Isomers of green leaf volatiles in Nicotiana attenuata and their role in plant-insect interactions
Allmann, S.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Oxylipin channeling in *Nicotiana attenuata*: Lipoxygenase 2 supplies substrates for green leaf volatile production

Silke Allmann\textsuperscript{1,2}, Rayko Halitschke\textsuperscript{3}, Robert C. Schuurink\textsuperscript{1}, Ian T. Baldwin\textsuperscript{2}

\textsuperscript{1} Department of Plant Physiology, Swammerdam Institute for Life Sciences, Science Park 904, 1098 XH, Amsterdam, NL
\textsuperscript{2} Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, DE-07745 Jena, Germany.
\textsuperscript{3} Department of Ecology and Evolutionary Biology, Cornell University, E443 Corson Hall, 14853 Ithaca, NY, USA


ABSTRACT

Lipoxygenases (LOXs) are key enzymes in the biosynthesis of oxylipins and catalyze the formation of fatty acid hydroperoxides, which represent the first committed step in the synthesis of metabolites that function as signals and defenses in plants. Hydroperoxides are the initial substrates for different branches of the oxylipin pathway and some plant species may express different LOX isoforms that supply specific branches. Here we compare isogenic lines of the wild tobacco *Nicotiana attenuata* with reduced expression of *NaLOX2* (irlox2) or *NaLOX3* (irlox3) to determine the role of these different LOX isoforms in supplying substrates for two different pathways: green leaf volatiles (GLVs) and jasmonic acid (JA). Reduced *NaLOX2* expression strongly decreased the production of GLVs without influencing the formation of JA and JA-related secondary metabolites. Conversely, reduced *NaLOX3* expression strongly decreased JA biosynthesis, without influencing GLV production. The temporal expression of *NaLOX2* and *NaLOX3* also differed after elicitation; *NaLOX3* was rapidly induced, attaining highest transcript levels within 1 h after elicitation, whereas *NaLOX2* transcripts reached maximum levels after 14 h. These results demonstrate that *N. attenuata* channels the flux of hydroperoxides through the activities of different LOXs leading to different direct and indirect defense responses mediating the plant’s herbivore resistance.

**Image description front page**

**Upper left panel:** procedure of mechanically wounding a leaf with a fabric pattern wheel. Image courtesy of A.P. van Doorn.

**Upper right panel:** trapping set-up in the greenhouse.

**Lower left panel:** Radial diffusion assay to determine trypsin protease inhibitor activity. Image courtesy of M. Hartl.

**Lower right panel:** Magnified areas of representative two-dimensional false-color chromatograms.
INTRODUCTION

Plants utilize diverse metabolic resources to defend themselves against attacks from herbivores. Often the production of different defense metabolites is activated by the same signaling pathway. Lipoxygenases (LOxs) are ubiquitous enzymes involved in the production of important signaling molecules and defense metabolites in plants, fungi, algae and mammals (Andreou et al., 2009). LOxs catalyze the dioxygenation of polyunsaturated fatty acids containing a (1Z,4Z)-pentadiene moiety (Gardner, 1991) to form fatty acid hydroperoxides (HPs). In plants linoleic acid and α-linolenic acid are the main substrates for this reaction. LOxs are divided in two major classes, 9-LOXs and 13-LOXs, introducing an oxygen group either at the C-9 (9-HPs) or C-13 position (13-HPs; Liavonchanka and Feussner, 2006). The resulting HPs are further metabolized via several metabolic routes and metabolites originating from these enzymatic reactions are commonly named oxylipins. One of these routes starts with the enzyme allene oxide synthase (AOS) which converts 13-hydroperoxy linolenic acid to an unstable allene oxide intermediate and in turn is modified by a cascade of enzymes finally leading to the formation of jasmonic acid (JA). Another route starts with the enzyme hydroperoxide lyase (HPL) which catalyzes the cleavage of 13-HPs to form C12- (12-oxo-(Z)-9-dodecenoic acid) and C6-compounds (hexanal and (Z)-hex-3-enal), commonly referred to as green leaf volatiles (GLVs).

JA and some of its precursors and derivatives are important plant signaling molecules involved not only in growth and developmental processes (Creelman and Mullet, 1997; Wasternack, 2007; Acosta et al., 2009) but also in mediating plant defense responses against herbivores and microbial pathogens (Browse, 2009; Koo and Howe, 2009). In unwounded leaves, JA is maintained at low levels. However, it quickly accumulates in plant tissue after mechanical damage or herbivory (Baldwin et al., 1994; Glauser et al., 2008; Paschold et al., 2008), and this accumulation is essential for the elicitation of defensive secondary metabolites to fend off diverse attackers (Halitschke and Baldwin, 2004; Glazebrook, 2005; Howe and Jander, 2008).

GLVs form an important group of herbivore-induced plant volatiles (HIPVs). This group consists of six-carbon-aldehydes, alcohols and their esters. GLVs are emitted only in trace amounts from healthy, undamaged plant tissue but they are rapidly released when a plant gets damaged (Turlings et al., 1995; D’Auria et al., 2007). GLVs can have diverse defense-associated functions. First, they can have a direct repellent or toxic effect on microbes, fungi (Hamilton-Kemp et al., 1992; Nakamura and Hatanaka, 2002; Prost et al., 2005), and insects (De Moraes et al., 2001; Kessler and Baldwin, 2001; Vancanneyt et al., 2001). However,
GLVs were also found to stimulate the feeding activity of some lepidopteran larvae (Halitschke et al., 2004; Meldau et al., 2009). Second, similarly to other HIPVs, GLVs can function as indirect defense responses by attracting foraging predators and host-seeking parasitoids to the plant and its attacker (Kessler and Baldwin, 2001; Shiojiri et al., 2006; Halitschke et al., 2008). However, while establishing indirect defenses, these volatiles can also serve as intra-plant signals eliciting systemic responses in adjacent branches or as inter-plant signals by inducing, or priming defenses in eavesdropping neighboring plants (Bate and Rothstein, 1998; Engelberth et al., 2004; Kost and Heil, 2006; Paschold et al., 2006; Mirabella et al., 2008).

Although the JA- and GLV- pathways are both dependent on the supply of HPs, some plant species possess different LOX isoforms that supply HPs only for specific oxylipin branches. In potato leaves, two distinct LOX genes (LOX-H1 and LOX-H3) have been identified that are induced after wounding and herbivory. Upon induction, LOX-H3 mRNA accumulated transiently with a peak at 30 min, whereas LOX-H1 transcripts reached maximum levels almost one day later (Royo et al., 1999). Moreover, transgenic plants with reduced expression levels of LOX-H3 exhibited a clear decrease in their GLV emission, while the release was normal in plants with reduced LOX-H3 expression. Interestingly, although both LOX-silenced lines were not impaired in their induced JA levels, they showed a clear reduction in their wound inducibility of proteinase inhibitors and other JA-responsive genes compared to wild type (WT) plants (Leon et al., 2002). In tomato plants, silencing TomLOXC expression led to a significant reduction of GLVs in fruits and leaves (Chen et al., 2004) while silencing TomLOXA and TomLOXB, which are expressed in seeds or roots (Ferrie et al., 1994), had no effect on the production of GLVs (Griffiths et al., 1999). While TomLOXC mRNA was not wound-inducible in tomato leaves, TomLOXD transcripts accumulated rapidly and transiently with the accumulation of JA, suggesting a possible role of TomLOXD in JA biosynthesis (Heitz et al., 1997). In Arabidopsis six different LOXs are known of which four possess 13-LOX activity (Andreou et al., 2009; Bannenberg et al., 2009). One of these 13-LOXs (AtLOX2) has been shown to be involved in the formation of JA. Co-suppression of AtLOX2 (Bell et al., 1995) or silencing via a nonsense mutation (Glauser et al., 2009) in the background of the Columbia (Col) ecotype reduced the wound-induced JA-accumulation. However, since the Col ecotype is unable to produce GLVs, due to a natural mutation in its hydroperoxide lyase gene (Duan et al., 2005), the impact of AtLOX2 in the formation of GLVs has not been studied. Finally, rice possesses at least five different LOXs: while OsHI-LOX channels 13-HPs specifically to the JA-branch (Zhou et al., 2009), another 9/13-LOX,
OsLOX1, has been shown to supply both, the JA- as well as the GLV-branch (Wang et al., 2008).

In *Nicotiana attenuata* three different LOX isoforms are known. Two of them, NaLOX2 and NaLOX3, are predicted to function as 13-LOXs and are mainly expressed in aerial tissues, while NaLOX1 most likely possesses 9-LOX activity and is specifically expressed in the roots. NaLOX3 has been shown to supply the JA pathway with 13-HPs (Halitschke and Baldwin, 2003). More recent work has demonstrated that the herbivore-induced JA burst in *N. attenuata* requires an increased flux of free linolenic acid (18:3) which likely results from the activation of a plastidal glycerolipase (GLA1) by *N*-linolenoylglutamate (18:3-Glu), an abundant fatty acid-amino acid conjugate (FAC) found in insect oral secretions. Surprisingly, an increase in free 18:3 after elicitation was not detectable suggesting a tight physical association between GLA1 and LOX3 in *N. attenuata* leaves (Kallenbach et al., 2010). Field studies revealed that NaLOX3-dependent induced defenses have a major influence on the plant’s herbivore load as well as the composition of the herbivore community. Plants with reduced expression of *LOX3* were not only more vulnerable to herbivores adapted to *N. attenuata*, they also attracted novel attackers not commonly observed on WT plants of this species (Kessler et al., 2004).

Here we characterize the LOX2 gene of *Nicotiana attenuata* by generating stable transgenic lines with reduced NaLOX2 expression (irlox2). To study which branches of the oxylipin pathway are supplied with LOX2-derived 13-HPs, we analyzed the GLV release and the levels of JA and JA-related secondary metabolites in irlox2 plants. By comparing the oxylipin profiles of irlox2 and NaLOX3-silenced (irlox3) plants, we examined the different physiological roles that the two LOX isoforms play.

**MATERIAL AND METHODS**

**Growing conditions**

We used seeds of the 30th generation of an inbred line of *Nicotiana attenuata* as the wild-type genotype in all experiments. The original seeds were collected in 1988 from a natural population at the DI ranch in southwestern Utah, USA. Seeds were germinated on agar plates containing Gamborg B5 media (Duchefa, Haarlem, The Netherlands). Plants were grown in the glasshouse in 1-L individual pots at 26°C to 28°C under 16 h of light as described by Krügel et al. (2002).
Plant treatments
All treatments were performed in the glasshouse with plants in the rosette stage of growth. The first fully elongated (+1 position) leaves were used, unless otherwise noted. Plants were wounded with a pattern wheel to punch three rows of holes on each side of the midrib. For a wounding + water (w+w) treatment, 20 µL of deionized water was immediately pipetted onto the wounded leaf and gently dispersed across the surface with a gloved finger. For a wounding + oral secretions (w+OS) treatment, 20 µL of Manduca sexta OS (diluted 1:3 in water, v/v) was applied to the wounds. Samples from untreated plants were used as controls. We used four to six replicates per genotype and treatment. Except for trapping volatiles, leaves were excised after specific time periods, flash frozen in liquid nitrogen, and stored at -80°C until use.

Generation of transgenic lines
A 498-bp fragment of the cDNA sequence of NaLOX2 was inserted into the pSOL3 transformation vector and a 467-bp fragment of the cDNA sequence of NaLOX3 was inserted into the pRESC5 transformation vector, both as an inverted repeat construct driven by the CaMV 35S promoter (Bubner et al., 2006). Both vectors were transformed into N. attenuata WT plants using Agrobacterium-tumefaciens-mediated transformation and homozygosity of T2 plants was determined by screening for resistance to hygromycin (Krügel et al., 2002). The lines were additionally tested for their ploidy level as described by Bubner et al. (2006). The number of insertions was determined as described by Berger and Baldwin (2009), by Southern blot hybridization of genomic DNA using a PCR fragment of the hptII gene as probe (Figure S2.1). Two T2 lines (52-2 and 67-4) of irlox2 and one line (562-2) of irlox3 were used in all subsequent experiments.

Analysis of silencing construct specificity
Pairwise alignment of the NaLOX3 sequence included in the irlox3 construct (sense and reverse complement orientation) and the NaLOX2 cDNA and the NaLOX2 sequence of the irlox2 construct (sense and reverse complement orientation) with the NaLOX3 cDNA were performed using ClustalX 2.0.7 (http://www.clustal.org; pairwise alignment parameters: slow-accurate; gap opening 15, gap extend 6.66). The resulting alignments were scrutinized for 21-nt stretches of sequence identity.
**Volatile collection and analysis**

Single leaves (+1 position) were enclosed immediately after treatment between two 50mL food-quality plastic containers (Huhtamaki, Bad Bertricher, Germany) secured with miniature claw-style hair clips. Ambient air was pulled through the collection chamber and a glass tube (ARS, Inc., Gainsville, FL, USA) packed with glass wool and 20mg of Super Q (Alltech, Düsseldorf, Germany). Airflow was created by a vacuum pump (model DAA-V114-GB; Gast Mfg; Benton Harbour, MI, USA) as described by Halitschke et al. (2000). SuperQ traps were eluted with 250 μL dichloromethane into a GC vial containing a glass insert after spiking each trap with 400 ng tetralin (Sigma-Aldrich, Germany) as an internal standard.

Samples were analyzed on an Agilent 6890N gas chromatograph equipped with an Agilent 7683 autoinjector (Agilent Technologies, Bübingen, Germany) and coupled with a LECO Pegasus III time-of-flight mass spectrometer with a 4D thermal modulator upgrade (LECO, Mönchengladbach, Germany) as described by Gaquerel et al. (2009). GLVs were identified and quantified using standard solutions of (Z)-hex-3-enal, (E)-hex-2-enal, (Z)-hex-3-en-1-ol, (E)-hex-2-en-1-ol, hexan-1-ol, [(Z)-hex-3-enyl] acetate, [(E)-hex-2-enyl] acetate, [(Z)-hex-3-enyl] propanoate, [(Z)-hex-3-enyl] 2-methylpropanoate, hexyl 2-methylpropanoate, [(Z)-hex-3-enyl] butanoate, hexyl butanoate, [(E)-hex-2-enyl] butanoate, [(Z)-hex-3-enyl] 2-methylbutanoate, [(Z)-hex-3-enyl] (Z)-2-methylbut-2-enoate) (Sigma-Aldrich, Germany) and [(E)-hex-2-enyl] propanoate (Bedoukian Research Inc, Danbury, USA).

**Phytohormone analysis**

JA and JA-Ile/Leu levels were measured as previously described by Wang et al. (2007). We treated two leaves (+1 and +2) per plant and harvested the older leaf (+2) to measure JA and JA-Ile. The younger leaf was harvested after three days to measure secondary metabolites (see analysis of secondary metabolites). Ethylene emissions were measured with a laser photoacoustic spectrometer (INVIVO, Sankt Augustin, Germany) as described by von Dahl et al. (2007). Results were determined by ‘stop-flow’ measurements of single leaves (+1 position) after wounding and directly treating the wounds with 20 μL of *M. sexta* OS (n=6; 1:3 diluted) or 20 μL of *N*-linolenoyl-L-Glu (18:3-Glu; n=5, 13.8ng/µl in 0.02% tween-20). The amounts of 18:3-Glu used for this experiment corresponded to the amounts found in *M. sexta* OS (Halitschke et al., 2001). Untreated leaves of each genotype served as control. Excised leaves were transferred to 250 mL cuvettes and ethylene was allowed to accumulate in the headspace for 5 h. The cuvettes were flushed with a flow of purified air at 130–150 mL min⁻¹, which afterwards passed through a - 190°C cooling trap to remove CO₂ and H₂O.
Analysis of secondary metabolites

Herbivore attack on *Nicotiana attenuata* rapidly increases the production and accumulation of two JA-related secondary metabolites; the anti-digestive trypsin proteinase inhibitors (TPI) and the toxic alkaloid nicotine. Nicotine was analyzed by HPLC as described previously (Keinanen et al., 2001) with the following modification of the extraction procedure: approximately 200 mg frozen tissue was homogenized in 1 mL extraction buffer utilizing the FastPrep® extractions system (Savant Instruments, Holbrook, NY, USA). Samples were homogenized in FastPrep® tubes containing 900 mg lysing matrix (BIO 101, Vista, CA, USA) by shaking at 6.0 m sec\(^{-1}\) for 45 seconds. TPI activity was analyzed by a radial diffusion activity assay as described previously (Van Dam et al., 2001).

Quantitative real-time PCR assay

Total RNA was extracted with TRI Reagent (Sigma-Aldrich, Germany) according to the manufacturer’s instruction. Reverse transcription of 1 µg of total RNA was performed using SuperScript II Reverse Transcriptase (Invitrogen, Germany) and a poly-T primer. Quantitative real-time PCR (ABI PRISM™7000, Applied Biosystems, Foster City, CA, USA and Stratagene Mx3005P, Waldbronn, Germany) was conducted with 40 ng cDNA using the qPCR™ core reagent kit (Eurogentec, Köln, Germany) and gene-specific primer pairs for each individual gene (*Table S2.1*). The PCR products were detected either by gene-specific double fluorescent dye-labeled TaqMan® probes (in combination with the ABI PRISM™7000) or with SYBR Green reactions (qPCR Core Kit for SYBR Green I; Eurogentec, Köln, Germany; in combination with the Stratagene Mx3005P). For calibration we used a duplicated dilution series of cDNAs which had been transcribed from induced RNA samples of the same experiment and calculated the relative transcript abundance by efficiency-correcting for each primer pair and normalizing to the *N. attenuata* actin gene according to Pfaffl et al. (2002). *NaLOX3* transcript levels were analyzed with two different pairs of primers (see *Table S2.1*), since we changed our qPCR-machine from ABI to Stratagene and the ABI NaLOX3 primers appeared to not work efficiently on the Stratagene machine. These two technical changes –different primers and different machines- might also explain the difference in the amplitude of the *NaLOX3* transcript levels. Data from *Figure 2.1* were obtained with new pair of LOX3 primers and the new machine; data from *Figure 2.2* were obtained with the ABI conditions.
Statistical analysis
All statistics were done using Excel (Microsoft Corporation, Redmond, Washington, USA), SPSS 17.0 (SPSS Inc., Chicago, IL, USA), or R (R-Project, http://www.r-project.org). Data were transformed, if necessary, to meet the requirements for homogeneity of variance. Unless otherwise stated, data were compared using analysis of variance (ANOVA) followed by a Scheffé post-hoc test or Student’s t-test.

RESULTS

Generation of transgenic plants with reduced expression levels of LOX2 or LOX3

The LOX2 cDNA of Nicotiana attenuata (NaLOX2) was previously identified in a flower cDNA library (Halitschke and Baldwin, 2003). In order to investigate the impact of NaLOX2 on the formation of GLVs we used a 498 bp fragment of the NaLOX2 (nucleotides 809-1306) in an inverted repeat construct to generate stable transgenic lines. Two independently transformed homozygous irlox2 lines (52-2 and 67-4) were selected and used for all experiments. These lines had a single T-DNA insertion (Figure S2.1) and showed a very high silencing efficiency of constitutive NaLOX2 expression (99%), and after wounding (98%) and treatment of the wounds with water (w+w) or oral secretion (OS) of Manduca sexta, a specialist herbivore of N. attenuata (w+OS; Figure 2.1a).

In this study we also addressed the different functions of the two 13-LOXs of N. attenuata, LOX2 and LOX3, by comparing plants that had reduced LOX2 or LOX3 transcript levels. Plants with reduced expression of LOX3 by means of antisense expression (aslox3) have been extensively characterized previously (Halitschke and Baldwin, 2003; Kessler et al., 2004). However, in order to directly compare the phenotypes of LOX2- and LOX3-silenced plants, we generated plants transformed with an inverted repeat LOX3 construct (467 bp; nucleotides 509-975). Since we have extensive knowledge about the phenotype of LOX3-silenced plants, we used only one irlox3 line in this comparative study. This line (562-2) had 81% and 83% reduced transcript levels after w+w and w+OS treatment, respectively (Figure 2.1b). LOX2 transcript levels were also affected in these plants with a 90% reduction of the constitutive levels (Figure 2.1a), still significantly less reduction than in irlox2 plants (99%).
NaLOX2 and NaLOX3 have different patterns of expression

To determine the effect of elicitation on the transcript levels of both LOXs, rosette stage leaves were harvested at different time points from non-wounded (control) and mechanically wounded WT plants that had been treated with water or *M. sexta* OS. NaLOX3 transcript levels peaked 1 h after OS elicitation, with transcript levels approximately two-fold higher than those in the water-treated leaves. NaLOX2 transcript levels did not increase during the first hours after elicitation, but, notably, OS-treated plants had lower LOX2 transcript levels than untreated plants. However, they increased in the evening and peaked after 14 h for both w+w and w+OS treatments. While in elicited plants the two LOXs had clearly different expression patterns, with NaLOX3 being induced early and NaLOX2 being induced late after elicitation, the transcript levels of both LOX isoforms displayed similar diurnal rhythms when plants were not damaged, starting with relatively low levels in the morning, rising in the afternoon and returning to initial levels during the night (Figure 2.2).
Reduced NaLOX2 expression decreases the release of GLVs

To investigate the involvement of NaLOX2 in the release of GLVs, we measured the wound-induced emission from individual leaves of WT plants and of two irlox2 lines in the first 20 minutes after elicitation. Both irlox2 lines were greatly impaired in their GLV-release. While line 1 (52-2) emitted less than 1% of the total GLV release of WT plants after w+w treatment, line 2 (67-4) emitted 10% of WT emissions (Table 2.1).

Figure 2.2. Mean (±SE) relative transcript abundance of NaLOX2 and NaLOX3.
Rosette stage leaves (+1 position) were harvested from non-wounded (control) or mechanically wounded wild type plants that had been treated with either water (w+w) or oral secretions of M. sexta (w+OS). Individual leaves from five replicate plants per treatment and time point were harvested at each indicated time after elicitation. The abundance of NaLOX2 (A) and NaLOX3 (B) transcripts were analyzed by qRT-PCR and normalized to an unregulated reference gene (actin). All data are expressed relative to the data of the unelicited WT at timepoint 0 (WT, control). Upper panels show the relative transcript levels of each gene in the first 3 h.
Table 2.1. Reduced green leaf volatile emission in irlox2 plants

<table>
<thead>
<tr>
<th>Class</th>
<th>IUPAC Name</th>
<th>RT (s)</th>
<th>VOLATILE RELEASE IN ng / g fresh mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT1</td>
<td>RT2</td>
</tr>
<tr>
<td>Aldehyde</td>
<td>hexanal</td>
<td>174</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>(Z)-hex-3-enal</td>
<td>174</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>(E)-hex-2-enal</td>
<td>246</td>
<td>2.90</td>
</tr>
<tr>
<td>Alcohol</td>
<td>(Z)-hex-3-en-1-ol</td>
<td>246</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>(E)-hex-2-en-1-ol</td>
<td>270</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>hexan-1-ol</td>
<td>276</td>
<td>2.56</td>
</tr>
<tr>
<td>Hexenylester</td>
<td>[(Z)-hex-3-enyl] acetate</td>
<td>570</td>
<td>2.89</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] propanoate</td>
<td>756</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] propanoate</td>
<td>774</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] 2-methylpropanoate</td>
<td>834</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>hexyl 2-methylpropanoate</td>
<td>840</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] butanoate</td>
<td>912</td>
<td>2.64</td>
</tr>
<tr>
<td></td>
<td>hexyl butanoate</td>
<td>918</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>[(E)-hex-2-enyl] butanoate</td>
<td>930</td>
<td>2.70</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] 2-methylbutanoate</td>
<td>990</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>[(Z)-hex-3-enyl] (Z)-2-methylbut-2-enoate</td>
<td>1140</td>
<td>2.92</td>
</tr>
</tbody>
</table>

Mean (± SE, n=5) release of GLVs in wild type (WT) plants and two independently transformed irlox2 lines (52-2 and 67-4). A single leaf (+1 position) of each rosette stage plant was mechanically wounded and treated with water. Plant volatiles were collected as described in Halitschke et al. (2000) the first 20 min after elicitation and samples were analyzed by GCxGC-ToF MS (Gauquerel et al 2009). Two-dimensional separations were attained using an RTX-5MS column (RT1) followed by a DB-17 column (RT2). Volatiles are listed by chemical classes with their retention times on the first and second dimension (RT1 and RT2). (n.d. = not detected)

In an additional experiment, we collected and compared the volatiles from single leaves of WT and irlox2 (52-2) plants with those of irlox3 (562-2) plants after w+w or w+OS elicitation. This experiment confirmed the results we previously obtained from irlox2 plants. While irlox2 plants emitted almost no GLVs, independent of the treatment, the wound-induced release of irlox3 plants did not differ from the WT (Figure 2.3a). However, when OS was applied to the wounds instead of water, irlox3 plants had the tendency to release less GLVs than WT plants. Since the pattern of release was similar for all GLVs, only the data for (Z)-hex-3-enol are presented (Figure 2.3).

Reduced NaLOX2 expression does not impair JA and JA-Ile accumulation

NaLOX3 supplies 13-HPs to the JA-pathway (Halitschke and Baldwin, 2003), but it is not known whether NaLOX2 additionally contributes to JA biosynthesis. To study the impact
of the two different LOX isoforms in the formation of JA we measured the basal and induced levels of JA and JA-Ile/Leu in WT and irlox2 (52-2 and 67-4) plants, and in a separate experiment in WT and irlox3 (562-2) plants. Leaves of WT and irlox2 plants accumulated similar amounts of JA and JA-Ile/Leu in unelicited as well as elicited plants (Figure 2.4a, c). In contrast, wound induced JA concentrations were significantly reduced (41% less) in irlox3 plants and this reduction became even more pronounced after wounds were additionally treated with M. sexta OS (64% less). The basal JA levels of irlox3 plants did not differ from the WT (Figure 2.4b). This is in accordance with previous data obtained from aslox plants (Halitschke and Baldwin, 2003) showing a significant reduction of JA accumulation in three independently silenced lines after w+w and w+OS treatment. Plants with reduced levels of LOX3 were not only impaired in their JA biosynthesis but also accumulated significantly less JA-Ile/Leu after w+OS treatment whereas wounded and unwounded irlox3 plants were not different from WT plants (Figure 2.4d). Although the absolute levels of JA-Ile/Leu in WT plants differed between the two experiments, the results demonstrate that the levels were reduced in irlox3 but not irlox2 plants.

![Figure 2.3. Silencing NaLOX2, but not NaLOX3 reduces elicited GLV emissions.](image)

Mean (+ SE) release of (Z)-hex-3-en-1-ol in wild type (WT) compared to LOX-silenced plants (irlox2 and irlox3). A single leaf (+1 position) of each plant was mechanically wounded and treated with either water (w+w; A) or OS of M. sexta (w+OS; B). Plant volatiles were trapped the first 20 minutes after elicitation. Asterisks indicate significant differences from WT plants (univariate ANOVA, w+w: F_{2,11} = 74.76, p ≤ 0.001 and w+OS: F_{2,12} = 71.77, p ≤ 0.001, followed by a Scheffé post-hoc test, p ≤ 0.001 (***)).
JA-mediated defense metabolites are not dependent on NaLOX2 expression

Herbivore-induced TPI activity as well as nicotine accumulation in leaves of *N. attenuata* are known to be JA-mediated. The induced increase of both metabolites occurs much slower than the upstream accumulation of JA (Baldwin, 1999; Van Dam et al., 2001). Leaf material was thus harvested 3 days after elicitation from WT and *irlox2* (52-2 and 67-4) plants and in a separate experiment from WT and *irlox3* (562-2) plants. Both, TPI activity and nicotine levels did not differ between WT and *irlox2* plants except for a small but significant decrease in TPI activity in line 67-4 after w+w treatment (Figure 2.5a, c). In *irlox3* plants, however, not only the basal and induced levels of TPI activity but also the OS-induced nicotine accumulation was significantly lower than in WT plants (Figure 2.5b, d).

*irlox3*, but not *irlox2* plants have reduced OS-elicited ethylene emissions

Herbivore attack or the application of *M. sexta* OS elicits an ethylene burst in *N. attenuata*. It has been shown that FACs are the active components in the OS causing the ethylene burst (von Dahl et al., 2007).

We measured the accumulated ethylene emissions from single leaves of WT and *irlox2* (52-2 and 67-4) 5 h after OS treatment with a laser photo-acoustic spectrometer. No differences were observed between the two *irlox2* lines and WT plants (Figure 2.6a). In an additional experiment we compared WT and *irlox2* (52-2) with *irlox3* (562-4) plants for their ability to emit ethylene in the first 5 h after treating an individual leaf of each plant with the most abundant FAC in OS of *M. sexta*, 18:3-Glu (Halitschke et al., 2001). Again, ethylene emissions from *irlox2* plants were similar to the emissions from WT plants. However, in *irlox3* plants ethylene emissions were significantly reduced by 36% (Figure 2.6b).

**DISCUSSION**

This study demonstrates the two different LOX isoforms of *N. attenuata*, NaLOX2 and NaLOX3, channel 13-HPs into two distinct branches of the oxylipin pathway, which have different functions in plant defense. By using stable transgenic plants with reduced expression levels of either *NaLOX2* or *NaLOX3* we showed that *irlox3* plants were heavily impaired in their JA-related direct defense metabolism, but released GLVs in amounts comparable to those released from WT plants, while *irlox2* plants released hardly any GLVs, but elicited WT
levels of direct defenses. Taken together, our data show that the induction of GLV emissions and JA-related direct defenses are separated by the action of different LOXs.

Figure 2.4. Basal and induced mean levels of jasmonic acid (JA) and jasmonic acid-isoleucin/Leucin (JA-Ile/Leu) in WT and LOX-silenced plants reveals that only the silencing of NaLOX3 results in dramatic reductions of OS-elicited JA and JA-Ile. Rosette stage leaves (+2 position) were harvested from non-wounded (control) plants or from mechanically wounded plants that had been treated with either water (w+w) or oral secretions of *M. sexta* (w+OS). Tissues were harvested 45min after elicitation. Asterisks indicate significant differences from equally treated WT plants. Statistical analyses were done for each treatment separate with genotype as factor. A, Mean (+SE) JA accumulation in WT plants and plants from two independently silenced irlox2 lines (52-2 and 67-4), (univariate ANOVA, control: $F_{2,12}=0.35$, $p=0.711$, w+w: $F_{2,12}=3.66$, $p=0.058$, w+OS: $F_{2,12}=1.79$, $p=0.208$). B, Mean (+SE) JA accumulation in WT and irlox3 plants (Student’s *t*-test, control: $t_8=1.52$, $p=0.166$, w+w: $t_8=-2.45$, $p<0.05$ (*), w+OS: $t_8=-4.67$, $p<0.001$ (***)). C, Mean (+SE) JA-Ile/Leu accumulation in WT plants and plants from two irlox2 lines. (univariate ANOVA, control: $F_{2,12}=5.29$, $p<0.05$ (*), followed by a Scheffé *post-hoc* test, $p<0.05$, w+w: $F_{2,12}=0.09$, $p=0.912$, w+OS: $F_{2,12}=1.53$, $p=0.257$). D, Mean (+SE) JA-Ile/Leu accumulation in WT and irlox3 plants (Student’s *t*-test, control: $t_8=-0.29$, $p=0.776$, w+w: $t_8=1.50$, $p=0.171$, w+OS: $t_{10}=-3.68$, $p<0.01$ (**)).
The different functions of the two LOXs were already suggested by Porta et al. (2008) as their phylogenetic analyses revealed that NaLOX3 grouped in a clade with LOXs that had previously been reported to be involved in the JA biosynthesis while NaLOX2 clustered with LOXs known to supply the GLV pathway. Most research has focused on the function of 13-LOX in JA biosynthesis and signal transduction and this function has been described for several plant species including potato (Royo et al., 1999), tomato (Heitz et al., 1997), Arabidopsis (Bell et al., 1995; Glauser et al., 2009), rice (Zhou et al., 2009), maize (Acosta et al., 2009), and the wild tobacco N. attenuata (Halitschke and Baldwin, 2003 and this study). In tomato plants the involvement of TomLOXD in the JA-pathway was inferred from the temporal patterns of transcript accumulation and ofJA-related defenses, but in N. attenuata, rice and Arabidopsis such reduced LOX expression could be directly linked to reduced levels of JA. Interestingly, in potato plants, reduced expression of LOX-H3 did not lead to a reduction of JA but only to a reduced activity of proteinase inhibitors (PIs). Clearly, NaLOX3 homologs are conserved players in plant direct defenses but the observed expression patterns and their implications for the spatial and temporal accumulation of its downstream phytohormone products seem to vary among different plant species.
LOXs that are involved in the production of GLVs have received less attention. Two close homologs of NaLOX2, potato LOX-H1 (Leon et al., 2002) and tomato TomLOXC (Chen et al., 2004), have been shown to deliver 13-HPs to the GLV pathway. However, the specificity of 13-HP supply by these LOXs to the GLV and JA pathway remain unclear: while JA levels and downstream metabolites were not analyzed in plants with reduced TomLOXC expression, silencing of the two potato LOXs (H1 and H3) did not affect JA levels, indicating redundancy or the existence of additional LOX isoforms responsible for JA biosynthesis in potato. This suggests that LOX genes may have acquired slightly different roles in the oxylipin metabolism of different plant species.

Our results, although clearly showing pathway specificity of the two different LOXs in *N. attenuata*, suggest that the two pathways are not completely independent of each other. Irlox3 plants had not only decreased LOX3 but also LOX2 transcript levels (Figure 2.1). However, this ‘co-suppression’ of LOX2 expression in irlox3 plants resulting in a 90% reduction of the constitutive LOX2 transcript levels did not lead to a decrease in wound-induced GLV emissions (Figure 2.3a), while a 99% reduction in the constitutive LOX2 transcript levels of the irlox2 lines (Figure 2.1) were sufficient to almost abolish the GLV emissions in these plants (Table 2.1 and Figure 2.3). Constitutive expression of LOX2, a late inducer (Figure 2.2), seems to be much higher than that of LOX3, an early inducer (Figure 2.2) since the Ct values in our Q-RT-PCR experiments were much lower (approx. six cycles) for LOX2 (data not shown). It thus seems that a very high reduction of LOX2 expression is necessary to diminish GLV emission. In any case, alignments of both LOXs revealed very little sequence similarity between the LOX3 silencing construct and the LOX2 sequence.

**Figure 2.5. Trypsin protease inhibitor (TPI) activity and nicotine concentration in WT and LOX-silenced plants reveals that only the silencing of NaLOX3 results in defense metabolite reductions.**

Rosette stage leaves (+1 position) were harvested from non-wounded (control) plants or from mechanically wounded plants that had been treated with either water (w+w) or OS of *M. sexta* (w+OS). Tissues were harvested three days after elicitation. Nicotine was only measured in OS-elicited leaves. Asterisks indicate significant differences from equally treated wild type plants. Statistical analyses were done for each treatment separate with genotype as factor. A, Mean (+SE) TPI activity in WT and irlox2 (52-2 and 67-4) plants (univariate ANOVA, control: F2,12=2.95, p=0.091, w+w:F2,12=4.57, p<0.05, followed by a Tukey post-hoc test, p<0.05 (*), w+OS: F2,12=1.87, p=0.196). B, Mean (+SE) TPI activity in WT and irlox3 (562-2) plants (t-test, control: t8=-3.21, p<0.05 (*), w+w: t8=-7.34, p≤0.001 (***)). C, Mean (+ SE) nicotine levels in OS-elicited WT and irlox2 (52-2 and 67-4) plants. (univariate ANOVA, F2,12=0.65, p=0.539). D, Mean (+ SE) nicotine levels in WT and irlox3 (562-2) plants (t-test, t10=-2.88, p<0.05
Aligning the NaLOX2 cDNA against the reverse complement strand of the NaLOX3 cDNA sequence used for the irlox3 construct, revealed only 60% homology but at least one homology stretch of 26bp which included two mismatches (Figure S2.2a; nucleotides 725-750 of NaLOX2). Similar effects have been observed in potato where LOX H1-depleted plants also had reduced LOX H3 transcript levels (Leon et al., 2002). The authors argued that, although LOX H3 transcript levels were lower than in the WT, the fold-induction of LOX H3 transcripts after elicitation was the same due to already decreased basal transcript levels. From this, the authors inferred that the kinetics of up- and down regulation, rather than absolute transcript levels, determined the effects on defense gene expression. Moreover, in a different study, plants with reduced LOX H3 expression exhibited decreased levels of LOX H1 as well. However, the decrease in transcript levels was not accompanied by a decrease in LOX H1 protein levels, suggesting a low rate of LOX H1 protein turnover (Royo et al., 1999). Clearly much remains to be learned about how fold-induction and absolute expression levels differentially contribute to changes in metabolite levels. In our study, although NaLOX2 transcript levels were reduced in both, irlox2 and irlox3 plants, the specific impact on the plant’s metabolic phenotype remained distinct between the two genotypes. Whatever the cause of the reduced NaLOX2 expression in irlox3 plants - the level of suppression of non-target LOX genes via post transcriptional gene silencing (Stam et al., 1997), ir-RNA-mediated chromatin silencing (Matzke and Birchler, 2005) of endogenous LOX gene sequences and/or transcriptional feedback regulation via changes in JA metabolism (Paschold et al., 2008) – it did not lead to a significant decrease in GLV emissions. However, we cannot exclude minor quantitative effects of these two genes on each other’s downstream phenotypes in WT plants.

Our data shows that the supply of 13-HPs by NaLOX2 is required for GLV release in *N. attenuata*. A 98% decrease in the LOX2 transcript levels after w+w treatment reduced GLV emissions by 99 and 90% in line 52-2 and 67-4, respectively (Table 2.1 and Figure 2.1). This reduction in GLV emissions clearly resulted from reduced NaLOX2 transcript level. NaHPL transcript levels did not differ between irlox2 and WT plants (Figure S2.3). However, in WT plants LOX2 transcript levels do not immediately increase after elicitation (Figure 2.2), while GLV release clearly does (Table 2.1). This phenomenon of a time delay in peak transcript levels is often seen for genes encoding enzymes of secondary metabolism. For example, while the emission of (Z)-3-hexenyl acetate from mechanically wounded Arabidopsis plants peaked between 4.5 and 5.5 min after wounding the levels of acetyl CoA:(Z)-3-hexenol acetyltransferase transcripts and enzymatic activity showed no increase in this time frame, but
instead increased much more slowly, attaining maximum values 3 h (transcript) and 6–12 h (enzyme activity) after damage was inflicted (D’Auria et al., 2007). Therefore, the GLVs emitted from the leaves in the first 20 min after damage must be synthesized by the LOX2 protein already present in the leaf prior to injury. The impaired release of GLVs after elicitation in irlox2 plants could thus result from reduced constitutive levels of LOX2 protein, implying that the initial GLV release in WT plants results from constitutive levels of LOX2, a hypothesis that needs to be tested by measuring protein levels. Similar expression patterns have been reported from potato where Leon and coworkers (2002) reported maximum transcript levels of LOX H1 24 h after elicitation. Similar associations are found with JA accumulations and NaLOX3 expression after elicitation, where the initial JA-accumulation is thought to result from pre-existing pools of enzymes and/or substrates (Ziegler et al., 2001). Recently we obtained evidence that flux of 18:3 substrates is the rate limiting step in JA biosynthesis (Kallenbach et al., 2010). If so, absolute expression levels of NaLOX3 may determine the strength of the early response while changes occurring after elicitation determine if these responses are subsequently regulated.

**Figure 2.6. Ethylene emissions in WT and LOX-silenced plants reveals the influence of NaLOX3 silencing.**

Ethylene emissions were determined by ‘stop-flow’ measurements, of single leaves (+1 position) after wounding and treatment of the wounds immediately with (A) *M. sexta* oral secretions (OS), or (B) *N*-linolenoyl-I-Glu (18:3-Glu). Untreated leaves of each genotype served as control. Asterisks indicate significant differences from equally treated WT plants. Statistical analyses were done for each treatment separate with genotype as factor. A, Mean (+SE) ethylene emissions in WT and irlox2 (52-2 and 67-4) plants (univariate ANOVA, control: $F_{2,15}=0.13$, $p=0.883$, w+OS: $F_{2,15}=1.47$, $p=0.262$). B, Mean (+SE) ethylene emissions of WT, irlox2 (52-2) and irlox3 (562-2) plants. (univariate ANOVA, control: $F_{2,12}=0.06$, $p=0.942$, w+18:3-Glu: $F_{2,12}=12.56$, $p<0.01$, followed by a Scheffé post-hoc test, $p<0.01 (**)$)
While the GLV emission of mechanically wounded *irlox3* plants did not differ from that of WT (Halitschke and Baldwin, 2003 and Figure 2.3a), *irlox3* plants emitted consistently, but not significantly, less GLVs when *M. sexta* OS was applied to the wounds. We propose that this persistent trend in decreased GLV emission resulted from a signaling (JA-mediated) rather than a flux based (13-HP-mediated) interaction. It is well known that application of OS leads to an amplification of the wound-induced JA-burst in *N. attenuata* (Halitschke *et al.*, 2001 and Figure 2.4) and that accumulation of JA and its amino acid-conjugates is essential for a wide range of defense responses in plants (Wang *et al.*, 2008). In fact, constitutive and induced *NaHPL* transcript levels were substantially decreased in *irlox3* compared to WT plants (Figure S2.3). Microarray analyses revealed that not only silencing *NaLOX3* (Halitschke and Baldwin, 2003) but also silencing of *NaAOS* (Halitschke *et al.*, 2004) reduced expression levels of HPL. Since the flux of 13-HPs into the GLV-pathway appeared to increase rather than decrease in *AOS*-silenced plants, it is likely that the reduced *HPL* transcript levels were due to a decrease in the JA-accumulation, which is known to feedback regulate other oxylipin pathway genes as well. If so, regulation of *HPL* expression may be regulated by JA levels but not by flux into the pathway. We therefore propose that JA not only acts as a regulator of transcript accumulation but also as a regulator of enzyme activity. If so, the loss of the OS-induced JA-amplification in *irlox3* plants could be responsible for the lower levels of GLVs.

In conclusion, our data show that JA-responses and GLV emission are separated by at least the function of two distinct LOXs in *N. attenuata*, enabling the plant to regulate these processes relatively independently. However, LOX3-dependent JA production exhibits some feedback control over the LOX2-dependent GLV emission although not likely via regulating induced gene expression.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: NaLOX2 (AY254348), NaLOX3 (AY254349), NaActin (EU273278) and NaHPL (AJ414400).

**ACKNOWLEDGEMENTS**

We thank Merijn Kant for critical reading and constructive comments on the manuscript, Markus Hartl for his help with the statistical analyses, Susan Kutschbach and Wibke Kröber for invaluable help in the lab, and the Max Planck Society for funding.
REFERENCES


Bate NJ, Rothstein SJ (1998) C-6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant Journal 16: 561-569


Heitz T, Bergey DR, Ryan CA (1997) A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiology 114: 1085-1093


OXYLIPIN CHANNELING IN TOBACCO

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 30: No. 9 e36, 1-10


Table S2.1. Primer and Probe sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
<th>Taqman® Probe (5’-3’)</th>
<th>SYBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaLOX2</td>
<td>TTAGTAGAAATGAGCACCACAAGG</td>
<td>TTGCACTTGTGTGTGATGTTGA</td>
<td>TGCTTTCCCATGGCAAGTTCA</td>
<td>X</td>
</tr>
<tr>
<td>NaLOX3</td>
<td>GGCAGTGAAATTTGGCAACCACAAC (1)</td>
<td>CCCAAAATTTGGAATCCACCAACA (1)</td>
<td>CAGTGAGGAACAGAACAGGAGATCTGAAG</td>
<td></td>
</tr>
<tr>
<td>NaLOX3</td>
<td>GAATTTGGAACAAGGAGAGTACTG (2)</td>
<td>CTGCAACTTTTCCTCTCAGATCTGCTC (2)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>NaActin</td>
<td>GGTCGTACCACCGTATTGTG</td>
<td>GTCAAGACGGAATGGCATG</td>
<td>TCACCCACCCGCTCCAATAATGAGG</td>
<td>X</td>
</tr>
<tr>
<td>NaHPL</td>
<td>CACCTAGACCTAGCTCCACCTGTGC</td>
<td>AACACAAACCTTTGAGGATCATCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) This NaLOX3 primer pair was only used in combination with the NaLOX3 Taqman probe and the ABI PRISM™7000
(2) This NaLOX3 primer pair was only used for SYBR Green Assays with the Stratgene Mx3005P

Figure S2.1: Verification of transgene insertion by Southern blot analyses. Genomic DNA of WT and two irlox2 lines, 52-2 and 67-4, and one irlox3 line, 562-2, were digested with XbaI (A) or with EcoRI (B). These enzymes were chosen, because both constructs harbor only a single restriction site for each enzyme. The blot was hybridized with a 32P-labeled probe specific for the hygromycin phosphotransferase gene hptII.
**OXYLIPIN CHANNELING IN TOBACCO**

---

**A**

**NaLOX2** versus **LOX3** PCR product in reverse complement direction: 60% homology

---

**B**

**NaLOX3** versus **LOX2** PCR product in sense direction: 53% homology

---

**Figure S2.2.** Alignment of *N. attenuata* cDNA sequences and silencing constructs. Searches for 21-nt identity stretches between the **NaLOX2** cDNA and the **LOX3-PCR product** (LOX3_PCR) used to create the irlox3 construct (A), as well as between the **NaLOX3** cDNA and the **LOX2-PCR product** (LOX2_PCR) used to create the irlox2 construct (B), were performed after aligning sequences using ClustalX (http://www.clustal.org/). The searches were done with LOX-PCR-products, in sense and reverse complement direction, but only those with the highest homology are displayed. (Rev.Compl, Reverse Complement)
Figure S2.3. *NaHPL* transcript abundance in *irlox2* and *irlox3* plants. Mean (+ SE) relative transcript abundance of *NaHPL* in (A) untreated (control), (B) w+w treated, and (C) w+OS treated leaves of WT, *irlox2* (52-2 and 67-4) and *irlox3* (562-2) plants. Relative transcripts were analyzed by qRT-PCR and normalized to an unregulated reference gene (actin). All data are expressed relative to the data of the unelicited WT (WT, control). Asterisks indicate significant differences from equally treated WT plants. Statistical analyses were done for each treatment and timepoint separate with genotype as factor (univariate ANOVA, control: $F_{3,12} = 6.12$, $p < 0.01$, followed by a Scheffé post-hoc test, $p < 0.05$ (*)); w + water: 1h: $F_{3,12} = 1.42$, $p = 0.286$, 14h: $F_{3,12} = 16.53$, $p < 0.001$ (**); w + OS: 1h: $F_{3,12} = 1.97$, $p = 0.173$, 14h: $F_{3,12} = 6.71$, $p < 0.01$, followed by a Scheffé post-hoc test, $p < 0.05$ (*)).