Initiation of root jasmonate biosynthesis

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Summary

The bioactive phytohormone jasmonoyl-L-isoleucine (JA-Ile) is a lipid-derived molecule that is widespread across higher plants. This signaling molecule protects plants against biotic and abiotic challenges, such as insect herbivores and mechanical wounding, and plays essential roles in reproductive development. Despite the crucial roles of JA-Ile in plant environmental responses, it remains unknown what cellular signals lead to JA-Ile biosynthesis initiation in plastids, and how JA-Ile plastidial enzymes are regulated. Furthermore, while the role of the jasmonate pathway has been intensely studied in green tissues, its functions in roots have received less attention.

As plastidial JA-Ile biosynthesis enzymes are basally present, and because jasmonic acid (JA) and JA-Ile levels increase within minutes following tissue damage, it is generally accepted that wound-induced JA-Ile biosynthesis relies upon the activation of pre-existing plastidial enzymes. Plastidial 13-LIPOXYGENASE (13-LOX) catalyzes one of the early steps in JA-Ile biosynthesis, and there are four genes in *Arabidopsis thaliana* contributing to wound-induced JA-Ile biosynthesis in leaves. This thesis will, therefore, focus on understanding their roles and regulation in root JA-Ile production.

To test if increasing the amount of 13-LOX enzymes leads to increased JA-Ile signaling under basal conditions, I developed overexpression (OE) lines. I found that LOX2 and LOX6 OE does not lead to higher basal expression of JA-Ile marker genes, while LOX3 and LOX4 OE does. These results suggest that LOX3 and LOX4 are regulated transcriptionally, while LOX2 and LOX6 are regulated at the protein level. Consistent with these results and with (Grebner et al., 2013), I found that LOX6 is essential to induce root JA-Ile signaling in intact plants. By developing and analyzing transcriptional and translational reporter lines, I next found that LOX6 is expressed in root xylem-associated procambial cells, indicating that these are the sites of initial hormone biosynthesis.

To uncover if LOX6 cell-type-specific localization or its specific enzyme activity is important for its function, I have developed and analyzed promoter swap lines in which LOX2, LOX3, or LOX4 were expressed under the promoter of *LOX6* in a *lox6* mutant background, which is unable to trigger JA-Ile signaling after root wounding. This approach showed that LOX3 and LOX4 can mimic the function of LOX6, while LOX2 cannot, indicating that both enzyme localization and regulation are critical for hormone synthesis. Furthermore, deleting the N-terminal POLYCYSTIN-1 LIPOXYGENASE ALPHA-TOXIN (PLAT) domain of LOX6 or replacing it with those of LOX3, LOX4, or LOX6 could not complement the *lox6* mutant phenotype, indicating an essential regulatory role of the PLAT domain for LOX6

activity in planta. Ongoing experiments are testing specific residues in the PLAT domain conferring this regulatory function.

Collectively, my efforts have been geared towards understanding the cell type-specific expression of 13-LOX enzymes and their regulation, which may provide novel strategies for future harnessing of plant defense responses.

Zusammenfassung

As nonnative German speaker, this text was translated by online DeepL website.
Das bioaktive Phytohormon Jasmonoyl-L-Isoleucin (JA-Ile) ist ein aus Lipiden gewonnenes Molekül, das in höheren Pflanzen weit verbreitet ist. Dieses Signalmolekül schützt Pflanzen vor biotischen und abiotischen Einflüssen, wie z. B. pflanzenfressenden Insekten und mechanischen Verletzungen, und spielt eine wichtige Rolle bei der Fortpflanzungsentwicklung. Trotz der wichtigen Rolle von JA-Ile bei pflanzlichen Umweltreaktionen ist nach wie vor unbekannt, welche zellulären Signale zur Einleitung der JA-Ile-Biosynthese in den Plastiden führen und wie die plastidialen JA-Ile-Enzyme reguliert werden. Während die Rolle des Jasmonat-Stoffwechsels in grünen Geweben intensiv untersucht wurde, wurde seinen Funktionen in Wurzeln weniger Aufmerksamkeit geschenkt.

Da plastidiale JA-Ile-Biosyntheseenzyme basal vorhanden sind und der Jasmonsäure- (JA) und JA-Ile-Spiegel innerhalb von Minuten nach einer Gewebeschädigung ansteigt, wird allgemein angenommen, dass die wundinduzierte JA-Ile-Biosynthese von der Aktivierung bereits vorhandener plastidialer Enzyme abhängt. Die plastidiale 13-LIPOXYGENASE (13-LOX) katalysiert einen der frühen Schritte der JA-Ile-Biosynthese, und es gibt vier Gene in *Arabidopsis thaliana*, die zur wundinduzierten Biosyntheseenzyme in Blättern beitragen. Diese Arbeit konzentriert sich daher auf das Verständnis ihrer Rolle und Regulierung bei der JA-Ile-Produktion in Wurzeln.

Um zu testen, ob eine Erhöhung der Menge an 13-LOX-Enzymen zu einer verstärkten JA-Ile-Signalisierung unter basalen Bedingungen führt, habe ich Überexpressionslinien (OE) entwickelt. Ich fand heraus, dass LOX2 und LOX6 OE nicht zu einer höheren basalen Expression von JA-Ile-Markergenen führt, LOX3 und LOX4 OE hingegen schon. Diese Ergebnisse deuten darauf hin, dass LOX3 und LOX4 transkriptionell reguliert werden, während LOX2 und LOX6 auf Proteinebene reguliert werden. In Übereinstimmung mit diesen Ergebnissen und mit Grebner et al. (2013) habe ich festgestellt, dass LOX6 für die Induktion des JA-Ile-Signals in intakten Pflanzen unerlässlich ist. Durch die Entwicklung und Analyse von Transkriptions- und Translationsreporterlinien fand ich als Nächstes heraus, dass LOX6 in Wurzel-Xylem-assoziierten prokambialen Zellen exprimiert wird, was darauf hindeutet, dass dies die Orte der ursprünglichen Hormonbiosynthese sind.

Um herauszufinden, ob die zelltypspezifische Lokalisierung von LOX6 oder seine spezifische Enzymaktivität für seine Funktion wichtig ist, habe ich Promotor-Austausch-Linien entwickelt und analysiert, in denen LOX2, LOX3 oder LOX4 unter dem Promotor von LOX6 in einem lox6-Mutantenhintergrund exprimiert wurden, der nicht in der Lage ist, nach einer Wurzelverletzung JA-Ile-Signale auszulösen. Dieser Ansatz zeigte, dass LOX3 und LOX4 die Funktion von LOX6 nachahmen können, während LOX2 dazu nicht in der Lage ist, was darauf hindeutet, dass sowohl die Lokalisierung

als auch die Regulierung des Enzyms für die Hormonsynthese entscheidend sind. Darüber hinaus konnte die Deletion der N-terminalen POLYCYSTIN-1 LIPOXYGENASE ALPHA-TOXIN (PLAT)-Domäne von LOX6 oder deren Ersatz durch die Domänen von LOX3, LOX4 oder LOX6 den Phänotyp der LOX6-Mutante nicht komplementieren, was auf eine wesentliche regulatorische Rolle der PLAT-Domäne für die LOX6-Aktivität in Pflanzen hinweist. In laufenden Experimenten werden spezifische Aminosäuren in der PLAT-Domäne getestet, die diese regulatorische Funktion verleihen.

Insgesamt zielen meine Bemühungen darauf ab, die zelltypspezifische Expression von 13-LOX-Enzymen und ihre Regulierung zu verstehen, was neue Strategien für die künftige Nutzung der pflanzlichen Abwehrreaktionen ermöglichen könnte.

The work presented in this thesis was conducted in frame of the Research Training Group RTG2498 '*Communication and Dynamics of Plant Cell Compartments*' of the Martin Luther University Halle-Wittenberg (MLU) chaired by Prof. Ingo Heilmann. Members of my PhD thesis supervisory committee were Dr. Debora Gasperini (main adviser), Prof. Ingo Heilmann, and Prof. Edgar Peiter.

Experimental work from this thesis was conducted at the Leibniz Institute of Plant Biochemistry (Halle, Germany), Department of Molecular Signal Processing in the research team 'Jasmonate Signaling' led by Dr. Debora Gasperini from 01 July 2019 to 31 May 2023.

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List of abbreviations

ABA	Abscisic acid
ACX	ACYL-CoA OXIDASE
ANOVA	Analysis of Variance
AOC	ALLENE OXIDE CYCLASE
AOS	ALLENE OXIDE SYNTHASE
ARF6	AUXIN RESPONSE FACTOR 6
bHLH	basic helix-loop-helix
BICAT	BIVALENT CATION TRANSPORTER
cDNA	Complementary DNA
CIT	CITRINE
COI1	CORONATINE INSENSITIVE 1
cTP	CHLOROPLAST TRANSIT PEPTIDE
CTS	COMATOSE
DAD1	DEFECTIVE IN ANTHER DEHISCENSE 1
dgd1	*DGDG SYNTHASE 1*
DGDG	digalactosyldiacylglycerols
DGL	DONGLE
DNA	Deoxyribonucleic acid
dn-OPDA	dinor-oxo-phytodienoic acid
DW	Dry weight
e.g.	exempli gratia – for example
FAD	FATTY ACID DESATURATION
Fig.	Figure
FW	Fresh weight
GFP	Green Fluorescent Protein
GH3	GLYCOSIDE HYDROLASE 3
GUS	β-glucuronidase
HDA6	HISTONE DEACETYLASES 6
i.e.	id est - that is to say
JA	jasmonic acid
jai1	*jasmonic acid-insensitive1*
JA-Ile	jasmonoyl-isoleucine
JAR1	JASMONATE RESISTANT 1
JAs	Jasmonates
JAT1	JASMONATE TRANSPORTER1
JAZ	JASMONATE ZIM-DOMAIN
LSM	Laser scanning microscope
MAPK	Mitogen-activated protein kinase
MED25	Mediator 25
MGDG	Monogalactosyldiacylglycerol
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog media
NASC	Nottingham Arabidopsis Stock Centre
NINJA	NOVEL INTERACTOR OF JAZ

NLS	Nuclear localisation signal
OE	Overexpression
OPC-4	3-oxo-2-(2-(Z)-pentenyl) cyclopentane-1 butanoic acid
OPC-6	3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-hexanoic acid
OPC-8	3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-oxo-phytodienoic acid
OPR	OXO-PHYTODIENOIC ACID REDUCTASE
PCR	Polymerase chain reaction
PI	Propidium iodide
PLA1	phospholipase A-type 1
PLAT	POLYCYSTIN-1 LIPOXYGENASE ALPHA-TOXIN
PUFA	polyunsaturated fatty acid
qRT-PCR	Real-time quantitative reverse transcription PCR
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RNA Pol II	RNA POLYMERASE II
SNP	Single nucleotide polymorphism
Tab.	Table
T-DNA	Transfer-DNA
TF	transcription factors
tnOPDA	tetranor-OPDA
TPL	TOPLESS
Ub	Ubiquitin
UBC21	UBIQUITIN-CONJUGATING ENZYME 21
VEN	VENUS
wt	wild type
α-LeA	α-linolenic acid
4,5-ddh-JA	4,5-didehydro JA
10,11-EOT	11(S)-10,11-epoxy-octadecatrienoic acid
11-HPHT	11(S)-hydroperoxy-hexadecatrienoic acid
12,13-EOT	13(S)-12,13-epoxy-octadecatrienoic acid
12-OH-JA	hydroxylation to 12-hydroxy-JA
13-HPOT	13(S)-hydroperoxy-octadecatrienoic acid
13-LOX	13-LIPOXYGENASE
35S	Cauliflower Mosaic Virus 35S promoter

INTRODUCTION

The jasmonate pathway

In response to tissue injury or infection, both plants and animals produce oxygenated polyunsaturated fatty acid (PUFA) derivatives (also known as oxylipins), acting as signalling molecules to mediate stress acclimation against biotic and abiotic stimuli, reviewed in (Wasternack and Hause, 2013). In vascular plants such as *Arabidopsis thaliana* (Arabidopsis), oxylipins include the bioactive hormone jasmonoyl-isoleucine (JA-Ile), its precursors (eg. 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA)) and derivatives (eg. hydroxylation to 12-hydroxy-JA (12-OH-JA)), collectively referred to as Jasmonates (JAs). JA-Ile is essential to protect plants against herbivores, necrotrophic pathogens, and RNA viruses, and to mediate tolerance against a broad range of stimuli, including mechanical wounding, extreme temperatures, drought, salt, and osmotic changes (Mielke et al., 2021; Wasternack and Feussner, 2018; Yang et al., 2020) (Fig. 1).

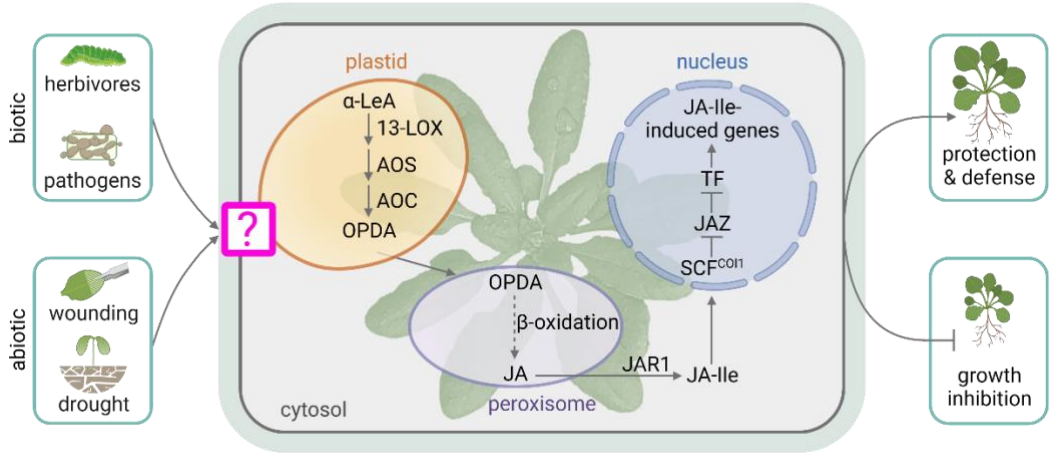

Figure 1. A wide-range of biotic and abiotic stimuli trigger JA-Ile biosynthesis and signalling, leading to an induction of plant defense and acclimation responses, often at the expense of growth. Despite the importance of the jasmonate pathway for plant survival and a detailed understanding of its biosynthesis and signalling occurring across different cellular compartments, the cellular and molecular events leading to its initiation in plastids have remained elusive. α-LeA (α-linolenic acid), 13-LOX (13-LIPOXYGENASE), AOS (ALLENE OXIDE SYNTHASE), AOC (ALLENE OXIDE CYCLASE), OPDA (12-oxo-phytodienoic acid), JA (jasmonic acid), JA-Ile (jasmonoyl-L-isoleucine), JAR1 (JASMONATE RESISTANT 1), SCF(SKP1-CULLIN3-F-box), COI1 (CORONATINE INSENSITIVE 1), JAZ (JASMONATE ZIM-DOMAIN), TF (transcription factor).

JA-Ile mediated signalling can protect plants against pathogens (such as fungi, oomycetes, bacteria, and viruses) and herbivorous insects (insects, invertebrate herbivores, larger animals). Necrotrophs are plant pathogens that live and feed on dead tissue imposing fatal diseases and economic losses (Glazebrook, 2005). After the first report revealed that mutants

involved in 18:3 PUFA biosynthesis unable to produce jasmonates are more susceptible to soil-borne pathogenic *Pythium jasmonium* when compared with wild type (Vijayan et al., 1998), numerous subsequent efforts have shown that JA-Ile signalling is essential for protection against necrotrophs. For instance, mutants compromised in JA-Ile perception show enhanced susceptibility to infection by fungal pathogens *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al., 1998). Similarly, mutants unable to synthesize the bioactive form of JA-Ile also show increased susceptibility to *Botrytis cinerea* (Ferrari et al., 2003). Furthermore, maize mutants defective in JA-Ile biosynthesis show increased susceptibility towards the root-rotting oomycete *Pythium ultimum* (Yan et al., 2012). Tomato mutants compromised in JA-Ile perception have reduced defense responses against oomycete *Pythium ultimum* (Campos et al., 2014). In addition to necrotrophic pathogens, JA-Ile also mediates defense responses against both specialist and generalist insect herbivores (Browse and Howe, 2008). For example, the specialist aphid *Myzus persicae* gains more weight when feeding on JA-Ile insensitive mutants, while *Myzus persicae* gain less weight when feeding on a mutant with constitutive JA-Ile signalling compared to wt plants (Ellis et al., 2002). Moreover, JA-Ile insensitive mutants show lower defense responses against thrips such as *Frankliniella occidentalis* (Abe et al., 2008), and chewing caterpillars such as *Spodoptera exigua* (Zhang et al., 2015). Other plant species also show similar phenotypes when infested with insect herbivores. For example, tomato and tobacco JA-Ile insensitive mutants show severely compromised resistance to spider mites *Tetranychous urticae* (Li et al., 2004) and the specialist hornworm *Manduca sexta* (Paschold et al., 2007), respectively. Strikingly, the jasmonate pathway also contributes to plant defense responses against vertebrate herbivores. Eastern Hermann's tortoise prefers to eat JA-Ile deficient mutants over wild-type plants (Mafli et al., 2012). Consistently, JA-Ile deficient tobacco plants in the field are more strongly damaged by the cottontail rabbit *Sylvilagus nuttallii* (Machado et al., 2016). Overall, JA-Ile is essential for regulating plant resistance to necrotrophic pathogens and insect herbivores.

Herbivory as well as necrotrophic pathogens often cause severe tissue damage, and similarly, mechanical wounding of plant tissues is a good elicitor of OPDA, JA and JA-Ile production, eg. (Glauser et al., 2009; Koo et al., 2009). Interestingly, transcriptional changes are similar between mechanically wounded plants and those challenged with insect herbivores

(Reymond et al., 2000). Nevertheless, insect oral secretion was reported to have additional roles in plant-herbivore interactions reviewed in (Kallure et al., 2022). Upon drought stress, wild-type plants accumulate JA-Ile which has been implicated in promoting stomatal closure to reduce transpiration-mediated water loss (Suhita et al., 2004; Zander et al., 2020). In addition, hypo-osmotic treatments can also trigger JA-Ile signalling, suggesting a correlation between osmoregulation with the JA pathway (Mielke et al., 2021). Interestingly, by studying the jasmonate pathway in the bryophyte *Marchantia polymorpha,* which uses the JA-Ile precursor dinor-oxo-phytodienoic acid (dn-OPDA) as the bioactive hormone, (Monte et al., 2020) found that OPDA and dn-OPDA molecules can regulate plant thermotolerance genes even in Arabidopsis. Furthermore, wound-induced activation of JA-Ile signalling can promote stem cell activation and regeneration of Arabidopsis roots (Zhou et al., 2019). Overall, the jasmonate pathway plays vital roles in defending plants against abiotic stresses.

The induction of defense responses mediated by JA-Ile is accompanied by a growth arrest in vegetative tissues. This phenomenon, known as the growth-defense trade-off, is dependent on the presence of JA-Ile. Mutants lacking JA-Ile fail to halt their growth in response to repeated shoot wounding, emphasizing the dependency on JA-Ile for the growth arrest observed in such circumstances (Yan et al., 2007; Zhang and Turner, 2008). The activation of JA-Ile signalling stunts leaf growth by reducing cell number and size, and by delaying the switch from the mitotic cell cycle to the endoreduplication cycle (Noir et al., 2013). Similarly, JA-Ile signalling activation in roots inhibits cell proliferation in the meristem as well as cell elongation, resulting also in root growth arrest (Chen et al., 2011). Interestingly, the stunted growth phenotype of constitutive JA-Ile signalling mutants can be restored by blocking the activity of the PHYTOCHROME B photoreceptor (Campos et al., 2016). The same group later showed that light and JA-Ile signalling act in parallel to regulate overall growth and defense phenotypes (Major et al., 2020).

The jasmonate pathway is also involved in the regulation of fertility in flowering plants. Both JA-Ile biosynthesis mutants as well as the JA-Ile signalling mutants are male sterile in Arabidopsis (Feys et al., 1994; Ishiguro et al., 2001; Park et al., 2002). In addition to Arabidopsis, JA-Ile controls sex determination in maize, stamen and spikelet development in

rice, and female fertility in tomato (Acosta et al., 2009; Cai et al., 2014; Dobritzsch et al., 2015; Li et al., 2001; Schubert et al., 2019). Unlike in vascular plants however, the jasmonate pathway does not regulate fertility in liverwort (Monte et al., 2018; Peñuelas et al., 2019), posing intriguing questions on the jasmonate pathway evolution (Chini et al., 2023).

JA-Ile biosynthesis

At the cellular level, the JA-Ile biosynthesis pathway is well characterized and takes place in three subcellular compartments: first in plastids, then peroxisomes, and finally in the cytosol, reviewed in (Wasternack and Feussner, 2018) (Fig. 2). JA-Ile biosynthesis starts from the de-esterification of 18:3 α-linolenic acid (LeA) from membrane galactolipids, mainly monogalactosyldiacylglycerols (MGDG) found in thylakoids and in the plastid inner membranes by the action of phospholipase A-type 1 (PLA1s) (Li and Yu, 2018). So far, DEFECTIVE IN ANTHER DEHISCENSE 1 (DAD1) is the only lipase that was shown to initiate JA-Ile production during Arabidopsis flower development (Ishiguro et al., 2001). Two abscisic acid (ABA)-regulated Plastid lipases (PLIP2 and PLIP3) were shown to induce JA-Ile production following ABA treatment, and to integrate ABA-JA crosstalk (Wang et al., 2018). However, it is still unknown which lipases control LeA release during mechanical wounding and insect herbivory.

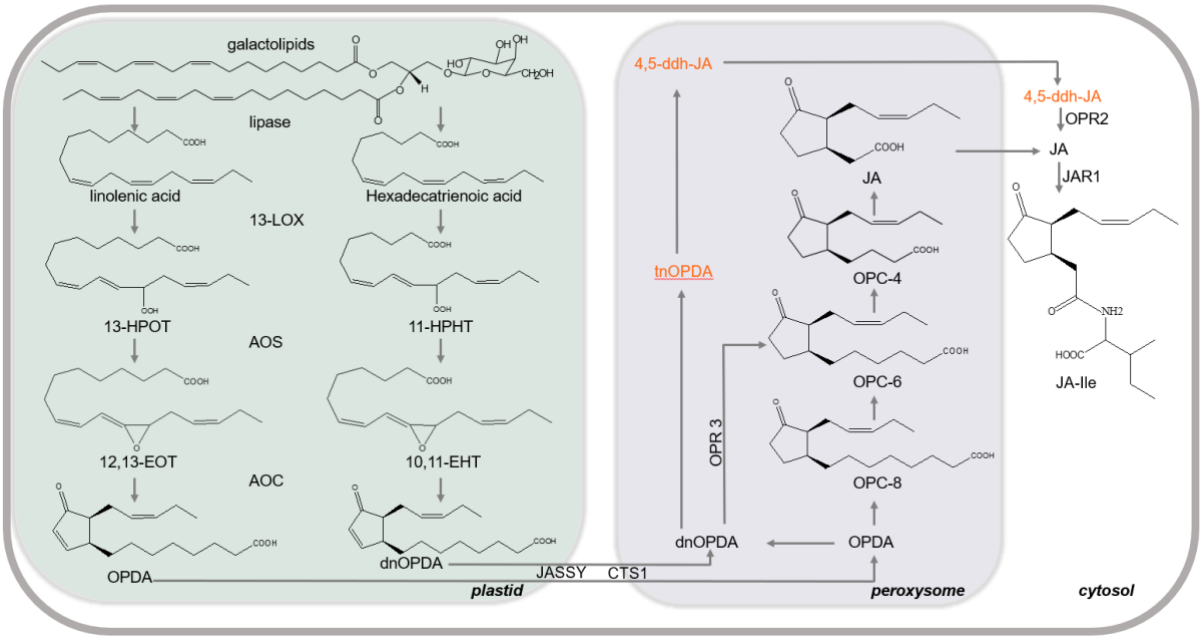

Figure 2. JA-Ile biosynthesis in Arabidopsis. The pathway initiates from de-esterification of plastidial membrane lipids (mainly galactolipids) to yield linolenic acid and hexadecatrienoic acid. Abbreviations: 13-HPOT (13(*S*)-hydroperoxy-octadecatrienoic acid), 11-HPHT (11(*S*)-hydroperoxy-hexadecatrienoic acid), 12,13-EOT (13(*S*)-12,13-epoxy-octadecatrienoic acid), 10,11-EOT (11(*S*)-10,11-epoxy-octadecatrienoic acid), dnOPDA (dinor-oxo-phytodienoic acid), CTS (COMATOSE), OPC-8 (3-oxo-2-(2-(*Z*)-pentenyl)-cyclopentane-1-octanoic acid), OPC-6 (3-oxo-2-(2-(*Z*)-pentenyl)-cyclopentane-1-hexanoic acid), OPC-4 (3-oxo-2-(2-(*Z*)-pentenyl) cyclopentane-1 butanoic acid), tnOPDA (tetranor-OPDA), 4,5-ddh-JA (4,5-didehydro JA), JA (jasmonic acid), JA-Ile (jasmonoyl-L-isoleucine), 13-LOX (13-LIPOXYGENASE), AOS (ALLENE OXIDE SYNTHASE), AOC (ALLENE OXIDE CYCLASE), OPR (OPDA REDUCTASE), JAR1 (JASMONATE RESISTANT 1).

The next step is the oxygenation of free LeA (18:3) or hexadecatrienoic acid (16:3) by 13-LIPOXYGENASES (13-LOX) at the C-13 position to form the corresponding hydroperoxides: 13(*S*)-hydroperoxy-octadecatrienoic acid (13-HPOT) and 11(*S*)-hydroperoxy-hexadecatrienoic acid (11-HPHT) (Feussner and Wasternack, 2002). 13-HPOT and 11-HPHT are then rearranged by ALLENE OXIDE SYNTHASE (AOS) to form allene oxides: (13*S*)-12,13-epoxy-octadecatrienoic acid (12,13-EOT) and (11*S*)-10,11-epoxy-octadecatrienoic acid (10,11-EOT) (Laudert et al., 1996). Because *AOS* is encoded by a single copy gene in Arabidopsis, the *aos* knock-out mutant lacks all compounds downstream and is hence deficient in all JA-Ile-mediated responses (Park et al., 2002). AOS products, 12,13-EOT and 10,11-EOT are extremely unstable compounds which are rapidly converted into OPDA and dnOPDA by ALLENE OXIDE CYCLASE (AOC) (Brash, 1999; Stenzel et al., 2012). There are four AOCs in Arabidopsis which have tissue and organ specific promoter activities and display *in vivo* heteromerization (Stenzel et al., 2012). Following OPDA export from the plastid, likely through the chloroplast outer membrane transporter JASSY, OPDA is then imported into peroxisomes partly through the ABC transporter COMATOSE1 (CTS1) (Guan et al., 2019; Theodoulou et al., 2005). In the peroxisome, OPDA is reduced to 3-oxo-2-(2-(*Z*)-pentenyl)-cyclopentane-1-octanoic (OPC-8) and hexanoic (OPC-6) acids, respectively. OPC-8 and OPC-6 undergo several rounds of β-oxidation by ACYL-CoA OXIDASE (ACX) enzymes to generate 3-oxo-2-(2-(*Z*)-pentenyl)-cyclopentane-1-butanoic acid (OPC-4) and finally JA (Breithaupt et al., 2006). Furthermore, OPDA can be converted to dnOPDA to form 4,5-didehydrojasmonate (4,5-ddh-JA) and thereafter JA, via an OPDA REDUCTASE 3 (OPR3)-independent shunt pathway, which contributes to a minor fraction of overall JA production (Chini et al., 2018). Upon export to the cytosol through an unknown route, JA is conjugated to isoleucine (Ile) by the GLYCOSIDE HYDROLASE 3 (GH3) enzyme JASMONATE RESISTANT 1 (JAR1) (Suza and Staswick, 2008).

JA-Ile signalling

The cytosolic JA-Ile can be translocated by the ABCG-type JASMONATE TRANSPORTER1 (JAT1) into the nucleus where hormone perception takes place (Li et al., 2017). Due to the high energy expenditure associated with the activation of JA-Ile dependent transcriptional and metabolic responses, the signalling pathway dependent on JA-Ile is typically suppressed during basal conditions (Barto and Cipollini, 2005; Zavala and Baldwin, 2006). Specifically, when JA-Ile levels are low, JA-Ile dependent transcription factors (TFs) of the basic helix-loop-helix (bHLH) family, chiefly MYC2, MYC3, and MYC4 (Fernández-Calvo et al., 2011; Lorenzo et al., 2004) are kept repressed by a modular repressor complex (Fig. 3). This complex is composed of JASMONATE ZIM-DOMAIN (JAZ) proteins, that either alone or in combination with NOVEL INTERACTOR OF JAZ (NINJA) adaptor protein recruits the transcriptional repressor TOPLESS (TPL) and TPL related proteins to repress JA-Ile responsive genes (Pauwels et al., 2010; Shyu et al., 2012). TPL in turn recruits histone deacetylases like HDA6 or HDA19, leading to a reduced chromatin accessibility and repression of gene expression (Pauwels et al., 2010; Zhu et al., 2011). When JA-Ile levels increase, the phytohormone serves as a molecular glue promoting the interaction between JAZs with the F-box protein CORONATINE INSENSITIVE 1 (COI1), which is part of an E3 ubiquitin ligase complex of the SKP1-CUL1-F-box type (Xie et al., 1998). COI1 targets JAZ repressors for ubiquitylation, leading to their 26S proteasome-mediated degradation (Chini et al., 2007; Thines et al., 2007). JAZ degradation results in liberated TFs that can now bind to the MEDIATOR 25 (MED25) subunit of the transcriptional mediator complex, recruiting RNA POLYMERASE II (RNA Pol II) to initiate the transcription of target genes such as *JAZ10* (Chen et al., 2012). *JAZ*s, being early responsive genes to JA-Ile, play a crucial role in replenishing the intracellular pool of transcriptional regulators and establishing a negative feedback loop to control JA-Ile signaling. (Yan et al., 2007).

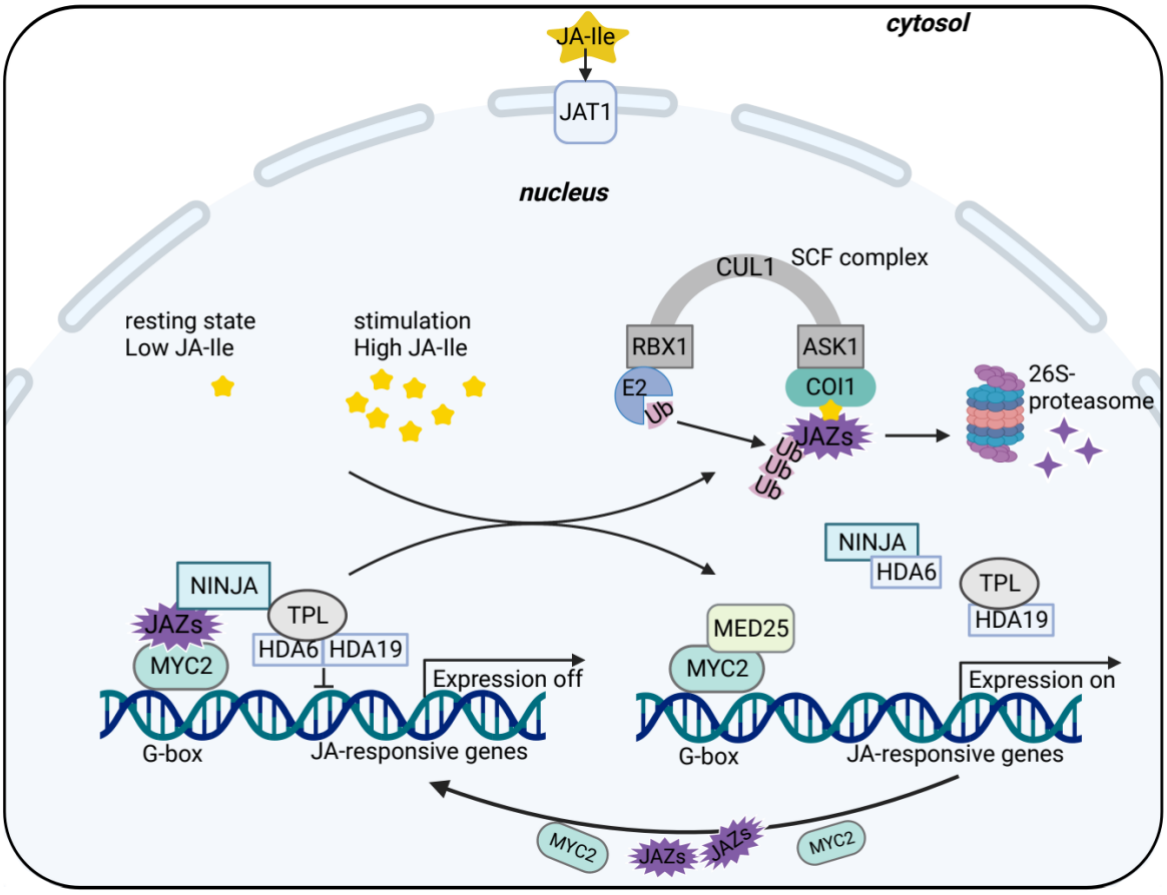

Figure 3. JA-Ile perception and signalling pathway. At low JA-Ile levels, the JAZ-NINJA-TPL repressor complex inhibits G-Box binding TFs, such as MYC2, MYC3, and MYC4, by recruiting histone deacetylases like HDA6 and HDA19. Upon developmental or environmental stimuli, JA-Ile levels increase and promote the direct interaction between JAZs and the F-Box protein COI1 which is part of an SCF-type E3 Ubiquitin ligase. Therefore, JAZs are polyubiquitylated and degraded by the proteasome, and the expression of JA-Ile responsive genes is mediated by the released TFs in cooperation with the MEDIATOR COMPLEX. JA-Ile (jasmonoyl-L-isoleucine), JAZ (JASMONATE ZIM-DOMAIN), NINIJA (NOVEL INTERACTOR OF JAZ), TPL (TOPLESS), HDA (HISTONE DEACETYLASE), SCF(SKP1-CULLIN3-F-box), COI1 (CORONATINE INSENSITIVE 1), MED25 (MEDIATOR25), CUL1 (Cullin 1), Ub (Ubiquitin), E2 (Ubiquitin-conjugating enzyme), RBX (RING-box), ASK1 (Arabidopsis Skp1-like). Figure created with BioRender.com.

Initiation of JA-Ile biosynthesis during flower development

In comparison to the initiation of JA-Ile production in vegetative tissues, the initiation of hormone biosynthesis in flower development is better understood and is under transcriptional regulation. Since the discovery that jasmonate biosynthesis mutants depleted in 18:3 PUFAS such as the FATTY ACID DESATURASE triple mutant (*fad3fad7fad8*) and the *coi1* signalling mutants are male sterile (Feys et al., 1994; Mcconn and Browse, 1996), many other JA-Ile biosynthetic and signalling Arabidopsis mutants have been uncovered by this typical phenotype, such as *opr3*, *aos* and the *lox3lox4* double mutant (Caldelari et al., 2011; Park et al., 2002; Stintzi and Browse, 2000). Specifically, JA-Ile has been found to coordinate stamen

filament elongation, anther dehiscence, and pollen viability during plant reproduction process (Browse and Wallis, 2019). Furthermore, treatment of flower buds before flower opening with exogenous JAs can rescue all three defects (McConn and Browse, 1996). Later, the characterization of *defective anther dehiscence1* (*dad1*) mutants revealed that the filament might be the major or sole site of JA-Ile synthesis, since this phospholipase A1 is only expressed in the stamen filament in Arabidopsis flower (Ishiguro et al., 2001). The transcriptional activation of *DAD1* transcripts by *AUXIN RESPONSE FACTOR6* (*ARF6*) and *ARF8* TFs is necessary for JA-Ile production in reproductive organs, and consistently external JA applications rescue the *arf6arf8* sterility phenotype (Tabata et al., 2010). After de-esterification of α-LeA from membrane galactolipids by DAD1, LOX3 and LOX4 oxygenate the substrate (Fig. 4).

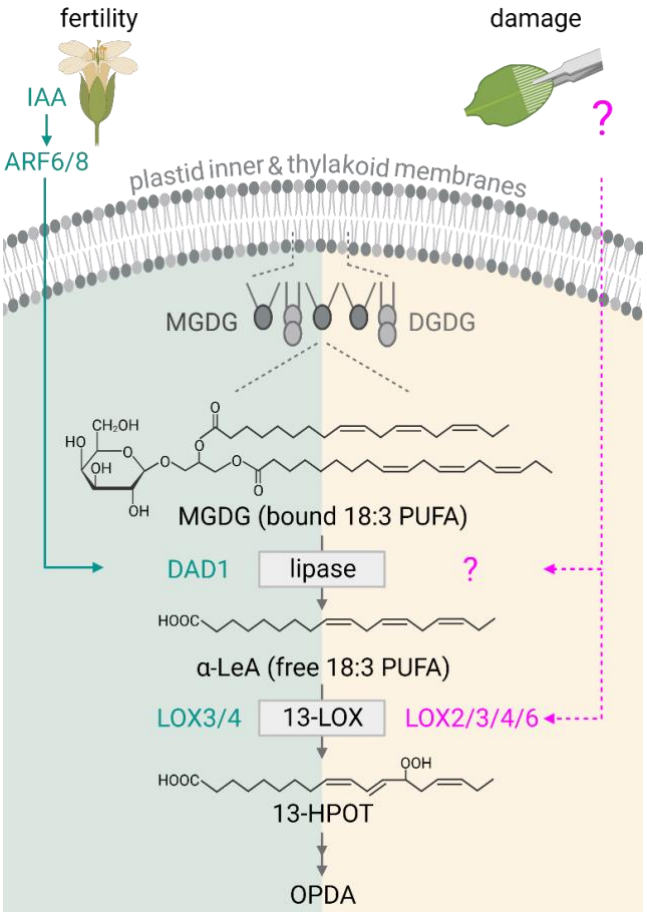

Figure 4. The initiation of JA-Ile biosynthesis during reproductive development is well studied, but not upon tissue damage. During stamen development, auxin (IAA) levels increase and stimulate the activity of AUXIN RESPONSE TRANSCRIPTION FACTORS ARF6 and ARF8 to directly or indirectly induce the expression of the phospholipase A1 (PLA1) DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1). DAD1 de-esterifies α-linolenic acid (α-LeA) from monogalactosyldiacylglycerol (MGDG)-bound to its free polyunsaturated fatty acid (PUFA) form, with α-LeA serving as substrate specifically for LOX3 and LOX4 enzymes. Thus, although it is still unknown what regulates the observed LOX3 and LOX4 specificity during Arabidopsis male fertility, hormone production initiates by the transcriptional induction of *DAD1*. Conversely, the observed rapid increase (1 min) of hormone levels upon tissue damage is likely the consequence of post-translational modifications of biosynthetic enzymes, rather than their transcriptional induction. However, neither the injury-induced signals leading to JA biosynthesis activation nor the identity of putative lipases acting upon MGDG are currently known. Furthermore, although all four Arabidopsis 13-LOX participate is JA-Ile biosynthesis upon tissue damage, their regulation still requires further studies. DGDG (Digalactosyldiacylglycerol); 13-HPOT (13(*S*)-hydroperoxy-octadecatrienoic acid). Figure reated with BioRender.com.

Indeed, *lox3lox4* double mutant is male sterile and can be complemented by overexpressing either *LOX3* or *LOX4* in the double mutant background (Caldelari et al., 2011). Surprisingly, expressing the co-receptor COI1 in the epidermal cells of the anther and filament is sufficient to complement the sterile phenotype of *coi1* mutants, indicating that the JA-Ile perception site is the epidermis (Browse and Wallis, 2019). Downstream *MYB21* and *MYB24* transcription factors were identified during the analysis of the *opr3* stamen transcriptome after JAs treatment (Song et al., 2011). In tomato, the jasmonate pathway also participates in promoting stamen development and pollen viability, but the sterility of the tomato *jasmonic acid-insensitive1* (*jai1*) mutant is caused by a defect in the maternal control of seed maturation (Li et al., 2004). The jasmonate pathway is also required for male sex determination and suppression of female reproductive organ biogenesis in maize (Acosta et al., 2009). Interestingly, both MpCOI1 and MpMYCs are not required for fertility in the early land plant *Marchantia polymorpha*, although the mutation in signalling pathway does affect the growth and defense phenotypes. Evidence suggests that the role of JA-Ile in regulating fertility was likely acquired or co-opted at a later stage of evolution (Monte et al., 2018; Peñuelas et al., 2019).

Initiation of JA-Ile biosynthesis upon mechanical damage

The initiation of JA-Ile biosynthesis in both shoots and roots remains rather unclear. JA and JA-Ile levels are quite low in vegetative tissues under basal conditions, while plants accumulate around 500-1000-fold of JA and 30-50-fold of JA-Ile 1 h after mechanical stresses and the hormone increase can be detected within 60 seconds after tissue injury (Glauser et al., 2009; Grebner et al., 2013; Koo et al., 2009; Schulze et al., 2019). This short time window suggests a rapid enzymatic substrate conversion and/or post-translational activation of the biosynthetic enzymes (Stenzel et al., 2003a; Wasternack, 2007). Consistently, biosynthetic enzymes such as the barley 13-LOXs and the tomato AOCs are basally expressed in respective plants, and their overexpression does not lead to constitutively higher hormone levels under basal condition (Bachmann et al., 2002; Stenzel et al., 2003a; Wasternack, 2007). Furthermore, inhibition of gene transcription or translation has no impact on wound-elicited JA-Ile biosynthesis (Kimberlin et al., 2022; Sharma et al., 2006; Staswick and Tiryaki, 2004). It has been therefore proposed that the first step of JA-Ile biosynthesis is the liberation of α-LeA

from galactolipids of plastid membrane by a phospholipase A1 (PLA1) (Bonaventure et al., 2011; Ryu, 2004). DAD1 is the first characterized lipase to be involved in JA biosynthesis during stamen development (Ishiguro et al., 2001). However, it has no role in production of the first rapid hormone burst and may be involved in JA production at later time points after wounding (>30min) (Ellinger et al., 2010; Kimberlin et al., 2022). DONGLE (DGL) is another PLA1 that was proposed to be involved in wound-induced JA-Ile biosynthesis in leaves (Hyun et al., 2008). However, another study later excluded this possibility, as DGL localizes to lipid bodies rather than plastids, and *dgl* mutants do not affect JA-Ile levels both under basal condition and after wounding (Ellinger et al., 2010). A PLA-Iγ3 knockout mutant was shown to have normal levels of wound-induced JA production but a greater than 50% reduction in basal levels of JA, OPDA, and dn-OPDA (Ellinger et al., 2010). Furthermore, PLA-Iγ3 is the only lipase shown to have a specific substrate preference for MDGD (Seo et al., 2009). Nevertheless, mutation of 16 lipases which have predicted plastid localization has no significant differences of JA-Ile levels after wounding compare with wt (Ellinger et al., 2010). Therefore, there are currently no lipase candidates involved in the initiation of wound-induced JA-Ile burst so far, possibly due to gene redundancy (Ellinger et al., 2010; Wasternack and Hause, 2013).

The presence of 13-LOXs in chloroplasts of resting leaves (Bachmann et al., 2002; Hause et al., 2003a; Stenzel et al., 2003a) suggests these enzymes may be activated in conjunction to unknown lipases post-translationally in response to a JA-Ile producing stimulus and hence initiate the first burst of hormone biosynthesis. 13-LOXs consist of two domains: an amino-terminal POLYCYSTIN-1 LIPOXYGENASE ALPHA-TOXIN (PLAT) mainly consisting of β-barrels, and a carboxyl-terminal LIPOXYGENASE (LOX) domain exerting the catalytic activity of the enzyme (Andreou and Feussner, 2009). Prior to their import into plastids, they also harbour a CHLOROPLAST TRANSIT PEPTIDE (cTP) that is then cleaved in the stroma upon import (Maynard et al., 2021; Springer et al., 2016) (Fig. 5).

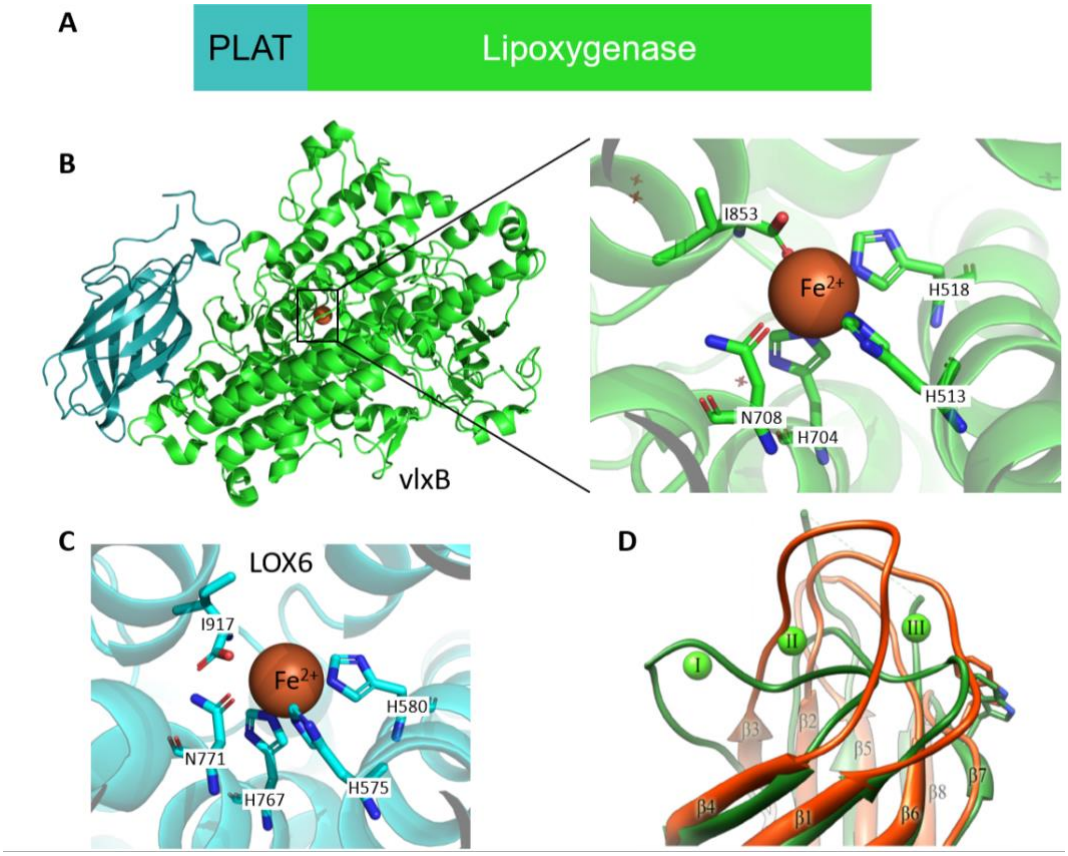

Figure 5. Protein structure and lipoxygenase features. (**A**) 2-D domain map of soybean lipoxygenase-B protein which has no chloroplast transit peptide. (**B**) Crystal structure of soybean lipoxygenase-B (PDB:2IUJ) and close up of the iron binding site, the PLAT domain is in blue and LOX domain in green. (**C**) Iron binding site in the Arabidopsis LOX6 protein, the protein structure is from AlphaFold protein structure database, the iron binding site was identified by sequence alignment with soybean lipoxygenase-B. The residues are conserved for all 13-LOXs. I917 is the last amino acid. (**D**) Superposition of the coral 8R-LOX Ca^{2+}-PLAT complex (green) and the 11R-LOX apo-PLAT (orange). Green spheres are calcium. Figure from (Eek et al., 2012).

The biochemical activity of the C-terminal LOX catalytic domain has been thoroughly characterized by the Feussner group (Hornung et al., 1999, 2008). The positional specificity of plant lipoxygenases is influenced by two crucial factors: the space available within the active site and the orientation of the substrate. These elements play significant roles in determining how the LOX enzyme interacts with its substrate and which specific positions on the substrate are targeted for oxygenation (Liavonchanka and Feussner, 2006). Several critical amino acids have been identified within the active site of selected lipoxygenase isozymes. These amino acids play essential roles in the catalytic activity and substrate specificity of the enzyme (Liavonchanka and Feussner, 2006). The catalytic domain of 13-LOXs contains one iron atom per protein molecule, which is coordinated by three histidines, one asparagine and one isoleucine (Andreou and Feussner, 2009).

The N-terminal PLAT domain is probably involved in regulatory functions. The soybean LOX1 was reported to bind membranes in response to calcium binding to its PLAT domain (Tatulian et al. 1998; Cho et al. 2015). In animal LOXs, the PLAT domain can regulate membrane interaction upon calcium binding (Walter et al., 2004), as well as substrate accessibility to the catalytic domain by removal of the obstruction lid which is mediated by π-cation and salt bridges between residues of the PLAT domain and helixes of the LOX domain (Eek et al., 2012, 2015). Consistently, biochemical studies have shown that plant 13-LOXs can bind calcium in *in vitro* assays to stimulate enzymatic activity (Mochizuki et al., 2018; Maynard et al., 2021). As mechanical wounding and herbivore attacks increase cytosolic calcium levels (Nguyen et al., 2018; Toyota et al., 2018), several works have proposed a direct link between elevated calcium concentrations and jasmonate production, reviewed in (Mielke and Gasperini, 2019). Among all the JA-Ile biosynthesis enzymes, 13-LOXs are the only ones harbouring a putative calcium binding domain. A possibility is that in response to wounding, increases in cytosolic calcium concentrations are transmitted to plastids, promoting calcium-PLAT binding of 13-LOX which in turn would stimulate catalytic activity, and/or membrane associations granting substrate accessibility. However, such possibilities remain untested. It has been also suggested that a tight channelling of metabolites through LOX2-AOS-AOC protein complex could increase the yield and the rate of *cis*-(+)-12-OPDA production with keeping reaction intermediates levels quite low (Pollmann et al., 2019). Since LOX2 is not present in roots and *lox2* mutants can still produce JA-Ile after wounding, this metabolon may only be relevant in leaves (Glauser et al., 2009).

The rapid production of JA-Ile could also derive from the conversion of a precursor into JA-Ile, such as arabidopsides, OPDA or jasmonic acid (Koo et al., 2009; Schaller and Stintzi, 2009). However, the majority of oxylipins (OPDA, JA, JA-Ile, arabidopsides) accumulate upon wounding (Glauser et al., 2009; Grebner et al., 2013; Schulze et al., 2019). JA-Ile biosynthesis initiation from MGDG does not preclude the possibility of other regulatory steps reinforcing JA-Ile production. Furthermore, it remains unclear if all cell types across all tissues are responsible to produce the first burst of JA-Ile after tissue injury, or if cellular specificity is also an important component of the whole-plant response.

Plant Lipoxygenases

Lipoxygenases are non-heme iron-containing dioxygenases that form fatty acid hydroperoxides from polyunsaturated substrates (Brash, 1999). They can be found in eukaryotes across plants, fungi, and animals. In mammals, different LOX isozymes are implicated in the metabolism of eicosanoids such as prostaglandins and leukotrienes (Ding et al., 2003). Plant LOXs are monomeric proteins of about 95-100kDa, of which 40kDa belong to the PLAT domain and about 55-65 kDa to the catalytic LOX domain (Schneider et al., 2007)(Fig. 5).

LOXs are classified with respect to their positional specificity of substrate oxygenation. In Arabidopsis, an unsaturated PUFA (e.g. α-LeA) can be oxygenated either at C-9 by 9-LOXs or at C-13 by 13-LOXs (Liavonchanka and Feussner, 2006) (Fig. 6). The two reactions form two groups of compounds: the (9*S*)-hydroperoxy- and the (13*S*)-hydroperoxy derivatives of PUFAs. The two LOX classes can be grouped also according to their spatial localization: 9-LOXs localize to the cytoplasm and 13-LOXs to plastids (Wasternack, 2007). Lipoxygenases with dual 9- and 13-lipoxygenase activity have also been identified in potato and soybean (Fukushige and Hildebrand, 2005; Hughes et al., 2001)

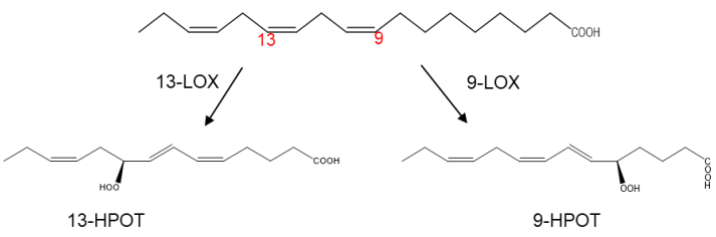

Figure 6. Arabidopsis 9- and 13-LOXs. 13-LOX (13-Lipoxygenase), 9-LOX (9-Lipoxygenase), 13-HPOT (13(*S*)-hydroperoxy-octadecatrienoic acid), 9-HPOT (9(*S*)-hydroperoxy-octadecatrienoic acid).

Arabidopsis 9-LOX enzymes (LOX1 and LOX5) use both linoleic acid and linolenic acid as substrates to produce 9-Hydroperoxy-octadecadienoic acid (9-HPOD), 9(*S*)-hydroperoxy-octadecatrienoic acid (9-HPOT), 9-ketooctadecatrienoic acid (9-KOT) (Boeglin et al., 2008). Most of the products derived from this pathway are involved in pathogen defense (Viswanath et al., 2020). Specifically, the *lox1* knock out mutant is more susceptible to *Pseudomonas syringae pv*. Tomato (Vicente et al., 2012), and participates in stomatal closure in response to both bacteria and the pathogen-associated molecular pattern flagellin peptide flg22

(Montillet et al., 2013). Oxylipins produced by 9-LOXs are also involved in lateral root development (Vellosillo et al., 2007). Importantly, although 9-LOX transcripts are JA-Ile responsive, 9-LOXs do not participate in OPDA nor JA-Ile biosynthesis (Wasternack and Hause, 2013).

There are four *13-LIPOXYGENASE* genes in *Arabidopsis thaliana* contributing to wound-induced JA-Ile biosynthesis: *LOX2, LOX3, LOX4* and *LOX6* (Chauvin et al., 2013, 2016; Glauser et al., 2009; Grebner et al., 2013). Phylogenetic analysis shows that while Arabidopsis LOX2 is more distantly related to the other three, LOX3 and LOX4 are very similar (Caldelari et al., 2011). All four enzymes oxygenate linolenic acid more effectively than linoleic acid (Bannenberg et al., 2009). *In vitro* studies show that all 13-LOXs reach their highest activity around neutral pH, which is reduced dramatically in alkaline condition with LOX2 being the most sensitive and LOX4 the least sensitive towards pH changes (Maynard et al., 2021). LOX6 has the highest apparent affinity to α-LeA while LOX2 has the lowest. LOX3 and LOX4 have relative higher velocities of product formation than LOX2 and LOX6 (Maynard et al., 2021). Divalent cations such as Ca^{2+} and Mg^{2+} could increase the 13-LOXs enzymatic activity while, on the contrary, compounds like OPDA and calcium chelators inhibited their activity (Maynard et al., 2021; Mochizuki and Matsui, 2018).

Physiological roles of 13-LOXs

While all four Arabidopsis 13-LOX enzymes catalyse the same enzymatic reaction (Bannenberg et al., 2009), the study of *lox* mutants revealed their individual physiological roles (Caldelari et al., 2011; Glauser et al., 2009; Grebner et al., 2013). LOX2 is essential for JA-Ile production upon wounding and insect herbivory in green tissues (Bell et al., 1995; Gasperini et al., 2015; Glauser et al., 2009), and plays minor roles in JA-Ile synthesis in undamaged leaves distal to wounds (Glauser et al., 2008). LOX2-derived hydroperoxides are also channelled into the synthesis of Brassicaceae-specific compounds, called arabidopsides, which are galactolipids that carry one or more esterified OPDA or dnOPDA molecules (Nilsson et al., 2012; Seltmann et al., 2010; Zoeller et al., 2012). In the *lox2* mutant, however, JA and JA-Ile are still synthesized in the first 5 min upon wounding (Glauser et al., 2009) indicating the activity of other 13-LOXs can sustain JA-Ile production. In addition, formation of five

carbon volatiles is also dependent on *LOX2* (Mochizuki et al., 2016), and this is regulated by calcium-dependent activation of LOX2 (Mochizuki and Matsui, 2018). *LOX3* and *LOX4* are indispensable and sufficient for Arabidopsis reproductive development, as demonstrated by the complete male sterility of the *lox3lox4* double mutant (Caldelari et al., 2011). LOX3 and LOX4 are also the principal 13-LOXs responsible for vegetative growth restriction after repetitive wounding (Yang et al., 2020b). *lox3* and *lox4* mutants behaved opposite towards nematodes infection, with LOX4 playing a major role (Ozalvo et al., 2014). LOX6 is essential for JA and JA-Ile accumulation in leaves distal to the wounded ones (Chauvin et al., 2013), and for basal and stress-induced OPDA, JA and JA-Ile accumulation in wounded roots (Grebner et al., 2013). (Chauvin et al., 2016) revealed a two-tiered, paired hierarchy in which LOX6, and to a lesser extent LOX2, control most of the early-phase of jasmonate response gene expression. LOX2 and LOX6 are active upstream of LOX3 and LOX4 in several responses (Chauvin et al., 2016).

Consistent with their different physiological roles, the promoters of the 4 different 13-LOXs are active in different cell types (Chauvin et al., 2016; Vellosillo et al., 2007). Under basal conditions, the *LOX2* promoter activity can be found in most cell types of above ground tissues but not in roots; the *LOX3* promoter is active in the shoot epidermis, shoot vasculature with the strongest activity at the xylem pole and lateral root primordia; *LOX4* promoter is active in the vasculature (more strongly at the phloem than the xylem pole) of above ground organs; and *LOX6* promoter is active in xylem contact cells of shoots although other studies found its transcripts specifically in roots (Chauvin et al., 2016; Grebner et al., 2013; Vellosillo et al., 2007). Interestingly, while *LOX2*, *LOX3* and *LOX4* transcripts are wound-inducible, LOX6 transcripts and protein levels are not wound-responsive in leaf tissue (Chauvin et al., 2013, 2016; Grebner et al., 2013). Chloroplast proteomic analysis revealed that LOX2 is localized in the chloroplast thylakoid and envelope membrane (Peltier et al., 2006).

Aims and objectives

Although JA-Ile biosynthesis as well as its perception and signalling are well characterized, reviewed in (Wasternack and Feussner, 2018), the initiation of hormone production in plastids and the regulation of plastidial JA-Ile biosynthetic enzymes is still unclear. The rapidity of JA-Ile production after tissue damage (Glauser et al., 2009) together with the basal presence of 13-LOX enzymes (Bachmann et al., 2002; Stenzel et al., 2003b) suggest that the post-translational activation of 13-LOXs might be critical for hormone biosynthesis initiation. To test this hypothesis, I will focus on 13-LOXs and their regulation in Arabidopsis roots as the pathway in this organ is less studied in comparison to leaves, with the overall aim of gaining new insights in JA-Ile biosynthesis initiation. Specifically, I aim to answer the following questions:

- Which of the four 13-LOXs contribute root induction of JA-Ile marker gene expression following root wounding in intact Arabidopsis plants?
- Which root cell types produce the first burst of JA-Ile after root wounding?
- Do 13-LOXs require activation to initiate JA-Ile production in planta?
- Can 13-LOXs complement one another, or they have specific functions?
- Do swaps between PLAT and LOX domains alter enzyme functionality in planta?

II. RESULTS

2.1 Which of the four 13-LOXs contribute(s) to root induction of JA-Ile marker gene expression following root wounding in intact Arabidopsis plants?

Mechanical wounding of Arabidopsis roots results in increased expression of JA-Ile marker genes such as *JAZ10* (Schulze et al., 2019). While basal OPDA levels are relatively high in roots (~30pmol/g FW), JA and JA-Ile levels are below the limit of quantification (Grebner et al., 2013; Schulze et al., 2019). When roots are detached from hydroponically grown 6-8-week-old plants, and then wounded five times along the entire root, their OPDA levels decrease while JA and JA-Ile levels increased in a LOX6- and time-dependent manner (Grebner et al., 2013). In fact, a *lox6* mutant was depleted in basal OPDA root levels and was unable to induce JA and JA-Ile levels after root wounding, suggesting that LOX6 is essential to synthesize basal OPDA pools, which are then used to produce JA and JA-Ile after wounding wild-type roots (Grebner et al., 2013). However, the experimental setup used by (Grebner et al., 2013) lacked mock controls (root separated from the plant and removed from hydroponic media but not subjected to wounding). To therefore clarify if LOX6 is essential to initiate root JA-Ile signalling in intact plants, I used several single and multiple order *lox* mutants (*lox2, lox3, lox4, lox6, lox23, lox24, lox26, lox34, lox36, lox46, lox234, lox236, lox246, lox346, lox2346*) described in (Caldelari et al., 2011; Chauvin et al., 2013; Glauser et al., 2009), evaluated JA-Ile marker gene expression before and after root wounding, as well as analysed the expression pattern of available *13-LOXp:GUS* reporter lines (Chauvin et al., 2016).

2.1.1 Optimizing root wounding in intact plants

To ensure that root wounding induces only a local response in the damaged organ, I first established a robust and reliable root wounding method that was then used across all my experiments. Because OPDA and JA are shoot-to-root mobile (Schulze et al., 2019), I wanted to avoid any induction of JA-Ile signalling in shoots to prevent the impact aerial organs might have on root JA-Ile signalling. Root wounding in young 5-d-old seedlings caused *JAZ10p:GUS* reporter induction only near the sites of damage, without spreading to aerial organs (Schulze et al., 2019). To confirm these findings and establish an optimized root wounding method in older plants, I wounded 2-week-old *JAZ10p:GUS* reporter plants (Gasperini et al., 2015) with 4 different methods (Fig. 2-1A). As a positive control, I wounded 4 leaves with fine forceps

which, as expected (Gasperini et al., 2015), provoked a strong reporter induction in shoots as well as in roots because of hormone precursor translocation (Schulze et al., 2019).

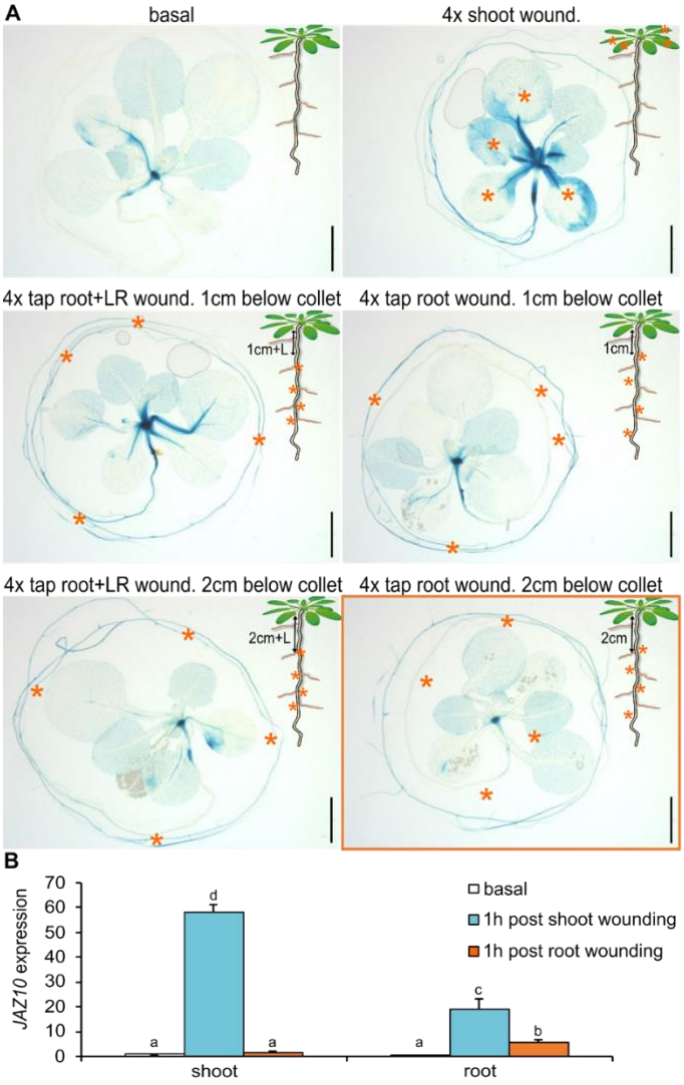

Figure 2-1. Root wounding optimization. (**A**) *JAZ10p:GUS* reporter activity in 2-week-old vertically grown plants at basal conditions and 2 h after wounding different locations (indicated by orange asterisks) with fine forceps. The top panel refers to basal condition and shoot wounding as a positive control; middle panel refers to 4 times root wounding at 1 cm below the collet region with or without lateral roots (LR); the bottom panel refers to 4 times root wounding at 2 cm below the collet region with or without lateral roots. Scale bars, 0.5 mm. (**B**) Quantitative qRT-PCR of *JAZ10* expression in wt shoots and roots at basal conditions, 1 h after shoot wounding, and 1 h post root wounding. Transcript levels were normalized to those of *UBC21* and calibrated to basal shoot or root levels. Bars represent the means of three biological replicates (±SD), each containing a pool of 5 organs from 2-week-old plants. Letters denote statistically significant differences among samples as determined by two-way ANOVA followed by Tukey's HSD test ($p<0.01$).

I then performed four equidistantly spaced root wounds, starting at different sites below the collet region (hypocotyl-root interface). When the first wound was performed 1 cm below the collet region and wounding included lateral roots, *JAZ10p:GUS* reporter induction was visible both in roots and shoots (although weaker). Repeating the same procedure but omitting wounding of lateral roots, still induced reporter activity in shoots. I therefore wounded roots at four equidistant locations starting 2 cm below the collet region. When the lateral roots were wounded together with tap roots, reporter activity was robustly triggered in roots and much less prominent in shoots. Finally, when the four wounds were performed only on the tap root (excluding lateral roots) starting 2 cm below the collet, reporter induction was visible

in the roots but did not reach aerial organs (Fig. 2-1A). Therefore, all my experiments were conducted by wounding the root with this last method.

To verify if *JAZ10p:GUS* reporter activity coincided with increased JA-Ile signalling, I evaluated *JAZ10* transcript induction in shoots and roots from shoot- and root-wounded wild-type plants, respectively (Fig. 2-1B). Shoot wounding induced a 60-fold *JAZ10* increase in shoots, and approximately a 20-fold increase in roots. On the other hand, in line with results from the *JAZ10p:GUS* reporter, my newly optimized root wounding method led to a 10-fold *JAZ10* increase in roots, while shoot induction was not statistically significant.

2.1.2 Evaluating root *13-LOX*s gene expression

All four 13-LOXs participate in JA-Ile production and signalling in wounded Arabidopsis shoots (Chauvin et al., 2013, 2016; Glauser et al., 2009). While the activity of LOX6 has been proposed to be critical for root JA-Ile production and signalling (Grebner et al., 2013), the involvement of the other enzymes is less clear. For example, transcriptional GUS reporter fusions of *LOX2*, *LOX3*, *LOX4*, and *LOX6* promoters showed basal root activity only for *LOX3p*, in agreement with its role in lateral root formation (Vellosillo et al., 2007). However, another study found that only *LOX6* transcripts are detectable in roots at basal conditions and not those of the other three genes (Grebner et al., 2013). Hence, I aimed to clarify the root expression pattern of all four genes and verify which is the main *13-LOX* involved in root JA production. To do so, I first tested basal *13-LOXp:GUS* promoter activities in available reporters (Chauvin et al., 2016) by performing a longer overnight β-glucuronidase (GUS) staining as opposed to the published 2 h in both 5-d-old seedlings and 2-week old adult plants(Fig. 2-2A, S1).

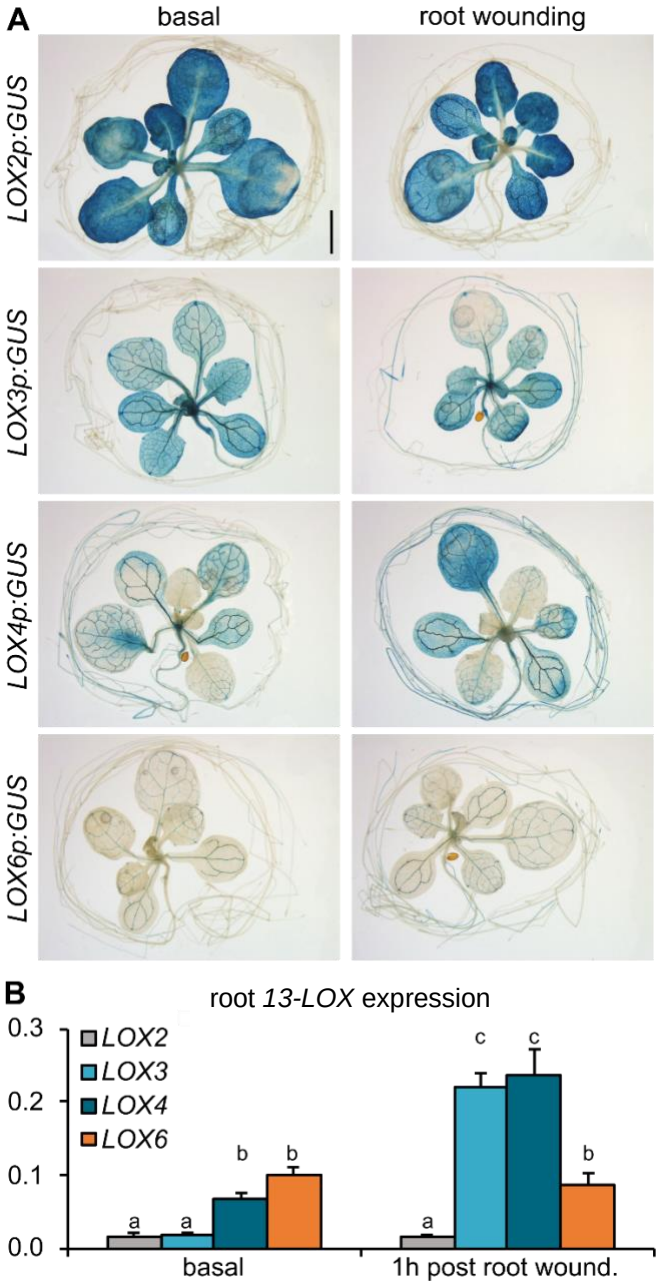

Figure 2-2. *13-LOX* expression pattern. (**A**) Histochemical detection of *13-LOXp:GUS* reporter activity in 2-week-old plants at basal conditions and 4 h after root wounding (single wound in the middle of the tap root followed by overnight GUS staining. Scale bars, 0.5mm (**B**) Quantitative qRT-PCR of *LOX2*, *LOX3*, *LOX4*, and *LOX6* expression in wt roots before and 1 h after root wounding. Transcript levels were normalized to those of *UBC21*. Bars represent the means of three biological replicates (±SD), each containing a pool of 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by two-way ANOVA followed by Tukey's HSD test ($p<0.01$).

The GUS staining confirmed that *LOX2* promoter is active only in the shoots (Gasperini et al., 2015). However, in addition to their predicted expression in shoots (Chauvin et al., 2016; Vellosillo et al., 2007), the activities of all three *LOX3*, *LOX4* and *LOX6* promoters were detectable in roots (Fig. 2-2A). To characterize if *13-LOX*s promoter activity is wound-inducible, I next analyzed their promoter activities after root wounding (Fig. 2-2A). I observed that the activity of *LOX2p:GUS* was still absent in roots following root wounding, but that the activities of *LOX3p* and *LOX4p* were clearly induced in the wounded organ.

The activity of *13-LOX* promoters was then validated by *13-LOXs* transcript expression via qRT-PCR (Fig.2-2B). As expected from (Grebner et al., 2013), the highest basal root transcript levels were those of *LOX6*. However, under our experimental conditions, *LOX4* transcripts were also detected in unwounded roots with similar levels to *LOX6* transcripts and were higher than those of *LOX2* or *LOX3*. I then analyzed their expression 1 h after root wounding. *LOX2*, *LOX3* and *LOX4* transcript levels are wound-inducible in shoots, while those of *LOX6* were not (Grebner et al., 2013). In agreement with root data from (Grebner et al., 2013), root wounding triggered the induction of *LOX3* and *LOX4* but not that of *LOX2* nor *LOX6* (Fig. 2-2B). Based on this gene expression analysis, the data suggest a possible role of *LOX3*, *LOX4* and *LOX6* genes, but not of *LOX2*, in root JA-Ile production and signalling.

2.1.3 Verification of available genetic material

To test which 13-LOX is essential for root JA-Ile production upon mechanical wounding, I took advantage of all the available *13-lox* single and multiple order mutants (Caldelari et al., 2011; Chauvin et al., 2013; Glauser et al., 2009), and first validated their genotype (Fig. 2-3A). The *lox2* mutant harbors a Trp630Stop mutation in LOX2 (Glauser et al., 2009), while *lox3A*, *lox4B* and *lox6A* single mutants are all T-DNA insertion lines (Caldelari et al., 2011). The double, triple and quadruple mutants were generated by crossing the single mutants (Chauvin et al., 2013). All the mutant genotypes were verified to be correct (Fig. 2-3B-E).

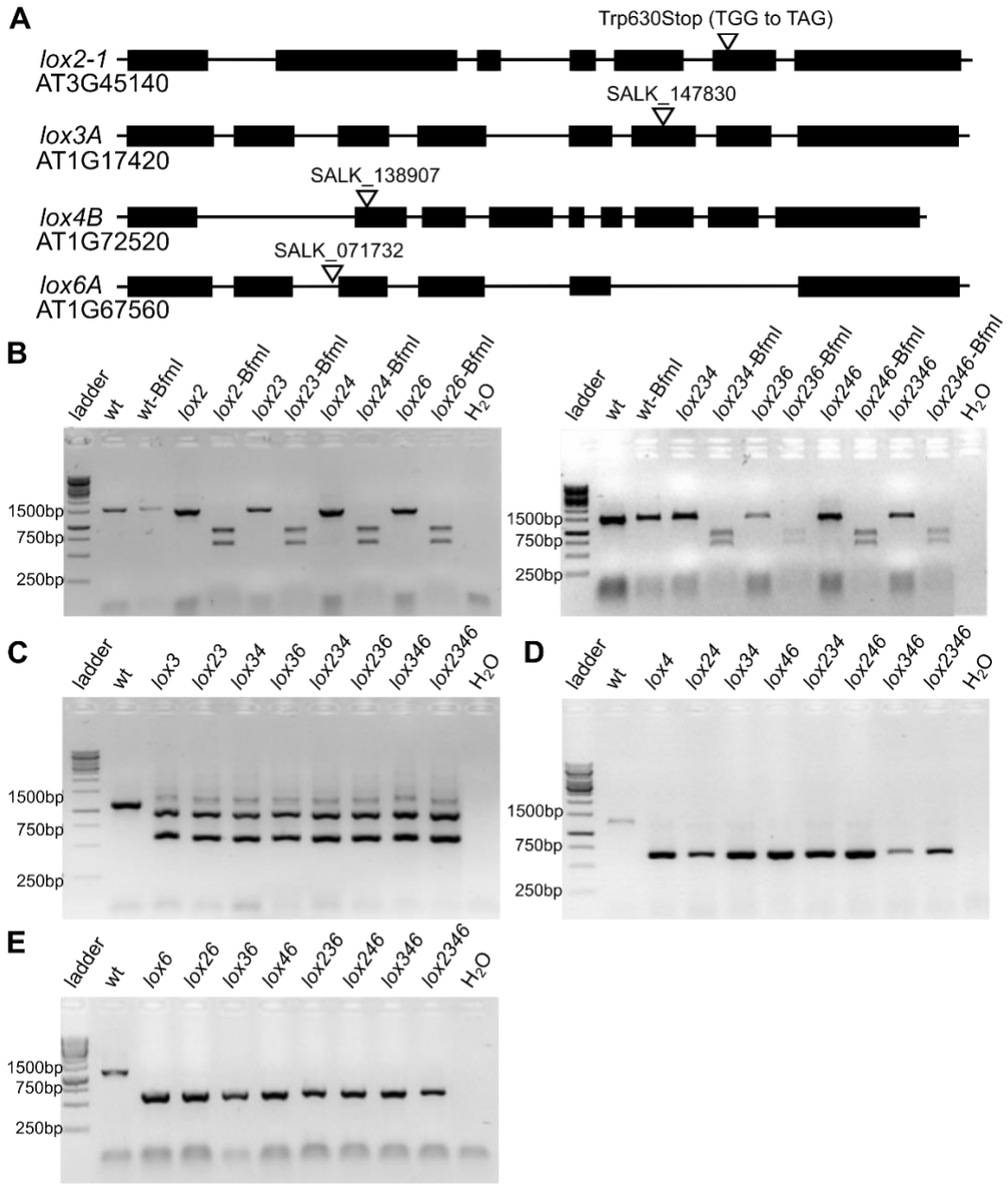

Figure 2-3. (**A**) Scheme of *13-lox* mutant alleles and mutation sites. Lines indicate introns and black boxes exons. (**B-E**) Genotyping of *13-lox* mutants. (**B**) *lox2-1* was amplified with LOX2.1F, LOX2.1R and digested with *BfmI*, with wt expected size of 1516bp and 864bp + 652bp in the *lox2-1* mutant. (**C**) *lox3A* was genotyped with SALK.LBb1.3 primer plus MAY136 and MAY137, the expected wt size is 1180bp, and the expected *lox3* mutant size is 490-790bp. (**D**) *lox4B* was genotyped with SALK.LBb1.3 plus MST266 and MST267, the expected wt size is 1246bp, and the expected *lox4B* mutant size is 556-856bp. (**E**) *lox6A* was genotyped with SALK.LBb1.3 primer plus MYJ003 and MYJ004, the expected wt size is 1263bp, and the expected *lox6A* mutant size is 578-878bp. Note that the images were cropped in B-C, and the negative control (H_2O) was re-aligned.

2.1.4 LOX6 is essential to induce root JA-Ile signalling after root wounding

To assess the individual contribution of each *13-LOX* to root JA-Ile signalling as a proxy for hormone production, I next analyzed *JAZ10* expression before and after root wounding in a series of single 1*3-lox* mutants (e.g. *lox2,* which has no LOX2 activity) and corresponding triple

mutants retaining only the activity of a single 13-LOX (e.g. *lox346,* which retains only LOX2 activity). Under basal condition, JA and JA-Ile levels are very low in Arabidopsis roots (Glauser et al., 2009; Grebner et al., 2013). Consistently, basal *JAZ10* root levels across all *13-lox* mutants were low and similar to the wt (Fig. 2-4A). in agreement with the lack of root *LOX2* promoter activity and low root *LOX2* transcript levels (Fig. 2-2A), wound-induced *JAZ10* levels in the *lox2* mutant were similar to the wt. While the *lox346* mutant, retaining only LOX2 activity, was unable to induce JA-Ile signalling (Fig. 2-4B), excluding a role of *LOX2* from root JA-Ile production and signalling.

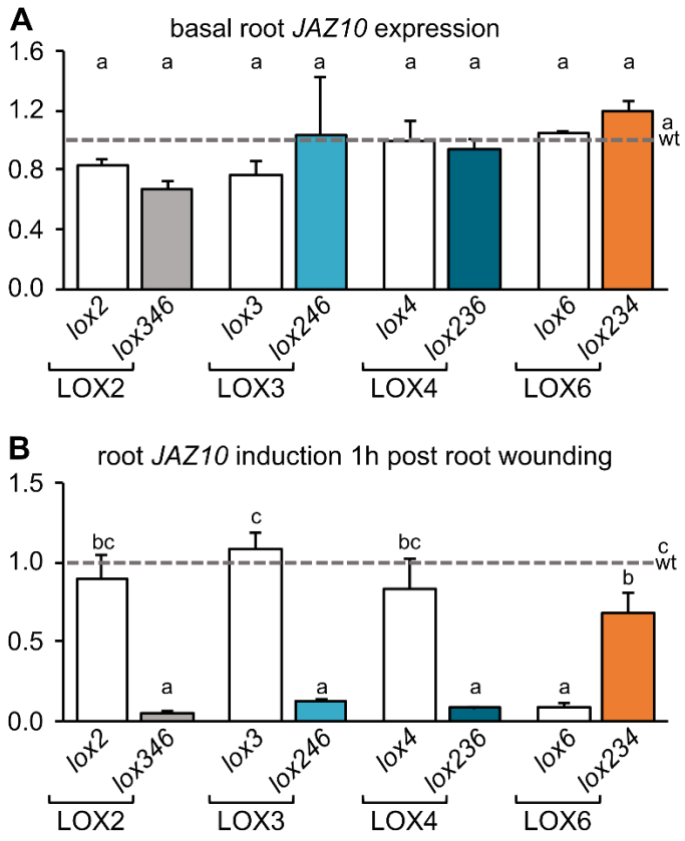

Figure 2-4. *LOX6* is essential for root JA production. Quantitative qPCR of *JAZ10* expression in roots of wt, *lox2*, *lox346* (retaining only LOX2 activity), *lox3*, *lox246* (retaining only LOX3 activity), *lox4*, *lox236* (retaining only LOX4 activity), *lox6*, *lox234* (retaining only LOX6 activity) at (**A**) basal and (**B**) 1 h after root wounding. *JAZ10* transcript levels were normalized to those of *UBC21* and are displayed relative to the expression of wt controls that are set to 1 and indicated with dashed lines. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

Similarly to the results for LOX2, wound induced *JAZ10* induction in *lox3* and *lox4* mutants was comparable to the wt, while the corresponding triple *lox246* and *lox236* mutants were unable to trigger *JAZ10* induction after root wounding (Fig. 2-4B). This indicates that individual activities of LOX3 and LOX4 cannot trigger root JA-Ile production after root wounding. Finally, the *lox6* single mutant was fully compromised in inducing root *JAZ10* expression following root wounding, with a nearly 100% reduction in *JAZ10* transcript levels compared to the wt (Fig. 2-4B). Consistently, the *lox234* mirror mutant retaining only LOX6 activity, was able to

induce around 70% of wt *JAZ10* induction, revealing that *LOX6* is essential but not sufficient to govern root JA-Ile production and signalling.

2.1.5 LOX3 and LOX4 aid LOX6 to reach wt *JAZ10* levels after root wounding

As LOX6 activity alone was not sufficient to induce root JA-Ile signalling to wt levels after wounding, I hypothesized that another 13-LOX participates in root JA-Ile production, following or in conjunction with LOX6. In leaves, the activity among the four different 13-LOX is hierarchically organized after wounding, with LOX2 and LOX6 producing the early burst of JA-Ile, which then induces the expression of *LOX3* and *LOX4* to further promote JA-Ile production and mount defense responses (Chauvin et al. 2016). To assess if LOX3, LOX4, or both assist LOX6 in triggering *JAZ10* expression in Arabidopsis roots upon mechanical root wounding, I analysed the contribution of *lox23* (retaining the activity of LOX6 and LOX4) and *lox24* (retaining the activity of LOX6 and LOX3) to *JAZ10* induction and compared it to that of *lox234* (with LOX6 alone). If both LOX3 and LOX4 aid LOX6 to fulfil a wt response, wound-induced *JAZ10* levels should be higher in *lox23* and *lox24*, compared to those induced in *lox234*.

In line with previous results, *JAZ10* transcript levels were very low in all genotypes under basal conditions (Fig.2-5). Following root wounding, wt plants had nearly 20 to 30-fold induction of *JAZ10* levels in 2 independent experiments, while those of *lox234* had about 50% of those of the wt (Fig. 2-5). On the other hand, wound-induced *JAZ10* levels were in the *lox24* mutant, which retains the activity of LOX3 and LOX6, between wt and *lox234* mutant. A similar trend was also observed for the *lox23* mutant which retains the activity of LOX3 and LOX6 (Fig. 2-5), indicating that either LOX3 or LOX4 aid LOX6 while if both are necessary at the same time still needs to be tested.

Figure 2-5. LOX3 and LOX4 aid LOX6 to reach wt *JAZ10* levels after root wounding. Quantitative qPCR of *JAZ10* expression in 2-week-old roots of wt, *lox23*, *lox24*, and *lox234* before and 1 h after root wounding. Transcript levels were normalized to those of *UBC21* and calibrated to basal root levels. Bars represent the means of three biological replicates (±SD), each containing a pool of 5 organs. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

2.2 Which root cell types produce the first burst of JA-Ile after root wounding?

At the tissue level, jasmonates can be detected in all organs containing plastids, such as leaves, roots and flower organs (Glauser et al., 2009; Grebner et al., 2013; Hause et al., 2000). Because OPDA and JA are cell-to-cell mobile (Li et al., 2020; Schulze et al., 2019), the cell types producing the first burst of JA-Ile after root tissue damage remain unknown. Specifically, which root cell type(s) initiate the first burst of hormone production in response to environmental stimuli such as mechanical damage? I hypothesized that the localization of JA-Ile biosynthetic genes, and specifically LOX6, should reveal the specific cell types that produce the first burst of JA and JA-Ile following root damage. Previous reports revealed that *LOX3* promoter is active in the shoot epidermis, and the shoot vasculature with the strongest activity at the xylem pole; while the *LOX4* promoter is active in the vasculature (more strongly at the phloem than the xylem pole) of above ground organs; and the *LOX6* promoter in xylem contact cells of shoot vasculature (Chauvin et al., 2013, 2016; Vellosillo et al., 2007). In addition, LOX, AOS and AOC were found within the phloem sieve elements in tomato (Hause et al., 2003a). Because these studies were confined to the shoots, little is known about the cellular localization of *13-LOX* promoters in the roots. I have therefore generated both transcriptional and translational 13-LOX reporters to characterize their cellular expression domains in the primary root.

2.2.1 Expression maps of *13-LOX* promoter activities

As *13-LOXp:GUS* reporters showed that only the promoters of *LOX3*, *LOX4* and *LOX6* were active in roots (Fig. 2-2A), I focused on those three. I have therefore generated 13-*LOXp:NUCLEAR-LOCALIZATION-SIGNAL(NLS)-3xVENUS* transcriptional constructs in wt background to detect nuclear fluorescence signals from cells expressing *13-LOX* promoters. To better visualize vascular cell types across the root, I have used the clear see protocol (Ursache et al., 2018) to clear the material and visualize cellular contours with calcofluor white which stains the cellulose component of plant cell walls.

Starting at the root tip meristem region of 5-d-old seedlings, *LOX3* promoter activity was present in the root cap and epidermal cells (Fig. 2-6A). Its expression domain remained in epidermal cells and started to appear in the vasculature of the early differentiation zone, with the vascular signal becoming more prominent in the middle of the root (late differentiation zone). While optical cross sections are still ongoing for *LOX3p:NLS-3xVENUS*, the fluorescence signal in the late differentiation zone appears to derive from pericycle cells (Fig. 2-6A).

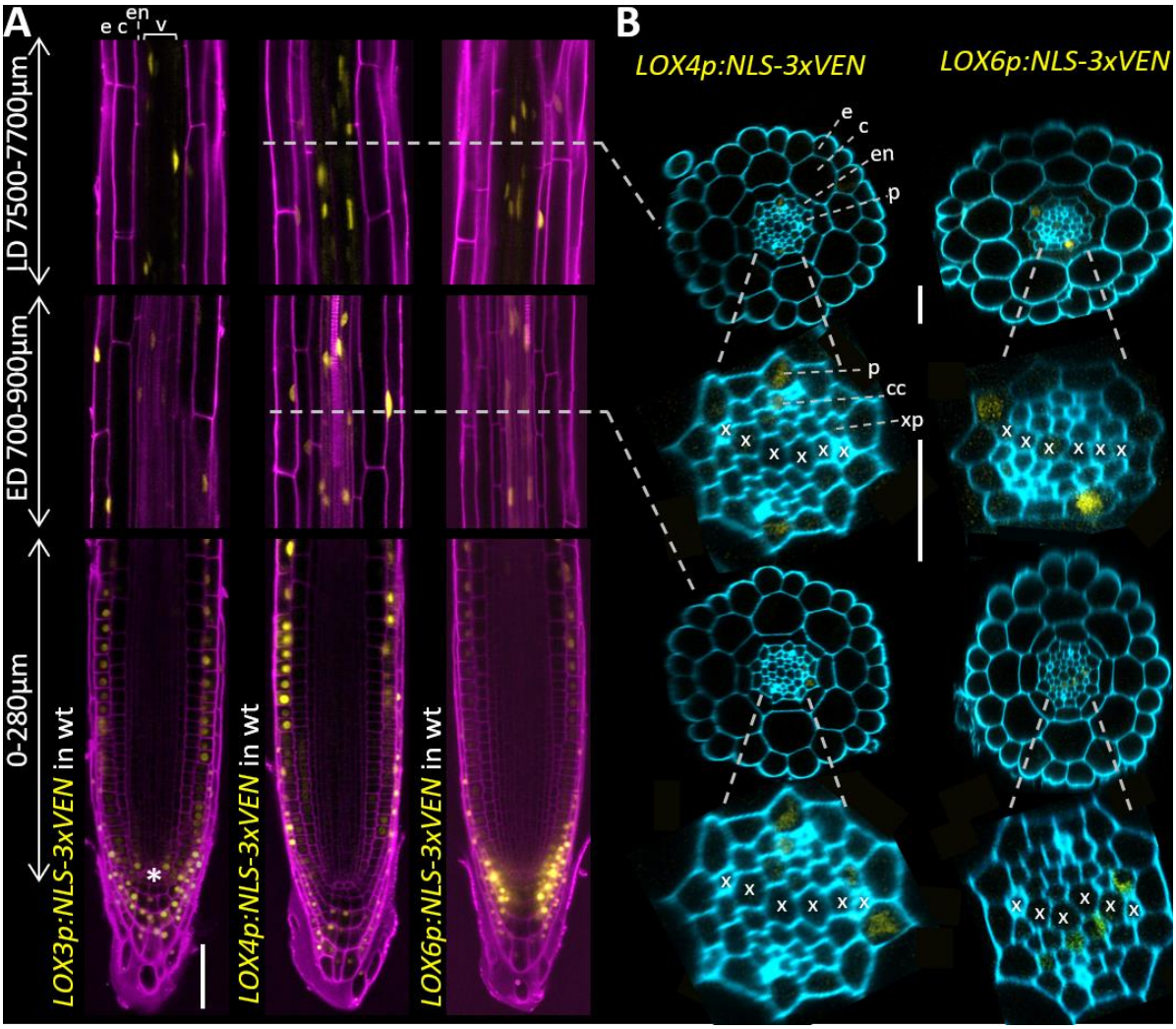

Figure 2-6. Promoter activity of *LOX3, LOX4* and *LOX6* in primary root. (**A**) Representative images of NLS-3xVENUS signals controlled by *LOX3*, *LOX4* and *LOX6* promoters in primary roots of 5-day-old seedlings stained with propidium iodide (magenta). ED: early differentiation zone, LD: late differentiation zone. Distances from the quiescent center (asterisk) are indicated on the left side. Scale bar, 50 µm. (**B**) Transverse section of *LOX4* and *LOX6* promoter activity in early and late differentiation zone stained with calcofluor white (cyan) and the below is the close-up of their promoter activity within the vasculature. e, epidermis; c, cortex; v, vasculature; en, endodermis; p, pericycle; cc, phloem companion cell; x, xylem; xp, xylem procambium. Scale bar, 20 µm.

Similarly to *LOX3p*, the *LOX4* promoter was active in the root cap and epidermal cells of the root meristem, with broader activity in the early differentiation zone encompassing epidermal, cortex, pericycle and vascular cells (Fig. 2-6A). The expression domain was then restricted to cortex and inner vascular tissues in the late differentiation zone (Fig. 2-6A). Optical transverse sections further revealed that *LOX4p* is expressed in phloem companion and pericycle cells and procambium cells associated with protoxylem in the early differentiation zone, and its expression domain extended into pericycle cells adjacent to the phloem pole (Fig. 2-6B).

In the division zone of the root meristem, the *LOX6* promoter was active in the root cap and in epidermal cells, with a prominent vascular expression starting to appear in the elongation zone which continued further into the early and late differentiation zone (middle) of the root including the endodermis (Fig. 2-6A). Optical transverse sections revealed that *LOX6p* was active in xylem procambium cells of the early differentiation zone and extended into pericycle cells of the late differentiation zone (Fig. 2-6B).

Collectively, while the expression domains of *LOX3p*, *LOX4p* and *LOX6p* overlap in the division zone of the root meristem and in the pericycle of the late differentiation zone, the analysis revealed that in vascular tissues *LOX3p* can be predominantly found in pericycle cells, *LOX4p* in phloem companion cells and pericycles cells adjacent to the phloem pole, while *LOX6p* is predominantly found in procambium cells associated to the xylem. Thus, the three promoters have both overlapping and distinct cell-type-specific expression domains.

2.2.2 Developing functional 13-LOX translational reporters

As promoter activity does not necessarily reflect protein translation, I have next generated 13-LOX protein fusion reporters with CITRINE (CIT) under the regulation of their native promoters to reveal the cell types in which the three JA-Ile biosynthesis enzymes are basally present, with the aim of uncovering which cells in the root can produce the initial burst of JA-Ile. I have therefore developed both N- and C-terminal fusion reporters, i.e. *13-LOXp:CIT-LOX* and *13-LOXp:LOX-CIT*, to test their respective functionality and analyze only functional reporters thereafter.

For N-terminal fusions, I inserted the reporter fluorophore between the chloroplast TRANSIT PEPTIDE (cTP) specific to each 13-LOX and the start of the PLAT domain to guide their localization to plastids. The functionality of N- and C-terminal fusion proteins were verified in planta by assessing the construct capacity to complement specific mutant phenotypes. Specifically, the *lox2* mutant has compromised *JAZ10* induction (Gasperini et al., 2015) and JA-Ile production after cotyledon wounding (Glauser et al., 2009). I transformed both N-terminal and C-terminal fusion constructs into *lox2* mutant to test which version could rescue the compromised shoot *JAZ10* induction upon cotyledon wounding. I have selected two independent T_3 transgenic lines for each construct. As expected, *JAZ10* expression was very low under basal conditions (Gasperini et al., 2015). Upon shoot wounding, the green tissues of the wt significantly induced *JAZ10* expression, while the *lox2* mutant was not able to induce wt *JAZ10* levels (Fig. 2-7A). Expressing the *LOX2p:CIT-LOX2* construct in the *lox2* mutant complemented the *JAZ10* phenotype upon shoot wounding, while the *LOX2p:LOX2-CIT* fusion could not do so and still exhibited a *lox2* mutant phenotype (Fig. 2-7A).

To test the functionality of LOX3 and LOX4 protein fusions, I used the sterility phenotype of the *lox34* double mutant (Caldelari et al., 2011), which was recovered by the N-terminal fusions of both LOX3 and LOX4 (CIT-LOX3 and CIT-LOX4), but not by C-terminal fusions (Fig. 2-7B). Finally, to test the functionality of LOX6 translational fusions, I tested if CIT-LOX6 or LOX6-CIT could rescue the inability of the *lox6* mutant to induce *JAZ10* expression after root wounding (Fig. 2-4). As for the other constructs, only CIT-LOX6 fusions could restore *JAZ10* levels induced by root wounding in *lox6* and not LOX6-CIT lines (Fig. 2-7C). Overall, the data indicate that N-terminal fusion proteins are functional for all *13-LOX*s, therefore, their protein localization was analysed in the N-terminally tagged native promoter lines.

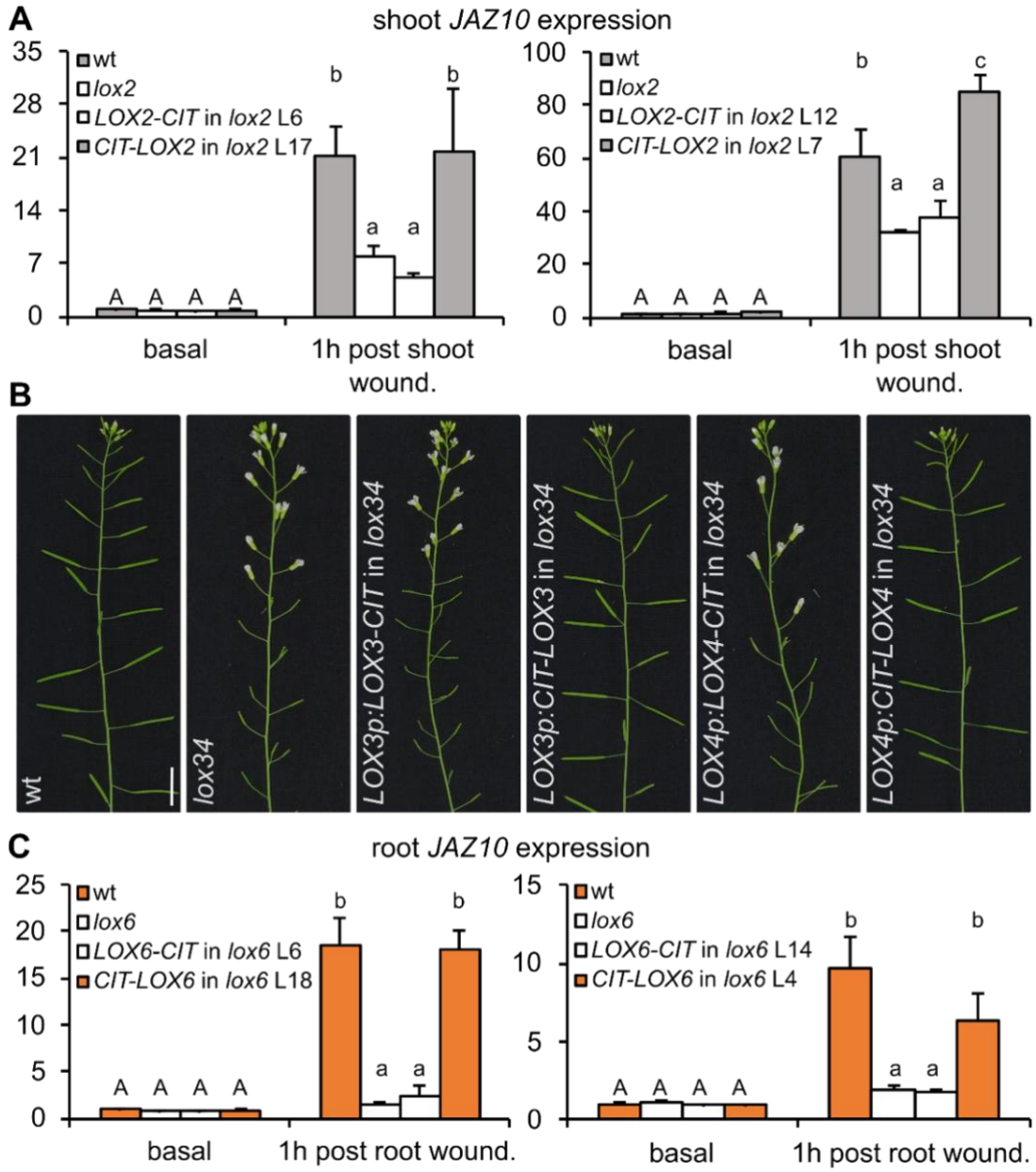

Figure 2-7. N-terminal CITRINE (CIT) fusions of 13-LOXs expressed under their native promoters are functional. (**A**) Quantitative qPCR of *JAZ10* expression in shoots of wt, *lox2*, and *lox2* complemented with either *LOX2p:LOX2-CIT* (LOX2-CIT) or *LOX2p:cTP-CIT-LOX2* (CIT-LOX2) in 5-day-old seedlings at basal conditions and 1 hour after shoot wounding. (**B**) Main inflorescences from 6-week-old plants of indicated genotypes. scale bar, 1cm. (**C**) Quantitative qPCR of *JAZ10* expression in roots of wt, *lox6*, and *lox6* complemented with either *LOX6p:LOX6-CIT* (LOX6-CIT) or *LOX6p:cTP-CIT-LOX6* (CIT-LOX6) 2-week-old plants at basal and 1 hour after root wounding. (**A, C**) Transcript levels were normalized to those of *UBC21* and calibrated to respective wt unwounded controls. Bars represent the means of three biological replicates (±SD), each containing a pool of organs from (**A**) 60 or (**C**) 5 plants. Letters in (**A, C**) denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test($p<0.01$).

2.2.3 CIT-LOX6 is basally expressed in the primary root, while CIT-LOX3 and CIT-LOX4 are induced after root wounding

After validating the functionality of the new *13-LOXp:CIT-LOX* translational reporters, I tested if the 13-LOX protein localization coincides with their promoter activities in the primary roots of living seedlings. Contrary to the presence of their promoter activities (Fig. 2-6), I could not observe any fluorescence signal for CIT-LOX3 nor CIT-LOX4 at basal conditions (Fig. 2-8A), despite analysing a minimum of 10 individuals in 4-5 selected T_3 families for each construct. However, CIT-LOX6 expression was visible in vascular cells starting in the root elongation zone and coincided with the expression observed with the *LOX6p:NLS-VENUS* transcriptional reporter (Fig. 2-6). The majority of the CIT-LOX6 expression domain was confined to the vasculature in punctuate structures, and fluorescence signals from other cell types could be observed only rarely in the early differentiation zone of the primary root. The diameter of the punctuated CIT-LOX6 fluorescent structures was 1±0.5µm, which is comparable to the size of root plastids (1.8-3.0 µm) (Köhler and Hanson, 2000), and therefore coincided with the expected subcellular CIT-LOX6 localization in plastids. Interestingly, fluorescent signals tended to associate closely to one another in the root elongation zone, perhaps forming associations with the nucleus. These associations then dissipated more broadly across the cells in later zones of the root.

As CIT-LOX3 and CIT-LOX4 constructs were functional but not visible under the conditions tested, and because *LOX3* and *LOX4* transcripts are wound-inducible (Fig. 2-2B), I tested if the translational reporters could be visualized after wounding the root. At 3 h after root wounding, CIT-LOX3 and CIT-LOX4 signals could be observed in punctuate structures in cells above the wounded tissue, with a stronger signal for CIT-LOX3 compared with CIT-LOX4. These signals were more abundant in vascular tissues for both constructs and included endodermal and pericycle cells. Conversely, wounding CIT-LOX6 did not alter its expression domain (Fig. 2-8B).

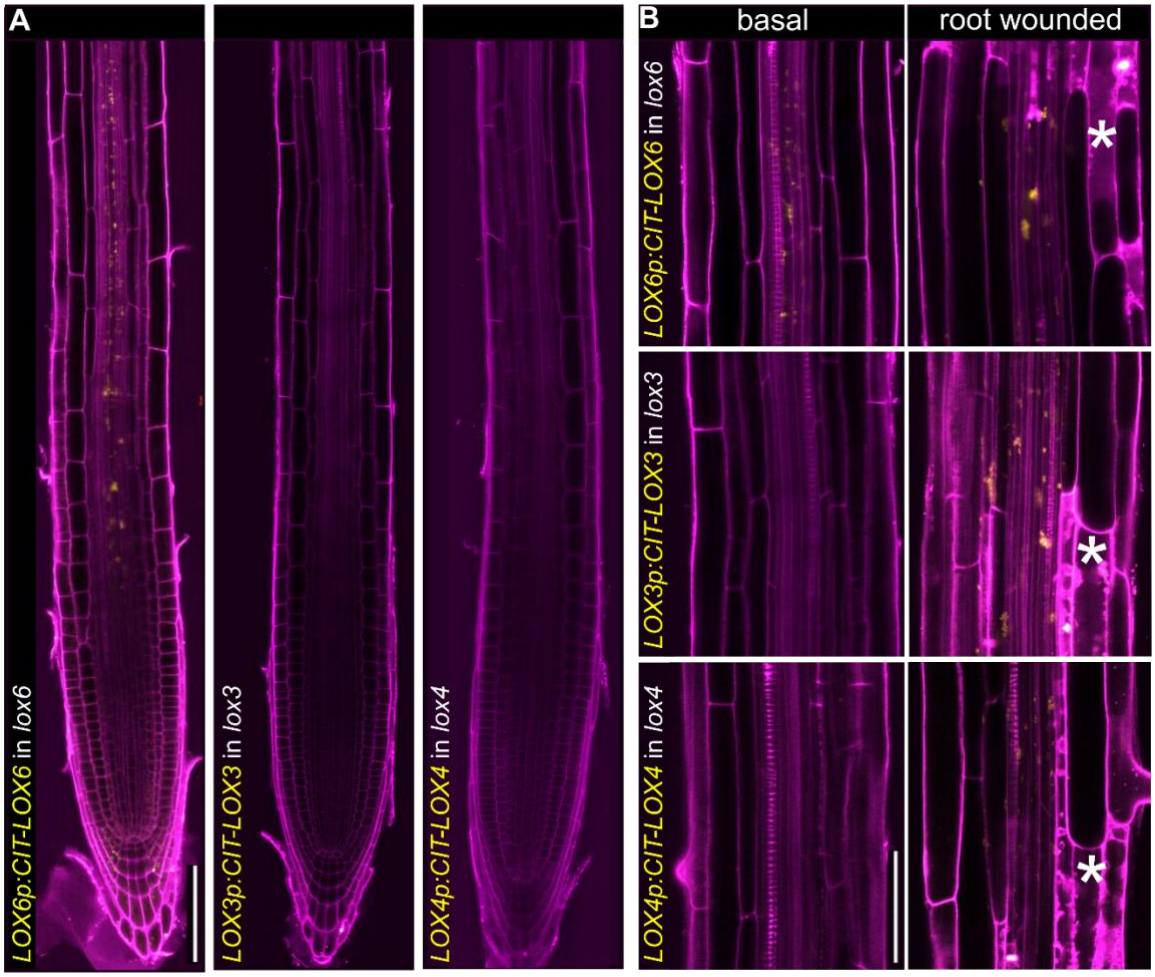

Figure 2-8. CIT-LOX6 can be visualized in the root at basal condition, while CIT-LOX3 and CIT-LOX4 only after wounding. (**A**) Representative images of CIT-LOX6, CIT-LOX3 and CIT-LOX4 expression controlled by their native promoters in primary roots of 5-day-old seedlings stained with propidium iodide (magenta). Scale bar, 100 µm. (**B**) Close-up of their basal localization in early differentiation zone and 3 h after root wounding. White asterisks indicate damaged cells in which propidium iodide could penetrate. Scale bar, 50 µm.

Collectively, the data indicate that while the promoters of *LOX3*, *LOX4* and *LOX6* are all active in the primary root, only LOX6 enzymes are basally present while LOX3 and LOX4 enzymes are wound-inducible. Given that LOX6 is essential for triggering JA-Ile signalling (Fig. 2-4), it is likely that xylem procambium cells are the first sites of root JA-Ile biosynthesis.

2.3. Does LOX6 require post-translational activation to initiate root JA-Ile production?

So far, my data indicate that JA-Ile signalling is very low in Arabidopsis roots, that LOX6 is essential for JA-Ile signalling induction after root wounding and that only the LOX6 enzyme can be localized in roots at basal conditions (Fig. 2-8A). In aboveground organs at basal conditions, JA and JA-Ile levels are very low, often below the detection limits of current instruments (<10 pmol/g FW), eg. (Glauser et al., 2009; Grebner et al., 2013; Kimberlin et al.,

2022; Schulze et al., 2019). Their levels increase as early as 30 seconds after tissue injury and reach 500-1000-fold for JA and 30-50-fold induction for JA-Ile at about 1 h after the wounding stimulus (Glauser et al., 2009; Koo et al., 2009). Due to this remarkably short time window, it is likely that hormone production results from the post-translational activation of biosynthetic enzymes, rather than their transcriptional regulation (Wasternack and Song, 2017). Consistently, numerous lines of evidence across multiple plant species indicate that JA-Ile biosynthesis enzymes, including 13-LOXs, AOCs and JAR1, are present under basal conditions in different tissues and cell types (Bachmann et al., 2002; Hause et al., 2003a, 2003b; Strassner et al., 2002; Swain et al., 2017). Their overexpression does not result in increased basal hormone levels, and plant treatments with general inhibitors of gene transcription or translation do not compromise wound-elicited JA-Ile biosynthesis (Kimberlin et al., 2022; Sharma et al., 2006; Staswick and Tiryaki, 2004). Furthermore, leaf wounding does not only trigger JA and JA-Ile induction, but also the increase of the plastidial precursor OPDA (Grebner et al., 2013; Schulze et al., 2019). Collectively, it is therefore generally accepted that the observed rapid JA-Ile increase in vegetative tissues relies upon the post-translational activation of pre-existing JA biosynthesis enzymes, including 13-LOXs. However, (Grebner et al., 2013) challenged this conclusion in roots, where LOX6 was found to be important to synthesize basal OPDA levels, which could then be converted to JA-Ile to trigger signalling after wounding. To test if this is the case in intact plants and not only detached roots, I overexpressed *LOX6* to analyse its impact on basal JA-Ile signalling. If LOX6 requires post-translational activation, I expect its overexpression does not influence basal JA-Ile signalling, but perhaps lead to higher JA-Ile signalling after wounding. Contrarywise, if LOX6 does not require post-translational activation, I would expect enhanced JA-Ile signalling at basal levels. I also developed *LOX2*, *LOX3* and *LOX4* overexpression lines to consolidate my experimental setup.

2.3.1 Developing 13-LOX overexpression lines

To overexpress (OE) functional CIT-LOX enzymes, I drove their expression under the *UBQ10* promoter (Grefen et al., 2010). Independent OE lines were selected for CIT-LOX2, CIT-LOX4 and CIT-LOX6, while it was not possible to do so for CIT-LOX3 after screening more than 100 independent T_1 plants. I therefore guided CIT-LOX3 OE under the *35S* promoter (Naru et al., 1985) and successfully recovered OE lines. Respective *13-LOX* transcript levels were first

verified for each construct in a minimum of two independent lines (Fig.2-9). Wild-type and single *lox2, lox3, lox*4, and *lox6* mutants exhibited very low levels of corresponding *13-LOX*s transcripts under basal condition, while *CIT-LOX2* overexpression resulted in around 800-fold increase of *LOX2* transcripts. Similarly, *LOX3* OE lines had more than 350-fold change of *LOX3* level, and *LOX4* OE lines had more than 140-fold induction of *LOX4* gene expression. Two independent *LOX6* OE lines displayed around 40-fold increased LOX*6* transcript levels (Fig. 2-9).

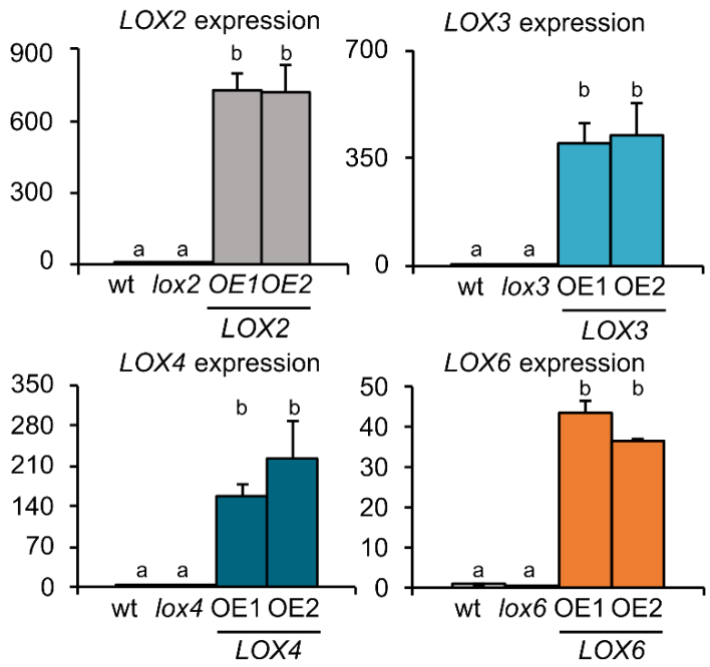

Figure 2-9. *13-LOX* transcript levels in *13-LOX* overexpression (OE) lines. Quantitative qPCR of basal *LOX2, LOX3, LOX4 and LOX6* expression in wt, single mutants and respective N-terminally CIT tagged OE lines (*UBC10p:cTP-CIT-LOX*). *LOX* transcript levels were normalized to *UBC21* and calibrated to respective wt controls. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

I next confirmed the presence of CIT-LOX proteins in primary roots of living plants with confocal microscopy. In all OE cases, CIT-LOX proteins were clearly visible across all cell types of the primary root in punctuate structure compatible with plastids (Fig. 2-10).

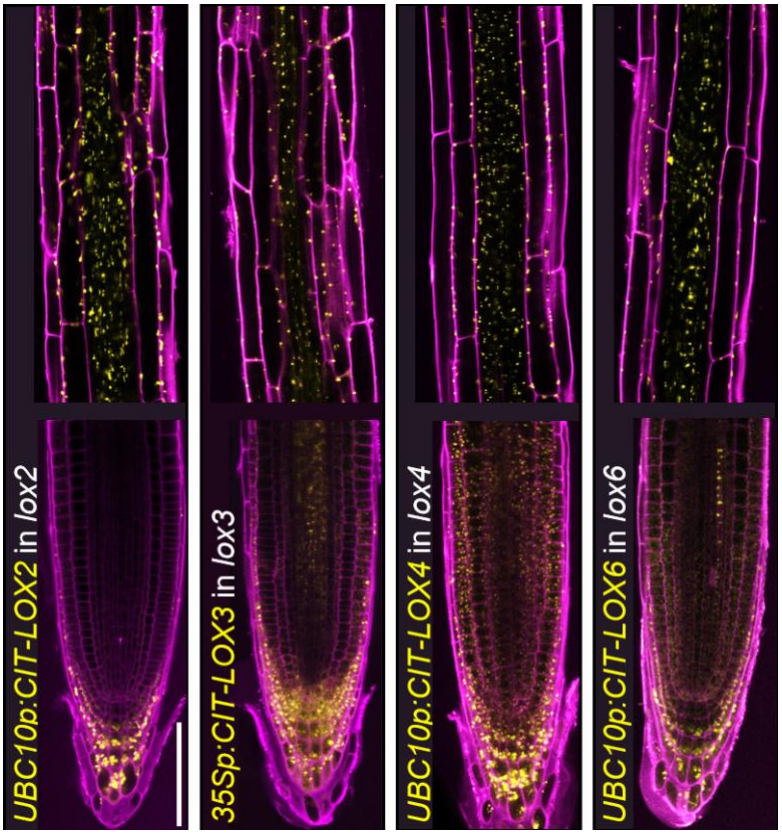

Figure 2-10. 13-LOX protein localization in 13-LOX OE lines. Optical sections through 5-day-old taproots stained with propidium iodide (magenta) showing CIT-LOX fluorescence and localization after overexpression. For comparison, refer to CIT-LOX expression under the control of native promoters (Fig. 2-8). Scale bar, 50 µm.

2.3.2 *LOX2* or *LOX6* overexpression does not lead to constitutive JA-Ile signalling in resting roots, while *LOX3* and *LOX4* does

After characterizing the newly developed CIT-LOX OE lines in detail, I could finally assess their impact on basal and wound-induced JA-Ile root signalling. Remarkably, CIT-LOX2 and CIT-LOX6 OE did not affect basal *JAZ10* root levels, which remained similar to the wt (Fig. 2-11A). On the other hand, CIT-LOX3 and CIT-LOX4 OE resulted in constitutive *JAZ10* expression which was 4-7-fold higher than in wt plants (Fig. 2-11A). Following root wounding, *JAZ10* level was induced in wt and reached to around 20-fold when normalized to *UBC21* and calibrated to control condition, which was calibrated to 1. *lox2, lox3, lox4* mutants, 2 independent *LOX2* OE lines and *LOX6* OE lines had similar induction of *JAZ10* expression as wt 1 h post root wounding (Fig. 2-11B). Interestingly, *JAZ10* levels in *LOX3* and *LOX4* overexpression lines was induced significantly higher than wt levels after root wounding. (Fig. 2-11B). The data indicate that while post-translational activation is needed to trigger LOX2 and LOX6 enzymatic activities to initiate root JA-Ile production (or those of a downstream JA-Ile biosynthesis enzyme), the enzymatic activities of LOX3 and LOX4 are likely regulated following transcriptional activation.

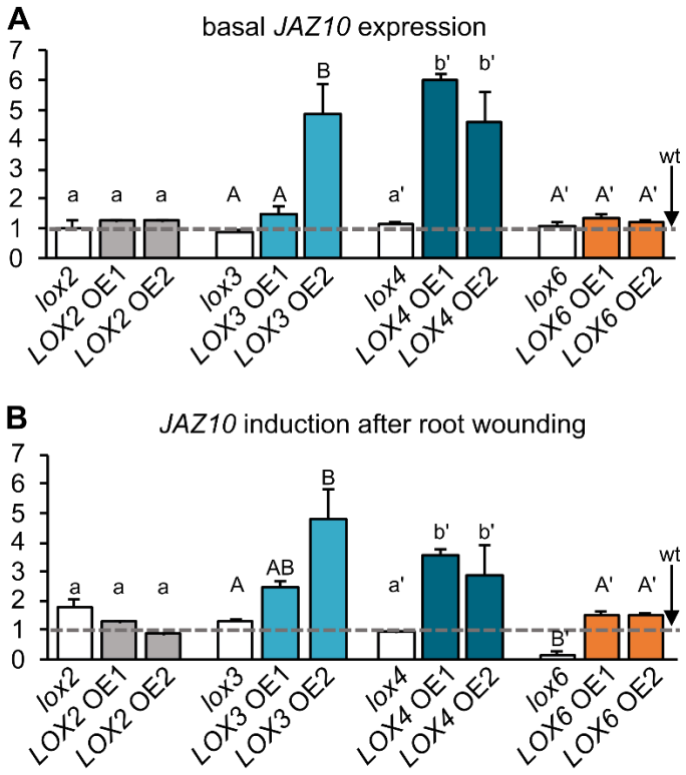

Figure 2-11. *LOX2* and *LOX6* OE does not result in higher *JAZ10* basally and after root wounding, while LOX3 and LOX4 OE does. qRT-PCR of (A) basal and (B) 1 h post root wounding *JAZ10* expression in roots of wt, *lox* single mutants, and *lox* single mutants transformed with corresponding *13-LOX* overexpression constructs (*UBC10p:cTP-CIT-LOX*). *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of wt that is set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

2.3.3 Preliminary efforts to study 13-LOX subcellular localization via immunogold

Previous LOX2 immunogold labelling experiments in barley leaves revealed that LOX proteins are basally expressed in chloroplasts, and that their quantity increases after long-term MeJA treatment (Bachmann et al., 2002). My newly developed transgenic lines can be therefore used to assess 13-LOX localization at the subcellular level in Arabidopsis roots and target the highly specific fluorescent fluorophore CIT tag. My preliminary efforts have first focused on embedding and sectioning root material (Fig2-12A). Unfortunately, root tissue embedding was not optimal and appeared squeezed. Immunogold labelling of CIT-LOX6 OE lines detected gold particles in plastids only occasionally, and not as abundantly as expected (Fig2-12B). Furthermore, gold particles were often detected in subcellular locations outside of plastids as well, though with a reduced frequency. While this research line remains preliminary and still requires optimization, I have successfully developed and characterized CIT-LOX translational reporters expressed both under the control of their native and overexpression promoters that will be used in future localization studies.

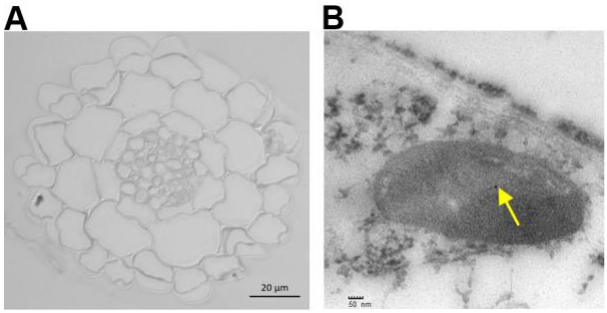

Figure 2-12. Preliminary efforts to study 13-LOX subcellular localization (**A**) Transversal section of the root division zone of a *LOX6* OE lines in resting 5-d-old seedlings. Scale bar, 20µm (**B**) Immunogold labelling for CIT-LOX6 protein in plastid from *LOX6* OE line in root late differentiation zone. Scale bar, 50nm.

2.4 Can 13-LOXs complement one another, or do they have specific functions?

All four Arabidopsis 13-LOXs can oxygenate the same substrate alpha-linolenic acid in *in vitro* assays (Bannenberg et al. 2009) while having different physiological roles (Chauvin et al. 2013; Chauvin et al. 2016; Grebner et al. 2013; Glauser et al., 2009). To test if LOX6 can replace the function of other 13-LOX when expressed under their specific promoters, I have designed promoter swap experiments and assessed their consequences in specific *lox* mutants. Similarly, to test if LOX6 root-specific function can be replaced by another one of the 13-LOXs, I have developed promoter-swap transgenic lines in which LOX2, LOX3 or LOX4 were expressed under the control of the *LOX6* promoter to test if they can complement the *JAZ10* phenotype of the *lox6* mutant (Tab. 1). These experiments should therefore verify if the biochemical activity among the four 13-LOXs may differ *in planta*, or if, contrarywise, their different physiological roles are predominantly due to their differential expression domains.

Table 1. Scheme of promoter swaps

Promoter	Fusion protein	Background
LOX2p	cTP-CIT-LOX6	*lox2, lox6*
LOX3p	cTP-CIT-LOX6	*lox34, lox6*
LOX3p	cTP-CIT-LOX2	*lox34*
LOX4p	cTP-CIT-LOX6	*lox34, lox6*
LOX4p	cTP-CIT-LOX2	*lox34*
LOX6p	cTP-CIT-LOX2	*lox6*
LOX6p	cTP-CIT-LOX3	*lox6*
LOX6p	cTP-CIT-LOX4	*lox6*

cTP:Chloroplast transit peptide CIT:CITRINE

2.4.1 LOX6 can complement the activity of LOX2, LOX3 and LOX4

To determine if LOX6 can complement the physiological function of other 13-LOXs, I expressed the functional CIT-LOX6 construct under the promoters of either *LOX2*, *LOX3* or *LOX4* in the respective mutant backgrounds. As described previously (Gasperini et al., 2015), LOX2 is important to induce JA-Ile biosynthesis and signalling in wounded shoots, as the *lox2* mutant has a comprised *JAZ10* induction compared to wounded wt plants. However, expressing *LOX2p:CIT-LOX6* in the *lox2* mutant background could partially complement JA-Ile signalling in the *lox2* mutant after shoot wounding as compared with the *LOX2p:CIT-LOX2* complemented line (Fig.2-13A).

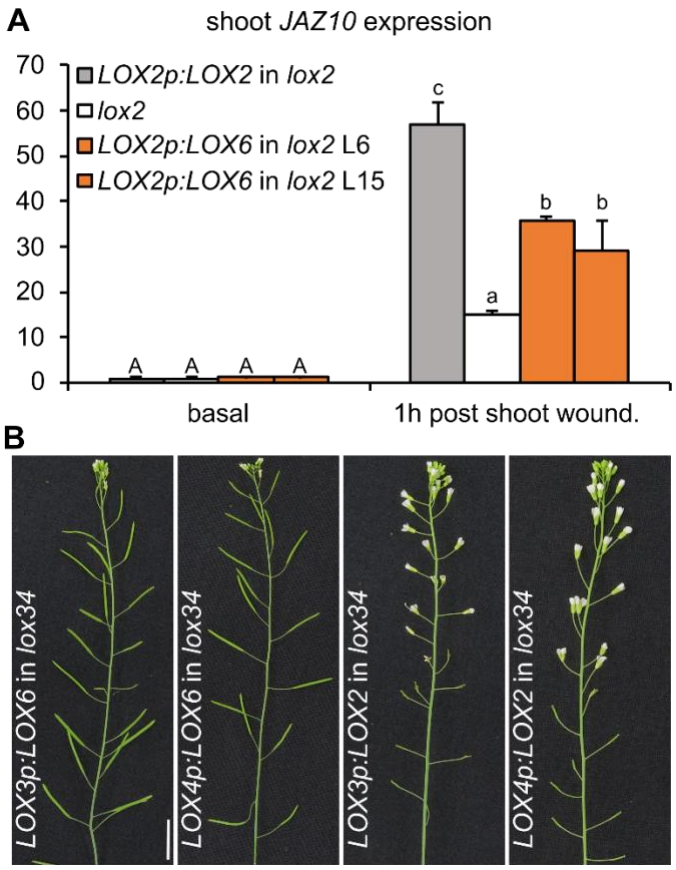

Figure 2-13. LOX6 can complement the function of other 13-LOXs when expressed under their promoters. (**A**) Quantitative qPCR of *JAZ10* expression in shoots of *LOX2p:CIT-LOX2* in *lox2*, *lox2*, and 2 lines of *lox2* complemented with *LOX2p:CIT-LOX6* in 5-day-old seedlings at basal condition and 1 hour after shoot wounding. Transcript levels were normalized to those of *UBC21* and calibrated to *LOX2* native promoter line unwounded controls. Bars represent the means of three biological replicates (±SD), each containing a pool of organs from 60 plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$). (**B**) Main inflorescences from 6-week-old plants of indicated genotypes. Scale bar, 1cm.

I next tested whether LOX6 can complement the function of LOX3 and LOX4 by introducing *LOX3p:CIT-LOX6* and *LOX4p:CIT-LOX6* in the sterile *lox34* double mutant. As described previously, *LOX3p:CIT-LOX3* and *LOX4p:CIT-LOX4* constructs could restore the fertility of the *lox34* mutant (Fig. 2-7B), serving as positive controls. Similarly, expressing CIT-LOX6 under *LOX3* and *LOX4* promoters could also rescue the sterility phenotype of the *lox34* mutant (Fig. 2-13B). Contrarywise, the *lox34* sterility phenotype could not be recovered by a functional

CIT-LOX2 fusion enzyme expressed under *LOX3* and *LOX4* promoters, as *lox34* plants transformed with *LOX3p:CIT-LOX2* and *LOX4p:CIT-LOX2* were still sterile (Fig. 2-13B). The data indicate that LOX6 can exert the same *in planta* biochemical activity as all other 13-LOXs at least to a certain degree but, due to the incapacity of LOX2 to complement the functions of LOX3 and LOX4 enzymes, that specific biochemical functions might differ among the four 13-LOX members.

2.4.2 The *in planta* enzymatic activity of LOX6 can be complemented by LOX3 and LOX4, but not by LOX2

I next evaluated if expressing LOX2, LOX3 or LOX4 enzymes under the *LOX6* promoter could restore the absence of wound-induced increase of JA-Ile signalling in *lox6* roots. After generating *LOX6p:CIT-LOX2, LOX6p:CIT-LOX3* and *LOX6p:CIT-LOX4* promoter swap lines in the *lox6* background, I verified that the localization of all CIT-LOX fusion proteins from independent families was present in vascular tissues with a similar pattern as CIT-LOX6 (Fig. 2-14A). I also evaluated *13-LOX*s transcript levels in promoter swap lines to ensure lack of possible overexpression. While *13-LOX* transcripts were slightly higher (5-12-fold) in promoter swap lines than their respective values in wt plants, they were in the same order of magnitude as in the *LOX6p:CIT-LOX6* complemented line (Fig. 2-14B). Promoter swap lines are therefore not overexpressing *CIT-LOX* constructs as in the case of previously described OE lines in which *13-LOX* transcripts were 40- to 700-fold higher than wt controls (Fig. 2-9).

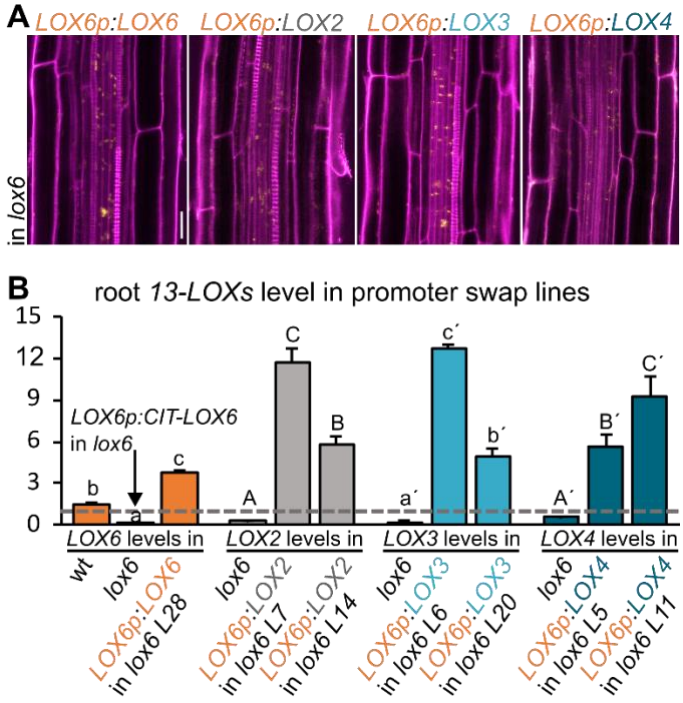

Figure 2-14. *13-LOX* transcript levels and protein accumulation in promoter swap lines. (**A**) Optical sections through 5-day-old taproots stained with propidium iodide (magenta) in their early differentiation zone, showing CIT-LOX fluorescence and localization under control of the LOX6 promoter in a *lox6* mutant background. Scale bar, 20 µm. (**B**) Quantitative qPCR of basal *LOX6, LOX2, LOX3* and *LOX4* expression in wt (only for *LOX6*), *lox6* mutant and respective N-terminally CIT tagged promoter swap lines (*LOX6p:CIT-LOX*) transformed in a *lox6* background. *13-LOX* transcript levels were normalized to *UBC21* and displayed relative to the expression of the *LOX6p:CIT-LOX6* native promoter line that was set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

Following these critical validations that both *13-LOX* transcript levels and protein localizations were similar to *LOX6p:CIT-LOX6* across all promoter swap lines, I next tested them in the *lox6* root wounding assay in which the *LOX6p:CIT-LOX6* complemented line was used as a positive control. Under basal conditions all promoter swap lines had similar *JAZ10* levels to the controls, except two independent *LOX6p:CIT-LOX3* lines which had a slight 3-5-fold, yet significant increase in *JAZ10* levels (Fig. 2-15A). While root wounding was effective in inducing root *JAZ10* levels in the control *LOX6p:CIT-LOX6* and in *the lox6* mutant transformed with *LOX6p:CIT-LOX3,* 2 independent lines of transgenic plants with *LOX6p:CIT-LOX4* all showed statistically higher *JAZ10* induction than *lox6* mutant, but not comparable with the positive control line. Furthermore, the *lox6* mutant phenotype was not rescued by *LOX6p:CIT-LOX2* (Fig. 2-15B).

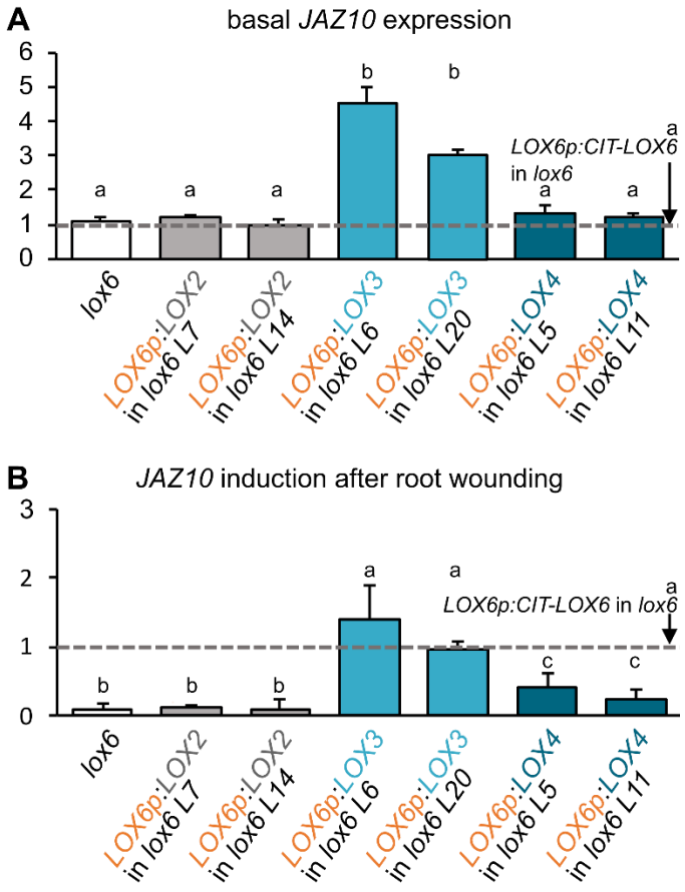

Figure 2-15. LOX3 can fully, LOX4 can partially while LOX2 cannot complement LOX6. qRT-PCR of root basal (**A**) and 1h post wounding (**B**) *JAZ10* expression in *lox6* mutant and promoter swap lines in which *LOX6* promoter drive the expression of other *13-LOXs* and transformed to *lox6* mutants. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of *JAZ10* in *LOX6* native promoter line that are set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.05$).

Overall, the promoter swap experiments indicate that LOX2 might have a different enzymatic activity or regulation compared to other 13-LOXs, and that the cell-type specific localization of LOX3, LOX4 and LOX6 is important for their function in the jasmonate pathway.

2.4.3 Cell-type specific LOX6 localization and its promoter regulation are essential for its function

As *LOX3p*, *LOX4p* and *LOX6p* were basally active (Fig. 2-6), but I could detect only CIT-LOX6 protein at basal conditions, I next tested if expressing CIT-LOX6 under *LOX2p*, *LOX3p*, or *LOX4p* could complement the *lox6* mutant phenotype. Consistently with the absence of the *LOX2* promoter activity in Arabidopsis roots (Fig. 2-2A), *LOX2p:CIT-LOX6* could not rescue the wound-induced lack of *JAZ10* increase in *lox6* roots (Fig. 2-16A). However, although *LOX3p* and *LOX4p* promoters were basally active, I could not detect fluorescence signals from *LOX3p:CIT-LOX6* nor *LOX4p:CIT-LOX6* in resting roots but only after root wounding (Fig. S3), in line with the above-reported lack of basal *LOX3p:CIT-LOX3* and *LOX4p:CIT-LOX4* root expression (Fig. 2-8A). Hence, it is not surprising that expressing CIT-LOX6 under the

promoters of *LOX3* and *LOX4* did not lead to a restoration of JA-Ile signalling in wounded *lox6* roots (Fig. 2-16B). Intriguingly, CIT-LOX3 and CIT-LOX4 proteins expressed under their native promoters could be induced in vascular cells 3h after root wounding (Fig. 2-8B), suggesting that analysing *JAZ10* levels 1h after root wounding may be too early.

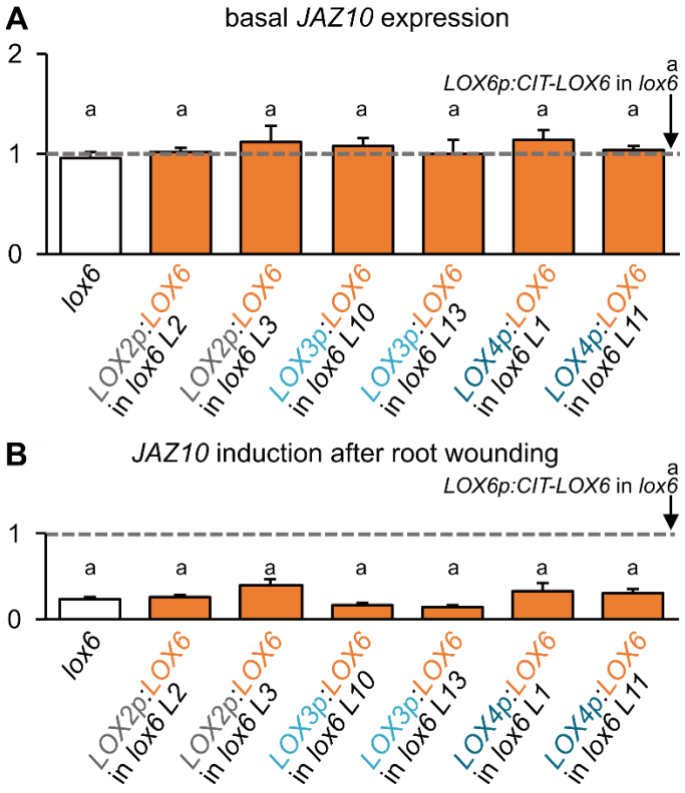

Figure 2-16. Expression of LOX6 under the promoter of other *13-LOX*s does not complement the *lox6* mutant phenotype after root wounding. qRT-PCR of (**A**) basal and (**B**) 1h post root wounding *JAZ10* expression in *lox6* roots in complemented and promoter swap lines in which *LOX2/3/4* promoters drive the expression of *LOX6*. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of *JAZ10* in *LOX6* native promoter line that are set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

These results suggest that *LOX6* promoter activity, along with transcript and/or protein stability, and with cell-type specific expression may be critical for root JA-Ile production and signalling.

2.5 Do domain swaps between PLAT and LOX domains alter LOX6 functionality?

13-LOXs contain a PLAT domain which putatively mediates membrane associations and/or calcium (Ca^{2+}) binding (Eek et al. 2012, Mochizuki and Matsui, 2018), and a catalytic LOX domain which is essential for the enzyme activity (Liavonchanka and Feussner, 2006; Maynard et al., 2021)(. My previous results indicated that LOX6 is indispensable in root JA-Ile biosynthesis upon local root wounding. To characterize if the role of LOX6 depends on the peculiarities present in its protein domains, I designed both PLAT and LOX domain swap lines. I exchanged the PLAT domain of LOX6 with those from other 13-LOXs. The same strategy was used to generate LOX domain swap lines. All these constructs (Tab. 2) were transformed into the *lox6* mutant, and resulting transgenic lines were assayed for their capacity to induce JA-Ile signalling after root wounding.

Table 2. Scheme of domain swap

Promoter	Protein Fusion	Background
LOX6p	cTP-CIT-PLAT ^{LOX6}-LOX^{LOX2}	*lox6*
LOX6p	cTP-CIT-PLAT ^{LOX6}-LOX^{LOX3}	*lox6*
LOX6p	cTP-CIT-PLAT ^{LOX6}-LOX^{LOX4}	*lox6*
LOX6p	cTP-CIT-PLAT ^{LOX2}-LOX^{LOX6}	*lox6*
LOX6p	cTP-CIT-PLAT ^{LOX3}-LOX^{LOX6}	*lox6*
LOX6p	cTP-CIT-PLAT ^{LOX4}-LOX^{LOX6}	*lox6*
LOX6p	cTP-CIT- LOX^{LOX6}	*lox6*

cTP:Chloroplast transit peptide CIT:CITRINE

2.5.1 Swapping the LOX catalytic domain with that of other 13-LOXs abolishes LOX6 function

To characterize if the LOX domain is important for the function of LOX6 in root JA-Ile production and signalling, I exchanged the LOX domain with that from LOX2, LOX3 or LOX4 in the *LOX6p:cTP-CIT-LOX6* construct, where LOX6 is comprised of the PLAT ^{LOX6} domain and the LOX^{LOX6} enzymatic domain. All the constructs were transformed in *lox6* mutant background and *LOX6p:cTP-CIT-LOX6* (equivalent to *LOX6p:CIT-PLAT^{LOX6}-LOX^{LOX6}*) was used as positive control. The *lox6* mutant was included as a negative control in my experimental setup.

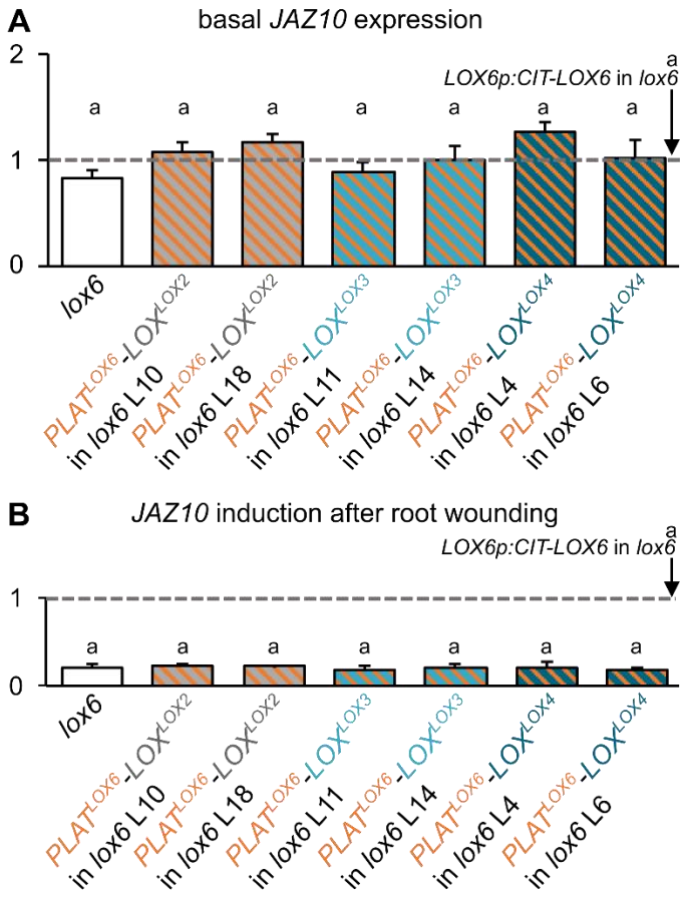

Figure 2-17. LOX domains from LOX2, LOX3 and LOX4 cannot replace the catalytic domain of LOX6. qRT-PCR of (**A**) basal and (**B**) 1h post root wounding *JAZ10* expression in *lox6* mutant roots transformed with PLAT domain swap constructs. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of *JAZ10* in *LOX6* native promoter line that are set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

Under basal conditions, root *JAZ10* expression was very low, and comparable to all domain-swap transgenic lines (Fig.2-17A). Following root wounding, none of the LOX domain swap lines (*LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX2}, LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX3} LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX4}*) could restore the lack of JA-Ile signalling in wounded *lox6* roots (Fig. 2-17B).

2.5.2 Replacing the LOX6 PLAT domain with that of other 13-LOXs abolishes LOX6 function

I next generated constructs in which the PLAT domain of LOX6 was swapped with the one from LOX2, LOX3 or LOX4 (*LOX6p:cTP-CIT-PLAT^{LOX2}-LOX^{LOX6}, LOX6p:cTP-CIT-PLAT^{LOX3}-LOX^{LOX6}, LOX6p:cTP-CIT-PLAT^{LOX4}-LOX^{LOX6}*) to test if the PLAT domain confers the specificity of LOX6 role in JA-Ile production after root wounding. Followed with the same strategy of LOX domain swap experiments, resulting transgenic lines in the *lox6* mutant background were then assayed for *JAZ10* expression after root wounding. *lox6* mutant was not able to induce *JAZ10* level to the positive control which again confirmed that *lox6* is essential for root JA-Ile production. Similarly to the LOX domain swap lines (Fig. 2-17), none of the PLAT domain swap lines could restore the lack of JA-Ile signalling in wounded *lox6* roots (Fig. 2-18).

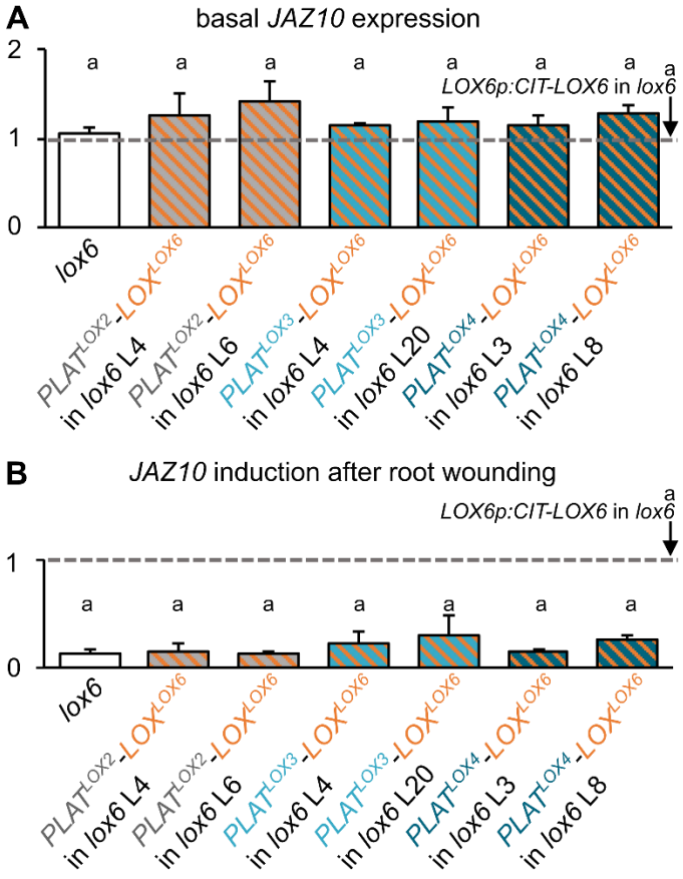

Figure 2-18. PLAT domains from LOX2, LOX3 and LOX4 cannot replace the PLAT domain of LOX6. qRT-PCR of root (**A**) basal and (**B**) 1h post wounding *JAZ10* expression in *lox6* mutant and PLAT domain swap lines in *lox6* mutant. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of *JAZ10* in *LOX6* native promoter line that are set to 1 and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

2.5.3 The PLAT domain is essential for LOX6 activity

In vitro biochemical studies with soybean LOX indicate that the PLAT domain is important for its activity, as the LOX$^{\Delta PLAT}$ variant lacking the PLAT domain shows lower stability but higher enzymatic activity (Venere et al. 2003). Hence, I tested if the PLAT domain was important for LOX6 activity in planta by transforming a *LOX6pcTP:CIT-LOX6*$^{\Delta PLAT}$ construct in *lox6* and evaluating the transgenic lines in root wounding assays. The sequence of PLAT domain was determined from Uniprot (https://www.uniprot.org/) and Intrepro (https://www.ebi.ac.uk/interpro/) and confirmed by putative protein structure from AlphaFold. The lack of the PLAT domain did not impact CIT-LOX6$^{\Delta PLAT}$ localization, as both CIT-LOX6 and CIT-LOX6$^{\Delta PLAT}$ could be visualized at the expected locations (Fig. 2-19A,B). Then, I utilized root wounding assay to test the functionality of the PLAT-deleted line. The positive control line which contains the full sequence of *LOX6* coding region was able to rescue the phenotype of *lox6* mutant. However, two independent PLAT domain deletion lines had no *JAZ10* induction after root wounding, maintaining the *lox6* mutant phenotype (Fig. 2-19C).

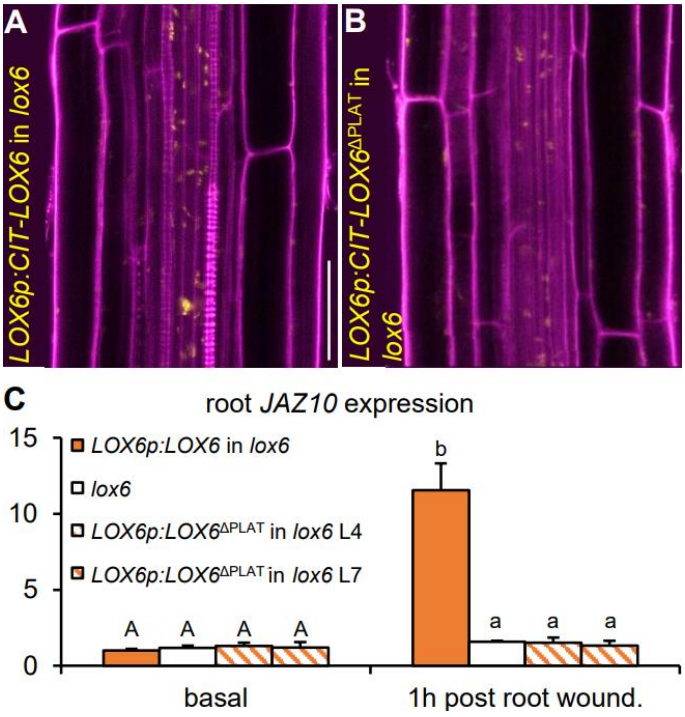

Figure 2-19. The PLAT domain is essential for LOX6 function *in planta*. (**A,B**) Optical sections through 5-day-old taproots stained with propidium iodide (magenta) showing CIT-LOX6 and CIT-LOX6 (ΔPLAT) fluorescence. Scale bar, 50 µm. (**C**) qRT-PCR of root basal and 1h post root wounding *JAZ10* expression in PLAT domain deletion lines. *JAZ10* transcript levels were normalized to those of *UBC21* and calibrated to *LOX6* native promoter line unwounded controls. Bars represent the means of three biological replicates (±SD), each containing 5 roots from 2-week-old plants. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

Collectively, enzymatic domain swap lines indicate that the PLAT domain is essential for LOX6 activity *in vivo*, and that interactions between the PLAT domain and the LIPOXYGENASE domain are critical for LOX6 enzyme activity. The coral 11R-LOX was shown to contain a π–cation bridge between the PLAT domain and the LOX enzymatic domain, which is critical to maintain folding stability and enzymatic function (Eek et al., 2015). In coral 11R-LOX, the π–cation bridge is formed between an electron-rich Trp residue on the PLAT domain and a positively charged Lys amino acid on the LOX domain, with this noncovalent molecular interaction exerting significant bonding energy and playing an important role in enzyme catalysis (Eek et al., 2012, 2015). Specifically, disrupting the π–cation bridge by replacing the PLAT Trp with Ala or Lys strongly reduced enzyme activity (Eek et al., 2015). The authors argue that the π–cation bridge between the PLAT and the LOX domains could serve as an allosteric "lid" over the putative substrate channel entrance in the LOX domain, which can adopt different conformations, thereby either opening or closing the orifice for substrate entry. If the π–cation bridge was indeed essential for 13-LOX activity even in Arabidopsis, my PLAT-LOX domain swap lines have likely disturbed its formation, and this would explain how come all domain swap lines failed to complement the *lox6* mutant phenotype.

I was therefore curious to assess if Arabidopsis 13-LOXs can also form such a π–cation bridge between their PLAT and LOX domains. By using the AlphaFold protein structure database (https://alphafold.ebi.ac.uk/), I have found that all four 13-LOXs are predicted to form π–cation interactions between their PLAT and LOX domains. Specifically, LOX2 has one π–cation bridge between the PLAT Trp184 and the LOX Arg299 in addition to one Hydrogen (H^+) bond between Trp184 and the LOX Asp300; LOX3 has one π–cation bridge (Trp207–Arg321) and one H^+ bond (Trp207–Asp322) between the two domains; LOX4 has one π–cation bridge (Trp213–Arg327) and one H^+ bond (Trp213–Asp328) between the two domains; and LOX6 has two π–cation bridges (Trp201–Arg314) and one H^+ bond (Trp201–Asp315) between the two domains. (Figure 2.20) shows a detail of the two π–cation bridges between the PLAT and LOX domains of LOX6.

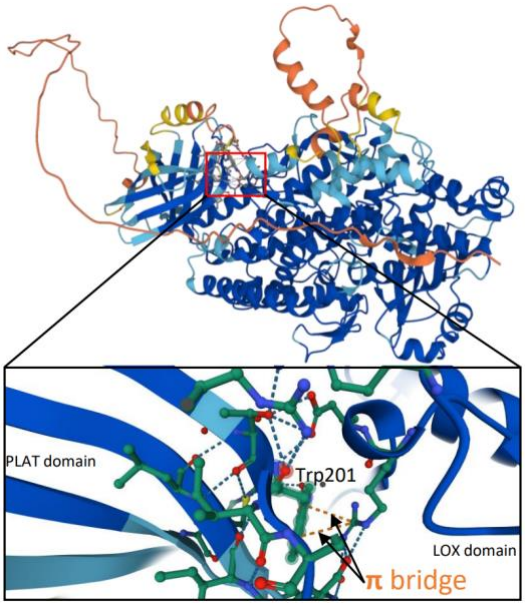

Figure 2-20. predicted LOX6 protein structure and two π –cation bridges between PLAT and LOX domains. The structure is from AlphaFold protein structure database. Orange dashed lines indicate the π–cation interactions between Trp201 and Arg314. The dashed blue lines are hydrogen bonds between the residues.

Given the high conservation of a specific Trp residue in forming both π–cation bridges and H^+ bonds between the PLAT and LOX domain, and given the essentiality of LOX6 in root JA-Ile signalling, I designed LOX6 transgenic with point mutations in the Trp201 residue to evaluate if specific substitutions can complement the *lox6* mutant phenotype. I therefore developed constructs which could potentially disrupt the PLAT-LOX interaction (*LOX6p:CIT-LOX6*^{W201A} and *LOX6p:CIT-LOX6*^{W201K}) as well as a conservative substitution (*LOX6p:CIT-LOX6*^{W201F}) serving as positive control. Resulting T_1 seedlings were already selected, and positively evaluated for the presence of chimeric proteins by confocal microscopy. Once stable

transgenic plants become available, I will perform root wounding assay to test the functionality of the mutated LOX6 proteins *in planta*.

2.5.4 Preparatory studies to test if perturbed plastidial calcium or manganese levels impact wound-induced jasmonate production *in planta*

Ca^{2+} levels increase in plant cytosol after mechanical wounding (Nguyen et al., 2018), and it is possible that Ca^{2+} changes then propagate to the stroma of plastids, where 13-LOXs are localized. BIVALENT CATION TRANSPORTER 1 (BICAT1) and BICAT2 are plastid-localized Ca^{2+}/manganese (Mn^{2+}) transporters in Arabidopsis, that determine the amplitude of dark-induced Ca^{2+} signals in the stroma. BICAT2 mediates high-affinity Ca^{2+} uptake across the chloroplast envelope, while BICAT1 operates in the thylakoid membrane (Frank et al., 2019). Hence, they represent invaluable tool to test if JA marker gene is impaired in *bicat1, bicat2* mutants basally and after wounding. To test if bivalent ion (Ca^{2+}) dynamics participate in the initiation of JA-Ile production, I analysed *JAZ10* expression in *bicat1*, *bicat2* mutants before and after cotyledon wounding. While *JAZ10* expression increased significantly in wounded wt samples of both shoots and roots, the *bicat2* mutant showed reduced *JAZ10* accumulation in both organs following wounding, and *bicat1* had reduced *JAZ10* induction in roots only (Fig. 2-21). Because root JA-Ile accumulation in shoot wounded samples depends on JAs translocation (Schulze et al., 2019), the data suggest that both *bicat1* and *bicat2* negatively impact JA-Ile production, possibly through decreased Ca^{2+} increases in the plastid stroma.

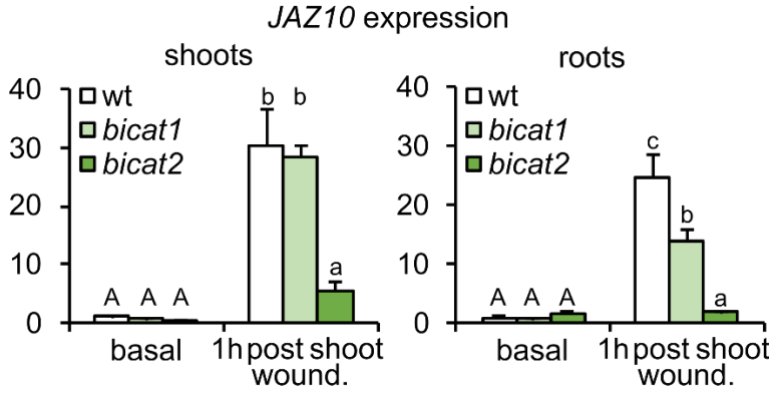

Figure 2-21. mutation of plastid calcium channel impacts JA-Ile signalling after shoot wounding. qRT-PCR of *JAZ10* expression under basal and 1 hour after shoot wounding in wt, *bicat1* and *bicat2* shoots. Transcript levels were normalized to those of *UBC21* and displayed relative to the expression of the wt control. Bars represent the means of three biological replicates (±SD), each containing a pool of organs from ~60 seedlings. Letters denote statistically significant differences among samples as determined by one-way ANOVA followed by Tukey's HSD test ($p<0.01$).

DISSCUSSION and OUTLOOK

Although the biosynthesis of JA-Ile has been characterized well at the molecular level (Wasternack and Feussner, 2018a), its initiation from 18:3 PUFAs in plant plastids has remained elusive (Kimberlin et al., 2022). In this thesis, I focused on 13-LOXs to determine how are they regulated, and indirectly, assess their contribution to JA-Ile biosynthesis initiation. I have shown that LOX6 is indispensable for initiating JA-Ile signalling after root wounding of intact plants, in line with (Grebner et al., 2013). Overexpressing 13-LOXs suggested that LOX2 and LOX6 require post-translational activation, and that LOX3 and LOX4 are regulated transcriptionally. Finally, 13-LOXs are also regulated at the protein level, the PLAT domain is of huge importance for the protein to exert a proper physiological function. Nevertheless, due to time limitation, there are remaining questions to be addressed in the future which will be discussed in the following paragraphs.

Shoot wounding induced higher JA-Ile marker gene expression than local root wounding

The levels of JA and JA-Ile are very low and close to the detection limit in vegetative tissues (Glauser et al., 2009; Grebner et al., 2013; Kimberlin et al., 2022; Schulze et al., 2019). This is in line with observed very low JA-Ile marker gene (*JAZ10*) levels under basal condition both in shoots and roots (Fig. 2-1B). 1 h after shoot wounding, JA level increases 500-1000-fold and 30-50-fold for JA-Ile in leaves (Glauser et al., 2009; Koo et al., 2009). 30 min after root wounding, JA and JA-Ile levels increase to 5 nmol g^{-1} dry weight and 1.7 nmol g^{-1} dry weight, respectively, around 2-5-fold induction (Grebner et al., 2013). Albeit the measurements are performed at different time points and with different methods, *JAZ10* induction after shoot wounding and root wounding in my experiment is comparable to hormone profiling from the literature. Interestingly, shoot wounding resulted in a higher root activation of JA-Ile marker gene expression compared to direct root wounding. Specifically, shoot wounding triggered a ca. 20-fold increase in root *JAZ10* accumulation compared to a 10-fold induction after direct root wounding (Fig. 2-1). Indeed, 1 h after shoot wounding, root JA and JA-Ile increase around 60-80-fold due to the translocation of OPDA and JA from shoot to root through the phloem (Schulze et al., 2019). While in my root wounding assay, *JAZ10* induction was induced only by local root wounding. Moreover, JA-Ile precursor levels are 10 times lower in roots compared

to shoots, with 18:3 leaf MGDG levels being 43.2 mol% while roots reaching only 3.4 mol% (Li-Beisson et al., 2013).

LOX6 is essential to induce root JA-Ile signalling after root wounding

In line with (Grebner et al., 2013), who grew plants hydroponically and wounded roots only after they were first separated from shoots, I found that *LOX6* is essential for inducing JA-Ile signalling after root wounding of intact plants. I have not only used the *lox* single mutants (*lox2*, *lox3*, *lox4*, *lox6*) to test if any sole 13-LOX is essential for root JA-Ile production, but also included corresponding mirror mutants (*lox346*, *lox246*, *lox236* and *lox234*) in which only a single 13-LOX enzyme activity remained. However, LOX6 alone was not able to induce 100 % wt *JAZ10* expression upon root wounding, as *lox234* mutant had around 50-70% of wt *JAZ10* levels across 3 independent experiments in this thesis (Fig. 2-5), indicating that other 13-LOXs is/are aiding LOX6. The absence of *LOX2* promoter activity from roots indicates that LOX2 has no role in root JA-Ile production after root wounding, which was confirmed by quantifying JA-Ile signalling in respective mutants. The analysis from *lox23* (is LOX4 helping LOX6?) and *lox24* (is LOX3 helping LOX6?) mutants could not reveal if any of them is enough or both are necessary. LOX3 and LOX4 seem to have redundant roles, and share 85% of full length sequence identity, including a 97% identity specifically in their substrate binding pocket (Caldelari et al., 2011). A double *lox3 lox4* mutant is male sterile and this can be complemented by expressing either LOX3 or LOX4 (Caldelari et al., 2011), further confirming a high degree of redundancy between LOX3 and LOX4. Furthermore, vegetative growth inhibition in leaves and defense response against nematodes in roots are also dependent both on LOX3 and LOX4 (Ozalvo et al., 2014; Yang et al., 2020). Hormone profiling across 5 genotypes of wt, *lox2*, *lox23*, *lox24* and *lox234* before and after root wounding should clarify if either or both LOX3 and LOX4 are necessary in addition to LOX6 to attain a full wt response. However, due to time limitations, I was not able to evaluate hormone and hormone precursor levels and have used JA-Ile signalling as a proxy of JA-Ile production throughout this thesis.

Alternative ways to induce root JA-Ile signalling independent of LOX6

There are also possibilities that LOX6 activation is not required for JA-Ile initiation. The existence of basal and wound inducible arabidopsides in leaves has been documented

(Stelmach et al., 2001), but the levels of all arabidopsides in roots were found to be undetectable, both under normal conditions and following root wounding (Grebner et al., 2013). Therefore, we can rule out the possible conversion from arabidopsides to JA and JA-Ile in Arabidopsis roots after root wounding. In the study by (Grebner et al., 2013) with plants grown in hydroponic system, basal levels of OPDA were LOX6 dependent which is approximately 1 nmol g^{-1} dry weight in both roots and shoots. After root wounding, OPDA levels decreased from 0.8 nmol to 0.3 nmol g^{-1} dry weight at 30 minutes which could be attributed to the conversion of OPDA to JA and JA-Ile in the roots. However, the decrease in OPDA (less than 0.7 nmol g^{-1} dry weight at 30 minutes) was insufficient to account for the significant increase in JA (more than 3 nmol g^{-1} dry weight). These findings suggest that *de novo* synthesis of OPDA is necessary to explain the observed increase in JA levels. Still, it might be that the first burst of JA-Ile production is converted from the basal OPDA pool. Contrary to these findings, (Hasegawa et al., 2011) demonstrated that root wounding does not alter the OPDA levels in roots. This apparent contradiction across the literature might be due to different growth conditions or developmental stages, rendering direct comparisons rather ineffective. Therefore, a time course of OPDA, JA and JA-Ile levels following root wounding should be performed also in our conditions.

Complementary possibilities to enzyme activation for JA-Ile biosynthesis initiation consider damage-induced substrate availability and substrate accessibility for both lipases and 13-LOXs (Lin et al., 2016; Yu et al., 2020). MGDG and digalactosyldiacylglycerols (DGDG) are among the predominant lipids found in plastidial membranes (Fig. 2) (Hölzl and Dörmann, 2019), and their typical MGDG:DGDG ratio of 2:1 is thought to confer an optimal lens-like plastid shape (Seiwert et al., 2017; Yu et al., 2020). Consistently, mutants in *DGDG SYNTHASE 1* (*dgd1*) with a higher than normal MGDG:DGDG ratio, exhibit plastids with more spherical shapes compared to the wt (Lin et al., 2016; Yu et al., 2020). Remarkably, the mutant also results in higher basal levels of OPDA, JA, and JA-Ile, and higher expression levels of JA-Ile biosynthesis genes (Lin et al., 2016; Yu et al., 2020). An interesting possibility is that an altered composition (or conformation) of the plastidial inner and thylakoid membranes permits easier steric accessibility of JA-Ile biosynthetic enzymes (lipases, 13-LOXs) to reach their substrate. Specifically, MGDG has a conical shape with a small head group of a single galactose moiety and flexible poly-unsaturated fatty acid tails and forms a hexagonal-II phase in aqueous

mixtures. In contrast, DGDG has a cylindrical shape with a larger head group of two galactose moieties, which allows for forming a lamellar bilayer phase common for packed bilayer membranes (Seiwert et al., 2017). As a consequence, a higher MGDG:DGDG ratio might create spaces within the bilayer allowing enzymes to gain easier accessibility towards their MGDG substrates.

The translocation of OPDA from plastids to peroxisomes by the ATP-binding cassette transporter COMATOSE (Theodoulou et al., 2005) could also be a possible regulatory step in JA-Ile production following wounding (Koo et al., 2009). After OPDA is transported into the peroxisomes, the OPR3 enzyme, which is basally present, carries out the reduction of OPDA (Browse, 2009; Schaller et al., 2005). It has been proposed that the activity of OPR3 *in vivo* might be controlled through reversible phosphorylation, as suggested by (Breithaupt et al., 2006). Despite OPR3 being located in the peroxisomes, making it less likely to be a substrate for cytosolic Mitogen-Activated Protein Kinase (MAPKs), there is a possibility that the enzyme can be indirectly regulated by a MAPK pathway. Interestingly, some protein kinases can be induced by wounding in the vicinity of the wound region thereby activating JA biosynthesis (Wu et al., 2007). The co-expression analyses of microarray datasets in Arabidopsis also have revealed a connection between JA biosynthesis and MAPK pathways (van Verk et al., 2011). As JA-Ile level is not completely abolished in *jar1-1* mutant after wounding (Suza and Staswick, 2008), and *gh3.10-2 jar1-11* double mutant also accumulates small amount of JA-Ile, the JA-Ile conjugating enzymes are also probably not the limiting factor of JA-Ile production (Delfin et al., 2022).

Xylem procambium cells might be the first sites of root JA-Ile production and signalling

To unveil which root cell type harbour 13-LOX promoter activities and ultimately enzyme localization, I generated transcriptional reporters containing *13-LOX* promoters driving the expression of NLS-3xVENUS. *LOX3* and *LOX4* promoters were active across the primary root, with *LOX3p* predominantly in root pericycle cells, and *LOX4p* in phloem companion cells starting in the early differentiation zone and continuing upwards towards the shoot. *LOX6p:NLS-3xVENUS* was visible in epidermal and cortex cells along the root, with the strongest signal in the vasculature. Optical cross sections revealed *LOX6* promoter activity in

xylem procambium cells in the root early differentiation zone (Fig. 2-6), which extended to pericycle cells in the middle of the root. Similarly, the *LOX6* promoter is active in and near developing xylem cells in leaves (Chauvin et al., 2013). The promoter activity of *LOX3*, *LOX4* and *LOX6* is also in line with recent single cell RNA-seq data that *LOX3*, *LOX4,* and *LOX6* are expressed in many cell types, such as pericycle, phloem, procambium, lateral root cap and xylem (Wendrich et al., 2020). However, caution must be taken when interpreting single cell RNA-seq data as the procedure requires protoplasting via cell wall degradation. This procedure might induce JA-Ile production (Mielke and Gasperini, 2019) and may affect *LOX3* and *LOX4* expression due to their JA-Ile inducibility (Grebner et al., 2013). Therefore my *in vivo* approach provides a detailed map in intact root tissues that were not subjected to external stress, and encompasses the entire root as opposed to only the meristem portion.

To define the protein localization of 13-LOXs, I have generated translational reporters to express functional N-terminally tagged 13-LOX proteins under their endogenous promoters. In fact, I tested the functionality of both LOX-CIT and CIT-LOX constructs in corresponding mutants' backgrounds according to their different physiological roles. In all cases, only N-terminal fusions were functional. According to the protein structure of 13-LOXs (AlphaFold protein structure database), the catalytic domain of 13-LOXs contains one iron atom as metal cofactor, which is coordinated by three histidines, one asparagine and one isoleucine, which is the last amino acid and conserved for all four 13-LOXs (Maynard et al., 2021; Wasternack and Feussner, 2018). Therefore, a C-terminal tag might affect the folding of the LOX domain and inactivate the enzyme (Fig. 5C).

With functional N-terminal CIT-LOX translational lines, I further analyzed the protein localization of CIT-LOX3, CIT-LOX4 and CIT-LOX6, for which promoter activities were basally present in Arabidopsis roots (Fig. 2-2). The transcript level of *LOX3* was quite low under basal conditions, *LOX4* was slightly higher, and *LOX6* had the most abundant levels in 2-week-old Arabidopsis roots (Fig. 2-2B), in line with (Grebner et al., 2013). Despite assessing more than 10 individual roots across their entire length in a minimum of 2 independent lines segregating Mendelianly for each *LOX3p:CIT-LOX3* and *LOX4p:CIT-LOX4* transgenics, I was not able to detect any basal signal by using state-of-of-the-art microscopy despite the constructs restored the fertility phenotype of the sterile *lox34* double mutants. Conversely, the

expression pattern of *LOX6p:CIT-LOX6* revealed a strong expression in punctuated structures of plant vascular cells. The size of the organelles harbouring fluorescence is comparable with root plastids, which is the expected site for 13-LOX localization due to the presence of their chloroplast transit peptide (Maynard et al., 2021). According to these results, LOX6 is the sole 13-LOX basally present in Arabidopsis roots (Fig. 2-8). In Arabidopsis leaves, immunohistochemical analysis of AOS, AOC and 13-LOX proteins shows that LOX proteins are basally present (Stenzel et al., 2003a). This is also true for 13-LOX detected by TEM immunogold in barley leaves (Bachmann et al., 2002). I therefore hypothesize that LOX6-expressing cells are the ones that make the first burst of JA-Ile upon wounding. The cell specific expression of other JA-Ile biosynthetic enzymes such as AOS and AOCs should also help to make solid conclusion of where the first burst of JAs is produced in Arabidopsis root upon root wounding.

Interestingly, although I could not detect *LOX3p:CIT-LOX3* and *LOX4p:CIT-LOX4* fluorescence signals in primary roots possibly due to a very low protein abundance or post-transcriptional regulation, both CIT-LOX3 and CIT-LOX4 fluorescence signals became visible 3 h after root wounding in cells near the wounding site (Fig. 2-8). This is in line with the wound inducibility of *LOX3* and *LOX4* transcripts (Grebner et al., 2013). Additionally, (Chauvin et al., 2016) revealed a two-tiered, paired hierarchy in which *LOX6*, with a relatively higher influence, and to a lesser extent *LOX2*, predominantly regulate the early-phase expression of jasmonate response genes in above ground tissues. In wounded leaves, the jasmonate precursors produced by LOX6 and LOX2 are converted into active jasmonate, which subsequently regulate the expression of *LOX3* and *LOX4* genes. It is thus possible that root wounding induces LOX6-dependent JA-Ile production and signalling which in turn elevates the expression of LOX3 and LOX4. Indeed, induction of *LOX3* and *LOX4* transcripts after root wounding were compromised in *lox6* mutant (Fig. S2).

Epidermal and root cap cells are in direct contact with the surrounding root environment and are thus intuitive candidates for initial JA-Ile production. However, while *LOX3*, *LOX4* and *LOX6* promoters were active in those cells, I detected LOX6 protein expression predominantly in root vascular cells and not in outer tissues. Protecting vascular tissues with JA-Ile production may be strategy to preserve water and nutrient transport, provide structural

support, long-distance signalling, and grant resource allocation, during root attacks. Proper vascular functioning is indeed vital for overall plant growth, development, and survival (Lucas et al., 2013). Specifically, for OPDA and JA which can travel through the root vasculature (Schulze et al., 2019), vasculature enables long-distance signalling between different parts of the plant, allowing coordinated responses to environmental stimuli and stressors (Takahashi and Shinozaki, 2019). It has been shown in leaves that JA precursors produced by LOX6 in leaf xylem contact cells can move to other cell populations, potentially facilitating long-distance signalling (Gasperini et al., 2015). At the same time, the *lox6* mutant has a diminished ability to synthesize JA rapidly in leaves distal to damage sites, and LOX6 plays a crucial role in protecting the shoot apical meristem against herbivory *Spodoptera littoralis* (Chauvin et al., 2013). While the role of LOX6-dependent root JA-Ile signalling will require future attention, previous work has shown that detritivorous crustaceans feed faster on roots of *lox6* than on the wt, and that the mutant is also less tolerant to drought (Grebner et al., 2013). While root insect bioassays are available to study the jasmonate pathway in crops such as maize (Erb et al., 2011), establishing a root insect bioassay for intact Arabidopsis plants has remained challenging.

LOX2 **or** ***LOX6*** **overexpression does not lead to constitutive JA-Ile signalling in resting roots, while** ***LOX3*** **or** ***LOX4*** **does**

To test if 13-LOXs require post-translational activation, I overexpressed *13-LOX*s under the promoter of *UBQ* for *LOX2*, *LOX4* and *LOX6*, while *LOX3* could only be overexpressed under the *35S* promoter. When I tried to overexpress *LOX3* with *UBQ* promoter, the CIT-LOX3 fusion protein were only observed in cytosol rather than in the plastids. I eventually obtained four *LOX3* OE lines with elevated *LOX3* levels, and detectable CIT-LOX3 fluorescence in the T_2 generation. However, two of them were silenced at the protein level in T_3. Moreover, only one of the *LOX3* OE lines displayed high basal *JAZ10* expression. Collectively this indicates that *LOX3* is very difficult to overexpress with my cloning strategy. Overexpression of *LOX4* also showed higher *JAZ10* expression under basal conditions. Whereas *LOX2* and *LOX6* OE lines displayed very low *JAZ10* expression basally, root wounding induced wt-like *JAZ10* expression for *LOX2* and *LOX6* OE lines, and higher *JAZ10* levels for *LOX3* and *LOX4* OE lines. *JAZ10* could only reflect JA-Ile signalling output, hence, hormone levels, such as OPDA, JA and JA-Ile need

to be measured in all the *13-LOX*s overexpression lines. It could be that *LOX6* OE lines accumulate higher OPDA since *lox6* mutant completely lacked basal OPDA in roots and leaves, indicating the significance of LOX6 in basal OPDA formation (Grebner et al., 2013). Arabidopsides levels could also be measured in *LOX2* OE lines, since LOX2 regulates their production in leaves (Glauser et al., 2009). In their study, (Caldelari et al., 2011) utilized the *35S* promoter to overexpress *LOX3* and *LOX4* in order to restore the male sterile phenotype of the *lox34* double mutant. When considering the whole plant images provided in the paper, the growth of the overexpressing plants resembled that of the wt. Overexpression of *LOX*s were also done in other plant species: *TomLOXD* is a tomato 13-LOX which responds to wounding, pathogen infection and JA treatment (Hu et al., 2013). Transgenic lines with high transcripts levels of *TomLOXD* have only a mild upregulation of JA content (Hu et al., 2013). Another study shows that plant growth, morphology, defense-related gene expression, and JA levels are the same among the wt and three *TomLOXD* OE lines under basal condition (Yan et al., 2013). Overexpression of barley *13-LOX* (*LOX2,2*) also does not result in any phenotypic difference between the transgenics and wt, while transcripts of a few JA-regulated genes are induced (Losvik et al., 2017). Overexpression of the rice *13-LOX* (*OsRCI-1*) gene reduces plant height and seed yield, and transgenic lines accumulate JA and JA-Ile higher than wt under basal conditions (Liao et al., 2022). Therefore, the effects of overexpressing 13-LOX may be LOX- and species-specific. Due to time limitations, I could not phenotype 13-LOX OE lines, such as root length, leaf area as well as their defense responses.

Possible LOX2 and LOX6 post translational activation means

Wt-like basal *JAZ10* levels in *LOX2* and *LOX6* OE lines suggest that post-translational activation is needed to trigger LOX2 and LOX6 enzymatic activities to initiate root JA-Ile production (or those of a downstream JA-Ile biosynthesis enzyme). Phosphorylation might be one possible post translational modifications that could activate LOX2 and LOX6, since it has been characterized recently that Ser^{600} phosphorylation inhibits AtLOX2 catalytic activity and damage by insects could dephosphorylate LOX2 Ser^{600} (Kaur et al., 2023; Thivierge et al., 2010). This amino acid is conserved for LOX6 and LOX3 while not for LOX4. In this case, possible mutants of protein phosphatases that dephosphorylate 13-LOXs should have inhibited JA

production. It is necessary to test if 13-LOXs can be phosphorylated or dephosphorylated *in vivo*.

There are also hints from LOXs in other organisms that calcium might be involved to regulate the enzymatic activity of LOX protein (Hammarberg and Rådmark, 1999; Kulkarni et al., 2002; Oldham et al., 2005). The loop region in the PLAT domain of mammalian 15-LOX is predicted to bind two Ca^{2+} ions (Hammarberg and Rådmark, 1999), which then help the enzyme anchor to the lipid bilayer (Oldham et al., 2005). As for soybean lipoxygenase 1, removing the PLAT domain improved enzyme activity and membrane binding ability in vitro, hence, the activity is not dependent upon calcium (Maccarrone et al., 2001), though high calcium concentrations have been shown to elicit structural fluctuations in the catalytic domain (Tatulian et al., 1998). In Arabidopsis, divalent cations such as Ca^{2+} and Mg^{2+} have been shown to increase the enzymatic activity of 13-LOXs (Maynard et al., 2021). Additionally, upon tissue damage in Arabidopsis, the enzymatic activity of LOX2, involved in the production of green leaf volatiles, is inhibited by a Ca^{2+} selective chelating reagent. However, the addition of Ca^{2+} alleviates this suppression (Mochizuki and Matsui, 2018). Therefore, it is necessary to verify if the PLAT domain of Arabidopsis 13-LOXs could bind to calcium and if calcium really affect the enzymatic activity *in vivo*.

Cell-type specific localization of LOX3, LOX4 and LOX6 is important for their function

The promoter swap experiments were designed to test if the protein function of 13-LOXs can be replaced by one another. I first tested if LOX6 can complement the function of LOX2, LOX3 and LOX4. Shoot wounding assays showed that LOX6 can partially function as LOX2 to induce shoot JA-Ile signalling upon cotyledon wounding. While expressing LOX2 under the promoter of *LOX6* could not rescue *lox6* mutant phenotype upon root wounding, LOX2 also did not complement the *lox34* male sterility when expressed under the promoters of *LOX3* and *LOX4*. LOX3, LOX4 and LOX6, on the contrary, could complement each other in fertility and root wounding assays. Phylogenetic trees of plant lipoxygenases show that LOX2 is in a separate cluster from LOX3, LOX4 and LOX6 (Mochizuki et al., 2016). This could explain to a certain extent the results from promoter swap experiments. On the other hand, when I expressed LOX6 under the promoters of *LOX2*, *LOX3* and *LOX4* and transformed them in the *lox6* mutant

background, none of these lines could complement *lox6* mutant phenotype in root wounding assay, indicating that the cell-type specific expression of LOX6 is key for its function. Interestingly, the basal expression of *LOX3p:CIT-LOX6* and *LOX4p:CIT-LOX6* was not detectable but could be visualized 3 h after root wounding (Fig. S3).

At the promoter level (https://agris-knowledgebase.org/AtcisDB), *LOX3* and *LOX4* promoter were predicted to contain *cis* elements that can be recognized by *MYB*, *MYC* and *WRKY* transcription factors. Mechanical wounding triggers the activation of early response genes in Arabidopsis, such as *WRKY* and *MYB*, and genes involved in the biosynthesis of JA-Ile (Yong et al., 2002). It is possible that the promoters of *LOX3* and *LOX4* can be regulated by these TF. In the future, one could first verify which transcription factor binds to the different *13-LOX* promoters by ChIP-qPCR.

Promoter swap experiments also indicated that the cell specific localization of 13-LOXs is essential for their function, since expressing LOX3 and LOX4 under the promoter of *LOX6* could rescue the *lox6* mutant phenotype. Similarly, LOX6 could restore the sterility phenotype of the *lox34* double mutant when expressed under the *LOX3* or *LOX4* promoters. Due to time limitation, I did not test if LOX3 and LOX4 could mimic the function of LOX2 in shoot wounding experiments when they are expressed under the promoter of *LOX2*.

13-LOXs are also regulated at the protein level

13-LOXs contain an N-terminal PLAT domain and a C-terminal catalytic LOX domain (Schneider et al., 2007). LOX6 is critical for the first burst of root JA-Ile production and signalling after root wounding, but its activity could be replaced by LOX3 and LOX4 when expressed under the *LOX6* promoter. Therefore, I was expecting that chimeric proteins such as $PLAT^{LOX6}$-LOX^{LOX3}, $PLAT^{LOX6}$-LOX^{LOX4}, $PLAT^{LOX3}$-LOX^{LOX6} and $PLAT^{LOX4}$-LOX^{LOX6} could also restore the *lox6* root phenotype. I was curious to test if exchanging the PLAT and LOX domain between LOX2 and LOX6 could render the LOX2 protein functional in roots ($PLAT^{LOX6}$-LOX^{LOX2}), or in-turn, abolish LOX6 activity ($PLAT^{LOX2}$-LOX^{LOX6}). However, none of the domain swap lines could complement the native LOX6 protein function. Given that *LOX6p:CIT-LOX3* could complement the *lox6* mutant phenotype, but *LOX6p:CIT-*$PLAT^{LOX3}$-LOX^{LOX6} and *LOX6p:CIT-*$PLAT^{LOX6}$-LOX^{LOX3}

could not mimic the function of LOX6, I hypothesized that the interaction between the PLAT and LOX domain is essential for the enzyme activity.

Consistently, it has been shown that a conserved π–cation bridge connects the PLAT domain and LOX domain and serves as a regulatory interface to the active site in the coral 11R-LOX (Eek et al., 2012). A π–cation interaction, refers to a noncovalent interaction between a positively charged cation and an aromatic system that possesses π-electrons (Gallivan and Dougherty, 1999). To test if the π–cation bridge exerts a physiological role for 13-LOXs in planta, especially for LOX6 which has two of the π–cation bridges, I have generated LOX6 point mutation lines to either mimic or disrupt the π–cation interaction. The conservative mutation of LOX6^{W201F} should behave similarly to the WT in terms of JA-Ile signalling, while LOX6^{W201K} and LOX6^{W201A} mutations that disrupt the π–cation interaction might not be able to rescue *lox6* mutant phenotype in response to root wounding. It would be interesting to assess the functional relevance of the π–cation bridge also for the other three 13-LOX.

The PLAT domain from mammalian 5-LOX, 15-LOX and coral 8R-LOX is predicted to bind Ca^{2+}, which provokes a conformational changes promoting the translocation of the enzyme to the membrane through its PLAT domain (Hammarberg et al. 2000; Droege et al. 2017; Oldham et al. 2005). In Arabidopsis, the LOX2 enzymatic activity is inhibited by Ca^{2+} chelators in the context of green leaf volatiles production upon tissue damage, which can be reverted by the addition of Ca^{2+} (Mochizuki and Matsui, 2018). It is well known that wounding triggers cytosolic calcium increase (Toyota et al., 2018; Nguyen et al., 2018). It is therefore possible that calcium concentration also increases in plastids in response to wounding (Navazio et al., 2020). Ca^{2+} could then bind the PLAT domain of 13-LOX to regulate their function, such as mediate their translocation from the stroma to plastid inner membranes to access their substrates and initiate JA-Ile biosynthesis. The results from chloroplast calcium channel *bicat* mutants suggest that disruption of calcium influx to plastids impact *JAZ10* induction after root wounding. To further test this hypothesis, one would need to characterize calcium dynamics between cytosol and plastids upon wounding. Finally, it would also be necessary to determine if the localization of 13-LOX protein would change within plastid after JAs-inducing stimuli, such as hypoosmotic stress, this can be achieved by TEM immunogold.

III. MATERIALS and METHODS

Key resources

Table 3: Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial Strains		
Escherichia coli Dh5α	Thermo Fisher	18265017
Agrobacterium tumefaciens Gv3101	GoldBio	CC-207-A
Chemicals, Peptides, and Recombinant Proteins/Enzymes		
Murashige & Skoog (MS) basal salt mix	Duchefa	M0221.0025
2-(N-morpholino)ethanesulfonic acid (MES)	Sigma	M8250
Plant agar	Applichem	A2111
Propidium Iodide (PI)	Sigma	P4864
Calcofluor white	Sigma	18909
Direct Red 23	Sigma	212490
Xylitol	Sigma	X3375
Urea	Sigma	U5378
Sodiumdeoxycholate	Sigma	D6750
Paraformaldehyde	Merck	8.18715
LR White	Agarscientific	R1280
Anti-GFP-antibody	Abcam	ab6556
NaH_2PO_4	Sigma	RDD007
Na_2HPO_4	Sigma	795410
Potassium ferricyanide (III)	Roth	7971.1
Potassium ferrocyanide (II)	Roth	7974.1
X-Gluc	Biomol	AG-CN2-0023-M001
Triton x100	Roth	9002-93-1
Methyl Jasmonate (MeJA)	Sigma	392707
Taq DNA Polymerase	Thermo Fisher	10342020
Phusion High-Fidelity DNA Polymerase	Life Technologies	F530
BfmI restriction enzyme	Thermo Fisher	FD1164
BP Clonase	Thermo Fisher	11789-100
LR Clonase	Thermo Fisher	11791-100
LR Plus Clonase	Thermo Fisher	12176590
Anti-GFP	Abcam	Ab6556
Anti-Rabbit IgG	Sigma	G7402
dNTPs	Promega	U1240
$MgCl_2$	Sigma	M0250
SYBR Green	Invitrogen	S7563
6-carboxy-X-rhodamine	Invitrogen	12223-012
Go Taq Polymerase	Promega	M7848
Critical Commercial Assays		
DNeasy Plant Mini Kit	Qiagen	69106
RNeasy Plant Mini Kit	Qiagen	74904
NuleoSpin Gel and PCR CleanUp	Marchery & Nagel	740609.25
QIAprep Spin MiniPrep Kit	Qiagen	27106
RNA extraction kit	Marchery & Nagel	740120.50
Experimental Models: Organisms/Strains		

**Arabidopsis thaliana:* Col-0	Widely distributed	N/A
**Arabidopsis thaliana: lox2-1*	NASC; (Glauser et al., 2009)	N3748
**Arabidopsis thaliana: lox3A*	NASC; (Caldelari et al., 2011)	SALK_147830
**Arabidopsis thaliana: lox4B*	NASC; (Caldelari et al., 2011)	SALK_138907
**Arabidopsis thaliana: lox6A*	NASC; (Caldelari et al., 2011)	SALK_071732
**Arabidopsis thaliana: lox23*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox24*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox26*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox34*	(Caldelari et al., 2011)	
**Arabidopsis thaliana: lox36*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox46*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox234*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox236*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox346*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: lox2346*	(Chauvin et al., 2013)	
**Arabidopsis thaliana: bicat1*	(Frank et al., 2019)	GK-166A05
**Arabidopsis thaliana: bicat2*	(Frank et al., 2019)	SALK-129037C
Arabidopsis thaliana: JAZ10p:GUS (JGP^{ns}) in Col-0	(Gasperini et al., 2015)	N/A
LOX2p:GUS in Col-0	(Gasperini et al., 2015)	N/A
LOX3p:GUS in Col-0	(Gasperini et al., 2015)	N/A
LOX4p:GUS in Col-0	(Gasperini et al., 2015)	N/A
LOX6p:GUS in Col-0	(Gasperini et al., 2015)	N/A
LOX2p:NLS-3xVEN in Col-0 (pMAY038)	This study	N/A
LOX3p:NLS-3xVEN in Col-0 (pMAY039)	This study	N/A
LOX4p:NLS-3xVEN in Col-0 (pMAY040)	This study	N/A
LOX6p:NLS-3xVEN in Col-0 (pMAY041)	This study	N/A
LOX2p:LOX2.1-CIT (pMAY006) in *lox2*	This study	N/A
LOX2p:LOX2.2-CIT (pMAY007) in *lox2*	This study	N/A
LOX3p:LOX3-CIT (pMAY034) in *lox34*	This study	N/A
LOX4p:LOX4-CIT (pMAY035) in *lox34*	This study	N/A
LOX6p:LOX6-CIT (pMAY047) in *lox6*	This study	N/A
LOX2p:cTP-CIT-LOX2.1 (pMAY043) in *lox2*	This study	N/A
LOX3p:cTP-CIT-LOX3 (pMAY044) in *lox3*	This study	N/A
LOX3p:cTP-CIT-LOX3 (pMAY044) in *lox34*	This study	N/A
LOX4p:cTP-CIT-LOX4 (pMAY045) in *lox3*	This study	N/A
LOX4p:cTP-CIT-LOX4 (pMAY045) in *lox34*	This study	N/A
LOX6p:cTP-CIT-LOX6 (pMAY046) in *lox6*	This study	N/A
UBQ10p:LOX2.1-CIT (pMAY012) in *lox2*	This study	N/A
UBQ10p:LOX2.2-CIT (pMAY013) in *lox2*	This study	N/A
UBQ10p:LOX3-CIT (pMAY036) in *lox3*	This study	N/A
UBQ10p:LOX3-CIT (pMAY036) in *lox34*	This study	N/A
UBQ10p:LOX4-CIT (pMAY037) in *lox4*	This study	N/A
UBQ10p:LOX4-CIT (pMAY037) in *lox34*	This study	N/A
UBQ10p:LOX6-CIT (pMAY042) in *lox6*	This study	N/A
UBQ10p:cTP^{LOX2}-CIT-LOX2.1 (pMAY050) in *lox2*	This study	N/A
UBQ10p:cTP^{LOX3}-CIT-LOX3 (pMAY051) in *lox3*	This study	N/A
UBQ10p:cTP^{LOX3}-CIT-LOX3 (pMAY051) in *lox34*	This study	N/A

UBQ10p:cTP^{LOX4}-CIT-LOX4 (pMAY052) in *lox4*	This study	N/A
UBQ10p:cTP^{LOX4}-CIT-LOX4 (pMAY052) in *lox34*	This study	N/A
UBQ10p:cTP^{LOX6}-CIT-LOX6 (pMAY053) in *lox6*	This study	N/A
UBQ10p:cTP^{LOX3}CIT-LOX3 (pMAY056) in *lox3*	This study	N/A
35Sp:cTP^{LOX3}-CIT-LOX3 (pMAY078) in *lox3*	This study	N/A
35Sp:cTP^{LOX3}-CIT-LOX3 (pMAY078) in *lox34*	This study	N/A
LOX6p:cTP-CIT-LOX2.1 (pMAY059) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX3 (pMAY060) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX4 (pMAY061) in *lox6*	This study	N/A
LOX2p:cTP-CIT-LOX6 (pMAY077) in *lox2*	This study	N/A
LOX2p:cTP-CIT-LOX6 (pMAY077) in *lox6*	This study	N/A
LOX3p:cTP-CIT-LOX6 (pMAY079) in *lox34*	This study	N/A
LOX3p:cTP-CIT-LOX6 (pMAY079) in *lox6*	This study	N/A
LOX4p:cTP-CIT-LOX6 (pMAY080) in *lox34*	This study	N/A
LOX4p:cTP-CIT-LOX6 (pMAY080) in *lox6*	This study	N/A
LOX3p:cTP-CIT-LOX2 (pMAY082) in *lox34*	This study	N/A
LOX4p:cTP-CIT-LOX2.1 (pMAY083) in *lox34*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX2}-LOX^{LOX6} (pMAY064) in *lox6*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX3}-LOX^{LOX6} (pMAY066) in *lox6*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX4}-LOX^{LOX6} (pMAY068) in *lox6*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX2} (pMAY072) in *lox6*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX3} (pMAY074) in *lox6*	This study	N/A
LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX4} (pMAY076) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX6$^{\Delta PLAT}$ (pMAY069) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX6 ^{W201F} (pMAY087) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX6 ^{W201K} (pMAY088) in *lox6*	This study	N/A
LOX6p:cTP-CIT-LOX6 ^{W201A} (pMAY089) in *lox6*	This study	N/A
Oligonucleotides		
primers used for cloning, genotyping, qRT-PCR can be found in Table 4		
Recombinant DNA		
Plasmid: *pUC57 (KpnI-XmaI)*	(Chauvin et al., 2013)	N/A
Plasmid: *pDONR221*	Invitrogen	Cat# 12536017
Plasmid: *pDONR-P2R-P3*	Invitrogen	Cat# 12537023
Plasmid: *pEDO 097*	(Chauvin et al., 2013)	N/A
Plasmid: *pR7m34gw*	Invitrogen, but then modified and gifted by Ivan Acosta	N/A
pDG121: *pEN-attL4-LOX2p-attR1*	(Chauvin et al., 2016)	N/A
pDG122: *pEN-attL4-LOX3p-attR1*	(Chauvin et al., 2016)	N/A
pDG123: *pEN-attL4-LOX4p-attR1*	(Chauvin et al., 2016)	N/A
pDG124: *pEN-attL4-LOX6p-attR1*	(Chauvin et al., 2016)	N/A
pDG29: *pEN-attL1-NLS-3x-VEN-attL2*	(Marhavý et al., 2019)	N/A
pMAY038: *pDEST-attB4-LOX2p:NLS-3x-VEN-attB2*	This study	N/A
pMAY039: *pDEST-attB4-LOX3p:NLS-3x-VEN-attB2*	This study	N/A
pMAY040: *pDEST-attB4-LOX4p:NLS-3x-VEN-attB2*	This study	N/A
pMAY041: *pDEST-attB4-LOX6p:NLS-3x-VEN-attB2*	This study	N/A
pMAY032: *pEN-attL4-LOX2p-cTP-attR1*	This study	N/A
pMAY019: *pEN-attL4-LOX3p-cTP-attR1*	This study	N/A
pMAY027: *pEN-attL4-LOX4p-cTP-attR1*	This study	N/A
pMAY033: *pEN-attL4-LOX6p-cTP-attR1*	This study	N/A

pDG006: *pEN-attL1-CIT (no*)-attL2*	(Gasperini et al., 2015)	N/A
pMAY031: *pEN-attR2-LOX2.1(no cTP)-attL3*	This study	N/A
pMAY030: *pEN-attR2-LOX3(no cTP)-attL3*	This study	N/A
pMAY028: *pEN-attR2-LOX4(no cTP)-attL3*	This study	N/A
pMAY029: *pEN-attR2-LOX6(no cTP)-attL3*	This study	N/A
pMAY043: *pDEST-attB4-LOX2p:cTP-CIT-LOX2.1-attB3*	This study	N/A
pMAY044: *pDEST-attB4-LOX3p:cTP-CIT-LOX3-attB3*	This study	N/A
pMAY045: *pDEST-attB4-LOX4p:cTP-CIT-LOX4-attB3*	This study	N/A
pMAY046: *pDEST-attB4-LOX6p:cTP-CIT-LOX6-attB3*	This study	N/A
pMAY001: *pEN-attL1-LOX2.1-attL2*	This study	N/A
pMAY024: *pEN-attL1-LOX3-attL2*	This study	N/A
pMAY025: *pEN-attL1-LOX4C-attL2*	This study	N/A
pDG132: *pEN-attL1-LOX6-attL2*	This study	N/A
pDG17: *pEN-attR2-CIT-attL3*	(Gasperini et al., 2015)	N/A
pMAY006: *pDEST-attB4-LOX2p:LOX2.1-CIT*-attB3*	This study	N/A
pMAY034: *pDEST-attB4-LOX3p:LOX3-CIT*-attB3*	This study	N/A
pMAY035: *pDEST-attB4-LOX4p:LOX4-CIT*-attB3*	This study	N/A
pMAY047: *pDEST-attB4-LOX6p:LOX6-CIT*-attB3*	This study	N/A
pMAY020: *pEN-attL4-UBQ10p-cTP^{LOX2}-attR1$^{\#}$*	This study	N/A
pMAY021: *pEN-attL4-UBQ10p-cTP^{LOX3}-attR1$^{\#}$*	This study	N/A
pMAY022: *pEN-attL4-UBQ10p-cTP^{LOX4}-attR1$^{\#}$*	This study	N/A
pMAY023: *pEN-attL4-UBQ10p-cTP^{LOX6}-attR1$^{\#}$*	This study	N/A
pDG158: *pEN-attL4-35Sp-attR1*	(Gasperini et al., 2015)	N/A
pMAY057: *pEN-attL1-LOX3cTP-CIT-attL2$^{\#}$*	This study	N/A
pMAY050: *pDEST-attB4-UBQ10p:cTP^{LOX2}-CIT-LOX2.1-attB3*	This study	N/A
pMAY051: *pDEST-attB4-UBQ10p:cTP^{LOX3}-CIT-LOX3-attB3*	This study	N/A
pMAY056: *pDEST-attB4-UBQ10p:cTP^{LOX3}CIT-LOX3-attB3*	This study	N/A
pMAY078: *pDEST-attB4-35Sp:cTP ^{LOX3}-CIT-LOX3-attB3*	This study	N/A
pMAY052: *pDEST-attB4-UBQ10p:cTP^{LOX4}-CIT-LOX4-attB3*	This study	N/A
pMAY053: *pDEST-attB4-UBQ10p:cTP^{LOX6}-CIT-LOX6-attB3*	This study	N/A
pMAY059: *pDEST-attB4-LOX6p:cTP-CIT-LOX2.1-attB3*	This study	N/A
pMAY060: *pDEST-attB4-LOX6p:cTP-CIT-LOX3-attB3*	This study	N/A
pMAY061: *pDEST-attB4-LOX6p:cTP-CIT-LOX4-attB3*	This study	N/A
pMAY077: *pDEST-attB4-LOX2p:cTP-CIT-LOX6-attB3*	This study	N/A
pMAY079: *pDEST-attB4-LOX3p:cTP-CIT-LOX6-attB3*	This study	N/A
pMAY080: *pDEST-attB4-LOX4p:cTP-CIT-LOX6-attB3*	This study	N/A
pMAY082: *pDEST-attB4-LOX3p:cTP-CIT-LOX2.1-attB3*	This study	N/A
pMAY083: *pDEST-attB4-LOX4p:cTP-CIT-LOX2.1-attB3*	This study	N/A
pMAY063: *pEN-attL1-CIT-PLAT ^{LOX2}-attL2$^{\#}$*	This study	N/A
pMAY065: *pEN-attL1-CIT-PLAT^{LOX3}-attL2$^{\#}$*	This study	N/A
pMAY067: *pEN-attL1-CIT-PLAT^{LOX4}-attL2$^{\#}$*	This study	N/A
pMAY070: *pEN-attL1-CIT-PLAT^{LOX6}-attL2$^{\#}$*	This study	N/A
pMAY071: *pEN-attR2-LOX ^{LOX2}-attL3*	This study	N/A
pMAY073: *pEN-attR2-LOX ^{LOX3}-attL3*	This study	N/A
pMAY075: *pEN-attR2-LOX ^{LOX4}-attL3*	This study	N/A
pMAY062: *pEN-attR2-LOX ^{LOX6}-attL3*	This study	N/A
pMAY064: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX2}-LOX^{LOX6}-attB3*	This study	N/A
pMAY066: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX3}-LOX^{LOX6}-attB3*	This study	N/A
pMAY068: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX4}-LOX^{LOX6}-attB3*	This study	N/A
pMAY072: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX2}-attB3*	This study	N/A
pMAY074: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX3}-attB3*	This study	N/A

pMAY076: *pDEST-attB4-LOX6p:cTP-CIT-PLAT^{LOX6}-LOX^{LOX4}-attB3*	This study	N/A
pMAY069: *pDEST-attB4-LOX6p:cTP-CIT-LOX6$^{\Delta PLAT}$-attB3*	This study	N/A
pMAY084: *pEN-attR2-LOX6*^{W201F}*-attL3*$^{\#}$	This study	N/A
pMAY085: *pEN-attR2-LOX6*^{W201K}*-attL3*$^{\#}$	This study	N/A
pMAY086: *pEN-attR2-LOX6*^{W201A}*-attL3*$^{\#}$	This study	N/A
pMAY087: *pDEST-attB4-LOX6p:cTP-CIT-LOX6*^{W201F}*-attB3*	This study	N/A
pMAY088: *pDEST-attB4-LOX6p:cTP-CIT-LOX6*^{W201K}*-attB3*	This study	N/A
pMAY089: *pDEST-attB4-LOX6p:cTP-CIT-LOX6*^{W201A}*-attB3*	This study	N/A
$^{\#}$ plasmids were obtained by GeneArt synthesis (Thermo Fisher). FASTA files are available in Appendix 1		

Table4: List of oligonucleotides used in this study.

Name	5' → 3' sequence	Objective
Primers for cloning		
MAY105	CGGGGTACCacctgaatgacaacaaaattcccga	*LOX2* promoter with cTP of 3147bp flanked by *XmaI* and *KpnI* sites amplify from pDG172
MAY106	TTCCCCCCCGGGattctgaatgggttctttcactgtgt	
MAY069	CGGGGTACCttgatacgcgataatcttacaagccc	*LOX3* promoter with cTP of 3182bp flanked by *XmaI* and *KpnI* sites amplify from pDG171
MAY070	TTCCCCCCCGGGccttcaccgccgctttctcct	
MAY109	CGGGGTACCagaagagaggccaaggacact	*LOX4* promoter with cTP of 3042bp flanked by *XmaI* and *KpnI* sites amplify from pDG174
MAY110	TTCCCCCCCGGGcttcaccgacttctcctcttcttc	
MAY107	CGGGGTACCtgcattttatcggcggcattgc	*LOX6* promoter with cTP of 3225bp flanked by *XmaI* and *KpnI* sites amplify from pDG173
MAY108	TTCCCCCCCGGGcgatcccgtatacttagatctttgcc	
MAY015	TGTACAAAAAAGCAGGCTGcatggccttagctaaagag	*LOX3* CDS no* of 2757 bp flanked by attB1 and attB2 sites
MAY016	TTTGTACAAGAAAGCTGGGTttatagatacactattaggtac	
MAY001	TGTACAAAAAAGCAGGCTGcatgtattgtagagagtccttg	*LOX2* CDS no* of 2688bp flanked by attB1 and attB2 sites
MAY002	TTTGTACAAGAAAGCTGGGTtaatagaaatactataaggaac	
MAY027	TGTACAAAAAAGCAGGCTGcatggctttagctaatgag	*LOX4* CDS no* of 2778bp flanked by attB1 and attB2 sites
MAY028	TTTGTACAAGAAAGCTGGGTtaatagatacactattaggcac	
MAY036	TGTACAAAAAAGCAGGCTGcatgttcgtagcatctccgg	*LOX6* CDS no* of 2751bp flanked by attB1 and attB2 sites
MAY037	TTTGTACAAGAAAGCTGGGTtaatggaaatgctgttgggaatac	
MAY001	TGTACAAAAAAGCAGGCTGcatgtattgtagagagtccttg	*LOX2* CDS of 2475bp flanked by attB2 and attB3 sites
MAY130	GGGGACAACTTTGTATAATAAAGTTGtcaaatagaaatactataag	
MAY015	TGTACAAAAAAGCAGGCTGcatggccttagctaaagag	*LOX3* CDS of 2511bp flanked by attB2 and attB3 sites
MAY132	GGGGACAACTTTGTATAATAAAGTTGttatatagatacactattagg	
MAY027	TGTACAAAAAAGCAGGCTGcatggctttagctaatgag	*LOX4* CDS of 2514bp flanked by attB2 and attB3 sites
MAY134	GGGGACAACTTTGTATAATAAAGTTGctaaatagatacactattag	
MAY036	TGTACAAAAAAGCAGGCTGcatgttcgtagcatctccgg	*LOX6* CDS of 2517bp flanked by attB2 and attB3 sites
MYAY128	GGGGACAACTTTGTATAATAAAGTTGctaaatggaaatgctgttggg	

MAY129	GGGGACAGCTTTCTTGTACAAAGTGGTtaagtcctacttgcctcccaaac	*LOX2* CDS without PLAT of 2091bp flanked by attB2 and attB3 sites
MAY130	GGGGACAACTTTGTATAATAAAGTTGtcaaatagaaatactataag	
MAY131	GGGGACAGCTTTCTTGTACAAAGTGGTgcagccgtatttgccgaatgag	*LOX3* CDS without PLAT of 2091bp flanked by attB2 and attB3 sites
MAY132	GGGGACAACTTTGTATAATAAAGTTGttatatagatacactattagg	
MAY133	GGGGACAGCTTTCTTGTACAAAGTGGTgcagccttacttgccgagcg	*LOX4* CDS without PLAT of 2094bp flanked by attB2 and attB3 sites
MAY134	GGGGACAACTTTGTATAATAAAGTTGctaaatagatacactattag	
MAY127	GGGGACAGCTTTCTTGTACAAAGTGGTgcaaccatgcctaccttccgag	*LOX6* CDS without PLAT of 2103bp flanked by attB2 and attB3 sites
MAY128	GGGGACAACTTTGTATAATAAAGTTGctaaatggaaatgctgttggg	
Primers for genotyping		
LOX2.1F	ggattatcatgatttgcttctacc	*lox2-1* and wt amplicons of 1516bp, digested with *BfmI* result in wt (1516 bp) and *lox2.1* (864bp+652bp)
LOX2.1R	tcaaatagaaatactataaggaacac	
MAY136	aattcgcacgtcaagccatag	*lox3B* (SALK_147830)
MAY138	tttgataaggttcgggtttgg	
MST266	gttggttccaaatacacacgg	*lox4A* (SALK_071732)
MST267	agccaaagaagagtaaagcgg	
MYJ003	cagtgaagaaatcgatcctgg	*lox6A* (SALK_138907)
MYJ004	cagtgaagaaatcgatcctgg	
MYJ005	tacgtaacgatctcagccacc	*bicat1* (GB-166A05)
MYJ006	agcttggtttgttaatcgcag	
MYJ007	cccattgaacctaaaaggacc	*bicat2* (SALK-129037)
MYJ008	atcagatgctggtgttggatc	
SALK.LBb1.3	attttgccgatttcggaac	For genotyping of all SALK T-DNA insertion lines
GABI.LB	atattgaccatcatactcattgc	For genotyping of all GK T-DNA insertion lines
Primers for qRT-PCR		
JAZ10.qF	atcccgatttctccggtcca	*JAZ10* (At5g13220) 222 bp fragment
JAZ10.qR	actttctccttgcgatgggaaga	
UBQ.qF	cagtctgtgtgtagagctatcatagcat	*UBC21* (At5g25760) 83 bp fragment
UBQ.qR	agaagattccctgagtcgcagtt	
MAY095	ccaagactgaccagcggatt	*LOX2* (AT3G45140) 117bp fragment
MAY096	ctcccactggtccaaagtct	
MAY097	cagccgtatttgccgaatga	*LOX3* (AT1G17420) 88bp fragment
MAY098	ctccacttccatctcctcgt	
MAY099	aatcagccttacttgccgag	*LOX4* (AT1G72520) 139bp fragment

MAY100	tgtcgttgtacacatcataatcg	
MAY101	cgatcctgaaactggtaaaggc	*LOX6* (AT1G67560) 99bp fragment
MAY102	tcggctgtgaatacgaggta	

Note, for genotyping of T-DNA lines, sizes of amplification products are given by T-DNA express (http://signal.salk.edu/cgi-bin/tdnaexpress). Three primer reactions will target a bigger wt amplicon (forward + reverse primer) and a smaller mutant amplicon (T-DNA left border primer + forward or reverse primer).

Plant material and growth conditions

The *Arabidopsis thaliana Columbia-0* (*Col-0*) accession was the wild-type genetic background used throughout this study. A list of the genetic material used is presented in Table 3. For assays on solid plant growth media, seeds were sterilized and stratified 2 days at 4 °C in the dark as described (Acosta et al., 2013). Seedlings were grown on 0.5x solid Murashige and Skoog (MS) media supplemented with 0.5 g/L 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate and 0.7% or 0.85% plant agar for horizontal or vertical growth, respectively. Horizontally grown seedlings were germinated on a nylon mesh (Lanz-Anliker AG, Rohrbach, Switzerland) with 200 µm pore size placed on top of the MS media as described (Acosta et al., 2013), and were used for shoot wounding. Controlled growth conditions were set at 21°C under 100 µE m^{-2} s^{-1} light, with a 14 h light/10 h dark photoperiod. For propagation and transformation, plants were also grown on soil at the same conditions but under continuous light.

Genotyping

Genomic DNA was extracted and purified with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Genotyping of T-DNA insertion lines was performed in 20 µL PCR reactions [20 ng genomic DNA, 500 nM each of specific forward and reverse primers, 500 nM of the general T-DNA primer left border primer, 200 µM Deoxynucleotide Triphosphates, 1× PCR buffer (Invitrogen), and 0.1 U of Taq DNA polymerase (Invitrogen) on a Thermocycler (Eppendorf™ Mastercycler™ PRO) with 95°C x 5' of initial denaturation and 35 amplification cycles consisting of 95°C x 30'' denaturation, 58°C x 30'' annealing, and 72°C x 60'' extension. Single nucleotide polymorphisms (SNPs) were genotyped with Cleaved Amplified Polymorphic Sequences (CAPS) markers (http://helix.wustl.edu/dcaps/). Briefly, PCR products were amplified as above but without a T-DNA primer and then digested with restriction enzymes according to the manufacturer specifications. Specific primers and restriction enzymes are

listed in Table 2. PCR products >300bp were separated by electrophoresis on 1% agarose gels, while <300bp products were separated on 2% agarose gels.

Histochemical detection of GUS activity

Aseptically and vertically grown 2-week-old plants were collected in 90% acetone, incubated for 60 min, and washed with 50 mM sodium phosphate buffer pH 7.0 for 5-10 min. The rinsing solution was replaced with the staining mix (50 mM sodium phosphate buffer pH 7.0, 0.1% Triton X-100, 0.5 mM K_4Fe (CN)6, 0.5 mM K_3Fe(CN)6, 0.5 mg/ml X-Gluc). Seedlings were then vacuum infiltrated for 5 min at room temperature and incubated overnight at 37°C in the dark. The reaction was stopped by replacing the staining solution with 70% ethanol, which was exchanged several times to remove the remaining chlorophyll. Seedlings were mounted in chloral hydrate: glycerol: water solution (8:2:1) and photographed with a Leica M165 FC stereomicroscope fitted with a Leica MC170 HD camera.

Plant treatments

2-week-old plants grown on vertical plates were used for root wounding by squeezing the primary root with forceps 4 times equidistantly. Wounding was performed 1cm below the collet while establishing the method, and 2cm below the collet for all optimized experiments. 5-day-old horizontally grown seedlings were used for single cotyledon wounding as described (Acosta et al., 2013). Plates were placed under a stereomicroscope and seedlings were wounded once in one of the two cotyledons with a 25G x 5/8" needle (0.5 mm x 16 mm). Plates were brought back to the growth chamber and incubated for different times before tissue collection depending on the type of experiment: 1 h for qPCR analysis of *JAZ10* and *13-LOX* expression; 2 h or overnight for normal GUS histochemical detection. Wounding experiments were performed in the morning between 8:00-11:00am.

Gene expression analyses

Total RNA was extracted with RNA extraction kit from MACHEREY-NAGEL. For qRT-PCR experiments of *JAZ10* and *13-LOXs,* 5-d-old horizontally grown seedlings or 2-week-old vertically grown plants, were separated in shoots and roots and collected for basal and 1 h after shoot/root wounding expression analysis. Total RNA was copied to cDNA with the M-MLV reverse transcriptase RNAse H(-) (Promega) according to the manufacturer's instructions.

The quantitative PCR reaction mixture contained 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.5× SYBR Green I (Invitrogen), 30 nM 6-carboxy-X-rhodamine, 0.5 units of GoTaq polymerase (Promega), and 0.25 µM of each primer in a total volume of 20 µL. The PCR program consisted of an initial denaturation step at 95°C for 2 min followed by 40 cycles of 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Real-time PCR was performed using the QuantStudio real-time PCR system (Thermo Fisher Scientific). Primer efficiency was optimized for each primer pair with a dilution series and kept with a 1.9 - 2.1 range. qRT-PCR primers are listed in Table 4.

Cloning and generation of transgenic lines

All transcriptional and translational reporter constructs were generated by double or triple Multisite Gateway Technology (Thermo Fisher). For transcriptional and translational reporters listed in Table X, inserts were amplified with primers listed in Table 4 or synthesized directly with GeneArt (Thermo fisher) Appendix1. pEN-attL4-*LOX2p*-attR1 (pDG121), pEN-attL4-*LOX3p*-attR1 (pDG122), pEN-attL4-*LOX4p*-attR1 (pDG123) and pEN-attL4-*LOX6p*-attR1 (pDG124) from (Gasperini et al., 2015) were recombined with pEN-attL1-*NLS-3xVEN*-attL2 into pEDO097, as described (Gasperini et al., 2015). For C-terminal fusion of *13-LOXp:LOX-CIT* constructs, coding DNA sequences (CDS) of *LOX2.1, LOX3, LOX4* and *LOX6* (primers MAY001/MAY002, MAY015/MAY016, MAY027/MAY028 and MAY036/MAY037, amplifying *13-LOX*s without the stop codon, respectively) were amplified from wt cDNA with oligonucleotides specified in parenthesis containing appropriate *att* sites and recombined with pDONR221 to obtain pEN-attL1-*LOX2/3/4/6*-attL2. The above mentioned pEN containing *13-LOX* promoters were recombined with pEN-attL1-*LOX2/3/4/6*-attL2 and pEN-attR2-*CIT*-attL3 into a modified pH7m34gw vector named pFR7m34gw, which harbours seed Red Fluorescent Protein (RFP) expression (*OLE1p:RFP*) for in planta selection. For N-terminal fusion of *13-LOXp:cTP-CIT-LOX* constructs, the entry plasmids containing *13-LOX* promoters with respective plastid transit peptides (pEN-attL4-*LOX2p-cTP*-attR1, pEN-attL4-*LOX3p-cTP*-attR1, pEN-attL4-*LOX4p-cTP*-attR1, and pEN-attL4-*LOX6p-cTP*-attR1) were amplified from wt genomic DNA with primers listed in Table 4 and cloned into pUC57. CDS inserts of *LOX2/3/4/6* with the stop codon were amplified with primers (MAY001/MAY130, MAY015/MAY132, MAY027/MAY134 and MAY036/MAY128 respectively) listed in Table 4, cloned in *pDONR-P2R-P3* to generate pEN-attR2-*LOX*-attL3. The fluorescent tag pEN-attL1-*CIT*-attL2 was inserted between promoter and 13-LOX CDS. To obtain *LOX2/4/6* overexpression constructs, pEN-

attL4-*UBQ10p-cTP*^{LOX2}-attR1, pEN-attL4-*UBQ10p-cTP*^{LOX4}-attR1 and pEN-attL4-*UBQ10p-cTP*^{LOX6}-attR1 were generated by gene synthesis (Thermo Fisher), appendix 1. The gene synthesized promoter region were then combined with respective *13-LOX* coding region (pEN-attR2-att*LOX2.1*-attL3, pEN-attR2-*LOX4*-attL3, pEN-attR2-*LOX6*-attL3) and pEN-attL1-*CIT*-attL2 into pFR7m34gw. The same strategy was performed to overexpress *LOX3* but without success even after screening more than 100 lines. I therefore next tried to only use *UBQ10* promoter and put cTP together with CIT to obtain pEN-attR2-*cTP*^{LOX3}-*CIT*-attL3 (gene synthesis) and recombined with and pEN-attL1-*LOX3*-attL2 into destination vector pFR7m34gw, I still did not get any lines that CIT-LOX3 were expressed in plastids. Finally, to overexpress *LOX3*, pEN-L4-*35Sp*-R1 was recombined with pEN-attR2-*cTP*^{LOX3}-*CIT*-attL3 and pEN-attL1-*LOX3*-attL2 into destination vector pFR7m34gw. To generate promoter swap constructs, pEN-attL4-*13-LOXp-cTP*-attR1 were recombined with pEN-attL1-*CIT*-attL2 and pEN-attR2-*LOX*-attL3 into pFR7m34gw. For domain swap constructs, 13-LOX CDSs (*LOX2.1, LOX3, LOX4* and *LOX6*) lacking the PLAT domain were amplified with primers (MAY129/MAY130, MAY131/MAY132, MAY133/MAY134 and MAY127/MAY128 respectively) listed in Table 4 and cloned into *pDONR-P2R-P3*. Resulting *pEN-attR2-LOX*^{LOXx}*-attL3* clones were recombined with pEN-attL4-*13-LOXp-cTP*-attR1 and pEN-attL1-*CIT-PLAT*^{LOXx}-attL2 (obtained by gene synthesis, Thermo Fisher) to generate *pDEST-attB4-LOXp:cTP-CIT-PLAT*^{LOXx}*-LOX*^{LOXx}*-attB3.* Similarly, pEN-attL4-*LOX6-cTP*-attR1 was recombined with pEN-attL1-*CIT*-attL2 and *pEN-attR2-LOX*^{LOX6}*-attL3* to yield *LOX6p:cTP-CIT-LOX6*$^{\Delta PLAT}$. For LOX6 point mutation constructs, *pEN-attR2-LOX6*^{W201F}*-attL3*, *pEN-attR2-LOX6*^{W201K}*-attL3,* and *pEN-attR2-LOX6*^{W201A}*-attL3* were obtained by gene synthesis and recombined with pEN-attL4-*LOX6p-cTP*-attR1 and pEN-attL1-*CIT*-attL2 into destination vector pFR7m34gw. All constructs were verified by Sanger sequencing and analysed with the DNASTAR Lasergene softwares SeqBuilder and SeqMan Pro. Transgenic plants were generated by floral dip with *Agrobacterium tumefaciens* strain GV3101. Transformed seeds expressing RFP in T_1, T_2, and T_3 generations were selected by fluorescence microscopy, and segregation analysis was performed in >12 independent T_2 lines. A minimum of two independent T_3 transgenic lines were used for each construct to perform experiments and verify reproducibility.

Confocal microscopy

Confocal laser scanning microscopy was performed on Zeiss LSM 880 or LSM 900 instruments. For live imaging, 5-d-old vertically grown seedling roots were mounted in 0.5x MS with 30 µg/ml propidium iodide (PI). To visualize 13-LOX expression domain within root vascular tissues, *13-LOXp:NLS-3xVEN* lines were fixed in 4% paraformaldehyde, cleared with ClearSee (10% Xylitol [w/v]; 15% Sodium deoxycholate [w/v] and 25% Urea w/v] in water), and stained with Calcofluor White as described (Ursache et al., 2018). Excitation / detection ranges were set as follows: VEN and CIT: 514/ 515-545 nm; Calcofluor White: 405/ 425-475 nm; PI: 561/ 600-700 nm. All images shown within one experiment were taken with identical settings, and by analysing at least 10 individuals from at least two independent lines.

Immunogold

Vertically grown 5-day-old Arabidopsis seedlings were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h under vacuum, and then washed 3 times with PBS. Samples were dehydrated through and EtOH series (15%, 30%, 50%, 70%, 90%) at 4°C, each shaken for 30 min, with three final replacements in absolute EtOH for 1 h. Samples were infiltrated with LR White according to the manufacturer's instructions. Embedded samples were sectioned with a diamond knife on a Microm HM355 microtome. Immunogold labelling was performed according to the procedure previously described (Müller et al., 2010). Ultrathin sections were blocked and subsequently incubated in a moist chamber with primary antibodies diluted 1:100 (Ab6556 rabbit polyclonal antibody against GFP (Abcam, Cambridge, UK) overnight at 4°C. This step was followed by rinsing and incubation with secondary antibodies goat anti-rabbit IgGs conjugated with 10-nm gold particles (Sigma Aldrich). Secondary antibodies were diluted 1:100 (Aurion) with PBS/BSA and incubated for 2 h at room temperature. Sections were extensively washed and examined with an LEO 912AB transmission electron microscope (Zeiss). Controls were performed using preimmune serum.

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Appendix

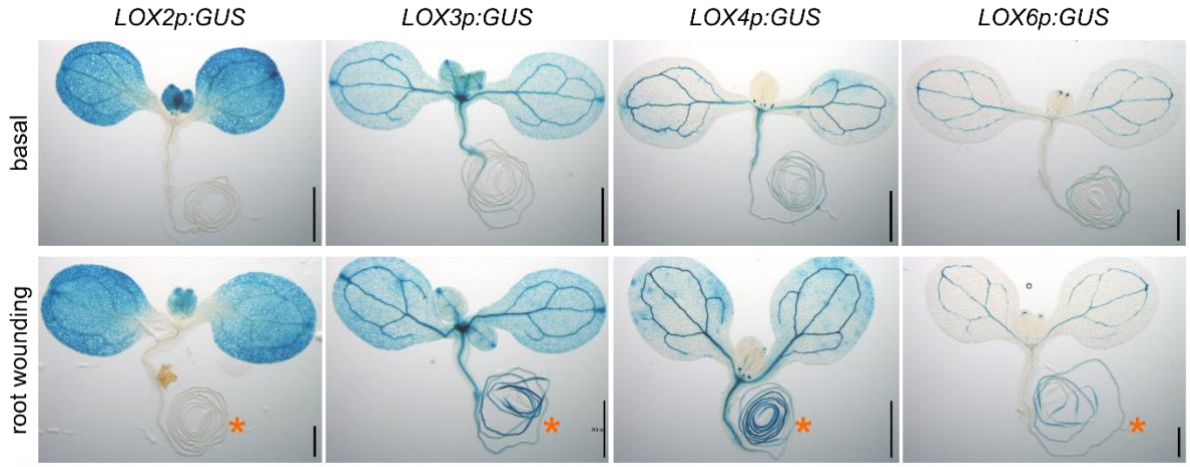

Figure S1. *13-LOX* promoter activity at basal and root wounding conditions. Histochemical detection of *13-LOXp:GUS* reporter activity in 7-day-old seedlings at basal conditions and 4 h after root wounding (single wound in the middle of the tap root indicated by asterisk) followed by overnight GUS staining. Scale bars, 0.5mm.

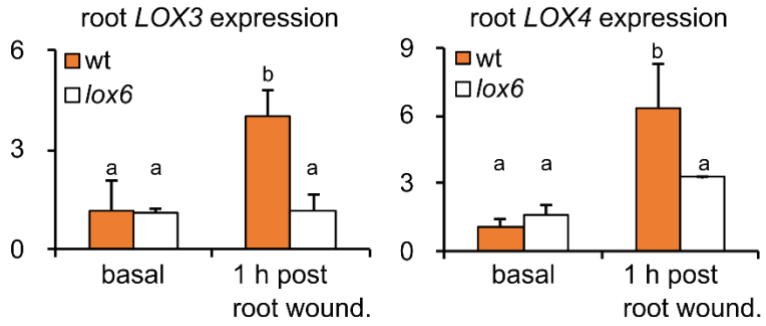

Figure S2. *LOX3* and *LOX4* induction after root wounding is LOX6 dependent. Quantitative qRT-PCR of *LOX3* and *LOX4* expression in wt and *lox6* roots at basal conditions, and 1 h post root wounding. Transcript levels were normalized to those of *UBC21* and calibrated to basal root levels. Bars represent the means of three biological replicates (±SD), each containing a pool of 5 organs from 2-week-old plants. Letters denote statistically significant differences among samples as determined by two-way ANOVA followed by Tukey's HSD test ($p<0.01$).

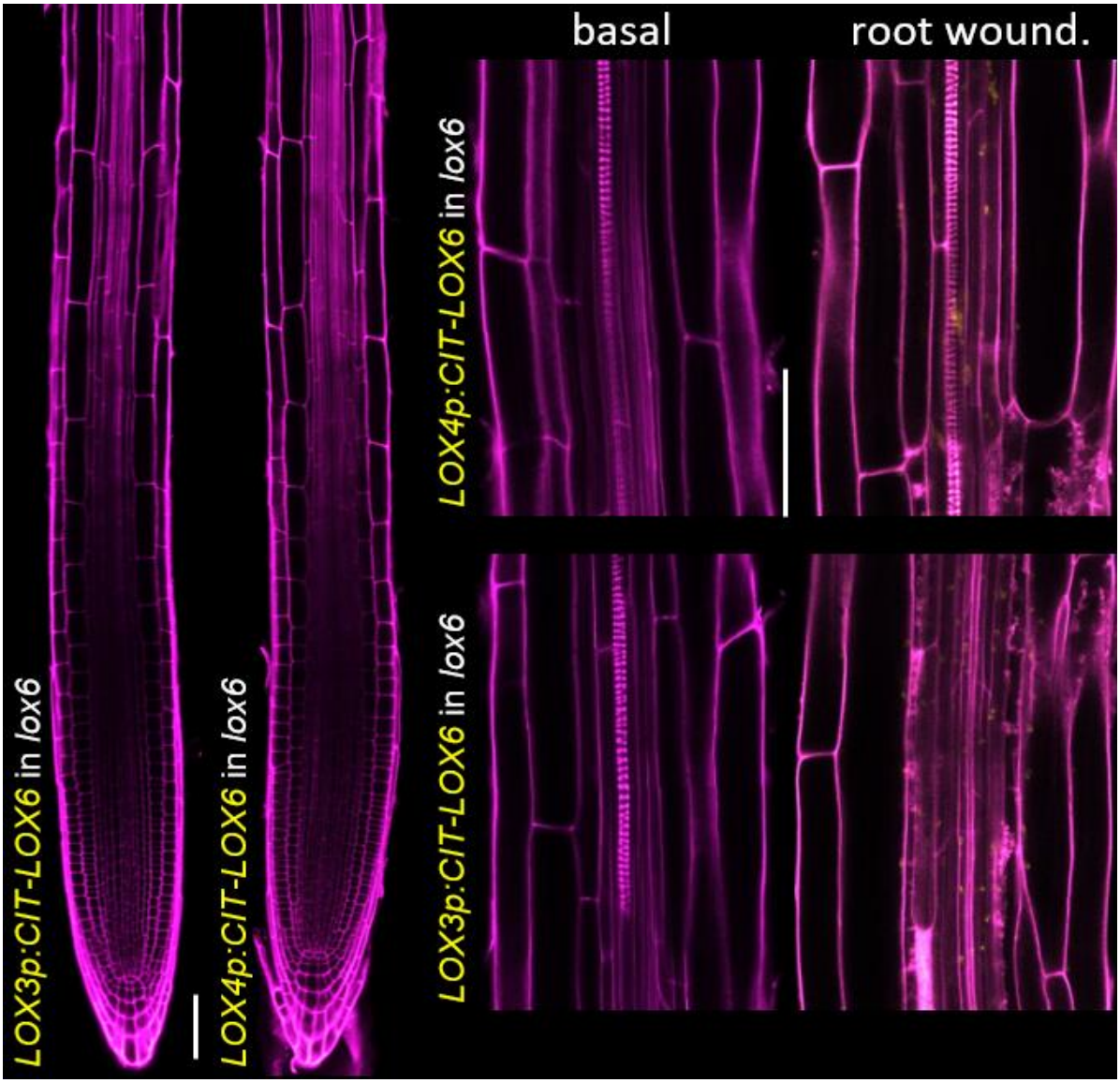

Figure S3. CIT-LOX6 controlled by *LOX3* and *LOX4* promoters can not be visualized in the root at basal condition, while can be induced by root wounding. Representative images of CIT-LOX6 expression controlled by LOX3 and LOX4 promoters in primary roots of 5-day-old seedlings stained with propidium iodide (magenta) on the left side. Close-up of their basal localization in early differentiation zone and 3 h after root wounding on the right side. Scale bars, 50 μm.

Fasta sequences for gene synthesis

CAP LETTERS indicate multisite GATEWAY attachment sites

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07/2018 – 08/2018	**Research assistant** Supervisor: Dr. Guiling Sun Henan University, Kaifeng, China
09/2015 – 06/2018	**Master student** College of Agriculture & Biotechnology **Zhejiang University**, China
09/2011 – 06/2015	**Bachelor student** College of Horticulture, **Northwest Agriculture & Forestry University** (NWAFU), China

Conference attendances

6/2023 poster presentation in ICAR 2023, Chiba, Japan

7/2022 poster presentation in Sixth international conference on Plant Vascular Biology 2022, Berlin, Germany

6/2022 oral presentation in Plant Science Student Conference, Halle, Germany

3/2022 online symposium organization in RTG (2498) plant cell compartments: stay connected

10/2019 poster presentation in 11th International PhD School Plant Development, Zellingen-Retzbach, Germany

Halle (Saale), June 2023

Yunjing Ma

List of publications

[1] **Yunjing Ma**, Mielke Stefan, Debora Gasperini. The initiation of jasmonate biosynthesis. 2022. New Phytologist, Tansley review in preparation

[2] Bai, Songling; Tao, Ruiyan; Tang, Yinxin; Yin, Lei; **Ma, Yunjing**; Ni, Junbei; Yan, Xinhui; Yang, Qinsong; Wu, Zhongying; Zeng, Yanling; Teng, Yuanwen. BBX16, a B-box protein, positively regulates light-induced anthocyanin accumulation by activating MYB10 in red pear. 2019. Plant Biotechnology Journal

[3] Yang Qinsong; Niu Qingfeng; Tang Yinxin; **Ma Yunjing**; Yan Xinhui; Li Jianzhao; Bai Songling; Teng Yuanwen. A member of pear GA-stimulated transcripts family, PpyGAST1, is potentially involved in bud dormancy release in 'Suli' Pear (*Pyrus pyrifolia* White Pear Group)". 2019. Environmental and Experimental Botany

[4] Jianzhao Li; Xinhui Yan; Qinsong Yang; **Yunjing Ma**; Bo Yang; Yuanwen Teng; Songling Bai. PpCBFs selectively regulate PpDAMs and contribute to pear bud endodormancy Process. 2019. Plant Molecular Biology

[5] Niu Qingfeng, Yang Qingsong, Li Jianzhao, Zheng Xiaoyan, **Ma Yunjing**, Bai Songling, Teng Yuanwen. Potential role of PpHB22 as a regulator of flower bud dormancy transition via its activation of PpDAM1 expression in 'Suli' pear (*Pyrus pyrifolia* white pear group)" . 2018. Plant Physiology and Biochemistry

[6] **Yunjing Ma**, Shashan Shu, Songling Bai, Ruiyan Tao, Minjie Qian, Yuanwen Teng. Genome-wide identification and analysis of the *TIFY* gene family and its potential role in anthocyanin synthesis in Chinese Sand Pear (*Pyrus pyrifolia*). 2018. Tree Genetics & Genomes

Eidesstattliche Erklärung (Statutory declaration)

Hiermit erkläre ich an Eides statt, dass ich mich mit der vorliegenden wissenschaftlichen Arbeit erstmals um die Erlangung des Doktorgrades bewerbe, die Arbeit selbstständig und ohne fremde Hilfe verfasst, nur die angegebenen Quellen und Hilfsmittel genutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Halle (Saale), den _____ _____

Yunjing Ma