Post-translational modification of Lipoxygenase (LOX2) in plant-insect interactions

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List of Abbreviations

ABA	abscisic acid
Ala	alanine
AOS	allene oxide synthase
AOC	allene oxide cyclase
APS	ammonium persulfate
Asp	aspartic acid
bHLHzip	basic-helix-loop-helix zipper
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CDPK	Ca^{2+}-dependent protein kinase
CJ	*cis*-jasmone
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic
FACs	fatty acid-amino acid conjugates
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GOX	glucose oxidase
HAMPs	herbivore-associated molecular patterns
HDA	histone deacetylase
HPOT	hydroperoxyocatrienoic
Ile	isoleucine
JA	jasmonate
JA-Ile	jasmonoyl-L-isoleucine

JAR	jasmonate-resistance
JAZ	JA-ZIM domain
JMT	jasmonic acid carboxymethyltransferase
LB	Luria-Bertani
LeA	linolenic acid
Leu	leucine
LOX	lipoxygenase
MAPK	mitrogen-activated protein kinase
MeJA	methyl jasmonate
Met	methionine
MS	Murashige and Skoog
NBT	nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
NINJA	Novel Interactor of JAZ
OPC-8	3-*oxo*-2-(2-pentenyl)-cyclopentane-1-octanoic acid
OPDA	12-*oxo*-phytodienoic acid
OPR3	OPDA reductase3
PCR	polymerase chain reaction
PDF1.2	*PLANT DEFENSIN1.2*
PIs	proteinase inhibitors
PLA1	phospholipase A1
PKs	protein kinases
PMSF	phenylmethylsulfonyl fluoride
PPs	protein phosphatases

PPP	phosphoprotein phosphotases
PTM	post-translational modification
PTP	protein tyrosine phosphatase
PVPP	polyvinylpolypyrrolidone
RuBiSCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
RT-PCR	reverse transcription polymerase chain reaction
SCF^{COI1}	Skp1/Cullin/F-box (SCF^{COI1})
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	serine
TEMED	tetramethylethylenediamine
TPL	TOPLESS
Ub	ubiquitin
VSP2	*VEGETATIVE STORAGE PROTEIN2*

Abstract

In response to biotic stress, plants respond with a series of morphological, chemical, and protein-based defenses to prevent further damage. Jasmonates (JAs) are a group of phytohormones that can regulate plant growth as well as defense against damage including chewing insect herbivory. 13-Lipoxygenases (13-LOXs) are involved in the first step of JA biosynthesis. LOX2, a member of this protein family, is mainly responsible for wound-induced plant defenses. According to previous research from Prof. Jacqueline Bede's laboratory, LOX2 can be phosphorylated; the enzyme is phosphorylated on a serine (Ser) on position 600 in unwounded plants and dephosphorylated when plants are wounded or attacked by caterpillars with impaired labial salivary secretions. To understand the role of this post-translational modification on LOX2 activity, we generated transgenic *Arabidopsis thaliana* plants that have a mutation of this Ser to produce either a phosphomimic (Ser to Asp or Ser to Met) or changing the Ser to an amino acid that cannot be phosphorylated (Ser to Ala). After obtaining T3 generation homozygous transgenic lines, *lox2* gene expression and protein analysis were performed on different lines. We identified at least two lines of each genotype that can express the *lox2* gene and produce lox2 protein. These lines will continue to be analyzed for phytohormone production and then can be used to further our understanding of plant-insect interactions.

Résumé

En réponse au stress biotique, les plantes réagissent avec une série de défenses aux niveaux morphologiques et chimiques ainsi qu'en utilisant des protéines permettant de prévenir d'autres dommages. Les Jasmonates (JAs) font un groupe de phytohormones capables de réguler la croissance des plantes servant à leur défense contre les dommages, notamment par les herbivores. La 13-lipoxygénase (13-LOX) est une famille d'enzymes impliquée dans la première étape de la biosynthèse des JAs. LOX2, un membre de cette famille de protéines, est particulièrement intéressant à étudier parce qu'il se révèle être principalement responsable de la défense des plantes contre les dommages. Selon des études antérieures au laboratoire de Prof. Jacqueline Bede, LOX2 peut être phosphorylée. Chez les plantes qui ne sont pas endommagées, le résidu sérine (Ser) en position 600 et phosphorylé de façon constitutive. Lorsque les plantes sont attaquées par des chenilles avec un dysfonctionnement des glandes salivaires labiales, ce résidu est déphosphorylé. Pour comprendre le rôle de cette modification post-traductionnelle sur l'activité de LOX2, nous avons généré des plantes transgéniques d'*Arabidopsis thaliana* portant une mutation à la position 600 afin de produire les mutants phosphomimétiques (Ser en Asp ou Ser en Met) ou lun mutant non phosphorylable (Ser en Ala). Après l'obtention de lignées transgéniques homozygotes de génération T3, des recherches sur l'expression des gènes et l'analyse protéique ont été effectuées. Les résultats ont montré que, pour chacun des génotypes, nous avions généré au moins deux lignées pouvant exprimer le gène *lox2* et produire la protéine lox2. Certaines lignées transgéniques n'exprimaient pas le gène *lox2* et ne produisaient de protéine lox2. Des recherches plus poussées seront nécessaires pour interpréter ces données. Des lignées transgéniques capables d'exprimer le gène *lox2* et de produire la protéine lox2 seront également utilisées pour des recherches ultérieures. En particulier, nous envisageons de faire des analyses des profils de phytohormones dans le contexte des interactions plantes-insectes.

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Contributions of Authors

The project presented hereafter was designed by myself and my supervisor Prof. Jacqueline C. Bede, Department of Plant Science, Macdonald Campus of McGill University, assisted by my co-supervisor Prof. Jean Rivoal, IRBV Université de Montréal. This project followed up research performed by Dr. Eryang Li and Mr. Julian Henao-Martinez. Dr. Li was responsible for cloning the transgene and Mr. Martinez performed *Agrobacterium* floral dip. I screened the transgenic lines and confirmed the homozygous T3 generation. Using plant material I collected, I accomplished gene expression analysis in Prof. J. C. Bede's lab and performed Western blots in the lab of Prof. J. Rivoal. Prof. J. C. Bede supervised the whole project and Prof. Bede and Prof. Rivoal helped me edit this thesis.

Contribution to Future Research

We have generated transgenic *Arabidopsis thaliana* lines with phosphor-deletion and phosphor-mimic at a serine at position 600 in the protein LOX2. I identified at least three homozygous lines of each genotype that express the appropriate mutant gene. These plants can be used as materials for further research. At least two lines of each genotype were shown to express the *lox2* gene and lox2 protein. These lines will be used for phytohormone analysis and plant-insect interaction experiments as well.

Potential protein degradation of lox2 in transgenic lines was found according to our protein analysis results. More research on this process is suggested, such as *in vitro* enzyme assays to assess protein degradation.

1. Introduction

1.1 General introduction

Plants respond to caterpillar herbivory with a series of morphological and chemical defenses to prevent further damage (Loon et al., 2006). Jasmonates (JAs) are important phytohormones that regulate plant defenses in response to mechanical damage and chewing herbivory (Halim et al., 2006). Upon damage, levels of jasmonic acid and its biologically active form, (+)-7-*iso*-jasmonyl-isoleucine (JA-Ile), increase which leads to induced defense gene expression (Howe and Jander, 2008). Lipoxygenase 2 (linoleate 13*S*-lipoxygenase, EC 1.13.11.12) (LOX2) is an important enzyme in the initial steps of JA biosynthesis (Bell et al., 1995). When plants are attacked or wounded by caterpillars, an increase in JA levels is expected; however, some caterpillars have effectors in their labial saliva that prevent this increase (Bede at al., 2006, Weech et al., 2008). The mechanisms involved in this response are not yet fully understood. However, previous research from Bede's laboratory has found that LOX2 is constitutively phosphorylated on a Serine at position 600 (Thivierge et al., 2010). LOX2 remains phosphorylated when plants are attacked by caterpillars but dephosphorylated in wounded plants or plants attacked by caterpillars with impaired labial salivary secretions. This suggests that the post-translational modification (PTM) of LOX2 on S^{600} may be important in the activation of this enzyme and that an effector in caterpillar saliva keeps LOX2 in the "constitutive" state to attenuate plant defense responses. To understand the role of this PTM on LOX2 activity, we used *Arabidopsis thaliana* mutant plants that produce a truncated form of the LOX2 protein (*lox2-1*, TAIR accession: polymorphism: 6531236264). The codon at position 630 which encodes a tryptophan in *LOX2* is changed to a stop codon, generating a shortened protein without activity (Glauser et al., 2009). The mutant was transformed with a plasmid containing *lox2* with different mutations of S^{600} to produce either an amino acid that cannot be phosphorylated (Ser to Ala) or a phosphomimic (Ser to Asp or Ser to Met). The original *LOX2* gene was also inserted into the vector and used to transform plants as a positive control. The pMDC32 alone is the empty vector control. Therefore, seven lines were used in these experiments: the wild type *A. thaliana* Col-0, the *lox2-1* mutant background line and the *lox2* mutant line transformed with either empty vector (Lox^{EV}), *AtLOX2* gene (Lox^{S600S}), *Atlox2*^{S600A} (Lox^{S600A}), *Atlox2*^{S600D} (Lox^{S600D}) or

Atlox2^{S600M}(Lox^{S600M}). The transgenic plants were self crossed and screened to identify at least three homozygous transgenic lines that were confirmed via checking the segregation ratio by exposing seedlings to the antibiotic hygromycin. Putative transgenic plants were further confirmed by amplifying the inserted gene through polymerase chain reaction (PCR) and sequencing the amplicons to ensure they contained the mutation sequence. Sequencing results confirmed that the mutations were present as predicted. All confirmed homozygous transgenic plants were grown for gene expression and protein analysis by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, respectively. Results suggested that for each construct at least two independent lines of transgenic plants can express the *LOX2* or *lox2* gene and can produce LOX2 or lox2 protein. These lines will be used as plant material for further research on the effect of LOX2 PTM in plant-insect interactions.

1.2. Hypotheses

Homozygous T3 transgenic lines transformed with vectors containing genes encoding mutant *lox2* forms will express their respective transformed versions of the *lox2* gene and lox2 protein.

1.3. Objectives

- To identify T3 transgenic *A. thaliana* plant lines: Lox^{S600A}, Lox^{S600D}, Lox^{S600M}, Lox^{S600S} and Lox^{EV} by antibiotic selection.
- To identify at least two independent homozygous transgenic lines of each genotype and confirm the construct (mutation) they carry to be correct.
- To perform RT-PCR to check *LOX2* or *lox2* gene expression level using leaf samples of each transgenic line.
- To perform Western blot analysis to check whether transgenic plants are able to produce the LOX2 or lox2 protein.

2. Literature Review

2.1. Plant-insect interactions

In nature, plants are faced with many stresses and attackers, such as microorganisms and insects. Insect damage is generally thought to be a serious threat for crop plants (Zhu-Salzman et al., 2008). To defend themselves against herbivorous insects, plants use morphological barriers, specialized metabolites and/or defensive proteins. Similar to mechanical wounding, damage caused by chewing insect herbivores results in the increase of stress hormones, such as jasmonates (JAs), that acts as signals to activate the expression of defense genes. Many of these genes encode enzymes that synthesize defensive metabolites, such as glucosinolates that are toxic for insects, or defensive proteins, such as proteinase inhibitors (PIs) that function to interfere with protein digestion needed for insect growth (Mewis et al., 2006; Ryan, 1990).

There are similarities between mechanical wounding and chewing insect herbivory, because insects, such as caterpillars, damage the plant when feeding. However, there are also differences. Indeed, caterpillars produce oral secretions that have specific effects on plants in addition to wounding. Plants can distinguish between simple wounding and insect herbivory by recognizing herbivore-associated molecular patterns (HAMPs) that reflect effectors from the insect, such as molecules and proteins in their oral secretions (Duran-Flores and Heil, 2016; Erb et al., 2012; Hogenhout and Bos et al., 2011; Howe and Jander, 2008). These effectors allow the plant to recognize and respond distinctly to different insect herbivores (Kessler and Baldwin, 2002; Hogenhout and Bos, 2011). For example, volicitin, identified in the regurgitation of *Spodoptera exigua* caterpillars, prompts plants to produce and release defense volatiles (Alborn et al., 1997). Fatty acid-amino acid conjugates (FACs), such as volicitin, are now known to be found in the regurgitation of many caterpillar species and can induce JA and ethylene production (Halitschke et al., 2000; Kessler and Baldwin, 2002). Caterpillars also produce saliva secretions from both labial and mandibular salivary glands (Peiffer and Felton, 2009; Zebelo and Maffei, 2012). *S. exigua* labial saliva may act to suppress plant induced defenses (Weech et al., 2008; Lan et al., 2014). The role of caterpillar labial saliva in plant-insect interactions has been shown by removing the labial salivary glands of the caterpillar or burning the spinneret from which the secretions are released

(Musser et al., 2002; Bede et al., 2006). Though there are many possible effectors in the labial saliva (Carolan et al., 2011; Felton et al., 2018), the best characterized one is glucose oxidase (GOX). GOX is believed to reduce JA-dependent gene expression by stimulating antagonistic phytohormone pathways, such as the salicylic acid-dependent systemic acquired resistance pathway, that acts to suppress the JA signaling pathway (Musser et al., 2012; Weech et al., 2008; Lan et al., 2014; Zhu-Salzman et al., 2008).

2.2. The role of jasmonates in plants

JAs are ubiquitous, endogenous phytohormones (Geyter et al., 2012). In addition to being involved in the regulation of plant development, JAs mediate plant responses to biotic and abiotic stress, particularly in response to chewing insect herbivores that damage the plant tissues (Wasternack and Hause, 2013; Howe, 2004). As plant growth regulators, JAs play an important part in the regulation of many developmental processes from seed germination to maturation of fruit to senescence (Corbineau et al, 1988; Ueda and Kato, 1980). JAs can positively regulate the formation or development of some parts of plants, such as induce tuber formation, flower development and trichome initiation (Yoshihara et al., 1989; McConn and Browse et al., 1996; Li et al., 2004; Falkenstein et al., 1991; Nakamura et al., 2006). JAs can also inhibit certain processes, such as the inhibition of seed germination and root growth (Corbineau et al., 1988; Dathe et al., 1981).

JAs are probably best known for their involvement in mediating plant responses to biotic stresses, such as caterpillar attack and pathogen infection, or abiotic stresses, such as drought, salt or cold (Wasternack and Hause, 2013; Avanci et al., 2010; Glazebrook et al., 2005). When plants are mechanically damaged or attacked by insect herbivores that wound the plant tissue, JA levels in the plant increase, leading to the expression of genes that encode defense-related proteins or biosynthetic enzymes that produce plant specialized metabolites (Howe and Jander, 2008). JAs act both locally at the site of attack and systemically throughout the plant (Kessler and Baldwin, 2002). An increase in JAs leads to enhanced levels of glucosinolates, defense-related specialized metabolites, in Brassicacea plants (Bodnaryk et al., 1994), as well as higher levels of vegetative storage protein and proteinase inhibitors (Bennett and Wallsgrove, 1994; Farmer and Ryan, 1990). Wound-induced JAs can also

induce plant morphological changes. For example, plant damage can lead to the inhibition of root growth (Schmidt et al., 2010). Trichomes, epidermal hairs that may act as a structural barrier or contain noxious specialized metabolites, can also be induced in response to wound-related JA production. This means that when a leaf is damaged, either mechanically or by chewing insects, there may be more trichomes on newly forming leaves, resulting in an increase in trichome density (Levin et al., 1973; Traw and Bergelson, 2003); this can act to reduce subsequent insect herbivory.

2.3. Jasmonate biosynthesis and signaling

The biosynthesis of JAs starts from galactolipids present in the chloroplast membranes (Wasternack and Hause, 2013). A phospholipase A1 cleaves galactolipids to release α-linolenic acid (α-LeA) (Ryan and Pearce, 1998), which is the fatty acid substrate of JAs (Fig. 2.1). Then, an enzyme from the lipoxygenase (LOX) family catalyzes the oxidization of α-LeA by adding an oxygen to the C-13 position, forming (13*S*)-hydroperoxyocatrienoic (13-HPOT). Allene oxide synthase (AOS) transforms 13-HPOT into a 13-hydroperoxy, an unstable allene oxide, followed by the formation of 12-*oxo*-phytodienoic acid (OPDA) catalyzed by allene oxide cyclase (AOC) (Vick and Zimmermann, 1987). OPDA is transferred from the chloroplast to the peroxisome.

The last step to get the bioactive form of jasmonic acid is catalyzed by jasmonic acid amino acid synthase 1 (JAR1). After jasmonic acid is transported into the cytoplasm, JAR1 adds the amino acid isoleucine (Ile) to make the bioactive form, (+)-7-*iso*-jasmonyl-isoleucine (JA-Ile). JAR is activated upon wounding and has a preference for Ile over other amino acids, such as leucine or valine (Suza et al., 2010). Other enzymes catalyze other JA conjugates. For example, jasmonic acid can be converted to a volatile form, methyl jasmonate (MeJA), by jasmonic acid carboxymethyltransferase (JMT) (Seo et al., 2001). Another biologically active volatile JA metabolite is *cis*-jasmone (CJ) that is currently thought to be formed through JA degradation and also involved in plant-insect interactions (Koch et al., 1997). JA can also be modified by other compounds to make sulfated- and glucosylated- jasmonates, but JA-Ile plays the key role in the JA signaling pathway especially in activating JA-dependent gene expression (Gidda et al., 2003; Staswick and Tiryaki, 2004;

Thines et al., 2007; Chung et al., 2008).

The “G-box”, located in the promoter region of JA-responsive genes typically containing the sequence CATGTG or AACGTG, is recognized and bound by a basic-helix-loop-helix zipper (bHLHzip) MYC transcription factor (Lorenzo et al., 2004). When JA-Ile levels are low, JA-ZIM domain (JAZs) proteins bind to the MYC2 transcription factor that is in association with the co-repressors Novel Interactor of JAZ (NINJA) and TOPLESS (TPL) (Pauwels et al., 2010) (Fig. 2.2). This protein complex prevents the activation of gene expression through histone deacetylase 6 (HDA6) and HDA19. In the presence of JA-Ile, JAZ proteins are brought together with the Skp1/Cullin/F-box (SCF^{COI1}) protein complex (Thines et al., 2007). The SCF complex acts as an E3 ubiquitin ligase and which leads to the detection and ubiquitination of JAZ proteins, targeting them for degradation by the 26*S*-proteosome. After released from the JAZ protein and co-repressor complex, the MYC2 transcription factor can then activate JA-responsive gene expression (Chini et al., 2007).

Expression of genes encoding enzymes that are involved in JA pathway, such as *LOX*, *AOC*, *AOS*, *OPR3* as well as *JAZ* and *MYC*, are JA-induced (Wasternack, 2013; Chung et al., 2008). However, overexpressing *AOC* or *AOS* does not lead to increased JA levels (Laudert et al., 2000). This suggests that there are other mechanisms involved in the regulation of JA levels.

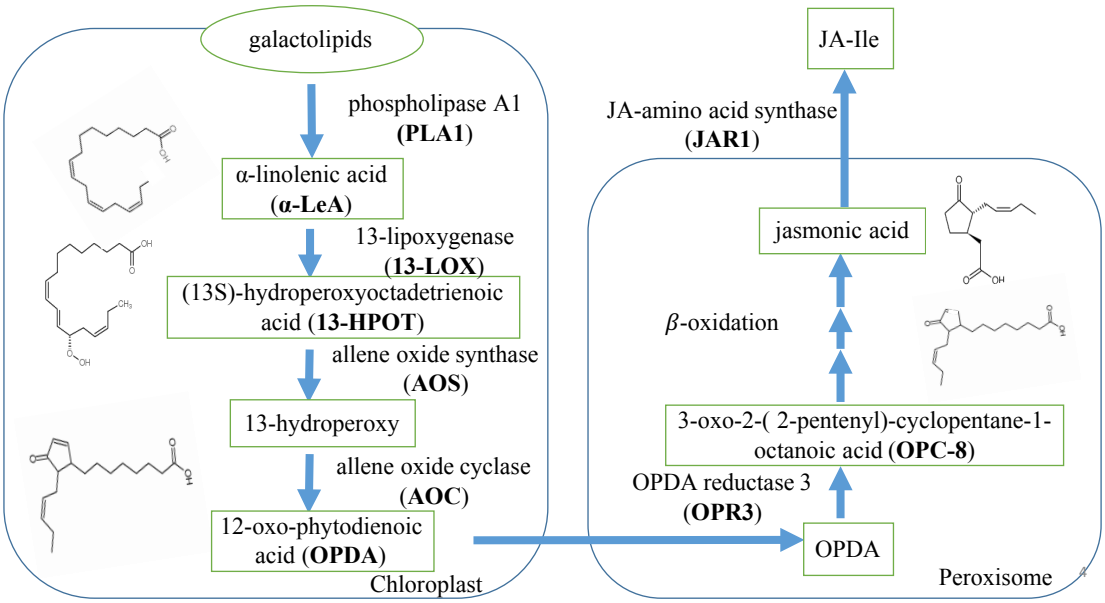


Figure 2.1. Biosynthesis of the jasmonic acid active form, 7-*iso*-jasmonyl-isoleucine (JA-Ile). The biosynthesis of JA starts from the galactolipids at the chloroplast membrane. Galactolipids are cleaved by PLA1 to release α-LeA, which is subsequently oxidized at C-13 position to form 13-HPOT. This step is catalyzed by 13-LOX. 13-HPOT is oxidized to 13-hydroperoxy by AOS and is further oxidized to OPDA by AOC. OPDA is then transfered to the peroxisome, where it undergoes reduction led by OPR3, followed by three steps of *β*-oxidation. Jasmonic acid is produced and is processed by JAR1 to form the bioactive form, JA-Ile. **Abbreviations of compounds**: α-LeA, α-linolenic acid; 13-HPOT, (13*S*)-hydroperoxyocatrienoic; OPDA, 12-*oxo*-phytodienoic acid; OPC-8, 3-*oxo*-2-(2-pentenyl)-cyclopentane-1-octanoic acid; JA-Ile, 7-*iso*-jasmonyl-isoleucine. **Abbreviations of enzymes**: PLA1, phospholipase A1; LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, OPDA reductase 3; JAR, JA amino acid synthase.

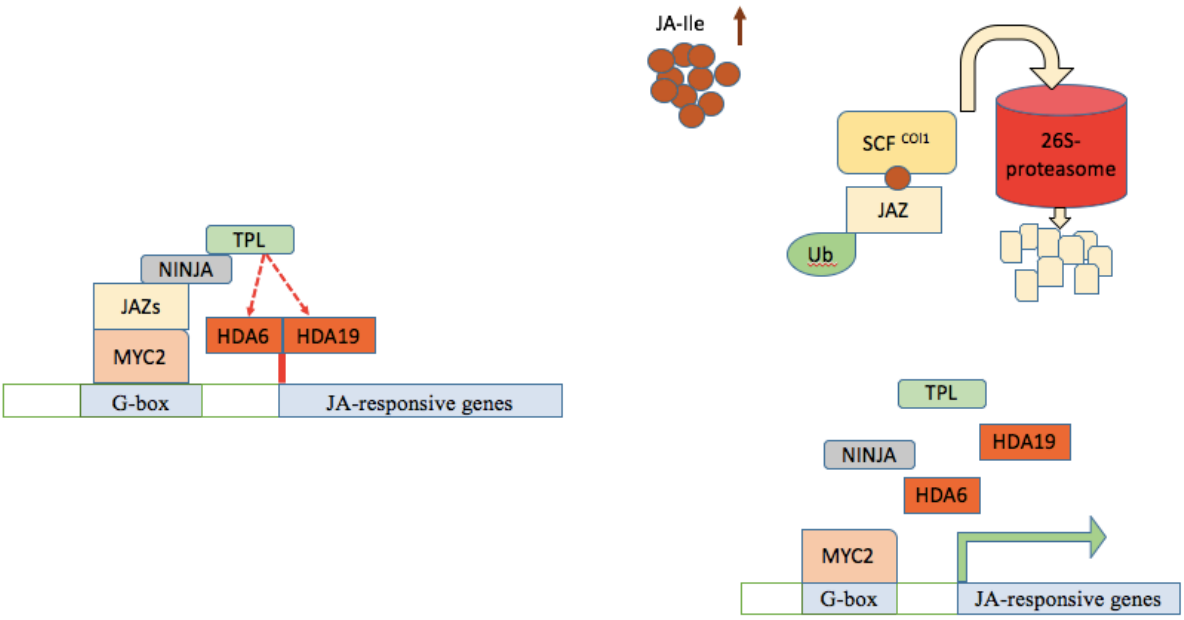


Figure 2.2. Role of jasmonyl-isoleucine in the activation of jasmonate-dependent gene expression. In unwounded plants, the MYC2 transcription factor at the promoter region of JA-responsive genes is bound by JAZ and its co-repressor, NINJA and TPL, which inhibit the expression of JA-responsive genes through HDA6 and HDA19. When plants are wounded, jasmonate biosynthesis is activated to produce more JA-Ile. Increased JA-Ile levels bring together the JAZ and SCF^{CO1} protein complex, JAZ is ubiquitinated and degraded by 26*S*-proteasome. MYC2 is released from repressors resulting in activation of gene expression. **Abbreviations**: JAZ, JA-ZIM domain protein; MYC2, basic-helix-loop-helix zipper (bHLHzip) transcription factor; NINJA, Novel Interactor of JAZ; TPL, TOPLESS; SCF^{COI1}, complex consisting of Skp1, Cullin-1 and F-box proteins; Ub, ubiquitin; HDA, histone deacetylase.

2.4. The *LOX* gene family in *Arabidopsis thaliana*

LOXs are ubiquitous, iron-containing enzymes that catalyze the hydroperoxidation of specific fatty acids in plants and animals (Vick and Zimmerman, 1987). In plants, they are categorized into two subfamilies, 9-LOXs and 13-LOXs, according to the position at which the enzyme adds an oxygen to the fatty acid substrate. LOXs can also be classified into two groups depending on sequence similarity (Feussner and Wasternack, 2002). The subgroup with high sequence similarity does not have transit peptide, which is named LOXs. The other subgroup has a putative chloroplast transit peptide at its N-terminal end. All of these enzymes belong to the 13-LOX family (Feussner and Wasternack, 2002). As is mentioned in Section 2.3, 13-LOXs are involved in the initial step of JA biosynthesis by converting α-LeA to 13-HPOT. In *A. thaliana*, there are six members of the LOX enzyme family (LOX1-6). LOX1 and LOX5 are 9-LOXs, whereas LOX2, LOX3, LOX4 and LOX6 are 13-LOXs (Bannenberg et al., 2009). These enzymes are expressed in different tissues and have different roles in plant physiology and defense (Table 2.1). The two 9-LOXs, LOX1 and LOX5, are not involved in JA biosynthesis but reported to regulate plant growth, such as lateral root development (Vellosillo et al., 2007).

AtLOX1 is mainly expressed in roots and young seedlings and can be induced by pathogen attack or exogenous application of the hormone abscisic acid (ABA) or MeJA (Melan et al., 1993, Vicente et al., 2012). LOX5 is involved in lateral root development (Vellosillo et al., 2007). Four 13-LOXs take part in wound-induced JA biosynthesis but their role is partly different, LOX2, LOX3, LOX4 and LOX6 have partially redundant roles in the wound response. LOX6 is involved in the immediate (within minutes) biosynthesis of JA in response to wounding (Chauvin et al., 2012), though overall LOX2 contributes more to the wound-dependent responses. *AtLOX2* is expressed on leaves whereas *AtLOX6* is highly expressed in xylem cells and leads to JA accumulation in root within 30 minutes after leaves are wounded. LOX6 is also involve in JA synthesis in response to drought stress and soil-dwelling pests (Grebner et al., 2013). LOX3 and LOX4 have much less of a function in plant defense. They are more involved in flower development and plant senescence (Calderlari et al., 2011). LOX3 also regulates lateral root development, like 9-LOXs (Vellosillo et al., 2007). Therefore, among all these proteins, LOX2 appears to be the isoform that is the most

responsible for sustained wound-induced responses (Chauvin et al., 2012).

AtLOX2 is localized in the chloroplast and the gene encoding this enzyme is expressed in leaves and flowers (Vick and Zimmerman, 1987; Bell et al., 1995). LOX2 is required for wound-induced JA biosynthesis since jasmonic acid accumulation was not found in damaged transgenic plants that has low *LOX2* expression (Bell et al., 1995). Early (within 1 hour) JA accumulation in foliar wounded *A. thaliana* plants was not observed in the *lox2-1* mutant, indicating the important role that LOX2 plays in the early JA increase in response to wounding (Glauser et al., 2009). The expression of *AtLOX2* is induced by jasmonic acid, MeJA, green leafy volatiles, wounding, caterpillar herbivory as well as some other stress factors and is down-regulated during leaf senescence. (Bell and Mullet, 1993; Kishimoto et al, 2005; Seltmann et al, 2010; Weech et al, 2008; He et al, 2002). LOX2 plays an important role in the plant responses to caterpillar herbivory. When caterpillars attack plants, they wound the plants and, just as in the response to mechanical damage, there is an increase in LOX2 activity and the production of JA phytohormones (Bricchi et al., 2010).

Table 2.1. ***Arabidopsis thaliana*** **lipoxygenase family: Tissue localization and physiological role.**

Lipoxygenase	Tissue	Role	Reference
LOX1	Root	Lateral root development	Vellosillo et al., 2007
LOX2	Leaf	Synthesis of oxylipins and green leaf volatiles in response to wounding	Creelman and Mullet, 1997; Mochizuki et al., 2016; Seltmann et al., 2010
LOX3	Leaf Flower	Senescence Male fertility (pollen)	He et al., 2002; Caldelari, 2011
LOX4	Leaf Flower	Senescence Male fertility (pollen)	He et al., 2002; Caldelari, 2011
LOX5	Root	Lateral root development	Vellosillo et al., 2007
LOX6	Xylem cells	Oxylipin synthesis in response to stress (wound, osmotic, drought), but in low levels compared to LOX2	Grebner et al., 2013

2.5. Caterpillar labial saliva-dependent changes in host plant proteins

Some caterpillar species have mechanisms to circumvent the rapid, high induction of JA in response to herbivory (Salzman et al., 2005). One such mechanism is related to the labial saliva of caterpillars, such as the *Spodoptera. exigua*. In response to attack by these caterpillars, there still is an increase in JAs but it is not as high as if the labial salivary secretions are impaired (Weech et al., 2008). The labial saliva from caterpillars of the beet armyworm, *S. exigua*, has high levels of GOX reflecting the diet that the caterpillars had been fed on (Afshar et al., 2010; Afshar et al., 2013). In a proteomic study, Thivierge et al. (2010) compared host plant protein PTMs in *A. thaliana* attacked by *S. exigua* caterpillars that had intact (normal) or impaired labial salivary secretions. This study showed that LOX2 was constitutively phosphorylated at a serine (Ser) at position 600 in unwounded *A. thaliana* plants and in plants attacked by caterpillars with intact labial salivary secretions. On the other hand, this protein was dephosphorylated in plants attacked by caterpillars with impaired labial salivary secretions. This led to the hypothesis that, in the constitutive state, LOX2 is phosphorylated at S^{600} and has low basal activity. In response to wounding, a phosphatase would be activated, thereby dephosphorylating LOX2. This would result in the activation of LOX2 and the biosynthesis of JA. When *S. exigua* caterpillars feed on the plant, the dephosphorylation of LOX2 does not occur, thus, it would remain phosphorylated with low activity. This is one way that caterpillars prevent the plant from mounting a strong JA-dependent induced defense response. At this stage, this scenario is still hypothetical, as the effects of phosphorylation of S^{600} on LOX2 activity is unknown.

2.6. Post-translational modification

Proteins can be modified after being made. PTMs, such as phosphorylation, methylation, ubiquitinoylation, acetylation, *S*-glutathionylation, *S*-nitrosylation and glycosylation, can lead to changes in protein activity, localization, regulation or protein-protein interaction (Seo and Lee, 2004). Phosphorylation and dephosphorylation are the most common and best characterized PTMs that can reversibly modulate enzyme activity, regulation, localization or protein-protein interactions. Phosphorylation is catalyzed by protein kinases (PKs) and can occur on protein serine, threonine or tyrosine amino acids as well as other amino acid

residues (histidine, aspartate) (Pawson, 2002; Swanson et al., 1994). Dephosphorylation is catalyzed by protein phosphatases (PPs). A large proportion of phosphatases and kinases belong to two groups, serine/threonine-specific or tyrosine-specific, depending on the amino acid residue that is modified (Luan, 1998). Representatives of serine/threonine-specific enzymes are Ca^{2+}-dependent protein kinases (CDPKs) and mitogen-activated protein kinase (MAPKs), important PK groups involved in JA-dependent signaling (Wasternack, 2013; van Verk et al., 2011; Yang et al., 2012). The most common group of PPs are serine/threonine PPs, consisting of PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7, which belong to the phosphoprotein phosphatases (PPP) family. PP2B is Ca^{2+}-calmodulin-dependent enzyme and PP2C belongs to the Mg^{2+}- or Mn^{2+}-dependent protein phosphatase family (PPM). A member of this family, PP7, is redox-dependent (Andreeva, 2001). Another group of PPs consists of the protein tyrosine phosphatases (PTPs) (Moorhead, 2007).

2.7. *Arabidopsis thaliana* as a model system

Arabidopsis thaliana is a popular model organism that is widely used in many areas of plant physiology and molecular genetics. It is a member of the Brassicaceae family, which encompasses many economically important horticultural crops, such as broccoli, cauliflower and kale (*Brassica oleracea*), canola (*Brassica napus*) and radish (*Raphanus raphanistrum*). *A. thaliana* is a self-pollinating plant species that can be easily genetically manipulated by *Agrobacterium*. Transgenic progeny can be obtained by a method called “floral dip” (Zhang et al., 2006; Clough and Bent, 1998). In this transformation technique, developing floral tissues are simply dipped into *Agrobacterium tumefaciens* solutions (*Agrobacterium* liquid culture resuspended in 5% sucrose and 0.05% surfactant). Transgenic seeds can be harvested three weeks later. These attributes make this plant species amenable to create transgenic plants as tools to better understand protein function. *A. thaliana* has become a good model to understand the regulation of JA-dependent signaling pathways (Truman et al., 2007). Many mutants in JA and other phytohormone pathways have been characterized to allow the study of their role in plant-insect interactions (Turner et al., 2002; Ishiguro et al., 2001; Stintzi and Browser, 2000). For instance, the *lox2-1* mutant of *A. thaliana* was characterized by Dr. E. E. Farmer’s lab in 2009 (Glauser et al., 2009). In this mutant line, the amino acid at 630 position

of the LOX2 sequence was changed from a tryptophan to a stop codon producing a truncated, inactive enzyme.

2.8. Importance of this work

The diverse and fascinating co-adaptation between host plants and their insect herbivores has resulted in complex interactions. Understanding how some caterpillar species manage to suppress the plant's natural defense responses is key to plant protection and can lead to future crop protection programs. The key pathway involved in the regulation of plant defenses against chewing insect herbivory is the JA pathway; yet, some insect species can circumvent these induced responses. In the caterpillar *S. exigua*, this may be due to effectors in its labial saliva. A caterpillar labial-saliva dependent response is the dephosphorylation of LOX2 that was seen when *A. thaliana* plants were attacked by caterpillars with impaired labial salivary secretions (Thivierge et al., 2010). LOXs are an enzyme family involved in the initial step of JA biosynthesis; however, the effect of this PTM on LOX2 is not clear. The focus of my thesis research is to generate transgenic plants that have phospho-deletion or phospho-mimic mutants to help understand the role of LOX2 phosphorylation in plant-insect interactions. This will enable us to have a more complete map of signal transduction in plant defense and a better understanding of plant-insect interactions.

3. Materials and Methods

3.1. *Arabidopsis thaliana* growth conditions

Seeds were surface-sterilized with 70% ethanol, centrifuged for 1 min at 9,000 rpm. The supernatant was discarded and the washing step was conducted with 10% (v/v) bleach (diluted from commercial bleach that contains 6.0% sodium hypochlorite). Seeds were thoroughly rinsed with multiple washes of sterile ddH_2O. Sterilized seeds soaked in ddH_2O were placed on Petri dishes containing Murashige and Skoog (MS) medium (Sigma product #M5524), 0.8% (w/v) agar. Seeds were stratified in the dark by wrapping plates with aluminum foil and chilling at 4℃. After 2 days, the plates were placed in a growth cabinet (Conviron, model #E15, serial #030075) with the following conditions: 14:10 h (light:dark) and 23:20°C. Light intensity was 250 μE m^{-2} s^{-1}. Fifteen-day-old seedlings were transplanted into pots (6.65 cm x 6.65 cm x 8.89 cm) containing Fafard agromix® PV20 (G6). Plants were watered from the bottom every 3 days and fertilized with every watering using All Purpose Fertilizer (Plant Products; total nitrogen 20%, available phosphoric acid 20%, soluble potash 20%); 1 g fertilizer/7 L water. For seed collection, plants were covered with plastic columns to avoid cross pollination when they started bolting. Mature seeds were collected in a cup that was put at the base of the plant and a sieve (0.250 mm pore size) was used to filter out plant tissue from seeds. When used, seed stocks were stored at room temperature to facilitate access. For long-term storage, seeds were kept in a tube and stored over dessicant at 4^{o}C.

3.2. Generation of *Arabidopsis thaliana* lines

Transgenic lines were made in a *lox2-1* mutant background that was previously characterized in the laboratory of Dr. E. E. Farmer (Glauser et al., 2009). To make the transgenic plants using this line as the background genotype, we first needed to make the *lox2* vectors with the different mutations that we need to transform the plants using the *Agrobacterium*-floral dip method (Clough and Bent, 1988).

Prior to my starting in the laboratory, the following was performed: the *LOX2* gene was modified using site-directed-mutagenesis (Norclone Biotech Laboratories, London, ON). Three different mutations of the S^{600} residue were made: one with a Ser to alanine (Ala) (S600A), one with Ser to aspartate (Asp) (S600D) and one with Ser to methionine (Met)

(S600M). Each of these different genes variants were made and inserted into the vector pBluescript. The genes were subcloned from pBluescript into the pCR™8/GW/TOPO® Gateway entry vector. This step was performed by Dr. Eryang Li. Using the attR1x attR2 combination, the entry vectors were used to insert the sequences into pMDC32 vectors, containing the Cauliflower Mosaic Virus (CaMV) 2x35*S* promoter (Fig. 3.1). This construction was designed to lead to the constitutive expression of the inserted gene. The recombinant plasmids were transformed into an *Escherichia coli* DH5-α strain by the heat shock protocol, in which the mixture of plasmid and bacteria is treated for 90 s in a 42°C water bath. Transformants were spread on plates with kanamycin (30 μg/mL) and incubated at 37°C overnight. Empty vectors were transformed into the *E. coli* ccdB-tolerant strain DB3.1 in the same way, because the destination vector we used in this study contained the ccdB gene, which encodes a toxic protein for bacterial cells (Bahassi et al., 1999). This ccdB gene is located in the region between attR1 and attR2. Transformed *E. coli* were incubated at 37°C with shaking for 1 h, then spread on Luria-Bertani (LB) agar (1% agar, 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, adjusted to pH 7) plates containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) and incubated overnight at 37°C. Single colonies were selected using a sterile toothpick and added to 2 x LB broth containing the antibiotics ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) and grown overnight at 37°C with shaking. Recombinant plasmids were extracted from *E. coli* and transferred into *Agrobacterium tumefaciens* GV3101 containing disarmed Ti plasmids by incubating the mixture of bacteria and plasmid for 5 min on ice followed by 5 min in liquid nitrogen, 5 min in a water bath at 37°C and then 30°C shaking for 2-4 hr. The transformants were plated on LB agar plates (1% agar, 10 g/L bacto-tryptone, 5 g/L yeast extract, 5 g/L NaCl; adjusted pH 7) containing the antibiotic spectinomycin (50 μg/mL) and incubated at 30°C for 48 hr.

Finally, the floral dip method was performed by Mr. Julian Henao-Martinez to transfer the vector into the mutant *lox2-1* background line. The transformed *Agrobacterium* cell suspension was grown in 5% (w/v) sucrose solution containing 0.05% (v/v) of Xiameter OFX-0309 (Mireault et al., 2014). The *Arabidopsis* flower bud was submersed in the *Agrobacterium* cell suspension, then placed horizontally and sealed in closed plastic bags overnight (Davis et al., 2009). Seeds were harvested three weeks after floral dip

transformation.

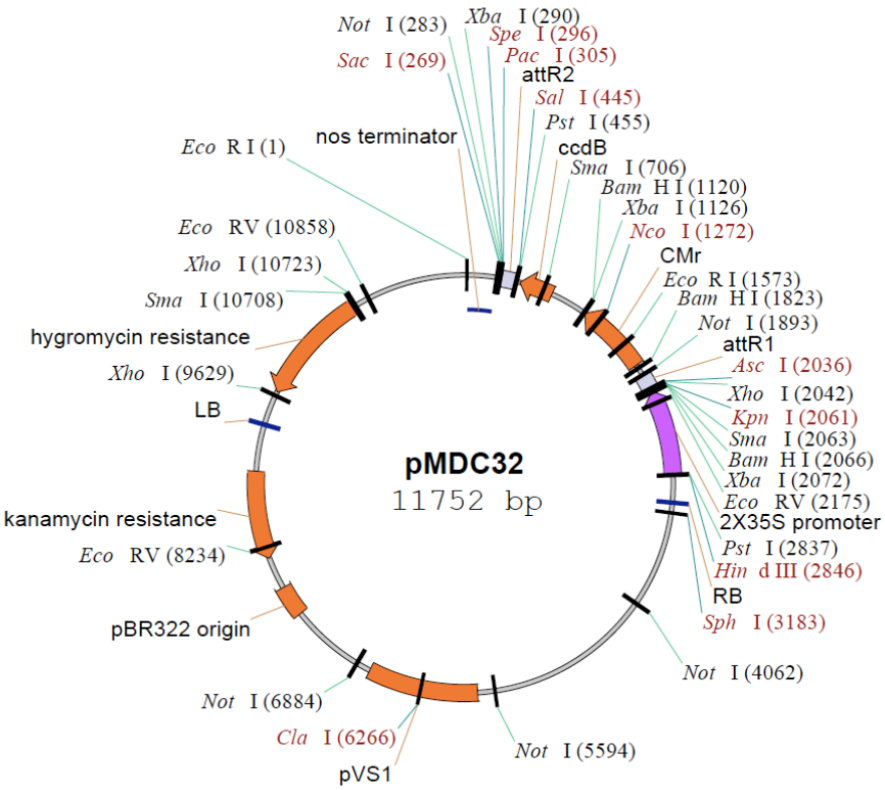


Figure 3.1. Destination vector pMDC32. The *lox2* gene was inserted into the attR1-attR2 region. This gene is constitutively expressed under the 2x35*S* promoter.

3.3. Selection of transgenic *Arabidopsis thaliana* lines

My project in the Bede laboratory began with selecting transformed transgenic plants. As described above, there were five different *lox2* transformed lines: Lox^{S600S}, Lox^{S600A}, Lox^{S600M}, Lox^{S600D} and the empty vector line (Lox^{EV}). Since the Gateway vector contains the hygromycin resistance gene, transgenic plants with a successful insertion are able to live on media contains the antibiotic hygromycin (Zalacain et al., 1986; Hellens et al., 2000). Therefore, selection plates were made by adding hygromycin B (Corning) into autoclaved MS media that was cooled to the touch, to get the final concentration of hygromycin at 20 mg/L. Approximately, 100 surface-sterilized seeds were spread evenly on each plate and germinated as explained above (Section 3.1). 15-day-old green seedlings that grew secondary leaves and had long roots were identified as being putatively transgenic and transplanted into soil in small black pots, otherwise seedlings were considered to be untransformed plants. These plants were grown and allowed to flower, self-fertilize and set seed. Seeds from T1 plants were collected for selecting transgenic plants of the next generation (T2). Seeds of T2 plants were again germinated, selected on selection media, allowed to flower, self-fertilize and set seed to generate the T3 population. This was, again, repeated to obtain the seed of T3 generation.

3.4. Segregation analysis

At the T3 stage, MS plates (MS media + 0.8% agar) containing 20 mg/L hygromycin selection plates were used for segregation analysis to investigate if the plants were homozygous. Approximately 100 sterilized seeds of putative transgenic plants were spread evenly onto each plate. Seeds from wild type Col-0 and *lox2-1* mutant plants were used as negative controls. Seeds were germinated in the same condition as outlined above (Section 3.1). At 15 days, the germination of the seeds was checked, and the number of seedlings that are green, can grow secondary leaves and have long roots were counted, as well as the number of dead seedlings. The live/dead seedling ratio was used to indicate segregation ratio.

3.5. DNA extraction and polymerase chain reaction (PCR) amplification

Two leaves of 40-day-old T2 plants were taken, snap frozen in liquid nitrogen and stored

in the -80°C freezer. Leaf samples were ground into a fine powder in liquid nitrogen using a mortar and pestle that had been autoclaved. Finely ground leaf powder was extracted with 400 µL extraction buffer (250 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), 200 mM Tris-HCl, pH 7.5 and 25 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) in a 2 mL Eppendorf tube and incubated for 2 min at room temperature. The SDS in the extraction buffer is used to disrupt cell membranes, denature proteins and release DNA. NaCl is used to stabilize DNA in the solution, as Cl^- ions bind to the phosphate groups in the backbone of DNA to allow precipitation. EDTA helps reduce DNA degradation as it binds Mg^{2+} which is needed for nuclease activity. Tris-HCl buffer controls the pH at a set value. After centrifugation at 13,000 rpm at room temperature for 3 min to pellet cellular debris, 300 µL of the supernatant was transferred to a new 2 mL Eppendorf tube. An equal amount (300 µL) of isopropanol was added to precipitate the DNA, because DNA is less soluble in isopropanol than in aqueous solution. After centrifuging at 13,000 rpm for 5 min, the supernatant was carefully discarded and the pellet was kept. The pellet was washed with 500 µL of 70% ethanol to remove salt and chemical residues. Samples were then recentrifuged to obtain a pellet. After two washes, the pellet was dried by removing the ethanol and inverting the tube on paper towel followed by air-drying for 20 min. DNA was resuspended in 60 µL $T^1/_{10}E$ buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored in the -20℃ freezer. DNA quality was checked by Infinite M200pro plate reader (Tecan, Männedorf, Switzerland) by measuring at wavelengths of 230 nm, 260 nm and 280 nm. DNA concentration was determined according to absorbance at 260 nm. The ratio of absorbance at 260 nm/280 nm and 260 nm/230 nm is used to assess DNA purity. Good quality DNA is considered to have an $A_{260/280}$ and $A_{260/230}$ ratios around 1.8. Low $A_{260/230}$ ratio is indicative of remaining chemical residues, such as EDTA, since it has an absorbance at 230 nm.

To verify that the plant lines were transformed with the correct plasmid, seven primer pairs targeted against the inserted plasmid were tested and the one that performed best was used in this study (Appendix 1 Table 1). Genomic DNA of each line was amplified using primers directed against the *nos* terminator site on the plasmid: NosabF: 5'- TGA ATC CTG TTG CCG GTC TT -3' and NosabR: 5'- GAC ACC GCG CGC GAT AAT TT -3' (designed using NCBI's Primer BLAST by Mr. Julian Henao-Martinez) (Table 3.1; Fig. 4.2A). Each

polymerase chain reaction (PCR) contained: 1 x buffer, 200 μM dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.025 unit/μL *Taq* polymerase, 1 μg DNA template per 50 μL reaction. Sterile ddH_2O water was added instead of DNA in the non-template control. The PCR program was as follow: initial denaturing stage 95℃ for 5 min. Denaturing stage 95℃ for 30 s, annealing stage 60℃ for 30 s, extending stage 72℃ 1 min for 40 cycles with a final extending stage 72℃ for 5 min and hold at 4℃. After PCR, 1 μL of 10 x loading dye was mixed with 5 μL PCR products for agarose gel electrophoresis. Ten x loading dye consisting of 39% (v/v) glycerol, 0.05% (w/v) SDS, 10 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, ddH_2O (Sambrook and Russell, 2006). Glycerol in the loading buffer is denser than water and used to make the DNA samples heavy so they remain in the well; SDS prevents the DNA from binding with proteins, EDTA protects the DNA from degradation as it binds Mg^{2+} which is needed for nuclease activity, bromophenol blue and xylene cyanol are dyes to track the progress of electrophoresis. The amplicon was separated on a 1.2% agarose gel (agarose, 1 X TBE buffer, 0.1 μL/mL SYBR safe gel stain (Thermofisher product #S33102)). A 100 bp DNA ladder (Invitrogen #1048805) was used to determine the amplicon size (199 bp). The gel was placed in 1 x TBE (0.1 M Tris-base, 0.1 M boric acid, 2 mM EDTA) buffer at 80-110 V for 20 min. The gel was visualized using a Gel Logic 200 Imaging System under ultraviolet (UV) light to obtain a picture of the migration pattern. DNA bands stained with SYBR safe gel stain could also routinely be detected by a standard UV transilluminator.

Table 3.1. Primers used for polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR).

Application	Target	Primer name and sequence (5'-3')	Annealing temperature/ (℃)	Amplicon size/ (bp)	Reference
PCR	*nos* terminator	NosabF: TGAATCCTGTTGCCGGTCTT NosabR: GACACCGCGCGCGATAATTT	60	199	Designed by using NCBI's Primer BLAST
PCR For sequencing	*AtLOX2* At3g45140	lox2end3F: TGAGGACTCATGCCTGTACG NosabR: GACACCGCGCGCGATAATTT	60	1310	Designed by using NCBI's Primer BLAST
RT-PCR Target gene	*AtLOX2* At3g45140	2-AtLOX2-F: GTCCTACTTGCCTTCCCAAAC 2-AtLOX2-R: ATTGTCAGGGTCACCAACATC	58	160	Weech et al., 2008
RT-PCR Reference gene	*AtACT2* At3g18780	B-Act2-F: TTGACTACGAGCAGGAGATG B-Act2-R: ACAAACGAGGGCTGGAACG	60	139	Bu et al., 2009

3.6. Generation of amplicons for sequencing

High-quality DNA was extracted from leaf samples using an EZ-10 column plant genomic DNA purification kit (Biobasic product #SK8262). Six hundred µL buffer PCB and 12 µL β-mercaptoethanol were added to 100 mg finely ground leaf samples. The solution was mixed thoroughly by vortexing and incubated at 65℃ for 25 min to lysis the cell. Six hundred µL chloroform were added into the tube and mixed by gently inverting 10 times, followed by 2 min centrifuge at 13,200 rpm at room temperature. Approximately 400 µL of the supernatant was then transferred to a new autoclaved 1.5 mL Eppendorf tube. To the supernatant, $^1/_2$ volume of buffer BD and $^1/_2$ volume of 100% (v/v) ethanol was added to precipitate the DNA. The solution was mixed by vortexing and transferred to an EZ-10 column placed over a 2 mL collection tube. The DNA in cell lysates selectively binds to the membrane in the column. After centrifuging at 12,000 rpm for 1 min, the liquid in the collection tube was discarded. The membrane was washed with 500 µL PW solution. The column was centrifuged at 12,000 rpm for 1 min and the flow-through was discarded. The washing step was repeated with the Washing Solution. Proteins and salts that did not bind to the column were removed by this step. Finally, the column was centrifuged at 12,000 rpm for 2 min to remove any remaining chemical residue and to dry the membrane. The column was transferred to a new autoclaved 1.5 mL Eppendorf tube. Sixty µL of $T^1/_{10}E$ buffer was added to the center of membrane and incubated at room temperature for 1 min, then centrifuged at 12,000 rpm at room temperature to elute the DNA. The elution step was repeated. DNA quality was checked using the Infinite M200pro plate reader as explained above (Section 3.5). A PCR reaction was performed, but this time using the primer set lox2end3F: 5'-TGA GGA CTC ATG CCT GTA CG-3' and NosabR: 5'-GAC ACC GCG CGC GAT AAT TT-3' which were designed to hybridize to the inserted *lox2* gene (Table 3.1; Fig. 4.2). Each PCR reaction contained: 1 x buffer, 200 µM dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.025 unit/µL *Taq* polymerase and 1 µg template DNA per 50 µL reaction. Sterile ddH_2O was used as the non-template control. The PCR program used is as follows: initial denaturing step 95℃ for 5 min. Denaturing step 95℃ for 30 s, annealing step 60℃ for 30 s, extending step 72℃ for 90 s for 40 cycles. Final extension step at 72℃ for 5 min and hold at 4℃. After PCR, the reaction was stopped and 5 µL of the PCR amplicon was transferred to another

sterile PCR tube, to which 1 µL 10 x loading buffer was added for agarose gel electrophoresis. The gel electrophoresis was repeated as outlined above (Section 3.5). The expected amplicon size was 1310 bp. Samples were stored at -20°C and sent to the ACGT Sequencing Corporation to be sequenced. Sequences were aligned to the wild type *AtLOX2* gene using NCBI's BLAST algorithm.

3.7. Wound treatment and sample collection

Expression of the transgene was determined by reverse transcription-polemerase chain reaction (RT-PCR) (to confirm gene expression) and Western blot analysis (to confirm protein levels). These analyses were done in both undamaged plants and in wounded plants. Plants for RT-PCR and Western blots were grown and collected at the same time in the same growth cabinet with condition outlined above (Section 3.1). Seeds of Col-0 wild type, *lox2-1* mutant background and confirmed homozygous T3 transgenic lines were germinated as described above (Section 3.1). Three plants of each genotype were sown. One plant was grown per pot and 3 plants per line were grown. Four weeks after transplanting, 3 leaves of each plant were pooled to generate the unwounded sample. They were frozen in liquid nitrogen and stored at -80°C. Leaves of the plants were then all wounded by a hole-puncher. Twenty-four hours later, 3 wounded leaves from same plant were taken, pooled, frozen in liquid nitrogen and stored at -80°C. As the transgene is controlled by the 2x35*S* promoter, it should be constitutively expressed; however, both situations were tested to fully understand the expression and protein patterns.

3.8. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Leaves were ground into fine powder in liquid nitrogen with a sterilized mortar and pestle. RNA was extracted with an EZ-10 Spin Column Plant RNA Miniprep Kit (Biobasic product #BS82314) according to the manufacturer's protocol. Four hundred and fifty µL of buffer Rlysis-PG was added to 50 mg finely ground leaf samples and mixed by vortexing to lyse cells. Mixed samples were incubated at room temperature for 5 min, then centrifuged for 5 min at 13,200 rpm. The supernatant was transferred to a new 1.5 mL RNase-free centrifuge tube, followed by the addition of $^1/_2$ volume of 100% ethanol and mixing by inverting. The

mixed solution was transferred to another column. This tube was centrifuged at 12,000 rpm for 30 second at room temperature and discarding the flow-through. The column was washed by adding 500 μL of Universal GT solution. The column was centrifuged as described above and then washed by adding 500 μL of Universal NT buffer. Finally, the empty column was centrifuged again to remove any residual ethanol. The column was transferred to a new 1.5 mL RNase-free centrifuge tube. Fifty μL RNase-free water was added to the center of the membrane of the column to elute the RNA. After incubating at room temperature for 2 min, the column was centrifuged at 13,200 rpm for 30 s at room temperature. The elution step was repeated to increase the RNA concentration. RNA samples were stored in the -80℃ freezer. RNA quantity and quality were checked by the Infinite M200pro plate reader. RNA samples were measured at wavelengths of 230 nm, 260 nm and 280 nm. The RNA concentration is determined according to the absorbance at 260 nm. The absorbance ratios 260 nm/280 nm and 260 nm/230 nm were used to assess RNA purity. Good quality RNA is considered to have an $A_{260/280}$ and $A_{260/230}$ ratio around 2.0. $A_{260/280}$ can indicate if there is protein or nucleic acid contamination in the RNA samples. A low $A_{260/280}$ ratio is indicative of protein contamination. A high $A_{260/280}$ ratio denotes a contamination by DNA. A low $A_{260/230}$ ratio suggests that the sample has chemical contamination. RNA quality was also checked by agarose gel electrophoresis. A 1% (w/v) agarose gel was used to separate the RNA sample. Six μL RNA of each sample was loaded in the well of the gel. A 1 kb standard DNA ladder was used to indicate the molecular weight of bands. Electrophoresis conditions were the same as described above (Section 3.5).

One μg of RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen product #205311). To 1 μg RNA sample (variable volumes used according to different concentrations) 2 μL of 7 x gDNA Wipeout Buffer and RNase-free water was added to make a final volume of 14 μL. Samples were incubated for 2 min at 42 ℃ to eliminate gDNA contamination then immediately placed on ice for addition of 6 μL reverse-transcription master mix which contains Quantiscript reverse transcriptase, 5 x Quantiscript RT Buffer and RT primer mixed in a 1:4:1 ratio. The reverse transcription PCR conditions were as follows: 15 min at 42 ℃ to synthesis the cDNA, 3 min at 95℃ to stop the reverse

transcription reaction and then hold at 4 ℃. The rest of the RNA was stored in the -80℃ freezer, cDNA was stored in the -20 ℃ freezer. To validate gene expression, the cDNA was amplified by PCR using the primer pair 2-AtLOX2-F: 5'- GTC CTA CTT GCC TTC CCA AAC -3' and 2-AtLOX2-R: 5'-ATT GTC AGG GTC ACC AAC ATC -3', which targets *LOX2* (At3g45140) (Table 3.1; Fig. 4.2). Each polymerase chain reaction (PCR) contained: 1 x buffer, 200 µM dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.025 unit/ µL *Taq* polymerase and 1 µg template cDNA per 50 µL reaction. Sterile ddH_2O was used as the non-template control. The PCR program used is as follows: initial denaturing step 95℃ 5 min. Denaturing step 95℃ 30 s, annealing step 58℃ 30 s, extending step 72℃ 30s for 30 cycles. Final extending step 72℃ 5 min, 4℃ hold. *AtACT2* (At3g18780) was used as the reference gene and amplified using the primer pair B-Act2-F: 5'-TTG ACT ACG AGC AGG AGA TGG-3' and B-Act2-R: 5'-ACA AAC GAG GGC TGG AAC AAG-3' with the same conditions (Dekkers et al., 2012; Bu et al., 2009). A 100 bp DNA ladder (either from Invitrogen #10488058 or New England Biolab #N3231S) was used to determine the amplicon size. The amplicons were separated on a 1.2% (w/v) agarose gel stained by RedSafe Nucleic Acid Staining Solution (FroggaBio #21141). The gel was visualized under UV after electrophoresis at 90V for 90 min in 1 x TBE buffer.

3.9. Protein extraction and Bradford assay

Leaf samples (450 mg) were ground in a precooled mortar and pestle with 1.5 mL of protein extraction buffer (150 mM NaCl, 10% glycerol, 20 mM Tris-HCl pH 7.5, 10 mM EDTA acid, 0.1% Triton X-100, 0.5% Na-deoxycholate, 5% polyvinylpolypyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM dithiothreitol (DTT)). In the extraction buffer, NaCl is used to limit protein binding and help keep proteins soluble. Tris-HCl controls the pH at level. Triton-X lyses the cells and increases protein solubility. EDTA can lyse the cells and acts as an inhibitor of metalloproteases. PMSF is a serine protease inhibitor. Na-deoxycholate helps dissociate protein-protein interactions. DTT reduces disulfide bonds in the protein structure. Finely ground samples were centrifuged for 20 min at 13,500 rpm. The supernatant was carefully transferred to a new tube avoiding the cell debris at the bottom of the tube. The protein concentration of the supernatant was

measured using a Bradford assay (Bradford, 1976). Bradford reagent (Bio-Rad product #5000006) was diluted from the 5 x commercial stock to make a 1 x solution. Coomassie brilliant blue G-250 dye in Bradford reagent can bind to protein and this changes its absorbance under wavelength of 595 nm. A VersaMax Microplate Reader (Molecular Devices, San Diego, CA, USA) was used. A standard curve was made by diluting a stock solution of 0.1 µg/µL bovine serum albumin (BSA) to cover the range of 0 to 5 µg protein per well. Standard BSA solutions for each concentration were added to a 96-well plate in triplicate and absorbance read at 595 nm. The standard curve was made with the average absorbance for each concentration. The concentration of the sample was determined by comparing the absorbance of the samples to the BSA standard curve. Samples were diluted 10 x, after which 15 µL of the diluted sample was mixed with 35 µL ddH_2O and 200 µL Bradford reagent in each well. The absorbance at 595 nm was compared with that of the BSA standard curve. The concentration of samples has to fit on the standard curve to have reliable results. When they did not, the concentration of the protein sample was adjusted and measured again. All samples were measured triplicated and the average was taken. From this, the volume of sample that contained 12 µg of protein was calculated and used in the Western blot analysis.

3.10. Western blot analysis

Each supernatant was adjusted to contain 1 x sample buffer (5% (v/v) glycerol, 0.125 M Tris-HCl pH 6.8, 1.25% (w/v) SDS, 0.25% (w/v) bromophenol blue and 0.25 M DTT) using a 4 x stock. The glycerol in sample buffer is used to increase the density of the sample. Tris-HCl maintains the pH level. Bromophenol blue is the tracking dye used to monitor the progress of electrophoresis. SDS binds to proteins non-covalently and eliminates the secondary structure of the proteins, leading to protein denaturation and dissociation from each other. Heating is necessary to denature the protein. The presence of DTT ensures that disulfide bridges are completely reduced. Samples were incubated for 5 min at 95°C. Denatured samples were store in -20°C. Variable volumes of the different samples was used for SDS-PAGE, but the amount of soluble protein analyzed was always 12 µg per lane.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

(Laemmli, 1970). The day before the electrophoresis, 10% (w/v) polyacrylamide gels were prepared. After combining the gel mold, the running gel was poured into glasses, consisting of 25% (v/v) Tris-HCl 1.5 M pH 8.8, 10% acrylamide, 0.1% (w/v) SDS, 0.75‰ (w/v) ammonium persulfate (APS), 0.75‰ (v/v) tetramethylethylenediamine (TEMED) and ddH_2O. After the running gel was polymerized, the top gel (stacking gel) was made and poured into the gel mold. The stacking gel contains 0.1% (w/v) SDS, 25% (v/v) Tris-HCl 0.5 M pH 6.8, 0.02‰ (w/v) bromophenol blue, 4% (w/v) acrylamide, 0.75‰ (w/v) APS, 1.5‰ (v/v) TEMED and water. In the stacking and running gels, the acrylamide solution can form polymers to make a gel. The polymerization is catalyzed by APS, which can decompose and release free radicals to help polymerization. TEMED further helps catalyze the polymerization. SDS is negatively charged and binds on the entire length of the protein chain. Proteins bound to SDS migrate from the cathode to the anode. In the running gel, proteins separate through electrophoresis according to their molecular weight, with small proteins migrating faster and big proteins moving slower. The samples (12 μg protein/well) were loaded into the wells. The volume was variable according to different protein concentration of the samples. A protein molecular weight standard ladder ranging from 14.4 to 97.4 kDa (Bio-Rad product #1610304) was used to indicate protein molecular weight. Electrophoretic separation was done by running the gel at 100 V for 30 min as the sample goes through the stacking gel and then 120 V for 1 h as the sample gets separated by the running gel in 1 x TGS buffer (25 mM Tris base, 0.2 M glycine, 0.1% (w/v) SDS). In the stacking gel, the pH value is lower so glycine is less charged and moves slower. Meanwhile, chloride ions become active and form a high electrical resistance zone. The proteins in the sample migrate behind the chloride ions and get concentrated into a tight band at the end of stacking gel.

After protein separation, a transfer "sandwich" was made by assembling in the following order: negative pole - sponge - Whatman filter paper - gel- nitrocellulose membrane (Amersham™ Protran™, 0.45 μm pore size) - Whatman filter paper-sponge-positive pole. Nitrocellulose membranes generally provide a low background. The transfer was done at 4°C in 1 x TGM buffer (25 mM Tris base, 0.2 M glycine, 20% (v/v) methanol) buffer, using a constant current of 70 V for 1 h. When negatively charged proteins are electroeluted from the gel, the presence of methanol favors their precipitation onto the membrane. Therefore, after

proteins are transferred onto the membrane, they stay on it. After transfer, the membrane was stained by slowly agitating it for 5 min in Ponceau red (2.5% (w/v) Ponceau red, 5% (v/v) acetic acid) using a gyrotory water bath shaker (model G76, New Brunswick Scientific). Ponceau red is negatively-charged and binds to positively charged amino acids. It is widely used to detect protein on membranes, but has a relatively low sensitivity. It can also be easily removed by washing in H_2O or aqueous buffers (Al-Amoudi et al., 2015). After staining, the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBiSCO) band at 55 kDa was seen and the membrane was cut to remove this protein since it cross-reacts with many antibodies due to its great abundance in leaf samples (Kolesinski et al., 2011). The protein markers in the ladder were marked by pencil and the stain was removed by washing the membrane with 1 x TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM NaN_3 and 0.05% (v/v) Tween 20) for 5 min under agitation. Tween 20 is a detergent that helps wet the membrane and can reduce unspecific binding. NaN_3 inhibits microbial growth, thus preserving proteins from degradation.

The top part of the membrane was used to detect the LOX2 protein (102 kDa) and the bottom part was used to detect glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (37 kDa) which was used as a loading control. In further experiment performed to investigate possible pattern of protein degradation, the whole membrane was probed with anti-LOX2 antibody except that the RuBiSCO bands were cut out and stained as loading control. The membrane was “blocked” by slow rocking in 5% (w/v) skim milk in TBST for 1 h. This step is used to reduce the background and prevent nonspecifically binding of antibodies to the membrane by using inert milk proteins to block the unbound areas on the membrane. Therefore, the antibody can only bind to the specific protein that it recognizes. This was followed by 3 x 10 min washes in 1 x TBST. The top membrane was incubated with primary antibody raised against *Arabidopsis* LOX2 protein, LOX-C (Agrisera product #AS07258, 1:5000 dilution in 1% (w/v) BSA in TBST) for 1 h under agitation. This antibody is produced in a rabbit and is polyclonal. It targets to the AtLOX2 protein at a loop (aa 257-450) (UniProt: P38418, TAIR: At3g45140). The bottom part of the membrane was incubated with a previously described primary antibody against GAPDH (1:20000 dilution in 1% (w/v) BSA in TBST (Marsolais et al., 2010)) for 1 h under agitation. Both membranes were subsequently

incubated 1 h with secondary antibody anti-IgG rabbit conjugated to alkaline phosphatase (1:10000 dilution in 1% (w/v) BSA in TBST, Promega product #S3731). The membrane was briefly washed with alkaline phosphatase (AP) buffer and incubated in visualization reagent which contains 10 mL AP buffer, 100 μL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 100 μL nitro blue tetrazolium chloride (NBT). AP removes the phosphate from BCIP to form an intermediate 5-bromo-4-chloro-3-indole which undergoes dimerization and shows up as a blue stain. The hydrogen ion released during dimerization reduces NBT to form NBT-diformazan, an insoluble dark blue precipitate.

4. Results

4.1. Isolation and confirmation of *Arabidopsis lox2* transgenic lines

To understand the role of phosphorylation on LOX2 activity, a series of constructs were made by Dr. E. Li that had site-specific modification at a phosphorylated serine to generate non-phosphorylatable (Ser^{600} to Ala) or phospho-mimic (Ser^{600} to Asp or Ser^{600} to Met) forms. These constructs were introduced into an *A. thaliana* plant line (*lox2-1*) that is unable to produce a functional LOX2 enzyme. The *lox2-1* mutant line was identified and characterized by Dr. E. E. Farmer (Glauser et al., 2009). Therefore, we make sure any alter of phenotypes in our transgenic plants is due to different function of inserted *lox2*. As controls, plants were also transformed with the empty vector and the wild type *AtLOX2* gene into the background *lox2-1* mutant line (Table 4.1).

To identify putative transgenic plants, T2 seeds collected from T1 plants were subjected to antibiotic selection. Hygromycin was used for antibiotic resistance since the inserted vector pMDC32 contains a gene that encodes the enzyme hygromycin B kinase that confers antibiotic resistance by adding a phosphate to hygromycin, making it less active (Zalacain et al., 1986; Curtis and Grossniklaus, 2003). Nine to 15 T2 plants from independent lines were selected for each transgenic construct (Table 4.1). From each T2 line, 3-5 seedlings were selected and transplanted for the T3 generation. However, some of them died when growing or produced low quality seeds. Therefore, only plants that had both leaf samples and good quality seeds were used to identify homozygous lines.

Putative transgenic plants of T3 generation were grown and the DNA isolated to verify the presence of the pMDC32 plasmid. The genomic DNA was extracted and amplified using primer NosabF and NosabR which directed against *nos* terminator on the plasmid (Table 3.1; Fig. 4.2A). Col-0 wild type and *lox2-1* plants are negative controls because they are not transformed with the plasmid. Plasmid DNA was the positive control (Fig. 4.1A; Appendix 1 Fig. 1A). Eleven to 30 seedlings of each transgenic construct were confirmed by PCR (Table 4.1).

To further confirm the transformation as well as to verify the presence of the mutated codon, the primer pair lox2end3F and NosabR, that targeted the *nos* terminator and the *Atlox2* gene spanning the mutation in the *lox2* sequence, was used (Table 3.1; Fig. 4.1 B; Fig. 4.2 A;

Appendix 1 Fig. 1B). Amplicons were sent for sequencing. The sequences were then aligned to the wild type *AtLOX2* gene (Fig. 4.2B). In all cases, the mutation was identified as being correct as expected: for Lox^{S600S} lines the codon at position 600 is AGT which encodes Ser; for Lox^{S600A} lines the codon at position 600 is GCT which encodes Ala; for Lox^{S600D} lines the codon at position 600 is GAT which encodes Asp; for Lox^{S600M} lines the codon at position 600 is ATG which encodes Met (Fig. 4.2B).

Table 4.1. Homozygous *Arabidopsis* lines containing the different constructs of the *Atlox2* transgene and confirmed by the polymerase chain reaction (PCR).

Name of transgenic line	Construct and function	Number of hygromycin-resistant T2 seedlings	Number of transgenic T3 seedlings confirmed by PCR[1]	Number of homozygous samples	Name of samples for subsequent research[2]
Lox^{EV}	Empty vector	9	12	3	lc.1 lc.2 lc.3
Lox^{S600S}	Ser →Ser	15	11	10	ls.1 ls.2 ls.3 ls.4 ls.5 ls.6 ls.7 ls.8 ls.9 ls.10
Lox^{S600A}	Ser →Ala non-phosphorylatable	15	17	4	la.1 la.2 la.3 la.4
Lox^{S600D}	Ser →Asp phosphomimic	12	16	5	ld.1 ld.2 ld.3 ld.4 ld.5
Lox^{S600M}	Ser →Met phosphomimic	13	30	4	lm.1 lm.2 lm.3 lm.4

[1]DNA extracted from leaf samples was amplified by PCR using primer sets: NosabF and NosabR; lox2end3F and NosabR to confirm the presence of both *nos* terminator and *lox2* gene.

[2]Name of selected samples was simplified for subsequent research as well as for easy reading: lc stands for Lox^{EV}(empty vector control), ls stands for Lox^{S600S}(Ser-Ser), la stands for Lox^{S600A} (Ser-Ala), ld sands for Lox^{S600D}(Ser-Asp), lm stands for Lox^{S600M}(Ser-Met).

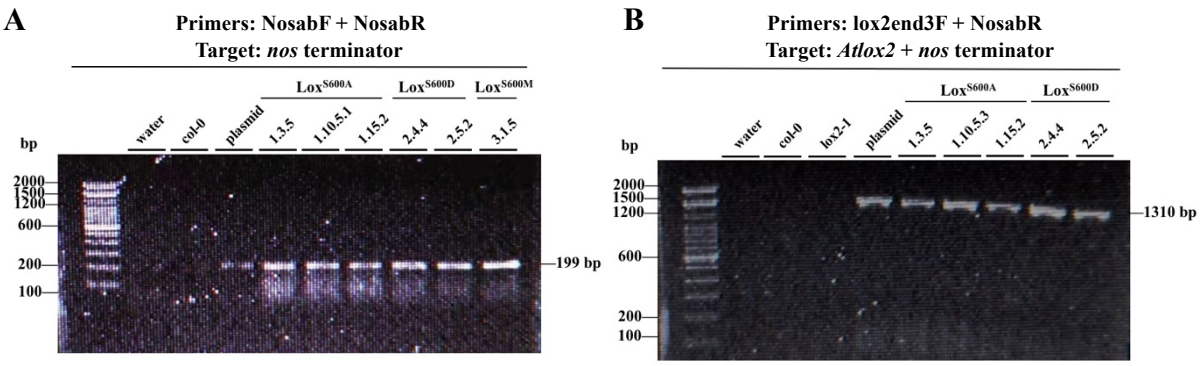


Figure 4.1. Representative gels confirming transgenic plants. Genomic DNA was extracted from Col-0 wild type, *lox2-1* mutant background and putative transgenic plants to identify transgenic T3 plants. Water indicates the non-template control. Col-0 wild type and *lox2-1* plants are negative controls for the transgene. The plasmid is the positive control. The molecular weight marker was a 100 to 2000 bp ladder (Invitrogen). Representative samples 1.3.5, 1.10.5.1, 1.15.2 were extracted from plants of independent transgenic Lox^{S600A} lines, samples 2.4.4, 2.5.2 were from independent transgenic Lox^{S600D} lines, sample 3.1.5 was from Lox^{S600M}. **A) Primers target the *nos* terminator site on the pMDC32 plasmid that was introduced to generate transgenic plants.** The expected amplicon size is 199 bp. **B) Primers target the the *LOX2* or *lox2* gene and *nos* terminator site on the recombinant vector.** The expected amplicon size is 1310 bp. Bands with correct molecular weight were seen in all representative transgenic samples. These samples were confirmed to be transgenic.

A

5'-ACCAGTGGGAGCCATCAAGATTCAGAACCAATACCATCGACAGTTGTTCCTCAAGGGGGTAGAGCTTAAATTACCCGGCGGGTCGAT

2-AtLOX2-F→

AACGTTTACATGTGAGTCATGGGTGGCCCCCAAGTCCGTTGACCCAACCAAGCGGATATTCTTCTCCGACAAGTCCTACTTGCCTTCCC
AAACACCAGAGCCTCTTAAAAAGTATCGAAAAGAGGAGCTCGAGACCTTACAAGGCAAGAACCGCGAGGAAGTTGGTGAATTTACCA
AGTTTGAGCGCATTTACGACTATGATGTGTACAACGATGTTGGTGACCCTGACAATGATCCTGAACTTGCTCGTCCGGTAATTGGAGGCC

←2-AtLOX2-R

TCACACATCCATATCCAAGGCGTTGCAAGACCGGTCGAAAACCTTGTGAGACTGACCCCTCTTCAGAGCAACGCTACGGGGGAGAGTT
CTATGTACCAAGAGACGAGGAGTTCTCTACAGCCAAGGGCACTTCATTCACAGGCAAGGCCGTACTGGCAGCTCTTCCCTCCATCTTCC
CTCAGATCGAGTCTGTTCTATTGAGTCCCCAAGAACCTTTTCCACACTTCAAGGCCATACAAAATCTCTTTGAAGAAGGCATTCAGCTTC
CCAAGGATGCTGGCCTCTTACCTCTGCTCCCCAGAATCATCAAAGCTCTTGGCGAAGCTCAAGATGATATTCTCCAGTTTGATGCCCCAG
TTCTCATTAACAGGGATAGATTTTCATGGTTACGAGACGACGAGTTTGCTCGCCAGACACTTGCCGGCCTTAATCCTTATAGCATTCAGCT
AGTTGAAGAGTGGCCGTTAATAAGCAAACTAGACCCGGCCGTTTATGGTGATCCCACCTCACTCATTACTTGGGAAATTGTGGAAAGAG
AAGTTAAAGGAAACATGACAGTTGATGAGGCTCTAAAGAATAAGAGATTGTTCGTGTTGGATTATCATGATTTGCTTCTACCATATGTGA
ACAAAGTGAGAGAGTTAAACAATACCACATTATATGCTTCTAGAACACTATTCTTCCTCAGCGATGATAGCACATTGAGGCCTGTTGCCA
TTGAGTTGACTTGTCCACCAAATATCAACAAGCCCCAATGGAAACAAGTCTTCACGCCAGGCTATGATGCTACGTCATGCTGGCTATGG

lox2end3F→

AATCTTGCTAAGACTCATGCTATCTCTCATGACGCCGGTTATCATCAGCTTATTTCCCATTGGTTGAGGACTCATGCCTGTACGGAGCCAT
ACATAATAGCGGCAAATAGACAACTAAGTGCCATGCATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCATGGAAATCAACGC
TCGTGCACGCCAAAGTCTTGTCAACGGAGGTGGAATCATTGAGACTTGTTTCTGGCCCGGGAAGTATGCATTAGAGCTAAGTTCAGCCG
TCTATGGTAAACTATGGAGGTTTGACCAGGAAGGCTTACCCGCGGATCTCATCAAAAGGGGGTTGGCTGAGGAAGATAAGACCGCAGA
ACATGGAGTACGTCTGACGATACCAGACTATCCATTTGCAAATGATGGTCTAATATTGTGGGATGCAATTAAAGAATGGGTGACAGACTA
TGTGAAACATTACTATCCTGATGAAGAACTGATCACATCGGACGAGGAACTCCAAGGATGGTGGAGTGAAGTGCGGAACATAGGCCAC
GGAGACAAGAAAGACGAACCTTGGTGGCCTGTCCTCAAAACACAAGATGACTTGATTGGTGTGGTAACTACGATTGCATGGGTCACCT
CAGGTCACCATGCAGCTGTAAACTTTGGACAGTACGGATATGGAGGATACTTTCCCAACCGACCAACGACAACAAGGATAAGAATGCC
AACGGAAGATCCGACAGATGAAGCCTTAAAAGAGTTCTATGAGTCGCCAGAAAAAGTGTTGCTTAAGACATACCCATCGCAGAAACAG
GCGACCCTAGTGATGGTCACGTTGGATCTTTTATCAACACATTCACCTGACGAAGAGTACATTGGAGAACAACAAGAAGCATCTTGGGC
CAATGAGCCTGTTATCAATGCTGCATTTGAAAGATTCAAAGGCAAGCTCCAATATCTAGAAGGAGTGATAGATGAGAGAAACGTGAACA
TTACTCTAAAGAATAGAGCTGGAGCTGGTGTTGTTAAGTATGAGCTTTTGAAGCCTACCTCTGAGCATGGTGTTACCGGAATGGGTGTTC

NosabF→

CTTATAGTATTTCTATTTGAGAGCTCGAATTTCCCCGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTC
TTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCACCTGAATAGATCTTGGACAAGCGTTA
GGCCTATCTGTGCACCTGAATAGATCTTGGACAAGCGTTAGGCCTATCTGTGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGT
CCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTA
GATCGGGAATTCG-3'

←NosabR

B

	600
Col-0 →... CATGCATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCATGGAAATCAACGCTCGTGCACGCCAA	**AGT** CTTG...
Lox^{S600S}→...CNTGCATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCATGGAAATCAACGCTCGTGCACGCCAA	**AGT** CTTG...
Lox^{S600A}→...NNTGNATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCNTGNNAATCAACGCTCGTGCACGCCAA	**GCT** CTTG...
Lox^{S600D}→...NNTGNATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCATGGAAATCAACGCTCGTGCACGCCAA	**GAT** CTTG...
Lox^{S600M}→...NNTGCATCCCATTTATAGGCTTTTGCATCCTCATTTCCGCTACACCATGGAAATCAACGCTCGTGCACGCCAA	**ATG** CTTG...

Figure 4.2. Partial sequence of the recombinant vector containing the *LOX2* gene and *nos* terminator. A) Part of the pMDC32 vector sequence containing the *LOX2* gene and *nos* terminator. Black boxes indicate primer bind sites and the arrows indicate the direction of the primers. Primer set NosabF and NosabR was used in PCR to validate the T3 transformants. Primer set 2-AtLOX2-F and 2-AtLOX2-R was used in RT-PCR to validate gene expression. **B) Validation of transformation with the mutant gene.** Primer set lox2end3F and NosabR was used to amplify the part of gene that had the mutation to validate the correct sequence. Grey box region (location circled in (A)) indicates partial amplicon sequence of each construct with the red box showing the different mutations at amino acid site 600. Nucleic acids marked in red indicate the mutations. The codon on amino acid site 600 in wild type Col-0 and Lox^{S600S} is AGT which encodes Ser; in Lox ^{S600A}: AGT changed to GCT which encodes Ala; Lox ^{S600D}: AGT changed to GAT which encodes Asp; Lox ^{S600M}: AGT changed to ATG which encodes Met.

4.2. Identification of homozygous lines

Homozygous transgenic lines are needed to test the role of LOX2 phosphorylation in plant-insect interactions. However, transgenic lines are expected to be heterozygous at their first generation. Their "selfed" progeny are expected to be either homozygous or heterozygous with a segregation rate of 1:2 as all homozygous plants without the transgene can not survive on the antibiotic treatment. Plants were crossed with themselves ("selfed") and their seeds were tested to identify homozygous lines. The seeds of homozygous plants should have 100% survival rate on both hygromycin antibiotic plates and MS-only plates (Table 4.2). Once the transgene in the transgenic plants was confirmed by PCR and the sequence confirmed the correctly inserted gene, seeds of T3 plants were used to calculate the segregation rate. Homozygous plants are expected to have a near 100% survival rate on the hygromycin antibiotic plates. Wild type Col-0 and the *lox2-1* mutant background were used as negative controls. Two confirmed lines ld.2 and lm.3 were used as positive controls. Screens were repeated twice. Eventually, 3 to 10 independent homozygous transgenic lines of each constructs were confirmed and were renamed according to the amino acid present at site 600: Lox^{EV} was renamed as lc.1-lc.3 (stands for empty vector); Lox^{S600S} was renamed as ls.1-ls.10 (indicates Ser at site 600 remains to be Ser), Lox^{S600A} was renamed as la.1-la.4 (indicates Ser at site 600 changes to Ala); Lox^{S600D} was renamed as ld.1-ld.5 (indicates Ser at site 600 changes to Asp); Lox^{S600M} was renamed as lm.1-lm. 4 (indicates Ser at site 600 changes to Met) (Table 4.1).

Table 4.2. Segregation rates of T3 homozygous *Atlox2* transgenic plants. Selection of plants was based on growth on MS plates containing 20 mg/L hygromycin and MS plates alone (germination control). "+" indicates seedlings survived, "-" means seedlings died.

Line	Construct	Replicate 1							Replicate 2						
		Name	Selection plate			MS plate			Name	Selection plate			MS plate		
			+	-	Survival rate	+	-	Survival rate		+	-	Survival rate	+	-	Survival rate
Lox^{EV}	Empty vector	lc.1	33	4	89.19%	31	2	93.94%	lc.1	44	5	89.80%	44	3	93.62%
		lc.2	33	3	91.67%	52	4	92.86%	lc.2	28	2	93.33%	38	2	95.00%
		lc.3	47	8	85.45%	72	10	87.80%	lc.3	47	11	81.03%	45	10	81.82%
Lox^{S600S}	Ser-Ser	ls.1	143	5	96.62%	120	0	100.00%	ls.1	121	4	96.80%	108	1	99.08%
		ls.2	90	0	100.00%	105	2	98.13%	ls.2	75	0	100.00%	74	0	100.00%
		ls.3	78	11	87.64%	86	6	93.48%	ls.3	132	4	97.06%	34	0	100.00%
		ls.4	58	0	100.00%	58	2	96.67%	ls.4	17	1	94.44%	30	3	90.91%
		ls.5	36	3	92.31%	35	2	94.59%	ls.5	34	2	94.44%	20	0	100.00%
		ls.6	25	1	96.15%	25	2	92.59%	ls.6	25	2	92.59%	25	1	96.15%
		ls.7	36	5	87.80%	45	7	86.54%	ls.7	24	2	92.31%	20	2	90.91%
		ls.8	40	2	95.24%	48	2	96.00%	ls.8	22	1	95.65%	30	2	93.75%
		ls.9	38	3	92.68%	34	2	94.44%	ls.9	19	1	95.00%	15	0	100.00%
		ls.10	39	6	86.67%	37	3	92.50%	ls.10	9	6	60.00%	37	3	92.50%
Lox^{S600A}	Ser-Ala	la.1	70	3	95.89%	34	2	94.44%	la.1	39	3	92.86%	40	4	90.91%
		la.2	82	2	97.62%	89	0	100.00%	la.2	103	2	98.10%	130	5	96.30%
		la.3	85	1	98.84%	109	3	97.32%	la.3	82	2	97.62%	77	2	97.47%
		la.4	44	2	95.65%	36	0	100.00%	la.4	50	3	94.34%	52	3	94.55%
Lox^{S600D}	Ser-Asp	ld.1	64	10	86.49%	48	6	88.89%	ld.1	105	5	95.45%	130	7	94.89%
		ld.2	51	6	89.47%	46	7	86.79%	ld.2	60	1	98.36%	55	1	98.21%
		ld.3	70	3	95.89%	67	3	95.71%	ld.3	64	5	92.75%	30	3	90.91%
		ld.4	65	5	92.86%	92	8	92.00%	ld.4	30	1	96.77%	36	1	97.30%
		ld.5	50	8	86.21%	55	6	90.16%	ld.5	24	2	92.31%	36	2	94.74%
Lox^{S600M}	Ser-Met	lm.1	109	0	100.00%	104	5	95.41%	lm.1	95	0	100.00%	28	3	90.32%
		lm.2	140	2	98.59%	70	2	97.22%	lm.2	114	3	97.44%	82	1	98.80%
		lm.3	122	3	97.60%	88	0	100.00%	lm.3	106	5	95.50%	72	1	98.63%
		lm.4	68	5	93.15%	90	6	93.75%	lm.4	46	0	100.00%	54	0	100.00%

4.3. Characterization of confirmed transgenic lines by reverse transcription-polymerase chain reaction (RT-PCR)

To verify that all samples were able to express the *LOX2* or *lox2* gene, RT-PCR was performed on wild type Col-0, the *lox2-1* mutant background and the 26 confirmed homozygous transgenic lines. Both unwounded and 24 hr mechanically-wounded leaf samples were taken from each plant. Total RNA was extracted and converted into cDNA. *LOX2* or *lox2* expression analysis was performed using the primers 2-AtLOX2-F and 2-AtLOX2-R which bind to the coding sequence and generate an amplicon size of 160 bp (Table 3.1; Fig. 4.2A). The no template control was performed to verify that the reagents were not contaminated. Wild type Col-0 and *lox2-1* mutant are positive controls as they express *AtLOX2* or *Atlox2*, respectively, and *AtACT2*. As expected, Col-0, *lox2-1* and all transgenic lines (Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M}) expressed the *AtLOX2* or *Atlox2* gene (Fig. 4.3A; Appendix 1 Fig. 2A). As a control for sample quality, the expression of the constitutive *AtACT2* gene was also analyzed using the primers B-Act2-F and B-Act2-R (Fig. 4.3B; Appendix 1 Fig. 2B). All samples expressed *AtACT2* as expected. This experiment was performed to verify that the *LOX2* or *lox2* gene is expressed.

Previous studies reported that *LOX2* gene expression is induced by wounding or treatment with MeJA (Bell and Mullet, 1993). Therefore, *LOX2* or *lox2* expression in Col-0 and *lox2-1*, respectively, is expected to increase in response to wounding. In the transgenic lines, the gene was expressed under a highly constitutive promoter. Therefore, in these plants even though there may be an increase in expression of the native gene, it is expected that the transgene expression will be much higher and mask it. From these RT-PCR results, it was difficult to ascertain if there was a wound-induced increase in gene expression in Col-0 or *lox2-1*; this may be because of the time point that the plant samples were taken that gene expression level were dropping back to basal levels. To understand gene expression dynamics, further experiments can be conducted using a time course and using quantitative real time polymerase chain reaction (qRT-PCR), which is more sensitive, to measure expression changes.

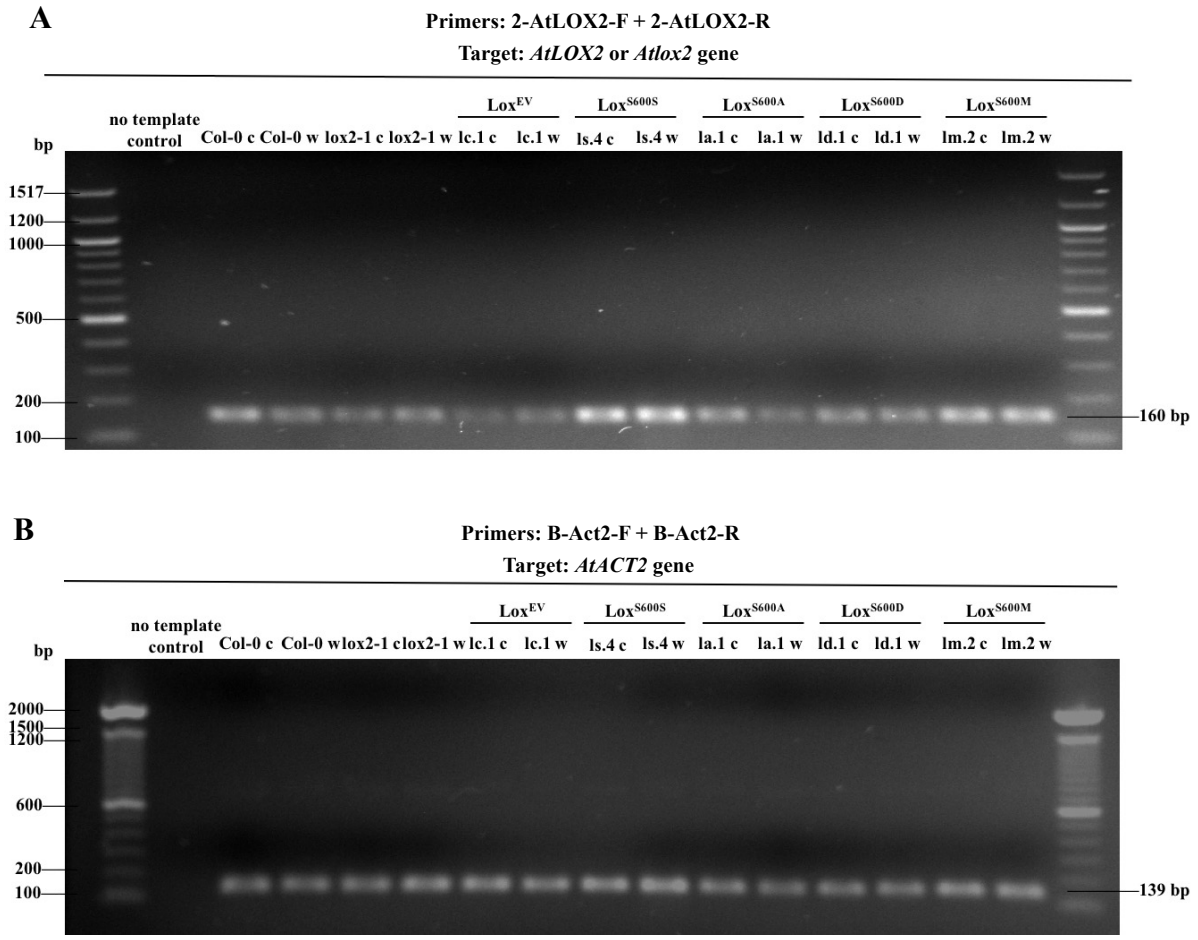


Figure 4.3. *Atlox2* expression in transgenic plants measured by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from unwounded/constitutive (c) or wounded (w) leaves of transgenic T3 plants and used to measure *Atlox2* (amplicon size 160 bp) and *AtACT2* (amplicon size 139 bp) expression. *AtACT2* is a constitutive gene used as a marker for sample quality. The 100 bp molecular weight marker (either from Invitrogen or New England Biolab) is indicated on the left side of the gel. **A) *AtLOX2* or *Atlox2* gene expression in samples: no template control, constitutive (c) and wounded (w) wild type Col-0 and *lox2-1* mutant (positive control) and one representative samples from each transgenic lines.** All samples show *AtLOX2* or *Atlox2* expression. **B) *AtACT2* gene expression in same samples as (A).** All samples show *AtACT2* expression.

4.4. Characterization of confirmed transgenic lines by Western blot analysis

To confirm the production of the LOX2 or lox2 protein, Western blot analysis using polyclonal antibodies against LOX2 was performed. Total protein was extracted from positive control wild type Col-0, negative control *lox2-1* mutant and transgenic plants of Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M} (Fig. 4.4; Appendix 1 Fig. 3 - 6). Both unwounded and 24 hr mechanically-wounded leaf samples were taken from each plant. Protein levels were detected using the antibody Anti-LOX-C (Agrisera #AS07258) that specific targets AtLOX2 protein at a loop (aa 257-450). Untransformed *lox2-1* mutant or Lox^{EV} transgenic lines are not expected to produce a protein that is recognized by the antibody because of its truncated C-terminal (Glauser et al., 2009; Cecchini et al., 2018). Therefore, the *lox2-1* mutant was used as negative control, although it rarely showed faint bind or a black spot (Fig. 4.4A; Appendix 1 Fig. 5B), which probably resulted from a cross contamination from the adjacent well that contained the protein sample from Col-0. A strong AtLOX2 band at 102 kDa is observed in the wounded and unwounded Col-0; this is the positive control on each blot. Just below this signal, a band is present in Col-0 unwounded and wounded samples. This band may represent a degradation product of LOX2, as it is absent from the *lox2-1* mutant and Lox^{EV}. Other faint bands that can be observed with lower molecular weight for all samples are interpreted as unspecific background binding. There is also a faint band above the LOX2 protein that possibly represents cross-reactivity with an unknown protein, this has also been observed in studies from other laboratories (Pilati et al., 2014).

lox2 proteins were detected in the following lines: Lox^{S600A} lines: la.1 and la.3, Lox^{S600D} lines: ld.1 and ld.5, Lox^{S600M} lines: lm.2 and lm.3, Lox^{S600S} lines: ls.4, ls.5, ls.6 and ls.7 (Fig. 4.4; Appendix 1 Fig. 3 - 6). Similar to the *lox2-1* mutant, we were unable to detect the protein in *Arabidopsis* lines transformed with the empty vector (Lox^{EV}), which is expected.

Even though this was not quantitative, it appears that some transgenic lines showed slightly different levels of lox2 protein between unwounded and wounded plants (Fig. 4.4B; Fig. 4.5A, B; Appendix 1 Fig. 3B). Differences in protein expression between different lines are to be expected because, even though the *lox2* gene is expressed under the 2x35*S* promoter, local insertion site effects can always occur (Rajeevkumar et al., 2015). Other

sources of variability in transgenic protein level may be linked to possible translational or post-translational regulatory mechanisms. Therefore, to identify putative lox2 degradation products, same amount (12 μg) of protein samples that were extracted for verifying lox2 protein production (as shown in Appendix 1 Fig. 5, Fig. 6) were separated on a new SDS-PAGE (Fig. 4.5). In this case, after proteins were transfer to a membrane, the membrane was again cut into 3 sections but this time the thin middle section that contained RuBiSCO was stained with Ponceau red as a loading control. The other 2 sections, represent the upper and lower part, making up most of the membrane were probed with the antibody against LOX2. In the Lox^{S600D} lines bands appeared at ~66 kDa, indicating that there may be protein degradation (Fig. 4.5 B; Appendix 1 Fig. 6B). This is particularly apparent in the analysis of extracts from lines ld.4 and ld.5 of Lox^{S600D} (Fig. 4.5B). Another band at ~31 kDa only appeared in all transgenic lines that were tested, which may also be protein degradation (Fig. 4.5). The intensity of the bands at ~ 66 kDa and ~31 kDa do not seem to vary between wounded and unwounded samples.

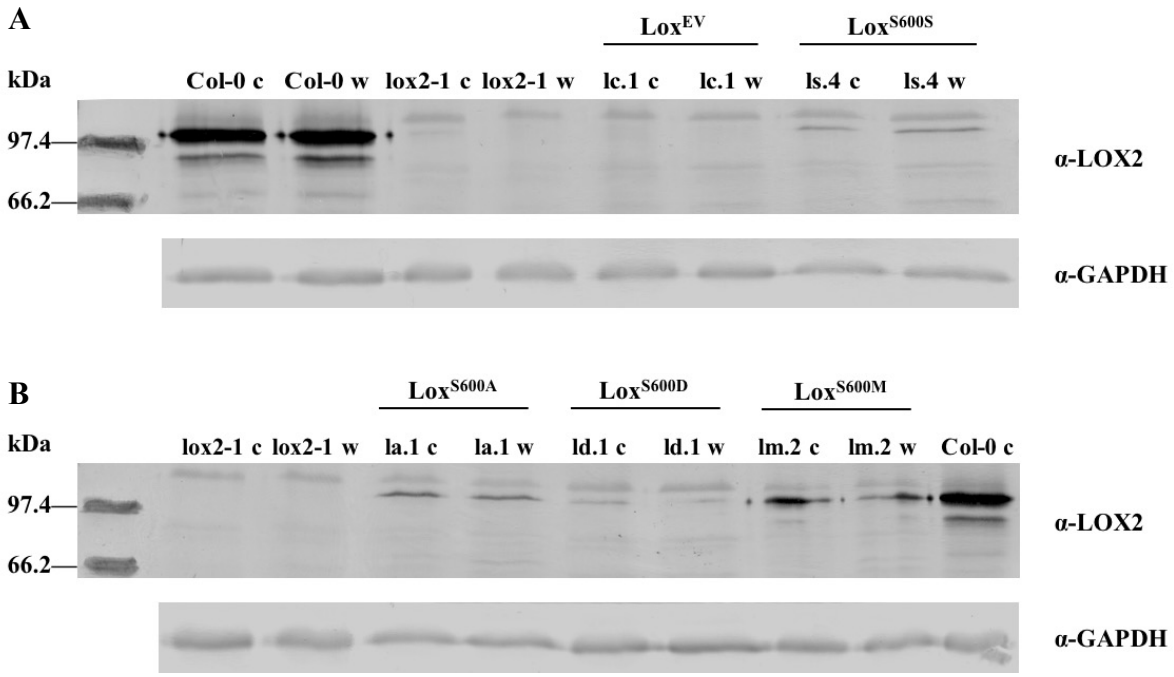


Figure 4.4. Western blot analysis of lipoxygenase 2 in leaf extracts of wild type Col-0, *lox2-1* mutant, and representative samples from each transgenic line expressing the various constructs. Twelve µg of soluble protein isolated from unwounded/constitutive (c) or wounded (w) foliar tissues of wild type Col-0, mutant background *lox2-1* and transgenic plants of Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M} were separated on a 10% (w/v) polyacrylamide denaturing gel. The protein molecular weight marker (Bio-Rad) bands at 97.4 and 66.2 kDa are indicated on the left side. After transfer to a membrane, the membrane was cut into 3 sections: the top section was probed with a polyclonal antibody against LOX2, the middle section with RuBiSCO was not used and the bottom section was probed with antibody against the constitutive protein GAPDH as a loading control (37 kDa). LOX2 and the transgenic lox2 proteins were seen as bands at 102 kDa. **A) LOX2 or lox2 expression in constitutive (c) and wounded (w) Col-0 (positive control), *lox2-1* (negative control), one transgenic plant from each of Lox^{EV} and Lox^{S600S} line. B) LOX2 or lox2 expression in constitutive (c) and wounded (w) *lox2-1* (negative control), one transgenic plants from each of Lox^{S600A}, Lox^{S600D}, Lox^{S600M} line and constitutive Col-0 (positive control).** All lines produced the reference protein GAPDH. As expected, Col-0 and the transgenic lines also produced LOX2 or lox2, respectively. The negative control *lox2-1* and Lox^{EV} did not produce a full length protein that could be detected by the antibody.

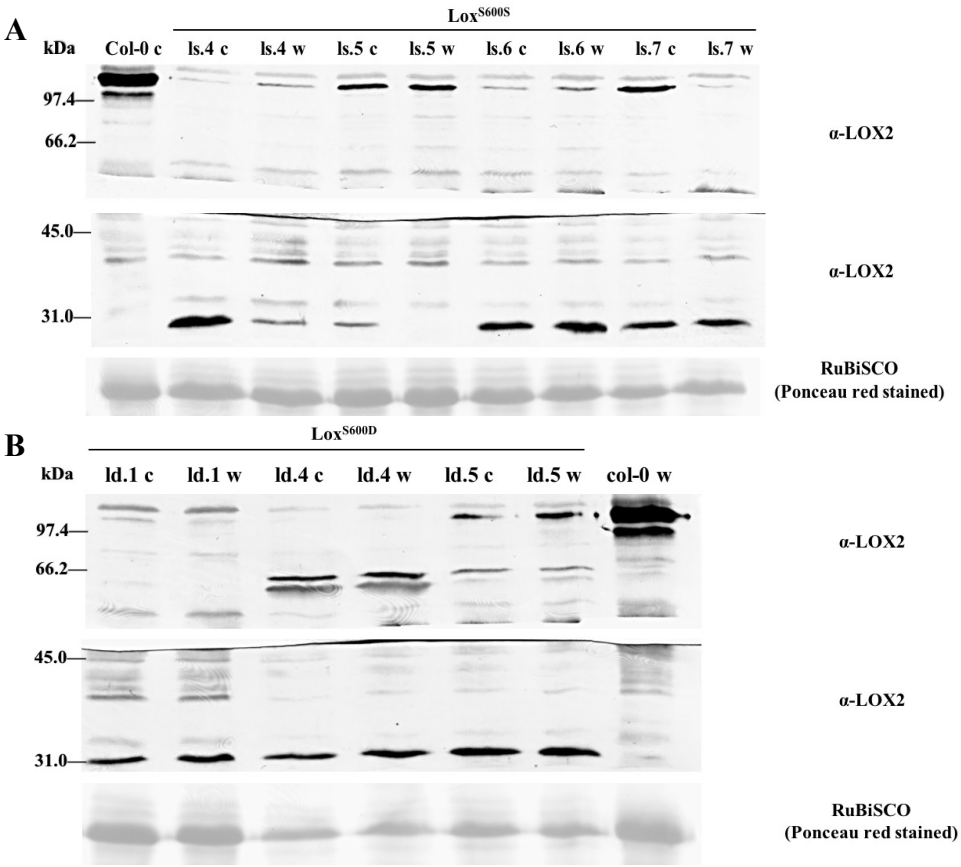


Figure 4.5. Western blot analysis of lipoxygenase 2 in leaf extracts of wild type Col-0 and representative transgenic samples showing a possible pattern of protein degradation. Twelve µg of protein samples that were extracted for verifying lox2 protein production (Appendix 1 Fig. 6) were separated on a new 10% (w/v) polyacrylamide denaturing gel. The protein molecular weight marker (Bio-Rad) bands range from 31.0 to 97.4 kDa are indicated on the left side. The membrane was cut into three sections after transference: the top and bottom parts were probed with a polyclonal antibody against LOX2, the middle section with RuBiSCO was stained with Ponceau red and scanned as a loading control. LOX2 and the transgenic lox2 proteins were seen as bands at 102 kDa. Strong bands at ~66 kDa and ~31 kDa suggest there is protein degradation. **A) LOX2 or lox2 expression in constitutive (c) Col-0 (positive control) and four independent transgenic lines of Lox^{S600S} with two treatments, constitutive (c) and wounding (w). B) LOX2 or lox2 expression in three independent transgenic lines of Lox^{S600D} with two treatments, constitutive (c) and wounding (w), and wounded Col-0 (positive control).** Col-0 and samples ls.4, ls.5, ls.6, ls.7 of Lox^{S600S} and ld.1, ld.5 of Lox^{S600D} produced Lox2 or lox2 as expected. All transgenic samples show protein degradation in variable degrees.

4.5. Summary of results

T3 transgenic plants of Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M} were produced. The mutations in transgenic plants were confirmed by amplification and sequencing. After verification of the segregation ratios of the transgenic plants, homozygous plants were selected and *lox2* gene expression and protein production were confirmed in these lines. For each transgenic construct, at least two independent homozygous lines have been identified. With this material, we are now in a good position to conduct further research to understand the role of this LOX2 phosphorylation on S^{600} in plant-insect interactions (Table 4.3). The identification of putative degradation products may also lead to studies to investigate the role of this PTM in protein dynamics and turnover.

Table 4.3. Summary of results for homozygous transgenic T3 line. Two to four independent homozygous transgenic line of different constructs (Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M}) will be used for future study were confirmed via determination of transgenic plants by PCR, verification of the mutation by sequencing and confirmation of gene expression by reverse transcription-PCR (RT-PCR) and presence of protein by Western blot. "✓" is detected, "X" is not detected, "NA" is not applicable because transgenic lines of Lox^{EV} were transformed with a vector that did not contain a *lipoxygenase 2* gene sequence.

Mutation	Name	PCR	Sequencing	RT-PCR	Western Blot
Lox^{EV}	lc.1	X	NA	✓	X
	lc.2	X	NA	✓	X
	lc.3	X	NA	✓	X
Lox^{S600S}	ls.4	✓	✓	✓	✓
	ls.5	✓	✓	✓	✓
	ls.6	✓	✓	✓	✓
	ls.7	✓	✓	✓	✓
Lox^{S600A}	la.1	✓	✓	✓	✓
	la.3	✓	✓	✓	✓
Lox^{S600D}	ld.1	✓	✓	✓	✓
	ld.5	✓	✓	✓	✓
Lox^{S600M}	lm.2	✓	✓	✓	✓
	lm.3	✓	✓	✓	✓

5. Discussion and Future Research Avenues

5.1. Screening transgenic lines; optimization of techniques

In this study, homozygous transgenic lines with the desired mutations were successfully generated. RT-PCR and Western blot were performed to confirm their ability to express the gene and the protein. There were numerous steps that required optimization. Different DNA extraction methods were used: chemical extraction and by a commercial kit. The chemical extraction method was used to screen transgenic plants because it is more efficient for large numbers of samples. However, the DNA for sequencing required a higher quality and a plant genomic DNA extraction kit (Biobasic EZ-10) was used for this purpose. Primers for different amplification products had to be tested and the conditions for PCR as well as RT-PCR had to be optimized (Appendix 1 Table 1).

5.2. Expression of the *lox2* gene in transgenic plants

LOX2 was expressed in the wild type Col-0, both constitutive and wounded (Fig. 4.3A, Appendix 1 Fig. 2A). Although we are not *a priori* expecting any difference between *lox2* gene expression of constitutive plants and wounded plant, we performed the wounding treatment. As expected, all plants, including Col-0 wild type, *lox2-1* mutant background line and transgenic lines are able to express *LOX2* or *lox2* gene. Based on Fig. 4.3A and Appendix 1 Fig. 2A, it appears that samples from transgenic plants showed some differences in band intensity for the *lox2* gene, whereas all samples showed similar band intensity for *ACT2* gene. This means the differences observed for *LOX2* expression are not due to differences in sample quality. The observed variation may reflect variable expression of genes depending on the region that they were inserted into (Prols and Meyer, 1992; Rajeevkumar et al., 2015). However, expression level was not assessed based on these experiments. One way to clarify this issue could be to analyze transcription of the transgenes using quantitative methods. In any case, these results showed that genes are successfully being expressed.

5.3. Protein analysis by Western blot

The ability of homozygous transgenic plants to produce the lipoxygenase 2 protein was confirmed by Western blot analysis (Fig. 4.4; Appendix 1 Fig.3). The wild type plant Col-0

had a band at 102 kDa, the predicted size of LOX2. The background *lox2-1* line has a mutation in the *lox2* gene that introduces a stop codon leading to a truncated protein with a predicted size of 65 kDa. This band was not detected in our lines; this is consistent with other published results and may indicate that this truncated protein is rapidly degraded or that the antibodies do not recognize the truncated protein (Cecchini et al., 2018). As expected, Lox^{EV} lines also did not produce the lox2 protein. It was expected that the transgenic lines of the different constructs were positive for both gene expression and protein expression. In the transgenic lines Lox^{S600A}, Lox^{S600D}, Lox^{S600M} and Lox^{S600S}, the *lox2* gene is behind a strong constitutive promoter, 2x35*S*. Therefore, we predicted seeing strong band at 102 kDa as well as a lower band at 65 kDa. Same as for the *lox2-1* mutant, the truncated band at 65 kDa was not observed in all the transgenic samples which is consistent with the literature (Cecchini et al., 2018).

However, some transgenic lines that expressed the *lox2* gene were found to not contain high amounts of the protein. This suggests the existence of regulation at post-translational level affecting protein stability. The level of the lox2 protein at 102 kDa was lower than expected in comparison to the LOX2 protein constitutively found in the Col-0 line. One possibility for these is that the protein encoded by the transgene could be degraded and that protein PTM may be related to regulation of protein levels.

To investigate further if the lox2 protein was degraded, the entire membrane was probed with the antibody raised against LOX2 except the section containing RuBiSCO bands (55 kDa), which were found by staining was cut out to avoid the cross-reaction of this protein with the antibody against LOX2. There was evidence of smaller proteins at ~66 kDa and ~31 kDa detected by the antibody against LOX2. The bands at ~66 kDa were particularly apparent in the transgenic Lox^{S600D} lines (Fig. 4.5B; Appendix 1 Fig. 6B). It also appeared that the samples containing strong signals for the smaller bands had a weaker lox2 band (Fig. 4.5). These smaller proteins were not strongly apparent in the wild type Col-0 or *lox2-1* mutant. Their presence appears to be linked to our attempt to overexpress LOX2 and/or to modify its sequence at the S^{600} position.

Post-translational phosphorylation of an enzyme could affect activity, localization, protein-protein interactions or stability (Trentini et al., 2016). The presence of the smaller

LOX2-immunoreactive peptides in the transgenic lines suggests that the protein PTM may affect protein stability leading to the degradation of LOX2. Changing Ser to Asp or Met means that these amino acids act as phosphomimics: Asp is chemically similar to phosphorylated serine because it is large and negatively charged (Sieracki and Komarova, 2013) and Met is large but lacks the negative charge. Our result shows that Lox^{S600D} lines contain more of the putative protein degradation product than transgenic lines carrying the construct for Lox^{S600M}. One possible reason is that Met is non-charged whereas Asp is negatively charged, which is thought to increase the possibility of protein degradation (Dice and Goldberg, 1975). However, the PTM of LOX2 may also be involved in other changes to the enzyme including affecting its activity, regulation or localization. In the presence of Ca^{2+}, LOX2 moves from the cytoplasm to the chloroplast (Cho et al., 2011). LOX2 PTM may affect this protein translocation.

5.4. Further studies: phytohormone analysis

Now that homozygous lines expressing both the *lox2* gene and protein have been identified, the next step would be to confirm an effect, if any, on levels of the JA phytohormones, such as OPDA, jasmonic acid and JA-Ile. Levels in undamaged and mechanically wounded plants should be compared 6 hours after wounding. As is reported in previous publications, this time point should be able to detect a JA burst in response to wounding (Bell, 1995). The predicted result would be that Col-0 and Lox^{S600S} transgenic line would have low constitutive levels of JA phytohormones but phytohormone levels would strongly increase after wounding. The *lox2-1* mutant background has low levels of phytohormones even after wounding. The transgenic lines Lox^{S600A} would show high JA levels in both unwounded and wounded plants. In comparison, the transgenic lines Lox^{S600D}, Lox^{S600M} and Lox^{EV} are expected to have constitutively low level JA phytohormones in unwounded and wounded plants.

5.5. Future studies: plant-insect experiments

After measuring the effect of the transgenic manipulation of LOX2 on phytohormones levels, an experiment verifying the role of the PTM in plant-insect interactions should be

conducted. The initial study from Bede's laboratory identified that LOX2 was phosphorylated constitutively and when caterpillars fed on *Arabidopsis* leaves but was dephosphorylated with plants were wounded or fed upon by caterpillars with impaired labial salivary secretions (Thivierge et al., 2010). Therefore, the different lines, Col-0, *lox2-1* and the five transgenic lines, Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D} and Lox^{S600M}, will be grown and subject to three different treatments: 12 hr herbivory by *S. exigua* with or without labial salivary secretions and the unwounded control. Samples will be collected to study JA-responsive gene expression by quantitative real-time polymerase chain reaction (qRT-PCR). Two examples of JA-dependent marker genes are the JA- or insect-/wound- responsive defense gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), and a JA-responsive pathogen defense gene *PLANT DEFENSIN1.2* (*PDF1.2*) (Dombrecht et al., 2007; Lorenzo et al., 2004; Lan et al., 2014).

The results expected from this experiment are shown in Table 5.1. Defense gene expression should be induced to the highest levels in the wild type Col-0 and Lox^{S600S} transgenic lines in response to herbivory by caterpillars with impaired labial salivary secretions. Defense gene expression is predicted to remain low in the *lox2-1* mutant and Lox^{EV} transgenic line. The transgenic line Lox^{S600A} should constitutively express defense genes at high levels. Transgenic lines Lox^{S600D} and Lox^{S600M} are expected to not express defense genes or they may be expressed at a low level. Caterpillar herbivory should not affect defensive gene expression in the transgenic plants. This experiment is designed to confirm our assumption that the phosphorylation of LOX2 at S^{600} reduces the activity of this enzyme. In addition, levels of the JA-induced defensive compounds glucosinolates are also worth studying. Through these experiments, we will get a more integrated understanding of plant-insect interactions.

Table 5.1. Expected results of the constitutive and wound-induced levels of jasmonate-related plant defenses in the wild type Col-0, *lox2-1* mutant background and *lox2* transgenic lines.

Treatment / Lines	Control (unwound)	Caterpillar herbivory with labial salivary secretion	Caterpillar herbivory without labial salivary secretion	wound
Col-0 wild type	--	++	++++	+++
lox2-1 mutant	--	--	--	--
Lox^{EV}	-	--	--	--
Lox^{S600S}	-	++++	++++	++++
Lox^{S600A}	++++	++++	++++	++++
Lox^{S600D}	-	-	-	-
Lox^{S600M}	-	-	-	-

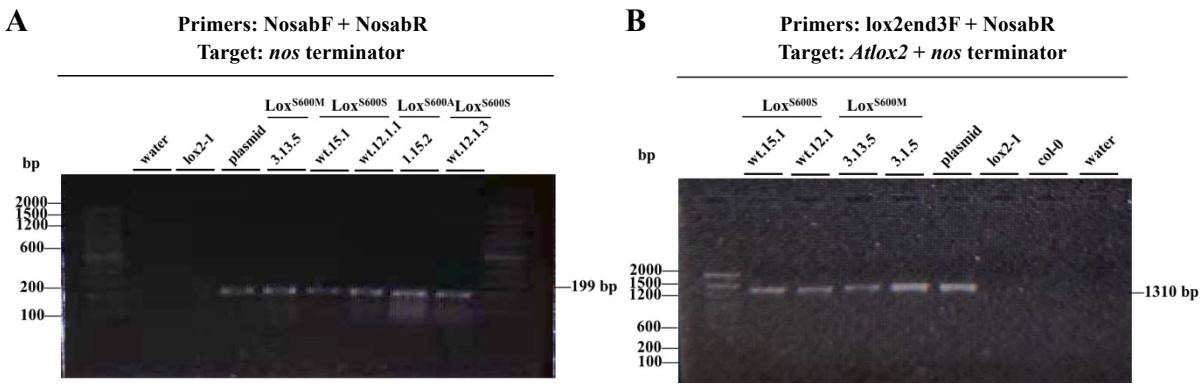
6. Conclusion

The main purpose of my study was to generate, screen and perform a preliminary characterization of *lox2* transgenic plants that express lox2 proteins with different mutations of the Ser at position 600. These lines could be used to further investigate the role of LOX2 PTM in the JA biosynthetic pathway. I obtained at least two transgenic lines for each construct. These lines were shown to contain the transgene with the correct mutation, they expressed the *lox2* gene and they produced the lox2 protein (Table 4.3). We found that all transgenic lines express *LOX2* or *lox2* gene. This is used to support the following protein analysis. However, the transformed genes expressed at a lower level than expected in spite of the exist of 2x35*S* promoter. This may be due to the fact that the insertion site where the gene has been integrated into the *Arabidopsis* genome is not well transcribed (Stam et al., 1997; Rajeevkumar et al., 2016). Other possibilities can also explain the interference with transgene expression, such as the copy number. By using Western blot analysis, we found that the transgenic plants only produce low amounts of protein, even though we could detect gene expression by RT-PCR. Levels that were, at least, comparable to the Col-0 line. Based on the results obtained after Western blot analysis of transgenic line extracts, it is reasonable to hypothesize that low protein expression levels could be due to protein degradation. More studies should, therefore, be performed to further understand if this is the case. In addition, the most important step now is to validate the effect of this transgene on JA phytohormone levels and then these plants can be used in plant-insect experiments.

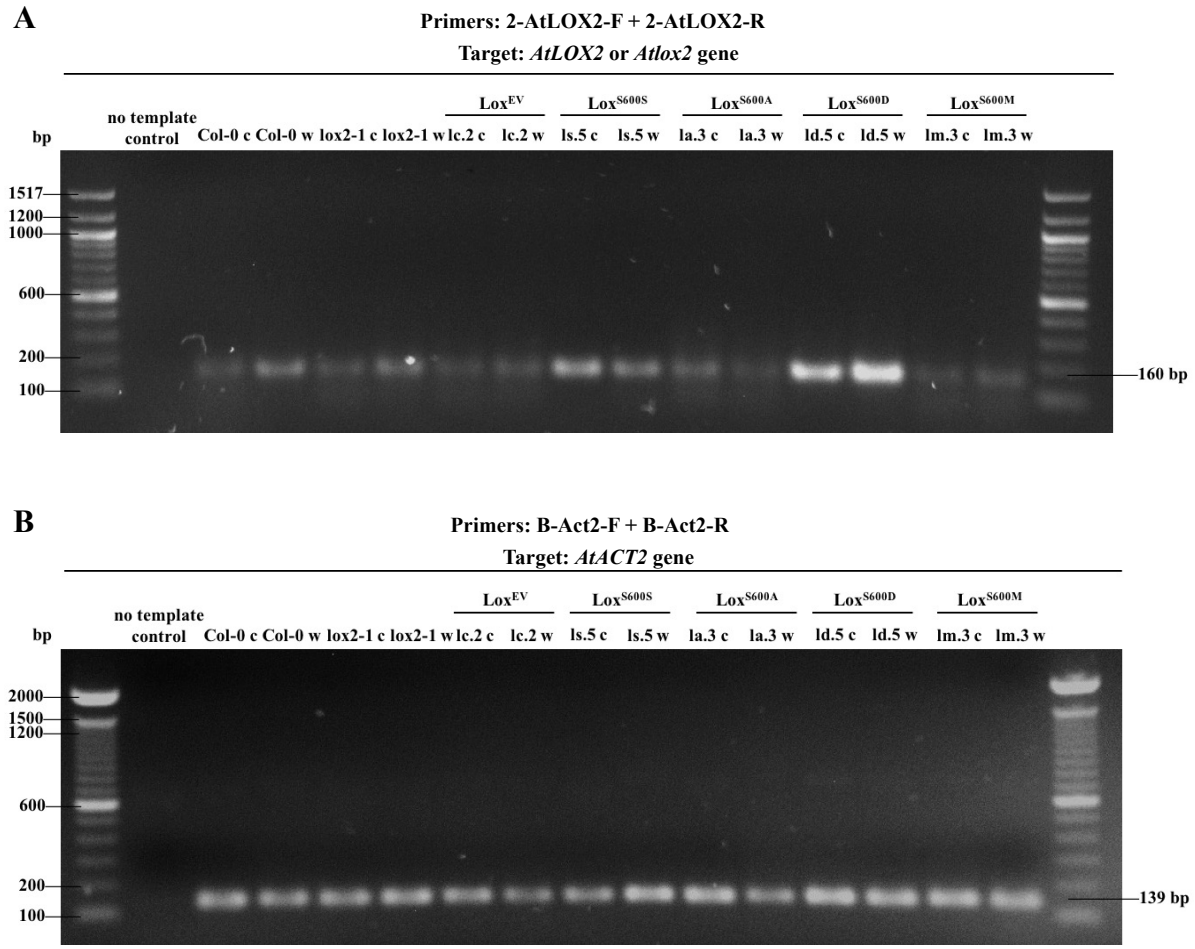
7. Appendix 1

Appendix 1 Table 1. Primer pairs used in PCR and RT-PCR. Primer pairs 1-7 were designed by NCBI's primer blast to confirm transgenic plants by PCR. Pair 2 and pair 7 performed well and were used in this study. Primer pair 6, 8 and 9 were designed for RT-PCR and pair 9 performed the best. Primer pair 10 was used to amplify the reference gene by RT-PCR.

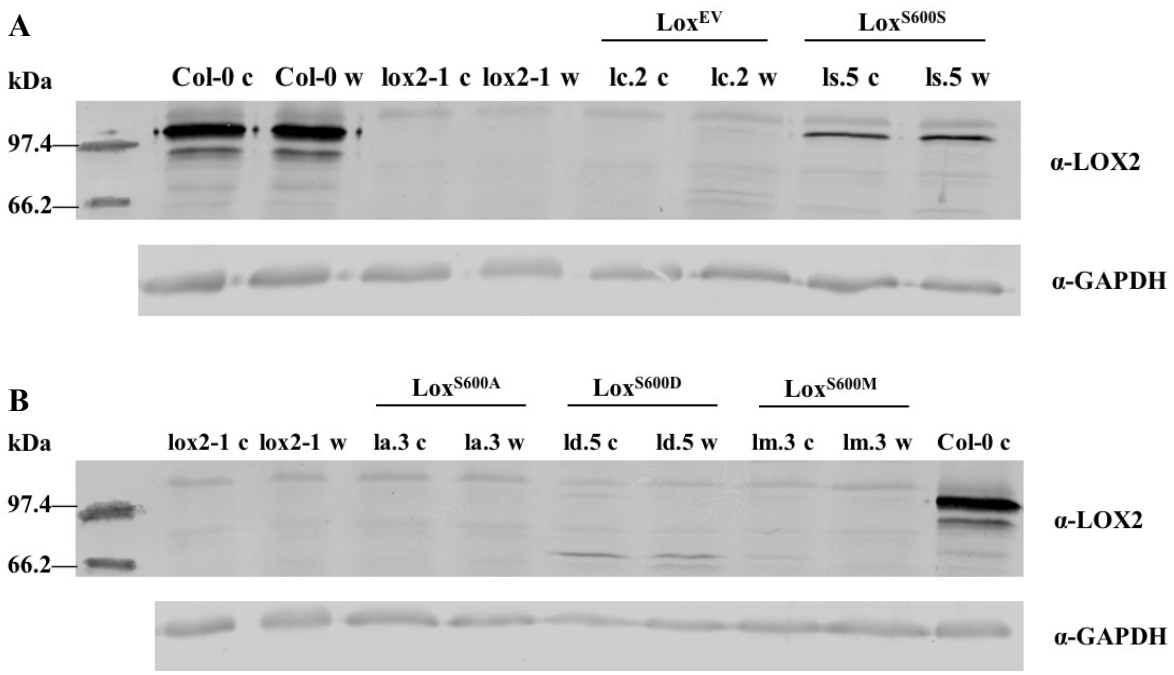
Pair	Target	Primer name	Primer sequence (5'-3')	Annealing temperature/℃	Amplicon size/bp
1	*nos* terminator	NosaF NosaR	TTTCCCCGATCGTTCAAACA TAATCATCGCAAGACCGGCA	58	74
2	*nos* terminator	NosabF NosabR	TGAATCCTGTTGCCGGTCTT GACACCGCGCGCGATAATTT	62	199
3	Hygromycin resistance	HygroFu HygroR	CAATGGCCGCATAACAGCGG GCTTCTGCGGGCGATTTGTGTA	62	319
4	*AtLOX2*	lox2mkf NosabR	GCTAGTTGAAGAGTGGCCGT GACACCGCGCGCGATAATTT	61	705
5	*AtLOX2*	lox2muf NosabR	CTATCTCTCATGACGCCGGT GACACCGCGCGCGATAATTT	60	1085
6	*AtLOX2*	lox2mf lox2mr	GGATTATCATGATTTGCTTCTACC TCAAATAGAAATACTATAAGGAACAC	54	1300
7	*AtLOX2*	lox2end3F NosabR	TGAGGACTCATGCCTGTACG GACACCGCGCGCGATAATTT	60	1310
8	*AtLOX2* cDNA	AtLOX2-F AtLOX2-R	TTGCTCGCCAGACACTTGC GGGATCACCATAAACGGCC	59	101
9	*AtLOX2* cDNA	2-AtLOX2-F 2-AtLOX2-R	GTCCTACTTGCCTTCCCAAAC ATTGTCAGGGTCACCAACATC	58	160
10	*AtACT2* cDNA	B-Act2-F B-Act2-R	TTGACTACGAGCAGGAGATG ACAAACGAGGGCTGGAACG	60	139



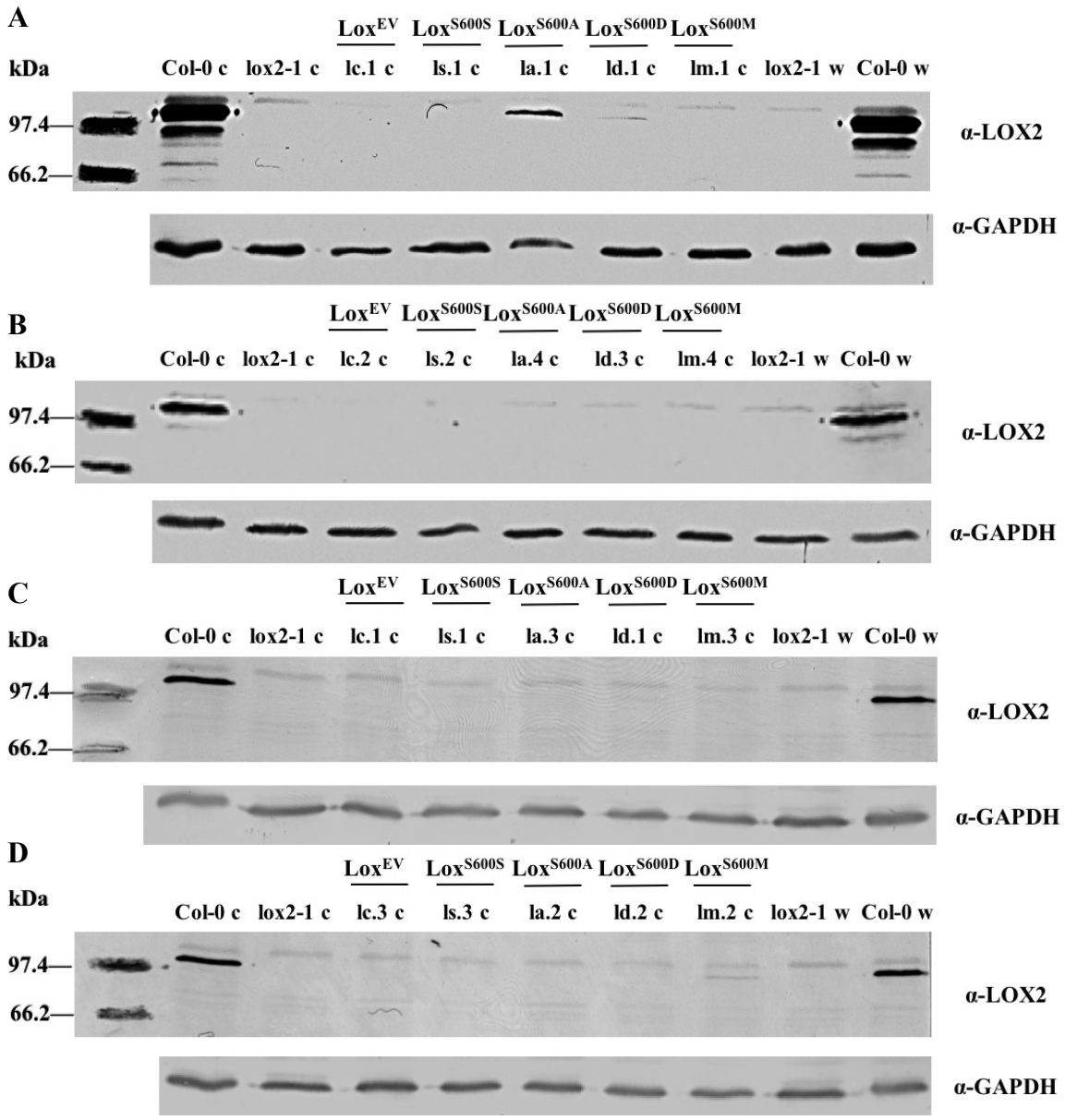
Appendix 1 Figure 1. Supplementary gels of amplicons confirming transgenic plants by PCR. Genomic DNA was extracted from Col-0 wild type, *lox2-1* mutant background and putative transgenic plants to identify transgenic T3 plants. Water indicates the no template control. Col-0 wild type and *lox2-1* plants are negative controls as they are not transformed. The plasmid indicates the positive control. The molecular weight marker was a 100 bp ladder range from 100 bp tp 2000 bp (Invitrogen). Representative sample 1.15.2 was extracted from plant of Lox^{S600A}, 3.1.5, 3.13.5 were from independent transgenic Lox^{S600M} lines and wt.15.1, wt.12.1.1, wt.12.1.3 were from independent transgenic Lox^{S600S} lines. **A) Primers were targeted to the *nos* terminator site on the pMDC32 plasmid that was introduced to generate transgenic plants.** The expected amplicon size is 199 bp. **B) Primers were targeted to the the *LOX2* or *lox2* gene and *nos* terminator site on the recombinant vector.** The expected amplicon size is 1310 bp. Bands with correct molecular weight were seen in all representative transgenic samples. These samples are considered to be transgenic.



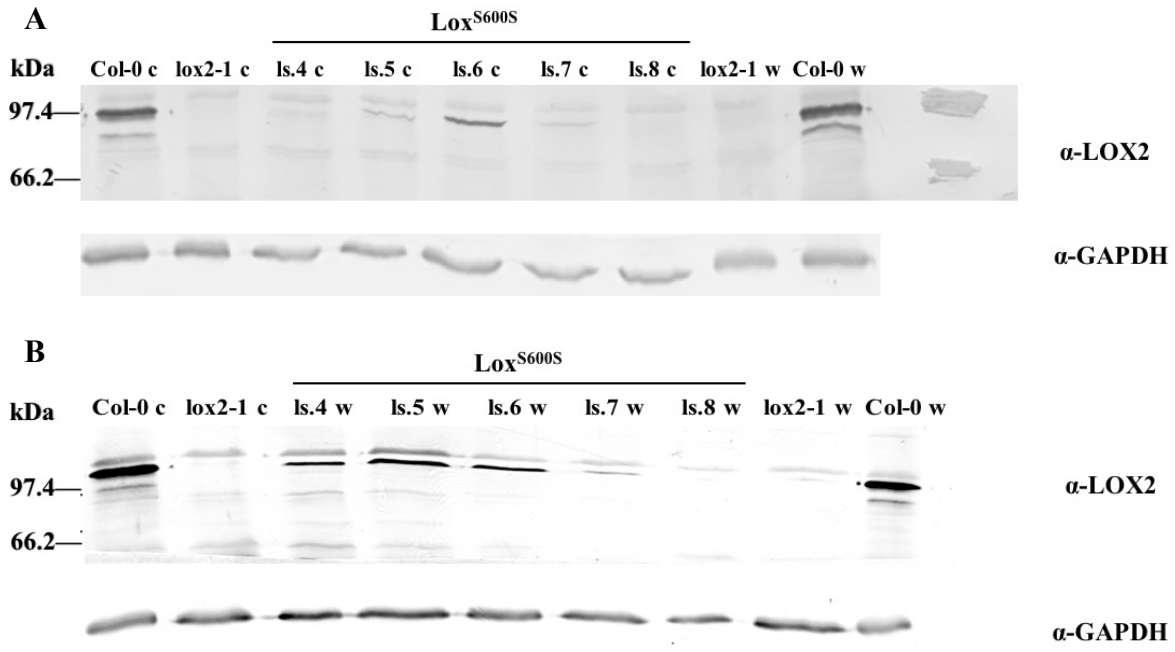
Appendix 1 Figure 2. Supplementary gels of *Atlox2* expression in transgenic plants measured by RT-PCR. Total RNA was isolated from unwounded/constitutive (c) or wounded (w) leaves of transgenic T3 plants and used to measure *Atlox2* (amplicon size 160 bp) and *AtACT2* (amplicon size 139 bp) expression. *AtACT2* is a constitutive gene used as a marker for sample quality. The 100 bp molecular weight marker (either from Invitrogen or New England Biolab) is indicated on the left side of the gel. **A) *AtLOX2* or *Atlox2* gene expression in samples: no template control, constitutive (c) and wounded (w) Col-0, *lox2-1* and one representative samples from each transgenic lines.** All samples show *AtLOX2* or *Atlox2* expression. **B) *AtACT2* gene expression in same samples as (A).** All samples show *AtACT2* expression.



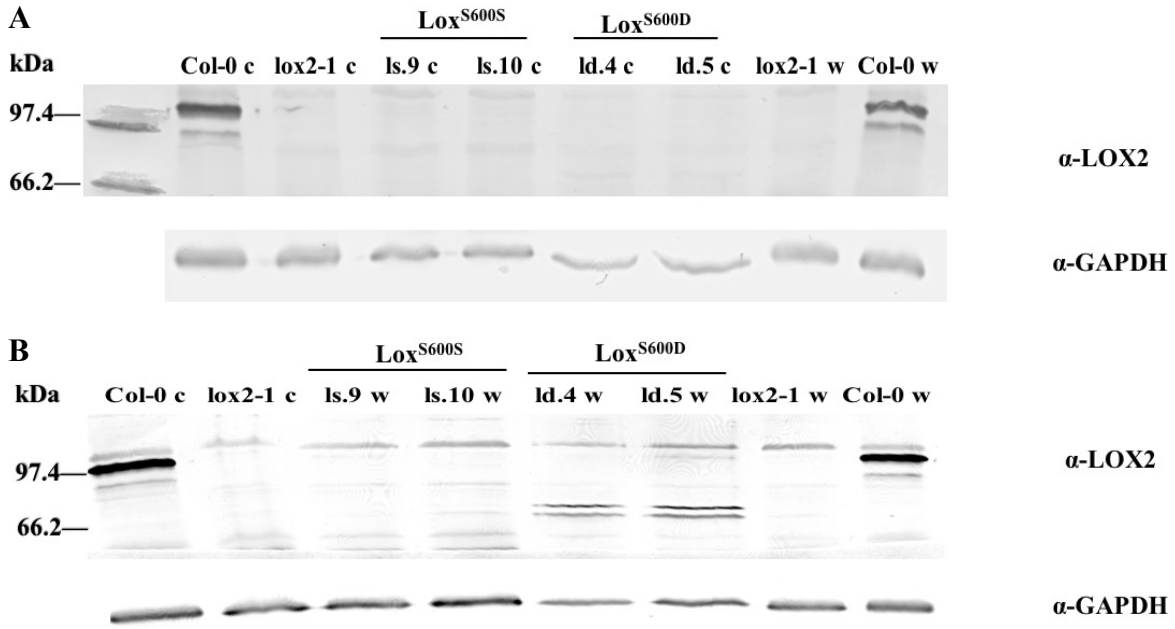
Appendix 1 Figure 3. Supplementary blots of lipoxygenase 2 expression in leaf extracts of wild type Col-0, *lox2-1* mutant, and representative samples from each transgenic line expressing the various constructs. Experiment condition is as described in the legend of Fig. 4.4. **A) LOX2 or lox2 expression in constitutive (c) and wounded (w) Col-0 (positive control), *lox2-1* (negative control) and one transgenic plants from each of Lox^{EV} and Lox^{S600S} line. B) LOX2 or lox2 expression in constitutive (c) and wounded (w) *lox2-1* (negative control) and one transgenic plant from each of Lox^{S600A}, Lox^{S600D}, Lox^{S600M} line and constitutive Col-0 (positive control).** All lines produced the reference protein GAPDH. As expected, Col-0 and the transgenic lines also produce LOX2 or lox2, respectively. The negative control *lox2-1* and Lox^{EV}, did not produce a full length protein that could be detected by the antibody.



Appendix 1 Figure 4. Supplementary blots in verifying lipoxygenase 2 expression in transgenic lines. Experiment condition is as described in the legend of Fig.4.4. **A) LOX2 or lox2 expression in constitutive (c) and wounded (w) Col-0 (positive control), *lox2-1* (negative control), one constitutive transgenic line from each of Lox^{EV}, Lox^{S600S}, Lox^{S600A}, Lox^{S600D}, Lox^{S600M} and wounded Col-0 (positive control). B, C, D) Expression of proteins extracted from different independent transgenic lines of each constructs with (A).** All lines produced the reference protein GAPDH. Col-0 and transgenic samples la.1 and la.3 from Lox^{S600A}, ld.1 from Lox^{S600D}, lm.2 and lm.3 from Lox^{S600M} produced LOX2 or lox2, respectively. The negative control *lox2-1* and Lox^{EV} did not produce a full length protein that could be detected by the antibody.



Appendix 1 Figure 5. Supplementary blots in verifying lipoxygenase 2 expression in transgenic lines. Experiment condition is as described in the legend of Fig. 4.4. **A) LOX2 expression in constitutive (c) Col-0 (positive control), *lox2-1* (negative control) and four constitutive samples extracted from independent transgenic lines of Lox^{S600S} and wounded (w) Col-0 (positive control). B) LOX2 expression in wounded (w) samples extracted from the same plants with (A).** All lines produced the reference protein GAPDH. The negative control *lox2-1* did not produce a full length protein that could be detected by the antibody. Col-0 and transgenic samples ls.4, ls.5, ls.6, ls.7 of Lox^{S600S} produced LOX2.



Appendix 1 Figure 6. Supplementary blots in verifying lipoxygenase 2 expression in transgenic lines. Experiment condition is as described in the legend of Fig. 4.4. **A) LOX2 or lox2 expression in constitutive (c) Col-0 (positive control), *lox2-1* (negative control), two constitutive samples extracted from independent transgenic lines of Lox^{S600S}, two constitutive samples extracted from independent transgenic lines of Lox^{S600D} and wounded (w) Col-0 (positive control). B) LOX2 or lox2 expression in wounded (w) samples extracted from the same plants with (A).** All lines produced the reference protein GAPDH. The negative control *lox2-1* did not produce a full length protein that could be detected by the antibody. Col-0 and transgenic sample ld.5 of Lox^{S600D} produced LOX2 or lox2, respectively.

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