to exert its greater potential.

DAD1-*Myc* protein was found to be more labile than other biosynthetic enzymes such as LOX, AOC or JAR1 (Fig. 7). Fast turnover is a hallmark of tight regulation. Plants may have to maintain certain level of lipases all the time for rapid response upon attacks. For this, lipases may be expressed at low levels all the time. However, plants seem to degrade them constantly to avoid their overaccumulation. The benefit must be outweighing the high cost of maintaining this system. DAD1 stability was also affected by the presence of α-LA and by wounding. Mutation in catalytic region could delay the degradation. While this does not explain, and may not even be directly linked to, the initiation of JA biosynthesis, it shows that the DAD1 stability is sensitive to factors occurring during wound-induced JA biosynthesis. More research is needed to understand the nature of this modulation of DAD1 protein stability by α-LA and wounding, and its relationship to the regulation of JA biosynthesis.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis was grown under long day conditions (16 h light) with 100 –120 μE m^{-2} s^{-1} light intensity in growth chambers kept at 22 °C. The wild-type (WT) used for all experiments was *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). *fad3fad7fad8* (McConn and Browse,

1996) was a gift from Dr. John Browse at Washington State University. Seedlings were either grown on solid Murashige and Skoog (MS) media (Caisson Laboratories) (0.7% w/v phytoblend agar, 0.7% w/v sucrose) or on soil. *Pisum sativum* var. little marvel (Green Seed Company, Springfield, MO) used for chloroplast isolation was surface sterilized by 50 % bleach and imbibed at 4 °C for two days prior to being sowed on a soil mixture of half perlite half soil. The peas were grown in short day conditions (10 h light / 14 h dark, 100 –120 μE m^{-2} s^{-1}) at 22 °C. All tissues were harvested and flash frozen in liquid nitrogen and stored in -80 °C until use.

Chemicals and antibodies

(±)-Jasmonic acid, methyl jasmonic acid (MeJA), coronatine (COR), α-LA ((9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid)), [$^{13}C_1$]-α-LA, dexamethasone (dex), cycloheximide (CHX), and cordycepin were purchased from MilliporeSigma (Burlington, MA). JA-Ile, [$^{13}C_6$]-JA-Ile, OPDA, [$^{2}H_5$]-OPDA, and dihydro-JA have been described previously (Koo *et al.*, 2009). The primary antibody for JAR1 was raised in rabbits following the company's instruction (Cocalico Biologicals, Stevens, PA). The full-length JAR1 cloned into a pET28a vector with an N-term His tag (Westfall *et al.*, 2012) was a gift from Dr. Joseph Jez from Washington University in St. Louis. The construct was transformed into Rosetta 2 (DE3) cells and the recombinant protein was purified using Ni-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) followed by desalting and concentration via ultrafiltration (Amicon Ultrafilters, MilliporeSigma) before sending to the company. Polyclonal antibody against *Myc* tag (rabbit), plastidial LOX, and peroxidase-conjugated anti-rabbit secondary antibody were purchased from Abcam (Cambridge, UK), Agrisera (Sweden) and MilliporeSigma, respectively. Anti-AOC (Hause *et al.*, 2000) was gift from Dr. Bettina Hause of Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany.

Plasmid vector constructs and transgenic lines

Sequence information for DNA primers used in this study is in Supplementary Table S1. The *Pdex:DAD1-Myc* plant binary vector construct was made by PCR-amplifying the full-length *DAD1* open reading frame (ORF) fused to 4×*Myc* epitope tag using overlapping PCR technique

using SpeI_DAD1_F and SpeI_FlagMyc_R primers and an equimolar mixture of *DAD1* ORF and 4×*Myc* DNA fragments as templates. The two template fragments were each individually prepared by PCR using primer sets SpeI_DAD1_F and ov_DAD1-Myc_R, and ov_DAD1-Myc_F and SpeI_Myc_R, respectively. The resulting *DAD1-Myc* was cloned into the *Spe*I sites of the glucocorticoid-inducible vector system (*Pdex*) (Aoyama and Chua, 1997, Koo *et al.*, 2009) to generate *Pdex:DAD1-Myc*. The site-directed mutagenesis of *Pdex:DAD1^{mut}-Myc* was done using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs, Ipswich, MA) following the manufacturers protocol. Mutagenic primers were designed using the NEBase Changer tool and can be found in Supplementary Table S1. Mutagenesis reactions were performed on the above generated *DAD1-Myc* ORF cloned in pGEM-Teasy vector (Promega, Madison, WI). The resulting construct with substitutes in the lipase consensus motif GSHLG to AAAAA was then subcloned into *Pdex* vector using the *Spe*I restriction site. The *OPR3pro:DAD1* was constructed by first putting the *DAD1* into a modified pBI121 binary vector (Schilmiller *et al.*, 2007) using the *Spe*I site. A 1.5 kb promoter region of the *OPR3* was amplified by PCR from the WT genomic DNA using ClaI_OPR3p_F and BamHI_OPR3p_R primers and the resulting PCR product was cloned in front of the *DAD1* using *Cla*I and *Bam*HI sites.

Above generated three plasmids were first transformed into the C58C1 strain of *Agrobacterium tumefaciens*, then into WT or *aos* backgrounds using the floral dip method (Clough and Bent, 1998). The flowers of *aos* were sprayed with 100 μM MeJA solution once every day beginning from 3 days prior to and 5 days post the floral dipping. Seeds harvested from resulting plants (T1) were screened for resistance to either glufosinate-ammonium (10 μg mL^{-1}) for *Pdex:DAD1-Myc* and *Pdex:DAD1^{mut}-Myc* or kanamycin (50 μg mL^{-1}) for *OPR3pro:DAD1-Myc*.

Wounding and chemical treatments

Wounding was administered by crushing across the midrib two to three times using a pair of serrated-tip hemostats. Systemic wounding was performed similarly except that two adult leaves (leaf 3,4) were wounded per rosette and two younger unwounded leaves (leaf 6,7) were harvested. Solution containing dex (30 μM in 0.01% Triton X-100) was either sprayed (to saturation) or applied as tiny droplets (20-30 μL per leaf) to the adaxial side of the leaf for the

indicated durations (typically 6-12 h). α-LA feeding of Arabidopsis seedlings was done by incubating the seedings in liquid MS media containing α-LA. α-LA was dried under N_2 gas with few droplets of ammonium hydroxide and reconstituted in liquid MS media (< 0.01% DMSO) to their final concentrations. For CHX treatment, 12-14-d old seedlings were transferred from MS plate to liquid MS media containing 0.2 mM CHX and incubated for the indicated times. Cordycepin treatment was carried out similarly in liquid MS media containing 1 mM of cordycepin 1 h before subsequent treatments. Wounding of CHX/cordycepin treated seedlings was done by indiscriminately but consistently crushing seedlings several times with hemostats while submerged in the media. COR (5 μM) was sprayed evenly on the surface of fully expanded mature leaves for indicated times.

Chloroplast incubation assay

Intact chloroplasts were isolated from 10-12-d old pea seedlings using continuous Percoll (GE Healthcare Life Sciences, Chicago, IL) density gradient method (Perry *et al.*, 1991). Chlorophyll content was determined according to method by (Arnon, 1949). α-LA feeding assay was carried out largely according to previously described methods (Koo *et al.*, 2004). The reaction mixture consisted of 200 μL incubation buffer containing 100 μM α-LA and 50 μg chlorophyll-equivalent chloroplasts. The reaction was initiated by adding chloroplasts to the reaction mixture in ambient temperature (25 °C) under light (80 –100 μE m^{-2} s^{-1}) while constantly shaking on a benchtop orbital shaker. Reaction was stopped by adding equal volume (200 μL) of 100% methanol containing internal standards for hormone analyses. Crude chloroplasts from Arabidopsis for immunoblot analysis were isolated based on procedures by (Salie *et al.*, 2016) with modification. About 10 g of freshly harvested 20-25-d old leaf tissues were homogenized in ice-cold grinding buffer (50 mM HEPES-KOH pH 8.0, 330 mM sorbitol, 1.5 mM $MnCl_2$, 2 mM $MgCl_2$, 2 mM EDTA, 0.1% (w/v) BSA) using a polytron homogenizer (Kinematica, Switzerland). The homogenate was passed through two layers of Miracloth, prewet with homogenization buffer, and then centrifuged at 2,600 ×*g* at 4 °C for 20 min. The supernatant was kept as the fraction containing no chloroplasts. Chloroplasts in the pellet fraction were lysed for 30 min in ice-cold lysis buffer (50 mM HEPES-KOH pH 8.0, 10% (v/v) glycerol,

680 0.5% (v/v) Triton X-100). Lysates were then further homogenized in a Dounce homogenizer on
681 ice and then centrifuged at 30,000 ×*g* for 20 min at 4 °C.
682
683 **Protein extraction and immunoblots**
684
685 Proteins were typically extracted with 50 mM sodium phosphate buffer (pH 7.0) containing
686 10% glycerol, 250 mM NaCl, 0.1% SDS, 1% Triton, and protease inhibitor tablets (Thermo
687 Scientific, Waltham, MA). Frozen tissue (50–100 mg) was ground into a fine powder using
688 TissueLyserII (Qiagen). About three times the tissue weight volume of extraction buffer was
689 added and upon brief vortex, samples were spun down for 1 min at 16,000 x *g* to remove debris.
690 Samples were incubated with sample buffer consisting of 2×Laemmli buffer and 6 M urea at 37
691 °C for 30 min before loading into 10% SDS-PAGE gel (20 μg per lane). For immunoblot
692 analysis, proteins were transferred to PVDF membrane and probed with primary antibodies
693 against AOC, *Myc* and JAR1 at a 1:3000 dilution and LOX at a 1:15,000 dilution. An anti-rabbit
694 secondary antibody (MilliporeSigma) conjugated to peroxidase was used at a 1:15,000 dilution.
695 Protein-antibody complexes were visualized with SuperSignal West Pico Chemiluminescent
696 substrate (Thermo Scientific) and exposed on an X-ray film (Midwest Scientific, Valley Park,
697 MO).
698
699 **RNA Analysis**
700
701 Total RNA was extracted from 50-100 mg of frozen tissues that were ground to a fine
702 powder while frozen. Ten times tissue volume of TRIzol reagent (Thermo Fischer Scientific,
703 Waltham, MA) was used for extraction followed by purification using the Direct-zol RNA
704 MiniPrep Plus Kit (Zymo Research, Irvine, CA) following manufacturers' instructions. One μg
705 of RNA was reverse transcribed using the iScript Reverse Transcription Supermix (BioRad,
706 Hercules, CA) following manufacturer's instructions. This was used as a template for semi-
707 quantitative PCR (qPCR) with the iTaq SYBR Green Supermix (BioRad) in a CFX96 Touch
708 real-time PCR detection system (BioRad). *ACT8* (At1g49240) was used as the internal reference
709 gene. The oligonucleotide primers for *JAZ7*, *JAZ8*, *OPR3*, *JAR1* and *ACT8* have been described

earlier (Poudel *et al.*, 2016, Zhang *et al.*, 2016, Kimberlin *et al.*, 2021). Primers for *OPCL1*, *DAD1* and other *DAD1-like PLA1s* are listed in Supplemental Table S1.

Quantification of anthocyanin, OPDA and JA

Anthocyanin measurement was according to (Poudel *et al.*, 2016) from 20-50 mg of leaf tissues from 30-d-old soil grown plants. Quantification of OPDA, jasmonic acid and JA-Ile was done using an electrospray ionization (ESI) triple quadrupole tandem mass spectrometer (Xevo T-QS, Waters, Milford, MA) operated at negative ion mode interfaced with ultraperformance liquid chromatography (ACUITY H-class, Waters) as described (Koo *et al.*, 2014). JA metabolites from the chloroplast incubation were centrifuged at 16,000 x *g* for 30 min at 4 °C and 60 µL of supernatant was mixed with the same volume of internal standard mixture. Five to seven µl of sample was separated on Ascentis Express C18 column (2.7 µm, 2.1 x 50 mm; Supelco, Bellefonte, PA) heated to 40 °C. The MRM method detects the following characteristic *m*/*z* transitions from precursor to product ions: 291>165 for OPDA, 296>170 for [$^{2}H_{5}$]-OPDA, 292>165 for [$^{13}C_{1}$]-OPDA, 209>59 for jasmonic acid, 211>59 for dihydro jasmonic acid, 322>130 for JA-Ile, and 328>136 for [$^{13}C_{6}$]-JA-Ile. LOQ with compounds mix with organic extracts from Arabidopsis, pea or isolated chloroplasts was 1 nM for jasmonic acid and OPDA and 0.1 nM for JA-Ile. Data acquisition and processing was done using MassLynx 4.1 and TargetLynx software (Waters).

Accession numbers

Accession numbers for genes appearing in this paper are as follows from Arabidopsis.org and National Center for Biotechnology Information library. *DAD1* (At2g44810, AEC10469.1); *OPR3* (At2g06050, AEC06000.1); *LOX2* (At3g45140, AEE77997.1); *AOS* (At5g42650, AED94842.1); *AOC1* (At3g25760, AEE77065.1); *CYP94C1* (At2g27690, AEC08026.1); *ILL6* (At1g44350, AEE32032.1); *OPCL1* (At1g20510, AEE29980.1); *JAR1* (At2g46370, AEC10684.1); *JAZ7* (At2g34600, AEC08997.1); *JAZ8* (At1g30135, AEE31184.1); *ACT8* (At1g49240, AEE32408.1); *DGL* (At1g05800, AEE27895.1); *PLA1-Iα2* (At2g31690, AEC08573.1); *PLA1-Iβ2* (At4g16820, AEE83808.1); *PLA1-Iγ1* (At1g06800, AEE28039.1);

PLA1-Iγ2 (At2g30550, AEC08407.1); *PLA1-Iγ3* (At1g51440, AEE32668.1). RNA-seq read data were based on supplemental data provided by (Attaran *et al.*, 2014).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Primers used in this study.

Supplemental Figure S1. Wound-induced JA biosynthesis spikes earlier than JA-biosynthetic or JA-responsive gene transcripts.

Supplemental Figure S2. Coronatine is not able to elicit nor enhance wound-induced JA biosynthesis.

Supplemental Figure S3. JA synthesis by exogenous α-LA feeding.

Supplemental Figure S4. JA and OPDA synthesis by pea leaves and chloroplasts.

Supplemental Figure S5. Wound-induced expression of *DAD1-like PLA1*s in the systemic undamaged leaves.

Supplemental Figure S6. Establishing the *Pdex:DAD1-Myc* lines.

Supplemental Figure S7. Effect of ectopic expression of DAD1 on endogenous JA-Ile accumulation and JAR1 protein levels.

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We thank Dr. Bettina Hause from Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany, Dr. Joseph Jez in Washington University in St. Louis, Dr. John Browse in Washington State University, and Dr. Douglas D. Randall from the University of Missouri for sharing AOC antibody, JAR1 vector construct, *fad3fad7fad8* seeds, and pea seeds, respectively. Taylor Wagner is acknowledged for technical supports. MU Small Molecules MS Lab is acknowledged for instrumental assistance with UPLC-MS/MS.

Figure legends

Figure 1. Inhibition of gene transcription or translation has no impact on wound-elicited JA biosynthesis. A-B, qRT-PCR analysis of *JAZ7* and *OPR3* (A), and jasmonic acid and JA-Ile levels (B) in unwounded and wounded plants after treating with mock or cordycepin (Cordy).

Arabidopsis seedlings (14-d) were incubated with mock (0.01% w/v ethanol in water) or 1 mM Cordy in liquid MS media for 1 h before wounding (1 h (A) or 0.5 h (B)). C-D, Protein immunoblot (C) of DAD1-*Myc* protein expression and JA levels (D) in *Pdex:DAD1-Myc* plants incubated in MS media with or without 0.2 mM cycloheximide (CHX). After 15 min of pre-incubation, DAD1-*Myc* expression was induced by adding 30 μM dexamethasone (dex) and incubated for shown duration of time. DAD1-*Myc* in the protein extract was probed with an antibody against *Myc* epitope tag. For JA measurements, the CHX-treated and untreated (mock) plants were either not wounded (UW) or wounded for another 1 h. Bar graphs represent mean ± SD of three biological replicates. Letters in graphs indicate statistical significance ($P < 0.05$) as determined by pairwise *t*-tests.

Figure 2. Expression of *DAD1-like PLA1* lipases is induced by wounding but not by COR or JA. A, Time course of *DAD1-like PLA1* expression by wounding. Total RNA from wounded Arabidopsis leaves was subjected to qRT-PCR analyses. B, RNA-seq analysis of *DAD1-like PLA1s*, *JAR1*, and *OPCL1* in Arabidopsis seedlings sprayed with 5 μM COR. Data are from publicly available RNAseq data by Attaran et al., (2014). Number in each cell indicates $\log_2$ of fold change (FC) over the untreated. Heat map is according to the color scale shown. C, qRT-PCR analyses of *DAD1* and *JAR1* in response to wounding or exogenous jasmonic acid (50 μM) treatment. *JAZ7* and *OPR3* show effectiveness of jasmonic acid treatment. Relative expression values are fold-difference compared to the mock. *ACT8* was used as an internal reference gene and bar graphs represent mean ± SD of three biological replicates. Letters above bar graphs indicate statistical significance ($P < 0.05$) as determined by pairwise *t*-tests.

Figure 3. Transgenic Arabidopsis expressing *DAD1* controlled by JA-responsive promoter displays symptoms of chronic JA accumulation. A, Photos of 4-week-old WT, *OPR3pro:DAD1* (three independent lines) and *OPR3pro:DAD1* in *aos* background grown under normal growth condition. B, Image of WT and *OPR3pro:DAD1* seedlings (12-d) grown on MS plates. C, RT-PCR analysis of *DAD1* expression in leaves of WT, *OPR3pro:DAD1* or *OPR3pro:DAD1/aos* treated with mock (0.01% ethanol in water) or 50 μM jasmonic acid. *ACT8* is an internal reference. D, Anthocyanin levels in WT, *OPR3pro:DAD1* and *OPR3pro:DAD1/aos* plants (4-week) with or without jasmonic acid treatment. Mock or 20 μM jasmonic acid was sprayed once

at 15-d stage. E, Jasmonic acid and JA-Ile in WT, *OPR3pro:DAD1* and *OPR3pro:DAD1/aos* grown as in (A). Data represent mean ± SD of three biological replicates with statistical significance (pairwise *t*-tests, $P < 0.05$) denoted by letters above the bars.

Figure 4. JA levels in plants transiently expressing *DAD1*. A-C, qRT-PCR (A), protein immunoblot (B), and hormone (C) analyses showing increases of *DAD1-Myc* transcripts, proteins, and jasmonic acid, respectively, over time after treating *Pdex:DAD1-Myc* plants with dex (30 μM). PCR primers target both native *DAD1* and recombinant *DAD1-Myc* (A). Two specific bands detected by anti-*Myc* antibody are the precursor (P) and the mature (M) forms of DAD1-*Myc* before and after cleavage of chloroplast transit peptide. Asterisk mark a nonspecific band detected by some commercial *Myc*-antibody batches (B). D and E, Wounding boosts DAD1-*Myc*-mediated JA synthesis. *DAD1-Myc* (plus endogenous *DAD1*) transcripts (D) and jasmonic acid levels (E) in WT or *Pdex:DAD1-Myc* plants treated either with wounding (W), dex or both (dex+W). Ten μL of 30 μM dex was added as small droplets on the adaxial surface of the leaf and incubated for 8 h. Wounding was administered by crushing leaves twice across the mid-rib using a hemostat at 6 h post dex treatment, and tissue was harvested after 2 h of wounding. *ACT8* was used as an internal reference and the relative expression values (A, D) are based on comparisons to the dex-treated WT. Data represent mean ± SD of three biological replicates with statistical significance (pairwise *t*-tests, $P < 0.05$) denoted by letters above the bars.

Figure 5. Induction of JA by DAD1-*Myc* expression in various developmental stages. (A-C) Levels of jasmonic acid (A), *DAD1-Myc* transcripts (B), and DAD1-*Myc* proteins (C) in 10, 15, 20, 25, and 30 d old *Pdex:DAD1-Myc* plants 8 h after sprayed with dex (30 μM). *ACT8* was used as an internal reference and the relative transcripts are based on comparisons to the mock (B). 'P' and 'M' detected by anti-*Myc* antibody denote the precursor and the mature (transit peptide cleaved) DAD1-*Myc*, respectively (C). D, JA levels in wounded and unwounded WT plants in shown aged plants. Wounding was administered to result in a quarter of leaf area being crushed by hemostats. E, Wound-elicited enhancement of JA synthesis by fully mature (30 d) WT and *Pdex:DAD1-Myc* plants treated either with wounding (W), dex or both (dex+W). Ten μL of 30 μM dex was added as small droplets on the adaxial surface of the leaf and incubated for 8 h. Wounding was administered by crushing leaves twice across the mid-rib using a hemostat at 6 h

833 post dex treatment, and tissue was harvested after 2 h of wounding. Data represent mean ± SD of
834 three biological replicates with statistical significance (pairwise *t*-tests, $P < 0.05$) denoted by
835 letters above the bars.
836
837 **Figure 6.** Wound-activated enhancement of systemic JA synthesis in *Pdex:DAD1-Myc* plants. A,
838 Immunoblot showing induction of DAD1-*Myc* in the local (loc) (leaf 3, 4) and systemic (sys) (6,
839 7) leaves after 6 h of 30 μM dex treatment (circle in the cartoon) on the systemic leaves of
840 *Pdex:DAD1-Myc*. WT leaves were treated with dex as control. 'P' and 'M' denote the precursor
841 and the mature (transit peptide cleaved) DAD1-*Myc*, respectively, and the asterisk (*) indicates
842 nonspecific detection. B, Wound-induced systemic JA accumulation w/o preinduction by dex.
843 Systemic leaves (leaf 6, 7) were pre-treated with mock (- dex) or dex (+ dex) for 6 h before
844 wounding the local leaves (leaf 3, 4). JA content in the systemic leaves was determined after 15
845 min of local wounding by crushing the leaves with hemostats twice perpendicular to the midvein.
846 Data in bar graphs represent mean ± SD of 3 biological replicates. Letters above bars indicate
847 statistical significance (Student's *t*-test, $P < 0.05$). Inset displays a magnified view.
848
849 **Figure 7**. DAD1 is an unstable protein whose stability is influenced by wounding and α-LA. A,
850 Immunoblot of protein extracts from *Pdex:DAD1-Myc* probed with antibodies against *Myc*,
851 LOX, AOC, or JAR1. Seedlings (14-d) that had been pretreated with dex (12 h) was submerged
852 in MS media containing 0.2 mM CHX for shown duration of time. B-C, *Pdex:DAD1-Myc*
853 seedlings were treated as in (A) except that they were either incubated with 0, 10, and 100 μM of
854 α-LA or wounded (Wnd). Seedlings were wounded indiscriminately but in a consistent manner
855 several times with a hemostat at the beginning of CHX treatment while submerged in the media.
856 Mock contained 0.01% w/v ethanol in water. D, A schematic of DAD1 protein with the
857 characteristic lipase domain including a Lid domain and three amino acid residues (S295, D352,
858 H416) of catalytic triad that were mutated to three Ala in *Pdex:DAD1*^{mut}*-Myc*. E, Jasmonic acid
859 level in the mutated (mut) and unmutated *Pdex:DAD1-Myc* in response to wounding (W), dex or
860 wounding and dex (W+dex). Data represent mean ± SD of three biological replicates. Letters
861 above bars indicate statistical significance (Student's *t*-test, $P < 0.05$). F, Protein stability
862 comparisons between mutated and unmutated DAD-*Myc*. Samples were treated as in (A).
863 Samples marked with asterisk were treated with dex for entire duration (18 h) without CHX.

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Fig. 1

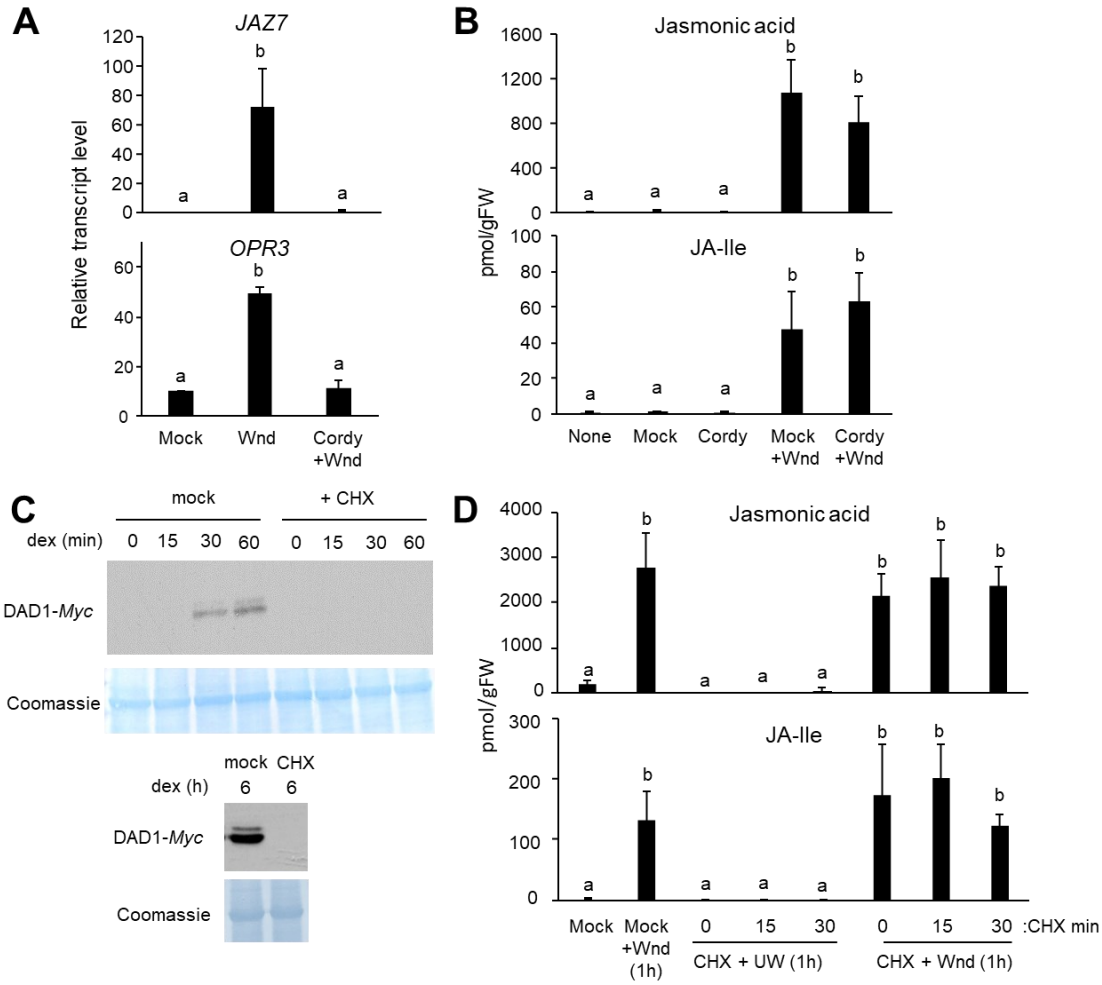

A
JAZ7
OPR3
Relative transcript level
Mock
Wnd
Cordy +Wnd
B
Jasmonic acid
JA-Ile
pmol/gFW
None
Mock
Cordy
Mock +Wnd
Cordy +Wnd
C
mock
+ CHX
dex (min)
0 15 30 60 0 15 30 60
DAD1-Myc
Coomassie
mock CHX
dex (h) 6 6
DAD1-Myc
Coomassie
D
Jasmonic acid
JA-Ile
pmol/gFW
Mock
Mock +Wnd (1h)
0 15 30
CHX + UW (1h)
0 15 30
CHX + Wnd (1h)
:CHX min

Fig. 2

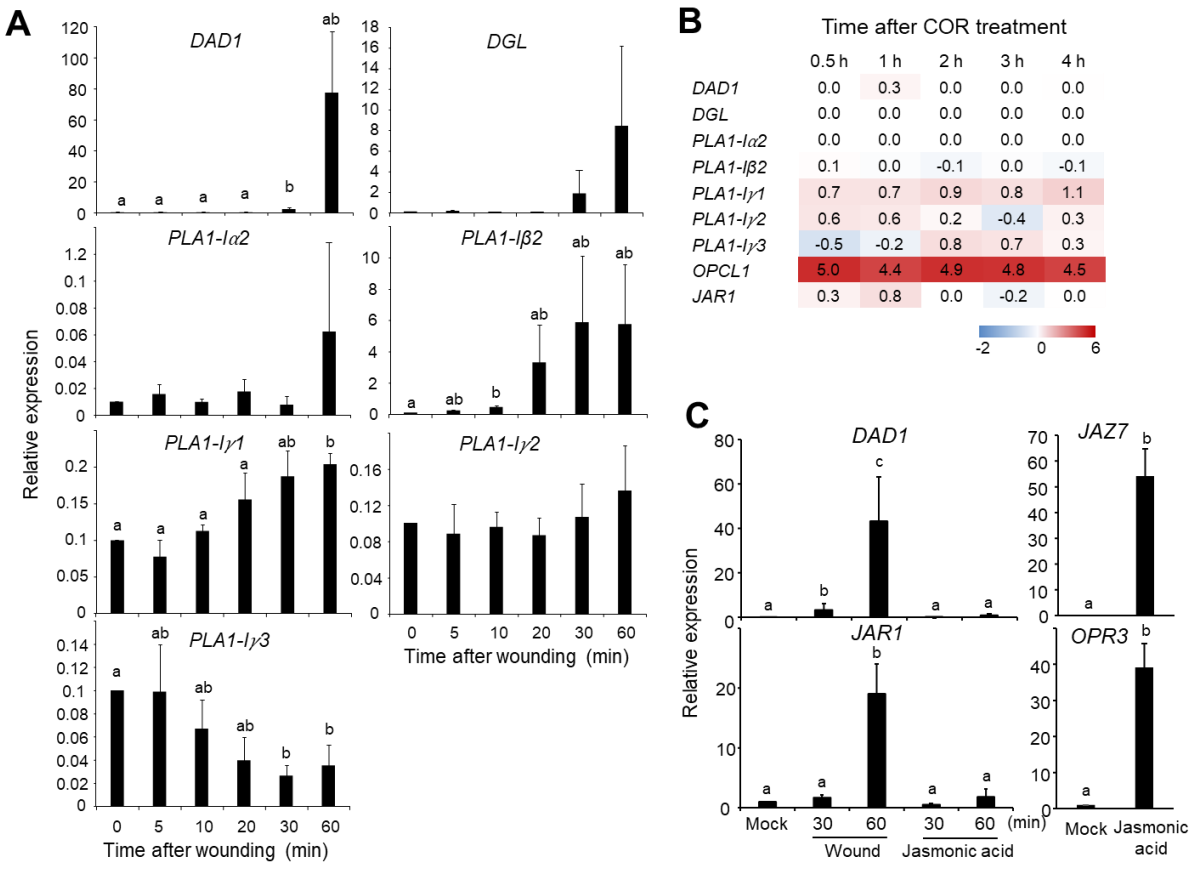

A
DAD1
DGL
PLA1-Iα2
PLA1-Iβ2
PLA1-Iγ1
PLA1-Iγ2
PLA1-Iγ3
Relative expression
Time after wounding (min)
B
Time after COR treatment
0.5 h 1 h 2 h 3 h 4 h
DAD1 0.0 0.3 0.0 0.0 0.0
DGL 0.0 0.0 0.0 0.0 0.0
PLA1-Iα2 0.0 0.0 0.0 0.0 0.0
PLA1-Iβ2 0.1 0.0 -0.1 0.0 -0.1
PLA1-Iγ1 0.7 0.7 0.9 0.8 1.1
PLA1-Iγ2 0.6 0.6 0.2 -0.4 0.3
PLA1-Iγ3 -0.5 -0.2 0.8 0.7 0.3
OPCL1 5.0 4.4 4.9 4.8 4.5
JAR1 0.3 0.8 0.0 -0.2 0.0
-2 0 6
C
DAD1
JAZ7
JAR1
OPR3
Relative expression
Mock 30 60 30 60 (min)
Wound
Jasmonic acid
Mock Jasmonic acid

Fig. 3

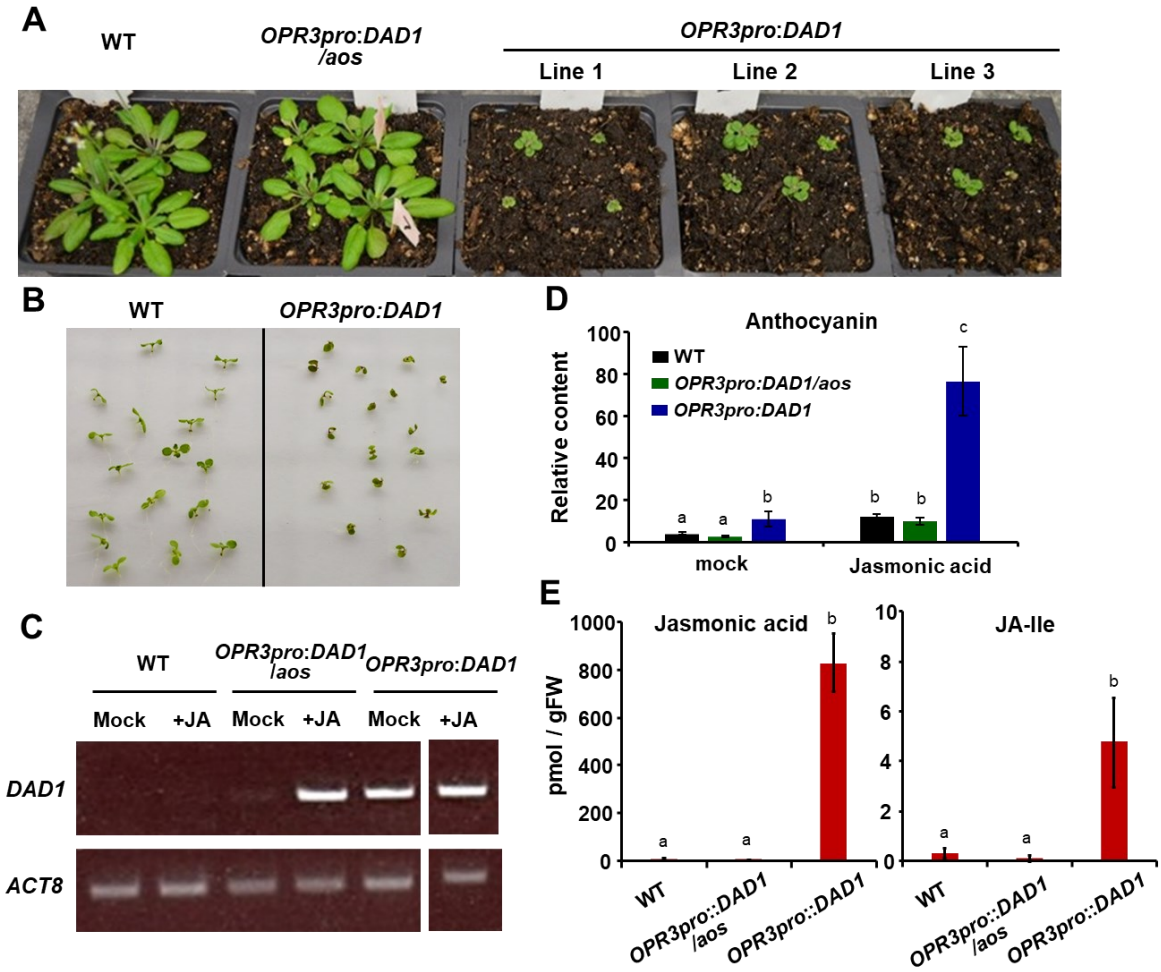

A
WT
OPR3pro:DAD1 /aos
OPR3pro:DAD1
Line 1
Line 2
Line 3
B
WT
OPR3pro:DAD1
C
WT
OPR3pro:DAD1 /aos
OPR3pro:DAD1
Mock
+JA
Mock
+JA
Mock
+JA
DAD1
ACT8
D
Anthocyanin
WT
OPR3pro:DAD1/aos
OPR3pro:DAD1
Relative content
mock
Jasmonic acid
E
Jasmonic acid
JA-Ile
pmol / gFW
WT
OPR3pro::DAD1 /aos
OPR3pro::DAD1

Fig. 4

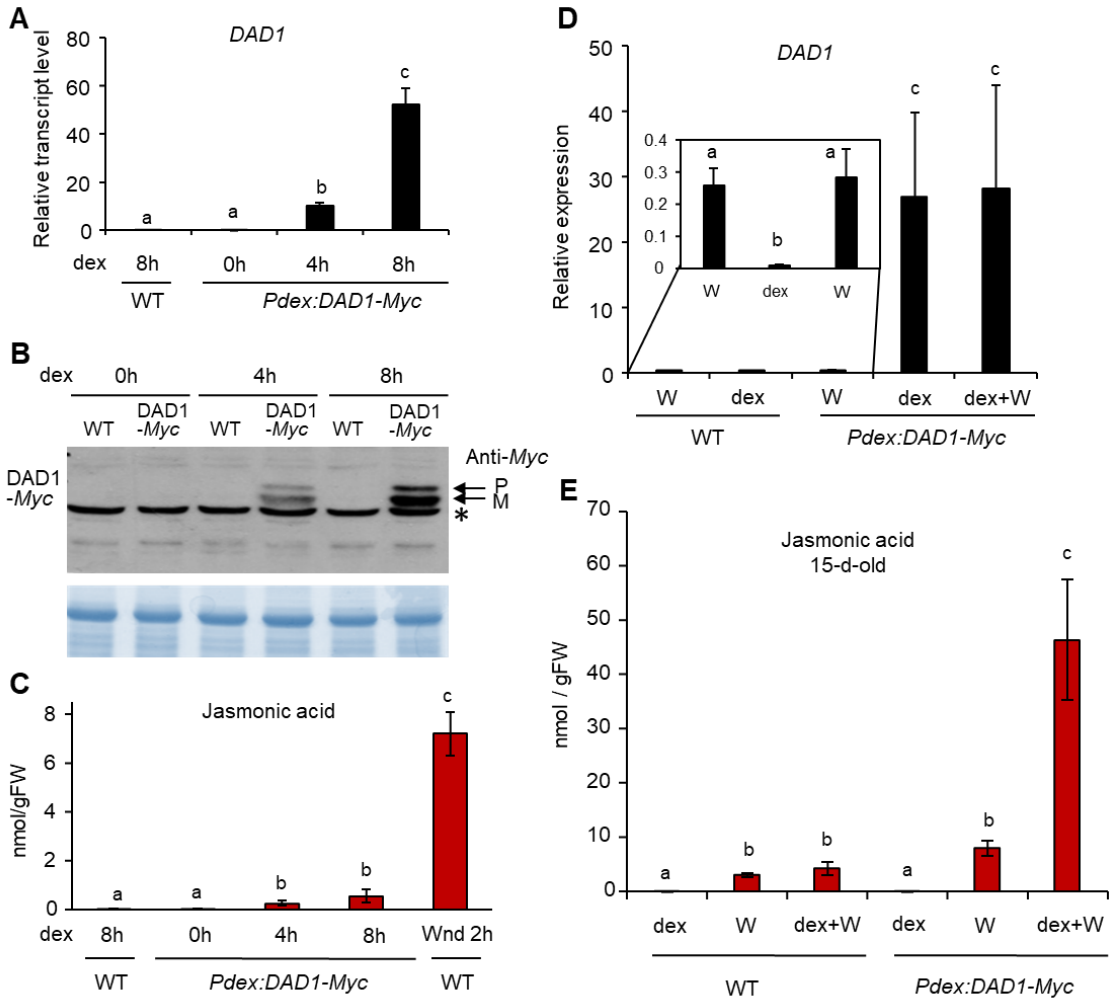

A
DAD1
Relative transcript level
dex
8h
0h
4h
8h
WT
Pdex:DAD1-Myc
B
dex
0h
4h
8h
WT
DAD1 -Myc
Anti-Myc
P
M
C
Jasmonic acid
nmol/gFW
Wnd 2h
D
DAD1
Relative expression
W
dex
dex+W
E
Jasmonic acid
15-d-old
nmol / gFW

Fig. 5

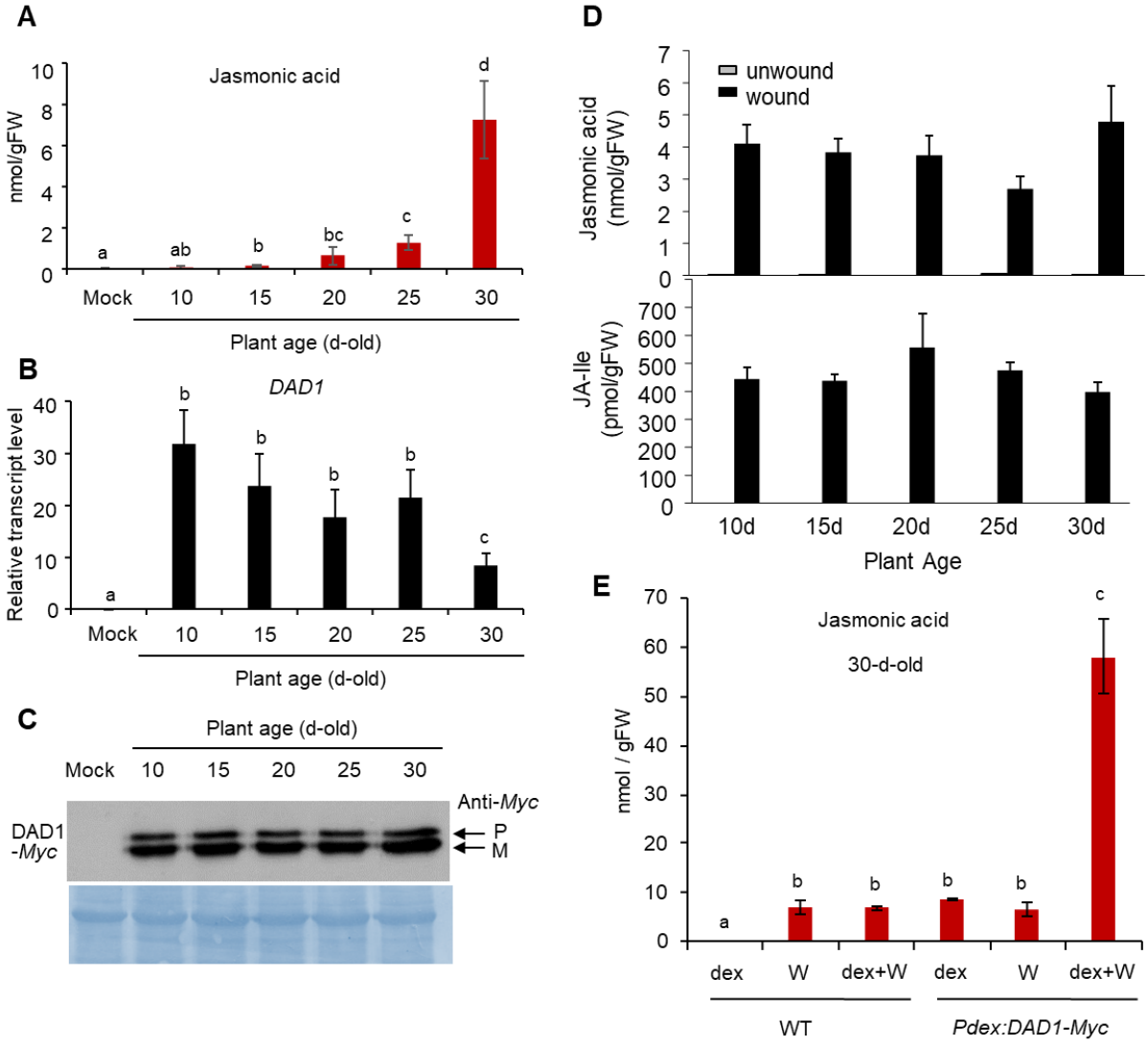
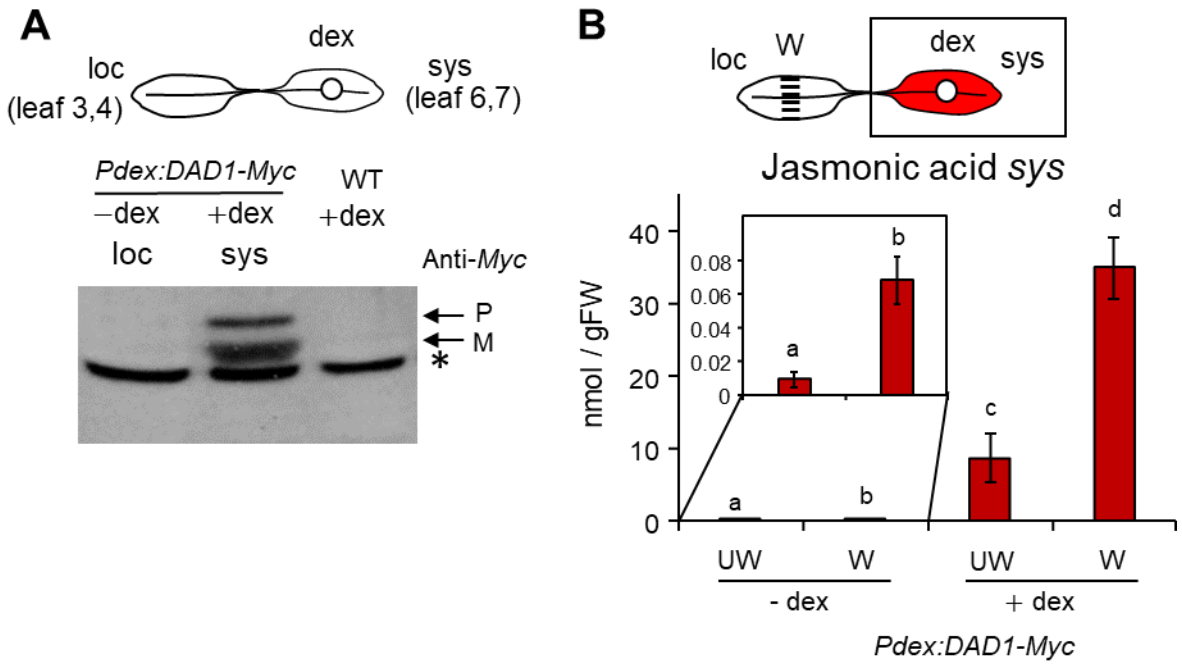

A
Jasmonic acid
nmol/gFW
Mock
10
15
20
25
30
Plant age (d-old)
B
DAD1
Relative transcript level
C
Plant age (d-old)
DAD1
-Myc
Anti-Myc
P
M
D
unwound
wound
Jasmonic acid
(nmol/gFW)
JA-Ile
(pmol/gFW)
10d
15d
20d
25d
30d
Plant Age
E
Jasmonic acid
30-d-old
nmol / gFW
dex
W
dex+W
WT
Pdex:DAD1-Myc

Fig. 6


A
dex
loc
(leaf 3,4)
sys
(leaf 6,7)
Pdex:DAD1-Myc
WT
−dex
+dex
+dex
loc
sys
Anti-Myc
P
M
*
B
loc
W
dex
sys
Jasmonic acid sys
nmol / gFW
40
30
20
10
0
0.08
0.06
0.04
0.02
0
a
b
a
b
c
d
UW
W
UW
W
- dex
+ dex
Pdex:DAD1-Myc

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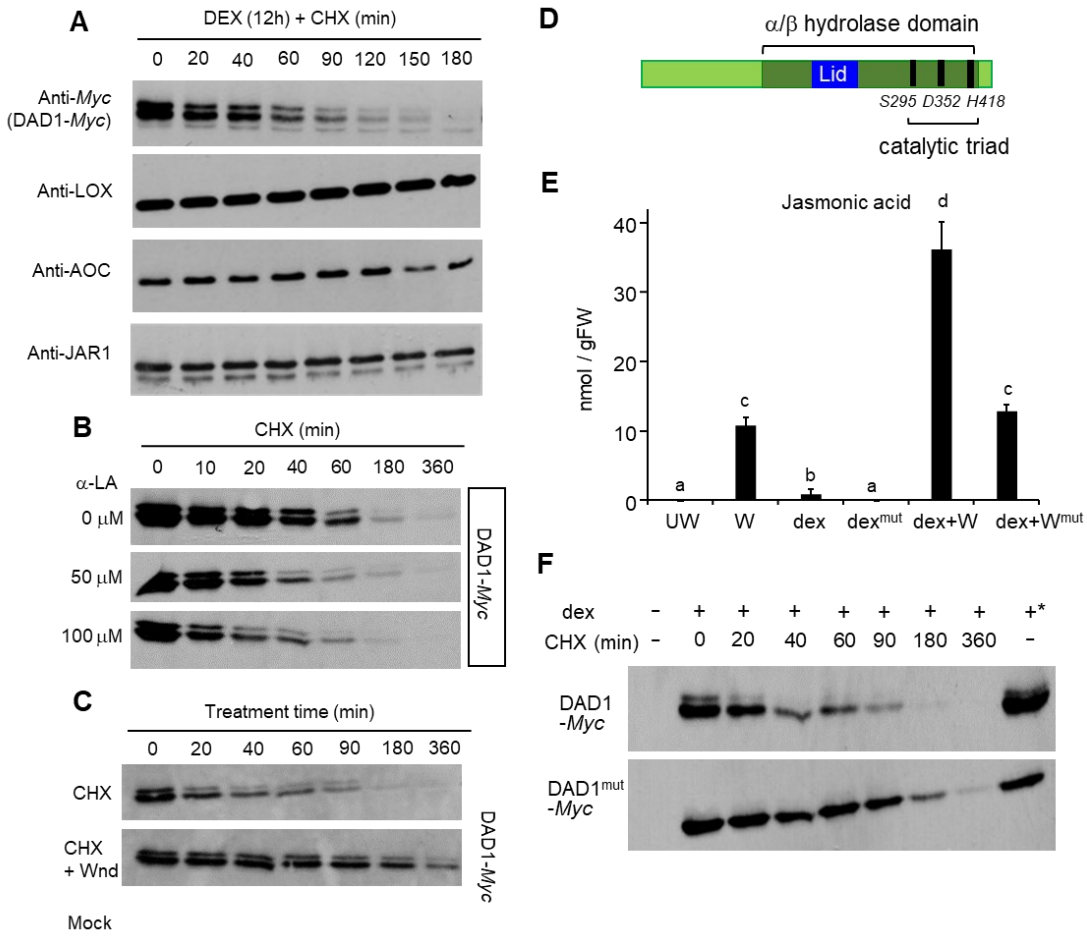
1143 Fig. 7



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