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N-Acyl-Homoserine Lactone Primes Plants for Cell Wall Reinforcement and Induces Resistance to Bacterial Pathogens via the Salicylic Acid/Oxylipin Pathway[C][W][OPEN]

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The ability of plants to monitor their surroundings, for instance the perception of bacteria, is of crucial importance. The perception of microorganism-derived molecules and their effector proteins is the best understood of these monitoring processes. In addition, plants perceive bacterial quorum sensing (QS) molecules used for cell-to-cell communication between bacteria. Here, we propose a mechanism for how *N*-acyl-homoserine lactones (AHLs), a group of QS molecules, influence host defense and fortify resistance in *Arabidopsis thaliana* against bacterial pathogens. *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) primed plants for enhanced callose deposition, accumulation of phenolic compounds, and lignification of cell walls. Moreover, increased levels of oxylipins and salicylic acid favored closure of stomata in response to *Pseudomonas syringae* infection. The AHL-induced resistance seems to differ from the systemic acquired and the induced systemic resistances, providing new insight into inter-kingdom communication. Consistent with the observation that short-chain AHLs, unlike oxo-C14-HSL, promote plant growth, treatments with C6-HSL, oxo-C10-HSL, or oxo-C14-HSL resulted in different transcriptional profiles in *Arabidopsis*. Understanding the priming induced by bacterial QS molecules augments our knowledge of plant reactions to bacteria and suggests strategies for using beneficial bacteria in plant protection.

INTRODUCTION

In plants, contact with microorganisms can induce local defense mechanisms based on the detection of either microbe-associated molecular patterns (MAMPs), which result in MAMP-triggered immunity (MTI), or effector proteins, resulting in effector-triggered immunity. In addition, plant tissues distal to sites of infection can develop systemic resistance. The two best understood mechanisms of systemic resistance are induced systemic resistance (ISR) and systemic acquired resistance (SAR), the former of which is a response to beneficial, soil-born microbes, whereas the later is associated with pathogen attack. Both require the presence of Nonexpressor of PR1 (NPR1) and the action of specific phytohormone signal cascades. SAR and ISR have been studied in depth and exhaustively reviewed (Van Wees et al., 2008; Dempsey and Klessig, 2012; Fu and Dong, 2013; Shah and Zeier, 2013). ISR requires components from the ethylene (ET) and jasmonic acid (JA) signaling cascades. In *Arabidopsis thaliana*, the *myb72* transcription factor mutant is impaired in ISR (Van der Ent et al., 2008), and the interaction between MYB72 and the ethylene-responsive EIN3-like transcription factor3 suggests that crosstalk exists between the JA and ET pathways. SAR, typically induced after infection with a pathogen, is dependent on the accumulation of salicylic acid (SA) at the infection site and on either or both MTI or effector-triggered immunity (Fu and Dong, 2013). Several genetic determinants of SAR have been identified, including SA binding proteins, methyl transferases, lipid transfer proteins, and transcription factors (for review, see Dempsey and Klessig, 2012; Shah and Zeier, 2013). Nevertheless, the molecular basis of the systemic signaling is not completely understood, since the beneficial bacteria *Pseudomonas fluorescens* strain 89B61 and *Bacillus pumilus* strain T4 induce resistance in a JA- and ET-independent or NPR1-independent manner, respectively (Ryu et al., 2003).

The multitude of low molecular weight metabolites and mobile proteins involved in establishing SAR is striking. Methyl salicylate (MeSA), dehydroabietinal, azelaic acid, a glycerol-3-phosphate–derived factor, pipecolic acid, as well as the putative lipid transfer proteins DIR1 and DIR1-like are proposed to be involved in the induction of systemic resistance (Park et al., 2007; Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Návarová et al.,

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2012; Champigny et al., 2013). The current model predicts that all signal cascades induced by these molecules require NPR1 (Dempsey and Klessig, 2012; Shah and Zeier, 2013). In addition, oxylipins other than JA and JA derivatives could play a role in plant defense mechanisms. For example, colneleic and colnelenic acids accumulate in potato (*Solanum tuberosum*) leaves infected with fungi or viruses (Weber et al., 1999), and together with other oxylipins are thought to act similarly to methyl jasmonate (Blée, 2002). Synthesis of active phytooxylipins is initiated either by lipooxygenases (LOX) that introduce an oxygen atom at the C9 or C13 position on the lipid chain (Andreou and Feussner, 2009) or by the nonenzymatic generation of structurally similar phytoprostanes (Sattler et al., 2006). A genome-wide analysis of *Arabidopsis* revealed that more than 150 genes responded to the application of 12-oxo-phytodienoic acid (*cis*-OPDA), though not to JA or methyl jasmonate (Taki et al., 2005). *cis*-OPDA–regulated genes encoded proteins involved in the stress response and signal transduction. Interestingly, expression of the majority of those genes depended on the TGACG motif binding transcription factors TGA2, TGA5, and TGA6 (Mueller et al., 2008), similar to genes regulated by SA and some pathogens.

Priming is yet another process related to defense mechanisms in plants. Primed plants respond in a stronger and/or faster manner to an infection than do nonprimed plants. This phenomenon, called "sensitization," has been used in agriculture since the early 1930s. Many potent "resistance inducers," including low concentrations of SA, benzothiadiazole, and β-aminobutyric acid (BABA), have been intensively studied in the last years (Conrath et al., 2002). Azelaic acid was shown to accumulate in local and systemic tissue upon SAR, priming the plant for enhanced SA production and conferring resistance to *Pseudomonas syringae* (Jung et al., 2009). Recently, two discoveries have shed light on the molecular basis of priming. The first was the accumulation of the inactive form of mitogen-activated protein kinases that could be activated upon secondary stimulation (Beckers et al., 2009); the second was histone methylation (H3K4me3 and H3K4me2) and acetylation (H3K9, H4K5, H4K8, and H4K12) in the promoter regions of the defense-associated *WRKY6*, *WRKY26*, and *WRKY53* transcription factors (Jaskiewicz et al., 2011). Whether the expression or action of mitogen-activated protein kinases and chromatin modification are connected remains unclear. The primed state can also be inherited. Transgenerational priming was observed in progeny of plants exposed to *P. syringae*, BABA, or herbivores (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012). Interestingly, while this phenomenon has an epigenetic basis, the priming induced by *P. syringae* or BABA depended on SA, whereas herbivore-induced transgenerational priming required COI1 and, thus, JA perception. Together, these findings suggest the importance of epigenetic mechanisms in induced resistance.

Recently, several reports have suggested that bacterial quorum sensing molecules also induce a primed state. Currently, *N*-acyl-homoserine lactones (AHLs) are the best-studied group of quorum sensing molecules having this activity. Many Gram-negative bacteria produce AHLs in order to monitor their population density. In plants, AHL application induces changes in gene expression, alters protein profiles, and modifies root development (Mathesius et al., 2003; Ortíz-Castro et al., 2008; von Rad et al., 2008; Schikora et al., 2011; Bai et al., 2012; Schenk et al., 2012). The first indication that AHLs play a role in plant immunity originated from a study of a *Serratia liquefaciens*–tomato (*Solanum lycopersicum*) interaction, in which the wild-type *S. liquefaciens* strain MG1, but not the corresponding AHL-negative mutant, induced resistance against the fungal pathogen *Alternaria alternata* (Schuhegger et al., 2006). Likewise, resistance against *P. syringae* induced by *Ensifer meliloti* (*Sinorhizobium meliloti*) in *Arabidopsis* plants depended on AHL accumulation (Zarkani et al., 2013). Usually bacteria produce high concentrations of quorum sensing molecules (e.g., AHLs) when growing in biofilms or mature colonies (above OD_{600} of 0.5). The production of AHLs on root tissues by bacteria growing in biofilms has been shown (Zarkani et al., 2013), and the production of AHLs in biofilms on leaves is likely to occur, but has not been demonstrated yet. In addition, the application of commercial AHLs induced resistance against bacterial and fungal pathogens in *Arabidopsis*. Our previous study suggested that this effect depends on a stronger and prolonged activation of MPK6 (Schikora et al., 2011). Moreover, plant responses to different AHL molecules appear to be AHL specific. Proteome analysis revealed ~150 differentially accumulated proteins in response to the application of either commercial oxo-C12-HSL or oxo-C16:1-HSL isolated from cultured *S. meliloti* (Mathesius et al., 2003).

In this article, we substantiate previous studies indicating that the plant responds to different AHLs at the transcriptional level. We also demonstrate that, after exposure to oxo-C14-HSL, a secondary challenge augments phenolic, lignin, and callose depositions in the cell wall. Furthermore, accumulation of oxylipins in distal tissues promoted stomata closure, thus enhancing plant resistance to bacterial infection. These data show how both local and systemic phenomena contribute to the enhanced resistance observed in AHL-primed plants.

RESULTS

N-Acyl-Homoserine Lactones Induce AHL-Specific Responses

Independent studies suggested that plants react to different AHL molecules in a specific manner (Mathesius et al., 2003; Ortíz-Castro et al., 2008; Schenk et al., 2012). To gain insight into plant responses, we performed a transcriptome analysis of *Arabidopsis* seedlings treated with three different AHLs know to have different effects. *N*-hexanoyl-L-homoserine lactone (C6-HSL) was selected because of its plant growth promoting effect (Ortíz-Castro et al., 2008; von Rad et al., 2008; Schikora et al., 2011; Liu et al., 2012; Schenk et al., 2012); *N*-3-oxo-decanoyl-L-homoserine lactone (oxo-C10-HSL) influences root morphology and root hair formation (Ortíz-Castro et al., 2008); and *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) induces plant resistance (Schikora et al., 2011) (Figure 1A). Two-week old *Arabidopsis* seedlings were transferred into six-well plates and floated on half-strength Murashige and Skoog (MS) medium supplemented with either 6 μM AHL or acetone (control), the solvent for AHLs. After 3 d, whole seedlings were harvested (Figure 1B). We interrogated the *Arabidopsis* (4x44k) Gene Expression Microarray from Agilent Technology to

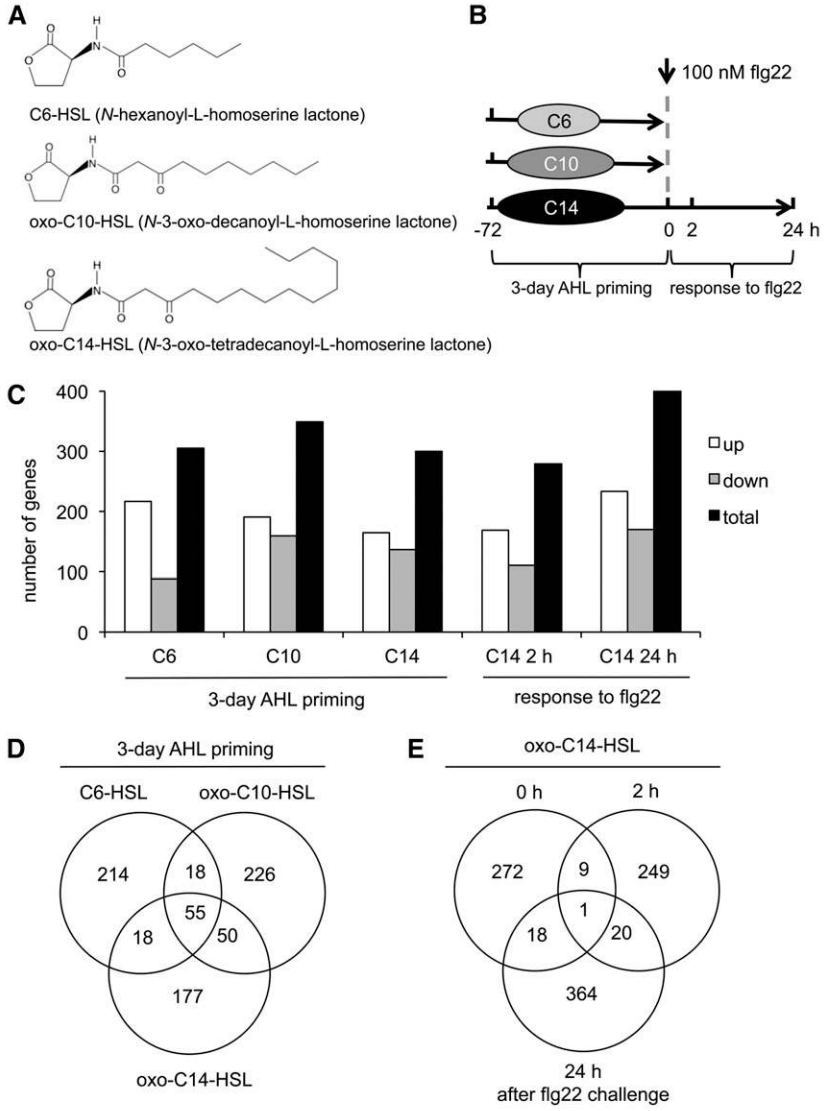

Figure 1. *Arabidopsis* Responds to Different AHLs with Diverse Transcriptional Reprogramming.

(A) AHLs were prepared as 60 mM stocks in acetone and used at a working concentration of 6 μM in half-strength MS medium for (pre)treatments.
(B) Experimental setup to analyze the transcriptional responses to treatments with 6 μM C6-HSL, oxo-C10-HSL, and oxo-C14-HSL as well as the response of oxo-C14-HSL–pretreated plants to secondary challenge with flg22. Two-week-old *Arabidopsis* seedlings were treated with three different homoserine lactones (AHL-priming). After 3 d, the oxo-C14-HSL–pretreated seedlings were challenged with 100 nM flg22 to trigger the defense response.
(C) Number of genes differentially expressed upon AHL (pre)treatment and the flg22 challenge. Differentially expressed genes were selected by a $\log_2$ fold change of 1 and a moderate P value of 0.05.
(D) Venn diagram of genes up- and downregulated after treatment with the three different AHLs. Only 55 genes were regulated by all AHLs, and ~200 genes were regulated by each AHL molecule.
(E) Venn diagram of genes differentially expressed in response to 100 nM flg22 in oxo-C14-HSL–pretreated seedlings after 0, 2, and 24 h. The response is very dynamic, as shown by the small overlap in differentially expressed genes at different time points after the secondary challenge.

identify differentially expressed genes showing a $\log_2$ fold change of 1 and a moderate P value of 0.05 (Smyth, 2004). Within these thresholds, we found 954 differentially regulated genes (Figure 1C; Supplemental Data Sets 1 to 4). Of the 55 genes that responded to all three AHLs, 45% (25 genes) were related to stress responses and signaling (Figure 1D; Supplemental Data Set 1). Furthermore, 214, 226, and 177 genes (total of 617 genes) responded only to the application of C6-HSL, oxo-C10-HSL, or oxo-C14-HSL, respectively, indicating that the different AHLs regulated a specific set of host genes. Among all of the genes responsive to a specific AHL molecule, 18 to 25% were assigned to the functional category "biotic stress" (Supplemental Figure 1), especially signaling-related

genes encoding kinases (but not MAP kinases), receptor kinases, and proteins involved in calcium signaling. Another prominent group included genes encoding proteins involved in protein degradation, such as ubiquitin-like and F-box proteins. This group was larger for oxo-C10-HSL–treated plants than for C6-HSL–treated plants. Interestingly, more glucanase genes were significantly ($P < 0.05$ in ANOVA) downregulated in oxo-C10-HSL–treated plants than in C6-HSL–treated plants (five genes versus one gene, respectively). Glucanases hydrolyze amylose and amylopectin to release glucose, suggesting that plants may reduce their carbohydrate metabolism in response to oxo-C10-HSL treatment, whereas C6-HSL–treated plants might conserve their level of stored carbohydrates. Taken together, the differentially expressed genes within the "biotic stress" functional category revealed similarities as well as important differences.

Response to Flg22 Challenge Differs in AHL-Primed Plants

The direct impact of oxo-C14-HSL application on resistance was shown in *Arabidopsis* and barley (*Hordeum vulgare*) plants, although the mechanism of resistance had not been resolved (Schikora et al., 2011). To address this point, we examined the transcriptional reprogramming that might occur during MTI in plants that were primed by AHL treatment versus those that were mock-primed. Oxo-C14-HSL–pretreated plants were challenged with 100 nM flg22 (a flagellin-derived 22–amino acid peptide perceived in *Arabidopsis* by the FLS2 receptor and thus initiating MTI) for 2 and 24 h. Plants pretreated with acetone and similarly challenged with flg22 were used as controls (Figure 1B). Between 279 and 403 genes were differentially expressed; however, only a few genes were differentially expressed in all or two of the three tested time points (Figure 1E; Supplemental Data Sets 4 to 6). Our findings indicate a transient transcriptional reprogramming. Nonetheless, at all tested time points, biotic stress remained the major functional category (Supplemental Figure 2). We also found an enrichment of genes related to signaling, including receptor kinases, calmodulin binding proteins, and G-proteins ($P = 0.042$ in Wilcoxon rank sum test) (Supplemental Data Set 7), cell wall ($P = 0.037$), and secondary metabolism and flavonoids ($P = 0.032$) (Supplemental Figures 2 and 3 and Supplemental Data Sets 6 to 9). Remarkably, there was no enrichment of genes related to the typical defense hormones such as SA, JA, or ET (Supplemental Figure 4).

Expression of Genes Related to the Cell Wall

The enrichment of cell wall–related genes in plants pretreated with oxo-C14-HSL and subsequently challenged with flg22 was of particular interest. Transcriptome analysis revealed the upregulation of genes encoding arabinogalactan proteins (*AT2G20520*, *AT4G40090*, *AT2G15350*) and proline-rich proteins (*AT3G62680* and *AT1G54970*) (Figure 2A; Supplemental Data Set 9). Both are structural components of plant cell walls and belong to the hydroxyproline-rich glycoprotein superfamily. Cross-linking of hydroxyproline-rich glycoproteins and glycosylation are key processes in the strengthening of the cell wall and contribute to plant resistance by limiting pathogen invasion (Wei and Shirsat, 2006). In addition, several other genes involved in the modification of polysaccharides were differentially regulated (*AT4G25820*, *AT428850*, *AT5G5730*). Cell wall–modifying expansins (*AT2G03090* and *AT5G39270*), responsible for relaxation and irreversible cell wall extension (McQueen-Mason and Cosgrove, 1995), were also differentially expressed. Moreover, we observed the differential expression of genes encoding pectinases, a class of cell wall–degrading enzymes (Figure 2A), cell wall–associated kinases (*AT1G16150*, *AT1G70450*, and *AT1G49270*), G-proteins, and genes related to flavonoid metabolism (Figure 3; Supplemental Data Set 7). This suggests that the application of oxo-C14-HSL is associated with the modification of cell wall structure and call wall–based defense.

Changes in Composition of Cell Wall after Oxo-C14-HSL Application

The deposition of callose upon pathogen attack generates a structural barrier to pathogen invasion. Recent studies showed the importance of stress-induced callose synthesis in defense mechanisms (Jacobs et al., 2003; Ellinger et al., 2013). The enrichment in genes belonging to cell wall–related functional categories encouraged us to analyze callose deposition in plants pretreated with oxo-C14-HSL and subsequently challenged with flg22. Experiments were performed with leaves floated on half-strength MS medium supplemented with AHL or acetone (control) for 3 d and challenged with flg22. After staining with aniline blue, leaves were ranked in four categories according to the amount of staining, where category I represented lowest callose deposition density (Figure 2B). Pretreatment with oxo-C14-HSL resulted in a reduction of the number of leaves in category I and an increase in the number of leaves in category IV (the highest callose deposition density) (Figure 2C). In addition to this local effect, oxo-C14-HSL induced changes in callose deposition in distal plant parts. Using a sterile systemic hydroponic system, which allowed separation between the AHL-pretreated root and flg22-treated leaf tissues, roots were pretreated with 6 μM oxo-C14-HSL for 3 d prior to challenge of the leaves with 1 μM flg22. Classification of distal leaves after aniline blue staining revealed that oxo-C14-HSL primed systemic tissues for enhanced callose deposition, as observed in the local response (Figure 2D).

Secondary metabolites have a significant role in plant defense; a prominent example is the phytoalexins (Treutter, 2005). The enrichment of genes related to flavonoid metabolism ($P = 0.032$) 24 h after challenge with flg22 in oxo-C14-HSL–pretreated plants motivated us to analyze the phenolic composition of cell walls. We measured the accumulation of soluble and cell wall–bound phenolic compounds as well as lignins in 2-week-old seedlings directly pretreated with AHL by floating on half-strength MS medium supplemented with oxo-C14-HSL or acetone (control) or plants grown in the sterile systemic hydroponic system, as described above. In accordance with the enrichment of genes related to secondary metabolism (Figure 3B), we observed an accumulation of soluble and cell wall–bound phenolic compounds in seedlings pretreated with AHL and challenged for 24 h with flg22 (Figure 3A). Similarly, the subsequent photometric analysis of the lignothioglycolic acid complex (lignin fraction) revealed increased lignification of cell walls in these plants (Figure 3A). The

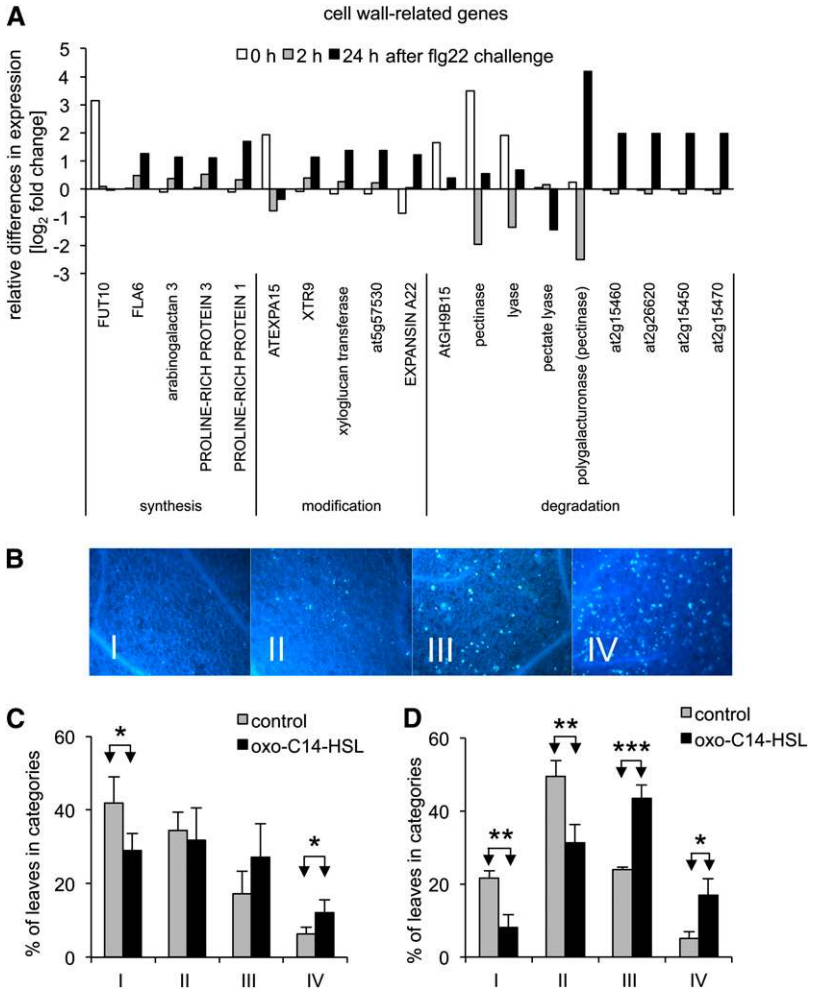

Figure 2. Cell Wall–Based Defense Mechanisms Are Influenced by AHL.

(A) Genes assigned to the gene ontology term "cell wall," regulated by a 3-day oxo-C14-HSL pretreatment and subsequent challenge with 100 nM flg22 for the number of hours indicated, as reveled by microarray analysis. The fold change was calculated in relation to the expression in plants pretreated with the AHL solvent, acetone.

(B) Callose deposition sites were counted and leaves were grouped accordingly into four categories. I represents 0 to 5 spots/area; II, 5 to 20; III, 20 to 100; and IV, >100. The observed area was 4.9 mm^2.

(C) Callose deposition in oxo-C14-HSL–pretreated leaves subsequently challenged with flg22. Detached leaves from 5-week-old plants were pretreated with 6 μM oxo-C14-HSL or acetone (solvent control) for 3 d prior to challenge with 100 nM flg22 for 24 h and stained with aniline blue. The experiment was performed five times with $n > 20$ leaves (four areas per leaf were analyzed). Asterisk presents $P < 0.05$ in Student's t test. Error bars represent SD of the means.

(D) Callose deposition in the systemic system. Assays performed as in **(C)**, except that leaves were from plants grown in sterile systemic hydroponic culture. Roots of those plants were pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d and subsequently challenged with 1 μM flg22. The experiment was performed four times with $n > 40$. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ in Student's t test. Error bars represent SD.

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increased accumulation of phenolic compounds was further visualized by autofluorescence (von Röpenack et al., 1998) using confocal laser scanning microscopy (Figures 3C and 3D). The impact of AHL on cell wall composition was visible not only in locally treated tissues, but in distal leaves as well. Analysis of phenolic compounds in leaves of plants in which only roots were pretreated with oxo-C14-HSL also showed an increased accumulation of the phenol fractions after a subsequent challenge (Figure 3E). In line with the enhanced callose depositions and accumulation of phenolic compounds in distal tissues, we observed an augmented expression of genes related to cell wall and flavonoid metabolism (Supplemental Figure 3). These findings support the notion that AHL primes the plant, both locally and systemically, to increase callose deposition and enhance accumulation of phenolic compounds for strengthening of the cell wall.

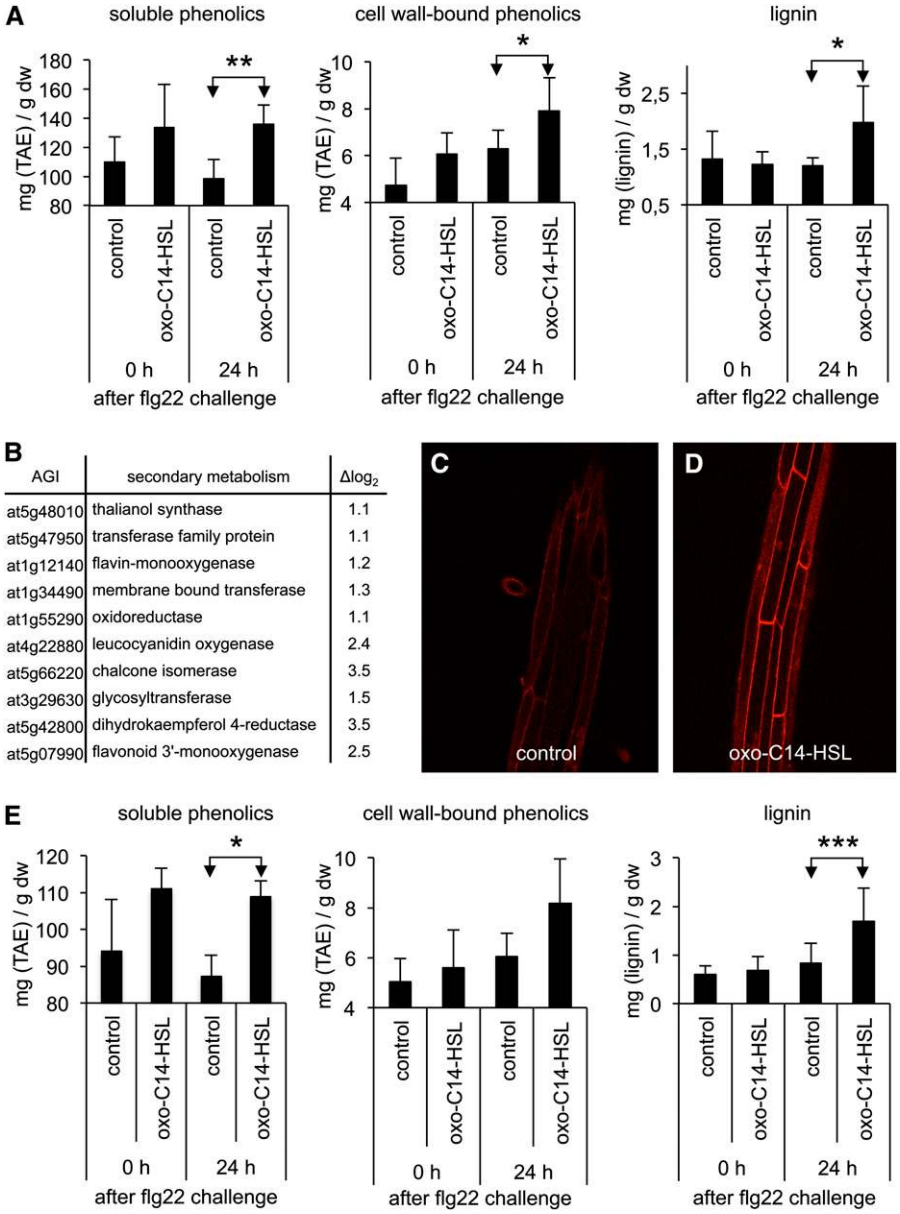

Figure 3. Application of Oxo-C14-HSL Has an Impact on Phenol-Related Metabolism.

(A) Colorimetric measurement of soluble phenolic compounds, cell wall–bound phenolic compounds, and lignin using the Folin-Ciocalteau method. Two-week-old *Arabidopsis* seedlings were pretreated with 6 μM oxo-C14-HSL or acetone (the solvent control) and subsequently challenged with 100 nM flg22 for 24 h. Both phenolic fractions were normalized to tannic acid equivalent (TAE). Quantification of lignins was done using alkaline lignin as a standard. *P < 0.05 and **P < 0.005 in Student's *t* test. Error bars represent SD.

(B) Genes assigned to the functional category "secondary metabolism" upregulated in oxo-C14-HSL–pretreated seedlings 24 h after challenge with 100 nM flg22 as revealed by the microarray analysis. The numbers represent the $\log_2$ fold change relative to the solvent control.

(C) and **(D)** Fluorescence of phenolic compounds in roots pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d. Experiments were performed five times with $n = 30$. Root tissue was excited at 488 nm, and the emitted light was collected between 577 and 624 nm.

(E) Phenolic compounds in distal tissues. Measurements performed as in **(A)**, except that leaves were from plants grown in sterile systemic hydroponic culture. Roots of those plants were pretreated with 6 μM oxo-C14-HSL for 3 d, and leaves were subsequently challenged with 1 μM flg22. The experiment was performed five times. *P < 0.05 and ***P < 0.0005 in Student's *t* test. Error bars represent SD.

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Oxo-C14-HSL–Induced Resistance Requires NPR1 and Is COI1 Independent

In the following experiments, we extended our studies of MTI to the more complex pathogen resistance induced by the application of oxo-C14-HSL shown in Schikora et al. (2011). Using the biomarker bacteria *Pseudomonas putida* FG117 pKR C12 GFP [*lasR*$^{+}$*lasB::gfp (ASV)*] (Steidle et al., 2001), oxo-C14-HSL was not detectable in leaves if added to roots (Schikora et al., 2011), suggesting that a systemic signal(s) is involved in AHL-induced resistance rather than the AHL itself. In this study, we tested the hypothesis that oxo-C14-HSL acts via a systemic signal. Hence, we sought to uncover the mechanism of this phenomenon by analyzing mutants compromised in the perception of two major defense-related plant hormones SA and JA. We compared the oxo-C14-HSL–induced resistance against *P. syringae* DC3000 pv *tomato* (*Pst*) in Columbia-0 (Col-0) wild-type plants and mutants impaired in either SA signaling (*npr1-1*) or JA perception (*coi1-16*) (Cao et al., 1994; Pozo et al., 2008). We used the sterile systemic hydroponic system to pretreat roots with oxo-C14-HSL and subsequently inoculated leaves with *Pst*. Bacterial proliferation was assessed during 96 h after inoculation (HAI) by counting colony-forming units. Unlike Col-0 wild-type plants (Figures 4A and 4B), the *npr1-1* mutant showed no difference in *Pst* proliferation in the absence of pretreatment or after oxo-C14-HSL or acetone pretreatments, suggesting that AHL-induced resistance requires NPR1 (Figure 4C). In contrast, oxo-C14-HSL pretreatment enhanced the resistance against *Pst* in the JA-insensitive *coi1-16* mutant (Figure 4D), in a manner similar to Col-0 plants. To substantiate this finding, we assessed the AHL-induced resistance in the *jar1-1* mutant, which is compromised in the synthesis of JA-Ile, the active compound in JA signaling (Staswick, 2008). Similar to Col-0 wild type and the *coi1-16* mutant, *jar1-1* showed AHL-induced priming (Figure 4E), demonstrating that oxo-C14-HSL-induced resistance is independent of JA.

cis-OPDA Is Involved in AHL-Induced Resistance

We measured the content of resistance-related hormones SA, JA, JA-Ile, and *cis*-OPDA in leaves of plants whose roots were pretreated with oxo-C14-HSL for 3 d prior to foliar inoculation with *Pst*. Application of oxo-C14-HSL had no effect on the relative accumulation of any of the plant hormones (Figure 5A), while inoculation with *Pst* induced accumulation of all hormones except JA-Ile. Importantly, leaves of plants challenged with *Pst* accumulated more SA and *cis*-OPDA if their roots were pretreated with oxo-C14-HSL (Figure 5A). Accumulation of SA in leaves primed with oxo-C14-HSL was consistent with the compromised AHL-induced resistance in the *npr1-1* mutant. The enhanced accumulation of *cis*-OPDA indicated that oxylipins are also involved in AHL-induced resistance. Oxylipins are known to function in different processes, including defense responses (Blée, 2002). To assess the function of oxylipins in oxo-C14-HSL–induced resistance, we analyzed the expression of selected OPDA-dependent, JA-independent genes encoding glutathione *S*-transferase 6 (GST6), and heat shock protein 70 (HSP70) (Mueller et al., 2008). The expression of *GST6* and *HSP70* was enhanced in the leaves of plants pretreated with oxo-C14-HSL when compared with control plants (Figures 5B and 5C). The AHL effect was especially strong at 24 HAI, which was in agreement with the enhanced

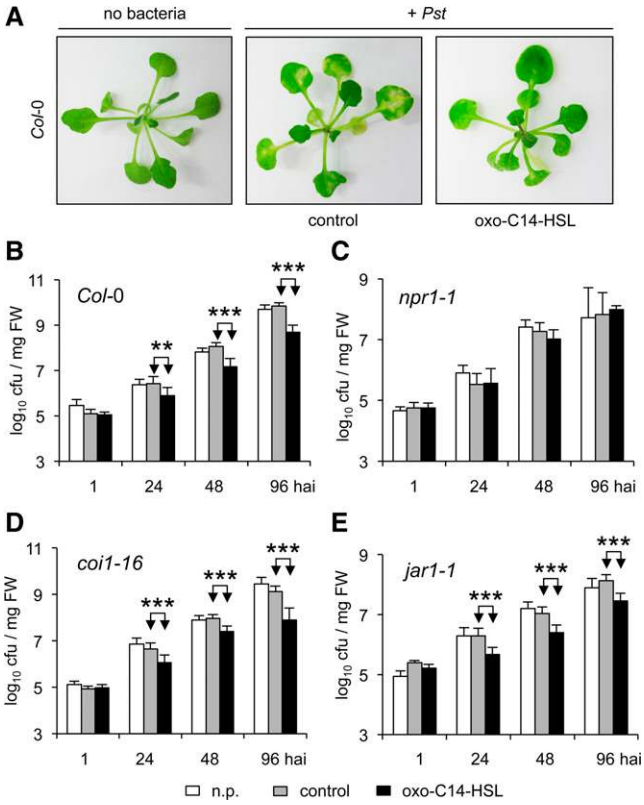

Figure 4. Resistance Induced by Oxo-C14-HSL Is JA Independent and NPR1 Dependent.

(A) Symptoms of *Pst* infection on wild-type Col-0 plants primed with AHL (oxo-C14-HSL) or pretreated with acetone (control).
(B) to **(E)** Pathogenicity assays with *Pst* on *Arabidopsis* Col-0 wild type and *npr1-1*, *coi-1-16*, and *jar1-1* mutant plants grown for 5 weeks in the sterile systemic hydroponic system. Plant roots were pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d or not pretreated (n.p.), and the leaves were subsequently spray-inoculated with *Pst* (OD_{600} = 0.1). Colony-forming units (cfu) were counted 1, 24, 48, and 96 HAI. **P < 0.005 and ***P < 0.0005 in Student's *t* test. Error bars represent SD.

accumulation of *cis*-OPDA at this time point after the secondary challenge with *Pst* (Figure 5A). To further test the hypothesis that oxylipins are involved in AHL priming, we examined the AHL-induced resistance against *Pst* in two *Arabidopsis* mutants, *lox2-1*, harboring a mutation in lipoxygenase 2 (the key enzyme in oxidation of unsaturated fatty acids leading to oxylipins) and the triple *tga2/5/6* mutant with mutations in *TGA2*, *TGA5*, and *TGA6*, which encode bZIP transcription factors involved in oxylipin-regulated responses (Mueller et al., 2008; Stotz et al., 2013). We observed no difference between the controls and plants pretreated with oxo-C14-HSL (Figures 5D and 5E), indicating that disruption of oxylipin biosynthesis or signaling eliminated the AHL effect. In summary, these results indicate that oxylipins have a key function in AHL-induced resistance.

AHL-Induced Resistance Requires the Conversion of SA to MeSA

We addressed the question of whether the effect of AHL in systemic tissues resembles the canonical systemic resistances and

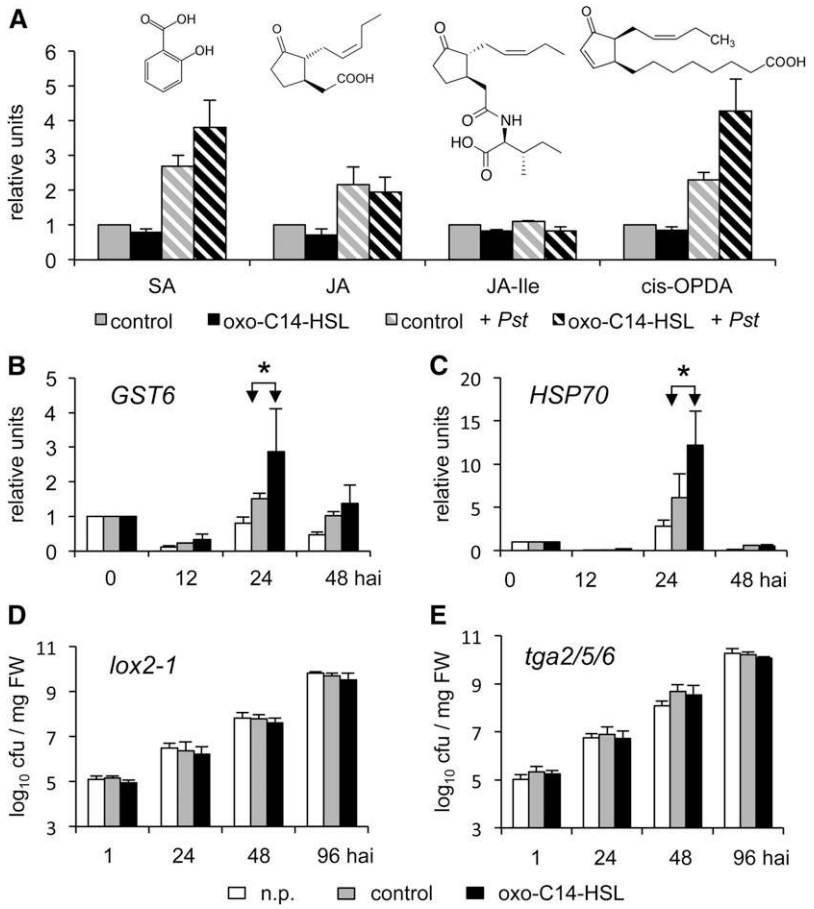

Figure 5. Oxylipins Are Involved in Systemic AHL-Induced Resistance.

(A) Accumulation of hormones determined by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry in *Arabidopsis* plants in which roots were pretreated with 6 μM oxo-C14-HSL for 3 d and leaves were subsequently spray-inoculated with *Pst*. Leaves were harvested at 24 HAI.

(B) and **(C)** Relative expression of *cis*-OPDA–regulated genes. *Arabidopsis* plants were grown in the sterile systemic hydroponic system for 5 weeks, pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d, or not pretreated (n.p.) and inoculated with *Pst* for the number of hours indicated. The relative level of mRNA was determined via quantitative RT-PCR using the expression of ubiquitin ligase *At5g25760* for normalization. *$P < 0.05$ in Student's *t* test. Error bars represent the SE.

(D) and **(E)** Pathogenicity assays on the *Arabidopsis lox2-1 mutant* **(D)** and the *tga2/5/6* triple mutant **(E)** challenged with *Pst*. Plants were grown for 5 weeks in the sterile systemic hydroponic system. The roots were pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d or not pretreated (n.p.). Colony-forming units (cfu) in leaves were counted 1, 24, 48, and 96 HAI. Error bars represent SD.

therefore requires either the conversion of SA to MeSA, which is important for SAR, or the transcription factor MYB72, which is required for ISR. For this purpose, we analyzed the expression of the two OPDA-regulated, oxo-C14-HSL–induced genes *HSP70* and *GST6* (Figures 5B to 5E) in two independent *myb72* mutants (*myb72-1* and *myb72-2*) impaired in ISR (Van der Ent et al., 2008; Segarra et al., 2009) and plants impaired in SAR, including the *bsmt1* mutant, which is deficient in benzoic acid/SA carboxyl methyltransferase 1 (Liu et al., 2010), and *med4-1*, a MeSA esterase knockdown line for multiple gene family members (Liu et al., 2011). The enhanced expression of *HSP70* and *GST6* in both *myb72* mutants pretreated with oxo-C14-HSL after *Pst* inoculation (Figure 6A) indicated that the MYB72-dependent pathway is not involved in AHL-induced resistance. However, in the *bsmt1* mutant and the knockdown *med4-1* line, the expression of *HSP70* and *GST6* was not enhanced in response to *Pst* (Figure 6A). To extend these findings, we analyzed the effect of AHL on resistance against *Pst* using the sterile systemic hydroponic system. Proliferation of *Pst* bacteria was assessed 24 HAI (Figure 6B). Consistent with the gene expression above, Col-0 and both *myb72* mutants showed reduced numbers of colony-forming units if pretreated with the AHL, whereas in the *bsmt1* and *med4-1* plants, the AHL-induced resistance was lost (Figure 6B). In summary, these results provide evidence that oxo-C14-HSL–induced systemic resistance depends on the conversion between SA and MeSA as well as on oxylipins, but not on MYB72.

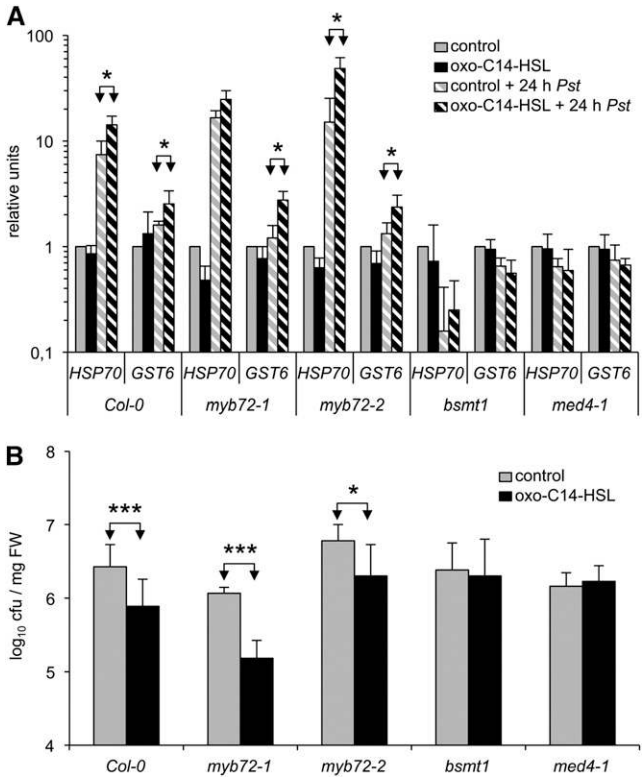

Figure 6. AHL-Induced Resistance Depends on SA Demethylation and Methylation but Not on the ISR Pathway.

(A) Expression pattern of oxylipin-regulated genes in ISR and SAR compromised plants. The roots of two *MYB72* mutants (*myb72-1* and *myb72-1*), a knockdown line harboring four RNA interference–silenced MeSA esterases (*med4-1*), a mutant in benzoic acid/SA carboxyl methyltransferase 1 gene (*bsmt1*), and the wild type Col-0 were pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d prior to inoculation with *Pst* for 24 h. Total RNA was extracted from leaves and quantitative RT-PCR was performed using the expression of ubiquitin ligase *At5g25760* for normalization. Values were normalized to the 0 h time point. Data represent the mean from three independent biological replications. Error bars represent SE. *P < 0.05 in Student's *t* test.

(B) Pathogenicity assays on *Arabidopsis myb72-1*, *myb72-2*, and *bsmt1* mutants and the knockdown line *med4-1* challenged with *Pst*. Plants were grown for 5 weeks in the sterile systemic hydroponic system. Roots were pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d. Colony-forming units (cfu) were counted in leaves at 24 h after foliar inoculation. *P < 0.05 and ***P < 0.0005 in Student's *t* test. Error bars represent the SD.

Stomatal Closure Is Involved in AHL-Induced Resistance against Bacteria

The function of stomatal closure in plant innate immunity is well understood and defined as the stomatal defense response (Melotto et al., 2008; Zeng et al., 2010). Recently, a study of flg22-induced stomatal closure suggested that this phenomenon depends on an OPDA- and SA-regulated pathway (Montillet et al., 2013). Since the AHL-induced resistance also depends on OPDA and SA, we assessed whether AHL pretreatment could trigger the stomata defense response. For this purpose, we counted the number of open and closed stomata in regions of leaves of plants pretreated with oxo-C14-HSL or the solvent control and subsequently challenged the plants with *Pst* for 2 or 24 h. The pretreatment with AHL alone had no impact on stomatal closure (Figure 7A). However, inoculation with *Pst* caused the closure of nearly 50% of the stomata in control plants at 2 or 24 HAI. In plants pretreated with oxo-C14-HSL, 60 and 70% of the stomata were closed at 2 and 24 HAI, respectively (Figures 7A to 7D). To further corroborate these results, we made use of the observation that stomatal closure causes an increase in leaf temperature (Hopkins and Hüner, 2009) and performed infrared imaging on plants pretreated with oxo-C14-HSL or acetone (control) prior to challenge with *Pst* for 24 h. The observed differences, represented in color-coded temperature images, confirmed a temperature shift between the control (20.0 to 20.5°C; dark blue) and oxo-C14-HSL–pretreated plants (20.5 to 21°C; light blue) (Figures 7E to 7H), consistent with a higher proportion of closed stomata. In summary, the increased stomatal closure in AHL-primed plants could be a second basis for the enhanced resistance against the pathogenic bacteria.

DISCUSSION

AHL Molecules Trigger Specific Transcriptional Reprogramming

The biological activity of AHLs in plants depends on the length of their lipid chains. AHLs with short chain (4 to 10 carbons) promote growth (Gao et al., 2003; von Rad et al., 2008; Liu et al., 2012); C10-HSL was found to alter root architecture in *Arabidopsis* (Ortíz-Castro et al., 2008) and after substitution with a ketone group at the C3 position, the derivative, oxo-C10-HSL, was capable of inducing adventitious roots in mung bean (*Vigna radiata*) (Bai et al., 2012). On the other hand, long-chain AHLs (12 to 16 carbons) were shown to induce resistance in *Medicago truncatula*, *Arabidopsis*, and barley plants (Mathesius et al., 2003; Schikora et al., 2011; Zarkani et al., 2013). Besides the numerous effects of the different AHLs on plant development, a few studies have reported global transcriptional changes upon AHL treatment. In *Arabidopsis* treated with the short-chain C6-HSL, 721 genes were differentially expressed in leaves and 1095 in root tissues (von Rad et al., 2008). These genes are mainly related to metabolism, translation, cell wall modification, and hormones, especially auxin and cytokinins. A proteome analysis of *M. truncatula* treated with two long-chain AHLs, oxo-C12-HSL and oxo-C16-HSL, showed changes in the quantity of 150 proteins related to plant defense, stress responses, protein degradation, flavonoid synthesis, the cytoskeleton, and various primary metabolic activities (Mathesius et al., 2003). Recently, a proteomic profile of *Arabidopsis* seedlings responding to oxo-C8-HSL revealed differential regulation of 53 proteins related to primary metabolism and energy status as well as to the cytoskeleton and defense (Miao et al., 2012). The data presented here provide a direct comparison of the changes in the *Arabidopsis* transcriptome after application of three AHLs (C6-HSL, oxo-C10-HSL, and oxo-C14-HSL). They revealed that around one-fifth of the differentially regulated genes (55 genes) are regulated in response to all three molecules. Functional annotation showed that many of those genes are related to the biotic stress response. Among them, G-proteins and proteins

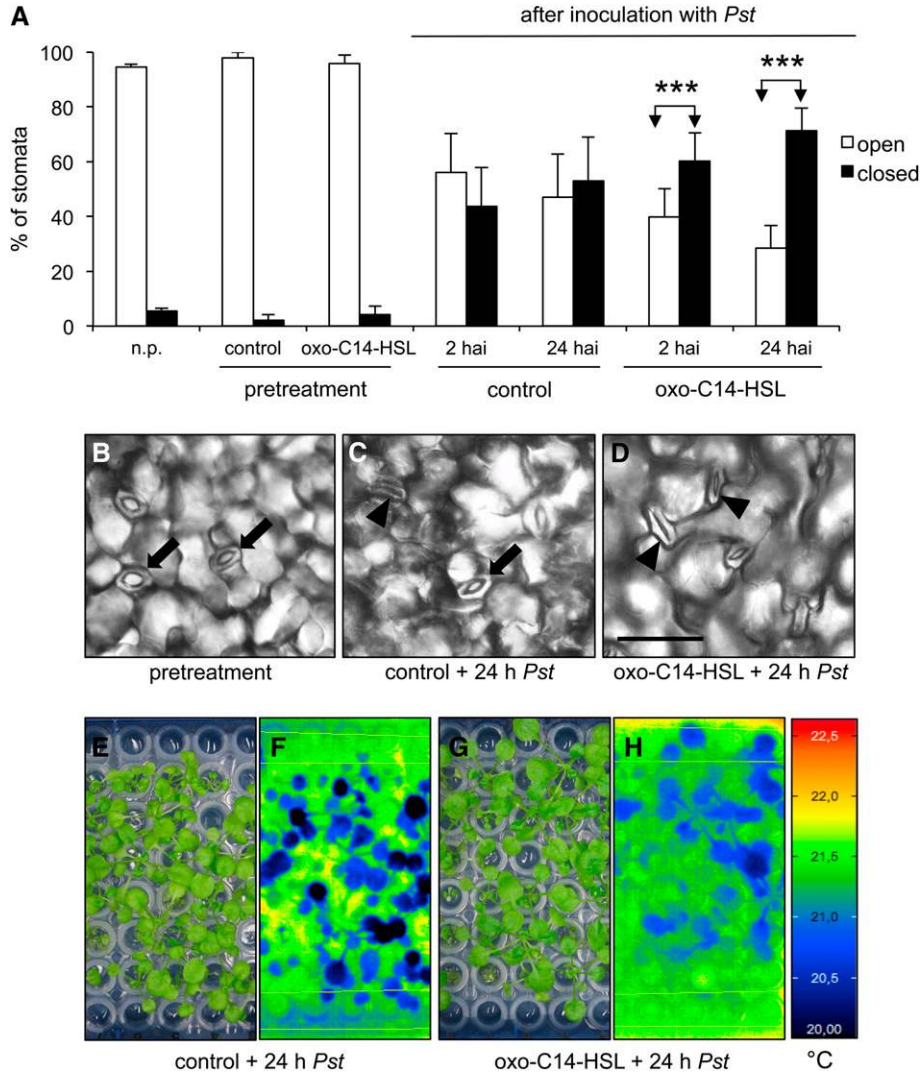

Figure 7. AHL-Induced Defense Acts via Stomatal Closure.

(A) The percentage of open and closed stomata was assessed in leaves of plants grown for 5 weeks in the sterile systemic hydroponic system and pretreated with 6 μM oxo-C14-HSL or acetone (control) for 3 d (pretreatment), followed by inoculation with *Pst* for 2 or 24 h. The experiment was performed three times, always in the light, with $n = 80$ in each treatment. ***$P < 0.0005$ in Student's t test.

(B) to **(D)** Exemplary images of stomata counted as described in **(A)**. Arrows point toward open stomata and arrowheads toward closed stomata. Bar = 50 μm.

(E) Assessment of temperature *via* infrared light images in leaves of plants treated as described in **(A)** and exposed to *Pst* bacteria for 24 h.

involved in calcium signaling were already proposed to play a role in the plant's response to AHLs (Song et al., 2011; Liu et al., 2012). Nonetheless, the majority of the differentially expressed genes were regulated by only one AHL (~200 genes by each AHL molecule). This suggests a very specific response and probably underlines the different macroscopic impact of these molecules on plants. The absence of enriched gene ontology terms related to hormones was remarkable, since others have reported AHL-induced changes in auxin level (Mathesius et al., 2003; von Rad et al., 2008; Bai et al., 2012). Nonetheless, in the case of the C6-HSL– and oxo-C10-HSL–regulated genes (both molecules modify root length and architecture), we observed the upregulation of numerous MADS box and other transcription factors, which could promote growth or alter root morphology. Furthermore, we observed that the AHL action in plants was timely restricted. A long-term experiment performed during this study revealed no significant alterations in plants treated with AHLs for 3 weeks or longer, implying either a transient effect or a saturation of the response system (Supplemental Figure 5).

Oxo-C14-HSL Primes Plants for Cell Wall Reinforcement

The plant cell wall is a dynamic arrangement of proteins, sugars, and phenols with a substantial structural complexity that plays an essential role in plant defense (Hückelhoven et al., 1999; Wei and

Shirsat, 2006; Hückelhoven, 2007; Deepak et al., 2010). Previously, we observed enhanced formation of papillae in barley plants pretreated with oxo-C14-HSL and challenged with the pathogenic fungus *Blumeria graminis* f sp *hordei* (Schikora et al., 2011). The formation of papilla results from the accumulation and reactive oxygen species–induced cross-linkage of glycoproteins and on deposition of callose and phenolic compounds at the formation site (Hückelhoven, 2007; Deepak et al., 2010). In this study, we observed an enrichment of genes in the cell wall and glycoproteins functional categories in oxo-C14-HSL–pretreated plants, particularly after challenge with flg22. Furthermore, we observed an increase of callose deposition, phenolic compounds, and lignins in flg22-challenged, AHL-primed plants. Together, these results suggest that AHL application primed plants for cell wall reinforcement, which could explain their enhanced resistance to bacterial pathogens.

Systemic Resistance in AHL-Primed Plants

In contrast to C6-HSL, long-chain oxo-C14-HSL is not transported within *Arabidopsis* plants (Schikora et al., 2011). A recent uptake and transport study of radioactive C8-HSL and C10-HSL showed that the intermediate-length lipid chain AHLs are taken up and transported into the lower shoot zone, although not into the upper leaf tissues of barley plants (Sieper et al., 2013). Therefore, a likely explanation of oxo-C14-HSL–induced resistance in distal tissue is a systemic induced resistance. The two best understood mechanisms of systemic resistance, ISR and SAR, depend on JA/ET and SA, respectively. However, JA- and SA-independent induction of resistance has also been reported (Ryu et al., 2003). Here, we speculated whether JA/ET and/or SA are involved in the establishment of AHL-induced resistance. On one hand, several lines of evidence indicate JA independence, including the induced resistance in *coi1-16* and *jar1-1* mutants and the expression of JA-responsive *MYC2* and *VSP2* genes, which was not affected by AHL application (Supplemental Figure 6). Similarly, the accumulation of JA or its bioactive derivative JA-Ile was not influenced by AHL pretreatment. Moreover, the enhanced expression of *HSP70* and *GST6* in both *myb72* mutants after pretreatment with oxo-C14-HSL as well as the lack of enhanced expression of the *MYB72* or ET-responsive genes in AHL-primed wild-type plants (Supplemental Figures 6 and 7) indicate that JA and the ISR mechanisms are not involved in oxo-C14-HLS–induced resistance. On the other hand, the loss of the AHL effect in the *npr1-1* mutant, the effect of oxo-C14-HSL on SA accumulation after inoculation with *Pst*, and the expression of *PR1* after oxo-C14-HSL pretreatment (Supplemental Figure 6) suggest that SA might be involved in AHL-induced resistance. However, the derivative MeSA and not SA per se was postulated to be the mobile signal (Vernooij et al., 1994; Park et al., 2007). Therefore, we monitored the expression of oxo-C14-HSL–induced genes in plants impaired in MeSA accumulation (*bsmt1*) (Liu et al., 2010) and its conversion back to SA (*med4-1*) (Liu et al., 2011). The oxo-C14-HSL–induced gene expression is lost in both mutants, suggesting the importance of MeSA and the conversion between MeSA and SA. Usually, SAR is associated with the expression of *Pathogenesis Related* (*PR*) genes. In addition, the *PAL* gene encoding phenylalanine-ammonia lyase is highly expressed in benzothiadiazole-primed plants after a subsequent challenge (Kohler et al., 2002). Nonetheless, neither *PAL* nor other SA-regulated genes (*PR2* and *PR5*) (Supplemental Figures 6 and 7) were differentially regulated in plants pretreated with AHL and inoculated with *Pst*, suggesting that the AHL-induced resistance could differ from the typical SA-dependent response. Recently, oxylipins (JA precursors) were postulated to be involved in stress responses (Stintzi et al., 2001; Blée, 2002; Taki et al., 2005). Besides an accumulation of SA, we observed an increased accumulation of the oxylipin *cis*-OPDA. OPDA has been shown to induce a set of genes that are COI1 independent (Stintzi et al., 2001; Mueller et al., 2008) and require the bZIP transcription factors TGA2, TGA5, and TGA6 (Stotz et al., 2013). Similarly, AHL-induced resistance required LOX2 lipoxygenase and TGA2/5/6 transcription factors and was independent of COI1. Moreover, the enhanced expression of OPDA-regulated genes (*GST6* and *HSP70*) (Mueller et al., 2008) in AHL-primed plants supports the notion that oxo-C14-HSL–induced resistance depends on OPDA. The three cytosolic lipoxygenases (*LOX2*, *LOX3*, and *LOX4*) involved in OPDA biosynthesis were not differentially expressed, suggesting that LOX activity is sufficient for the AHL effect (Supplemental Figure 8).

A recently published study suggested that OPDA and SA are involved in flg22-induced stomatal closure (Montillet et al., 2013). Pathogen-induced stomatal closure plays a central function in innate immunity, since it controls one of the major bacterial entry routes into the plant (Melotto et al., 2008; Zeng et al., 2010). Abscisic acid (ABA) usually regulates stomatal closure; however, flg22-induced stomatal closure appears to be independent of ABA (Montillet et al., 2013). A similar situation was observed in plants treated with oxo-C14-HSL, which show enhanced stomatal closure after *Pst* inoculation, while the expression of genes regulated by ABA (*RD22*, *RD29*, and *RAB18*) was not affected (Supplemental Figure 6). Based on these and the above observations, we postulate that the effect of AHL on stomata depends on the oxylipin-SA pathway. Moreover, the higher percentage of closed stomata in AHL-primed plants suggests that the JA-independent AHL priming possibly protects the plant from the bacterial toxin coronatine, which induces reopening of stomata and depends on JA perception (Melotto et al., 2006). Interestingly, mycorrhizal roots typically allow high colonization densities of selected plant growth–promoting rhizobia strains, a phenomenon commonly referred to as "the mycorrhizosphere effect." It was recently suggested that these mycorrhizosphere bacteria could contribute to mycorrhiza-induced resistance (Cameron et al., 2013). In this context, it is tempting to speculate that high densities of mycorrhizophere bacteria can give rise to the production of resistance-inducing long-chain AHLs.

Overall, our results and those of others suggest that plants respond to the presence of different AHL molecules in a specific way. The resistance inducing effect of the long-chain AHLs is based on reinforcement of the cell wall with callose, phenolic compounds, and lignins as well as enhanced stomatal closure, and the latter effect is driven by the increased concentration of *cis*-OPDA and SA. Taken together, we present here insight into the role of quorum-sensing molecules in the interaction between plants and bacteria and propose a mechanistic explanation of how those molecules influence plant resistance against bacterial pathogens.

METHODS

Plant Growth

Arabidopsis thaliana Col-0 (N60000) and *Arabidopsis* mutant plants were grown in three different systems. For microarrays and local-induced experiments, seeds were surface-disinfected and grown on half-strength MS plates for 2 weeks. Thereafter, seedlings were transferred to six-well plates with 5 mL half-strength MS per well for the AHL (pre)treatment and the subsequent challenge with 100 nM flg22. Whole seedlings were harvested for extractions of total RNA or phenolic compounds. For all systemic analyses, *Arabidopsis* plants were grown in a sterile systemic hydroponic system, which allows the separation of roots and shoots. Surface-disinfected seeds (3 min with 50% ethanol/0.5% Tritron X-100 mix and briefly rinsed with 95% ethanol) were germinated and grown for 5 weeks at 22°C with 150 μmol/m^2/s light in 8/16-h day/night photoperiod. Seeds were directly planted onto perforated 96-well plates filled with half-strength MS medium supplemented with 0.5% agar. Plates were placed on 200 mL liquid half-strength MS in sterile boxes. Medium was exchanged every third week. For the callose deposition experiment, *Arabidopsis* plants were grown on soil under short-day conditions (8/16-h day/night photoperiod) at 21°C for 5 weeks and used to obtain detached leaves or plants were grown as for systemic analyses.

AHL Treatment

Plants were pretreated with AHLs: C6-HSL, oxo-C10-HSL, or oxo-C14-HSL (Sigma-Aldrich) for 3 d. AHLs were dissolved in acetone as 60 mM stocks and used at a concentration of 6 μM or lower if indicated (Supplemental Figure 9). All experiments were performed with the solvent (acetone) as a control at an end dilution of 1:10,000. Additionally, in pathogenicity and quantitative RT-PCR tests, a nonpretreated control was included.

Microarrays

Total RNA was extracted with the RNeasy plant mini kit 74903 (Qiagen) and purified with the RNeasy kit 74104 (Qiagen). RNA quality checks were performed using the Agilent 2100 Bioanalyzer. The microarray experiment was performed using the *Arabidopsis* (4x44k) Gene Expression Microarray from Agilent Technology with the Low Input Quick Amp Labeling Kit One-Color and the Gene Expression Hybridization Kit from Agilent. The labeling and hybridization procedures were performed according to the One-Color Microarray-Based Gene Expression Analysis Low Input Quick Amp Labeling Protocol from Agilent Technology. The image files of the scanned microarrays were analyzed using Agilent's Feature Extraction software version 10.7.3.1. To assess the quality of the slides, diagnostic plots were generated. For each spot, the median signal and background intensities were obtained. Systematic biases within the data were standardized using quantile normalization. To select differentially expressed genes between different treatments, the fold change and the moderate t-statistic implemented in the limma package of Bioconductor were calculated. Differentially expressed genes were selected based on a moderate P value of 0.05 and a $\log_2$ fold change of 1. The R environment software (http://www.r-project.org/) was used for data analysis. Gene Ontology terms analysis was performed with the help of MapMan (Thimm et al., 2004). The microarray experiment consisted of three independent biological replications and was validated using quantitative RT-PCR of 16 selected genes. The data are stored in Gene Expression Omnibus (accession number: GSE52979) and comply with MIAME (minimal information about a microarray experiment) guidelines.

Callose Deposition

Detached leaves from 5-week-old soil-grown *Arabidopsis* plants were floated in half-strength MS media supplemented with 6 μM oxo-C14-HSL or acetone for 3 d. After pretreatment, leaves were challenged with 100 nM flg22, briefly vacuum infiltrated, and incubated on wet filter paper for 24 h. The leaves were destained in 1:3 acetic acid/ethanol for 24 h and thereafter stained with 0.01% aniline blue in 150 mM K_2POH_4, pH 9.5. Callose deposition assays in distal tissues were performed on leaves of plants grown for 5 weeks in the sterile systemic hydroponic system, where roots were pretreated with oxo-C14-HSL for 3 d and leaves subsequently challenged with 1 μM flg22 for 24 h.

Phenolic Compound Extraction

Two-week-old *Arabidopsis* seedlings were pretreated with 6 μM oxo-C14-HSL or acetone in half-strength MS liquid media for 3 d and subsequently challenged with 100 nM flg22. For distal tissues, phenolic compounds were measured in leaves of plants grown for 5 weeks in the sterile systemic hydroponic system, in which roots were pretreated with oxo-C14-HSL for 3 d and leaves subsequently challenged with 1 μM flg22 for 24 h. Samples were taken 0 and 24 h after challenge, freeze-dried, and crushed with metal beads in a TissueLyser. All phenolic compounds were measured via a colorimetric assay based on the Folin-Ciocalteau method (Eynck et al., 2009) with some modifications. The soluble phenolic compounds were extracted twice with 80% aqueous methanol for 1 h. The phenolic compounds were measured in the merged supernatants. The cell wall–bound phenolic compounds in the remaining pellet were washed with subsequently: 80% aqueous methanol, distilled water, and acetone. Pellets were dried and alkaline hydrolysis was performed with 1 M NaOH at 80°C for 1 h with continuous shaking. After 12 h incubation at room temperature, the solution was acidified with 86% H_3PO_4 and incubated for 30 min with ethyl acetate. The samples were centrifuged and the supernatant collected. After a second incubation with ethyl acetate, the supernatants were merged and dried. Residual pellet was resuspended in 80% methanol. All phenolic compounds were measured via a colorimetric assay based on the Folin-Ciocalteau method.

Lignin Extraction and Quantification

Cell walls after extraction of bound phenolic compounds were washed sequentially with 80% methanol, distilled water, and acetone, followed by drying. The pellets were dissolved in 2 M HCl and thioglycolic acid, incubated for 4 h at 95°C with continuous shaking, and centrifuged for 5 min at 13,000*g* and 4°C. The supernatant was discarded. The remaining pellets were washed twice with water and incubated in 0.5 M NaOH for 12 h. After centrifugation, the supernatant was collected and 0.5 M NaOH was readded to the residues for repeated incubations. Pooled supernatants were acidified with 32% HCl and precipitated as lignothioglycolic acid complex. Samples were incubated for 4 h at 4°C and centrifuged, and the lignin pellets were resuspended in 0.5 M NaOH. The lignin content was measured at 340 nm. Quantification was done according to a calibration curve obtained for alkaline lignin.

Hormone Measurements

Arabidopsis Col-0 plants grown in the sterile systemic hydroponic system for 5 weeks were inoculated with *Pseudomonas syringae* DC3000 pv *tomato* (OD_{600} = 0.1) for 0 and 24 h and frozen in liquid N_2. Frozen tissue (250 mg) was used for hormone extraction and quantification via liquid chromatography–tandem mass spectrometry according to Nakamura et al. (2013) with some modifications. Finely ground leaf material was extracted with 1 mL of methanol containing 40 ng of 9,10-D_2-9,10-dihydrojasmonic acid, 40 ng D_4-salicylic acid (Sigma-Aldrich), 40 ng D_6-abscisic acid (Santa Cruz Biotechnology), and 8 ng of jasmonic acid-^{13}C6-isoleucine conjugate as internal standards. Jasmonic acid-$^{13}C_6$-isoleucine conjugate was synthesized as described by Kramell et al. (1988) using $^{13}C_6$-Ile (Sigma-Aldrich). The homogenate was mixed for 30 min and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was collected. The homogenate was reextracted with 500 μL methanol, mixed, and

centrifuged, and supernatants were pooled. The combined extracts was evaporated in speed-vac at 30°C and redissolved in 500 μL methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm; Agilent). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0 to 0.5 min, 5% B; 0.5 to 9.5 min, 5 to 42% B; 9.5 to 9.51 min 42 to 100% B; 9.51 to 12 min 100% B, and 12.1 to 15 min 5% B. The mobile phase flow rate was 1.1 mL/min. The column temperature was maintained at 25°C. An API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards, where available. The ion spray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700°C. Nebulizing gas was set at 60 p.s.i., curtain gas at 25 p.s.i., heating gas at 60 p.s.i., and collision gas at 7 p.s.i. Multiple reaction monitoring was used to monitor analyte parent ion → product ion: *m/z* 136.9 → 93.0 (collision energy [CE] −22 V; declustering potential [DP] −35 V) for salicylic acid; *m/z* 140.9 → 97.0 (CE −22 V; DP −35 V) for D_4-salicylic acid; *m/z* 209.1 → 59.0 (CE −24 V; DP −35 V) for jasmonic acid; *m/z* 290.1 → 165.1 (CE −24 V; DP −45 V) for 12-oxo-phytodienoic acid; *m/z* 213.1 → 56.0 (CE −24 V; DP −35 V) for 9,10-D2-9,10-dihydrojasmonic acid; *m/z* 322.2 → 130.1 (CE −30V; DP −50 V) for JA-Ile conjugate; *m/z* 328.2 → 136.1 (CE −30 V; DP −50 V) for jasmonic acid-13C6-isoleucine conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of 12-oxo-phytodienoic acid, *cis*-OPDA, 9,10-D_2-9,10-dihydrojasmonic acid was used as the internal standard, applying an experimentally determined response factor of 1.0.

Pathogenicity Tests

Plants grown in the sterile systemic hydroponics system were spray inoculated with *Pst* grown overnight in King's B medium supplemented with rifampicin (50 mg/mL), washed in 10 mM $MgSO_4$, and adjusted to an optical density (OD_{600}) = 0.1 in 10 mM $MgSO_4$ with 0.02% Silwet 77. After 1, 24, 48, and 96 h, 100 mg of leaves was harvested and homogenized. Serial dilutions of the homogenate were plated onto King's B media plates with selective antibiotics (rifampicin and kanamycin 50 mg/mL). Colony-forming units were counted after 2 d. Each of three independent biological experiments was performed using four technical repetitions.

Quantitative RT-PCR

Total RNA extraction was performed using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA concentration was measured photometrically using NanoDrop (Thermo Fisher Scientific). Two micrograms of total RNA from each sample was treated with DNase-I (Quanta BioScience). cDNA was synthesized using the qScript cDNA Synthesis Kit (Quanta BioScience). The efficiency of the reverse transcription was verified with semiquantitative amplification of the *actin2* transcript. Quantitative RT-PCR was performed using the Applied Biosystems 7500 real-time PCR system using primers listed in Supplemental Table 1. Values were normalized to the housekeeping gene *At5g25760* (ubiquitin ligase) and to the 0 HAI time point.

Stomata Closure

Arabidopsis Col-0 plants grown in the sterile systemic hydroponic system for 5 weeks were pretreated with 6 μM oxo-C14-HSL or acetone (control) and inoculated with *Pst* bacteria. Stamata were observed 2 or 24 h after inoculation. Counting of closed or open stomata was performed after at least 6 h of light.

Accession Numbers

The accession numbers for the genes discussed in this article are as follows: *PR1* (At2g14610), *Pdf1.2* (At5g44420), *GST6* (At2g47730), *HSP70* (At3g12580), *VSP2* (At5g24770), *MYC2* (At1g32640), *PR2* (At3g57260), *PR5* (At1g75040), *RD29* (At5g52300), *RD22* (At5g25610), *RAB18* (At5g66400), *MYB72* (At1g56160), *PAL1* (At2g37040), *LOX2* (At3g45140), *LOX3* (At1g17420), *LOX4* (At1g72520), *ETR1* (At1g66340), *ERF5* (At5g47230), *PR3* (At3g12500), *XTR9* (At4g25820), *XTH26* (At4g28850), *EXP22* (At5g39270), *flavonoid 3′-monooxygenase* (At5g07990), *flavin-monooxygenase* (At1g12140), *UDP-Glycosyltransferase* (At3g29630), *actin2* (At3g18780), and *ubiquitin ligase* (At5g25760). The microarray data are stored in the Gene Expression Omnibus under accession number GSE52979.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. "Response to Biotic Stress" Is the Gene Ontology Term with the Highest Number of Genes Regulated upon AHL Treatment.

Supplemental Figure 2. Response to a Secondary Challenge in Oxo-C14-HSL–Pretreated Plants Varies from the Response in Non-Pretreated Plants.

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Supplemental Figure 4. Hormones-Associated Gene Ontology Terms Are Not Enriched within the Differentially Expressed Gene Groups.

Supplemental Figure 5. Long-Term Observation of the Influence of AHLs on Plants Development and Defense System.

Supplemental Figure 6. Pretreatment with Oxo-C14-HSL Has No Impact on the Expression of JA-, SA-, ET-, and ABA-Regulated Genes.

Supplemental Figure 7. Neither the *MYB72* nor the *PAL1* Gene Was Induced upon AHL Priming.

Supplemental Figure 8. Expression of *LOX* Genes.

Supplemental Figure 9. Effect of Different Concentrations of Oxo-C14-HSL on Induced Resistance.

Supplemental Table 1. List of Primers Used in Quantitative RT-PCR.

Supplemental Data Set 1. Genes Regulated by the Three AHLs.

Supplemental Data Set 2. Genes Differentially Expressed after 3-d Treatment with C6-HSL.

Supplemental Data Set 3. Genes Differentially Expressed after 3-d Treatment with Oxo-C10-HSL.

Supplemental Data Set 4. Genes Differentially Expressed after 3-d Treatment with Oxo-C14-HSL.

Supplemental Data Set 5. Genes Differentially Expressed after 3-d Pretreatment with Oxo-C14-HSL and Additional Challenge with 100 μM Flg22 for 2 h.

Supplemental Data Set 6. Genes Differentially Expressed after 3-d Pretreatment with Oxo-C14-HSL and Additional Challenge with 100 μM Flg22 for 24 h.

Supplemental Data Set 7. Differentially Regulated Genes Related to Signaling in Oxo-C14-HSL–Pretreated Plants after Challenge with Flg22.

Supplemental Data Set 8. Differentially Regulated *PR* Genes in Oxo-C14-HSL–Pretreated Plants after Challenge with Flg22.

Supplemental Data Set 9. Differentially Regulated Genes Related to the Cell Wall in Oxo-C14-HSL–Pretreated Plants after Challenge with Flg22.

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AUTHOR CONTRIBUTIONS

S.T.S., C.H.-R., B.S., A.B., and A.S. designed the experiments. S.T.S., C.H.-R., E.S., C.N., M.S., and M.R. performed the experiments. S.T.S., C.H.-R., B.S., M.S., A.M., A.B., and A.S. analyzed the experiments. S.T.S., C.H.-R., B.S., M.R., A.M., A.B., K.-H.K., and A.S. wrote the article.

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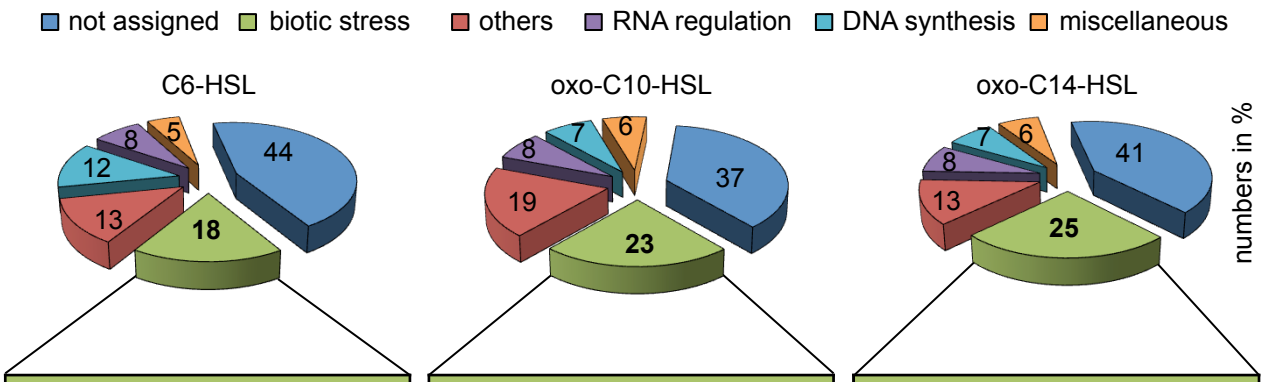
Functional Categories:



subgroup	genes	%
signaling	16	30
protein degradation	13	24
cell wall	7	13
defense proteins	6	11
transcription factors	5	9
secondary metabolism	3	6
hormone metabolism	3	6
redox	1	2

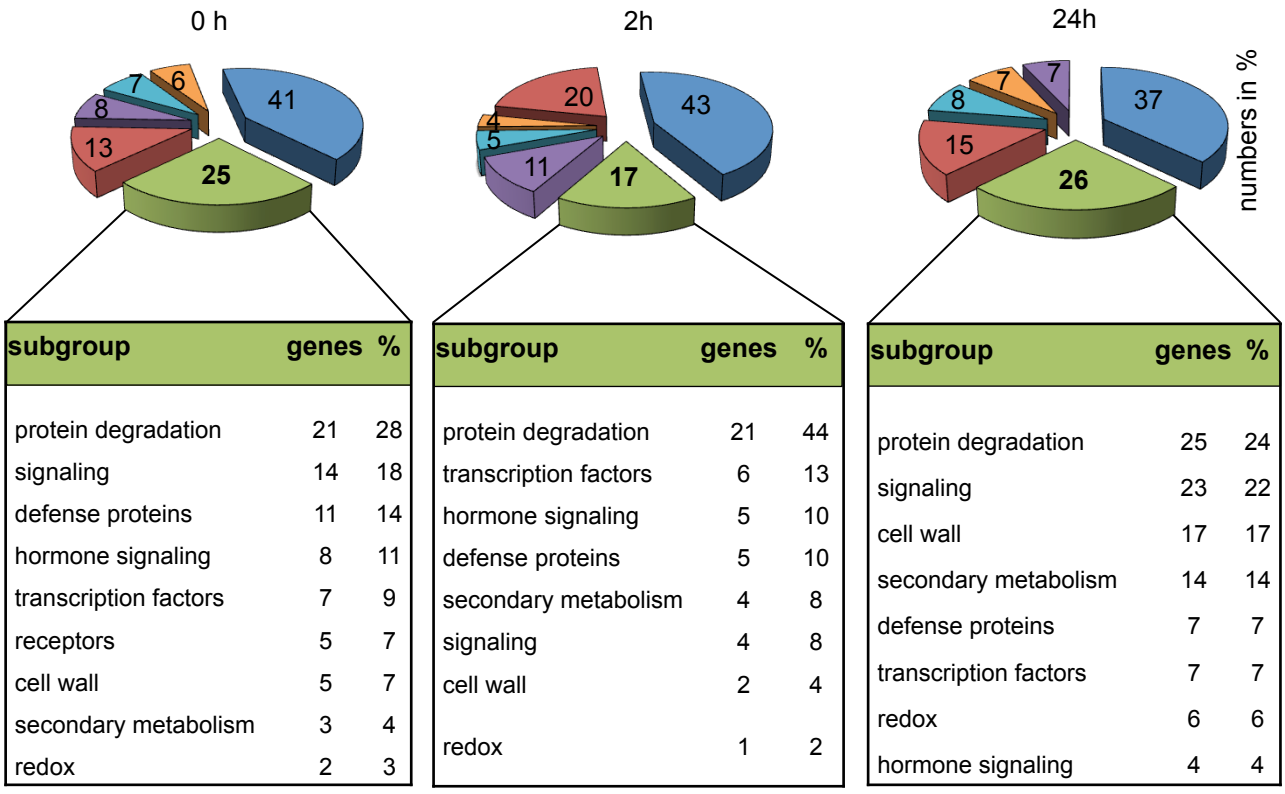
subgroup	genes	%
protein degradation	28	35
signaling	15	19
transcription factors	7	9
defense proteins	7	9
cell wall	7	9
secondary metabolism	5	6
glucan hydrolases	5	6
hormone metabolism	4	5
redox	2	3

subgroup	genes	%
protein degradation	21	28
signaling	14	18
defense proteins	11	14
hormone signaling	8	11
transcription factors	7	9
receptors	5	7
cell wall	5	7
secondary metabolism	3	4
redox	2	3

Supplemental Figure 1. "Response to Biotic" Stress Is the Gene Ontology Term with the Highest Number of Genes Regulated upon AHL Treatment

Biotic stress is the functional category with the highest number of genes regulated in AHL-treated plants. The functional annotation was performed using the MapMan application software (Thimm et al., 2004). The pie charts represent the number of genes in particular functional categories among all differentially expressed genes. The tables below the pie charts present a further analysis of the differentially expressed genes belonging to the "biotic stress" category, divided into subgroups, showing the number of genes and their percentage within the "biotic stress" functional category.

response to flg22 in oxo-C14-HSL pretreated plants



subgroup	genes	%
protein degradation	21	28
signaling	14	18
defense proteins	11	14
hormone signaling	8	11
transcription factors	7	9
receptors	5	7
cell wall	5	7
secondary metabolism	3	4
redox	2	3

subgroup	genes	%
protein degradation	21	44
transcription factors	6	13
hormone signaling	5	10
defense proteins	5	10
secondary metabolism	4	8
signaling	4	8
cell wall	2	4
redox	1	2

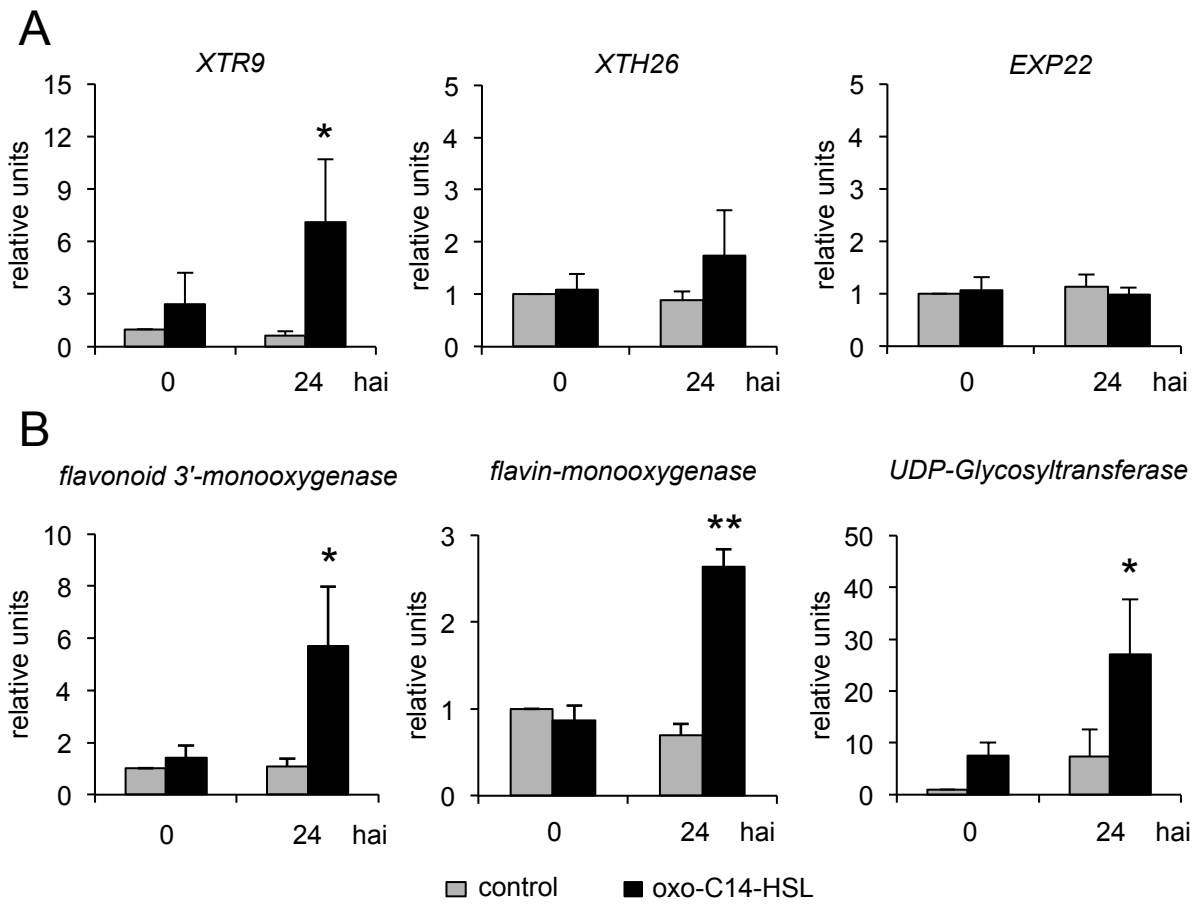
subgroup	genes	%
protein degradation	25	24
signaling	23	22
cell wall	17	17
secondary metabolism	14	14
defense proteins	7	7
transcription factors	7	7
redox	6	6
hormone signaling	4	4

Functional categories:

not assigned | biotic stress | others | RNA regulation | DNA synthesis | miscellaneous

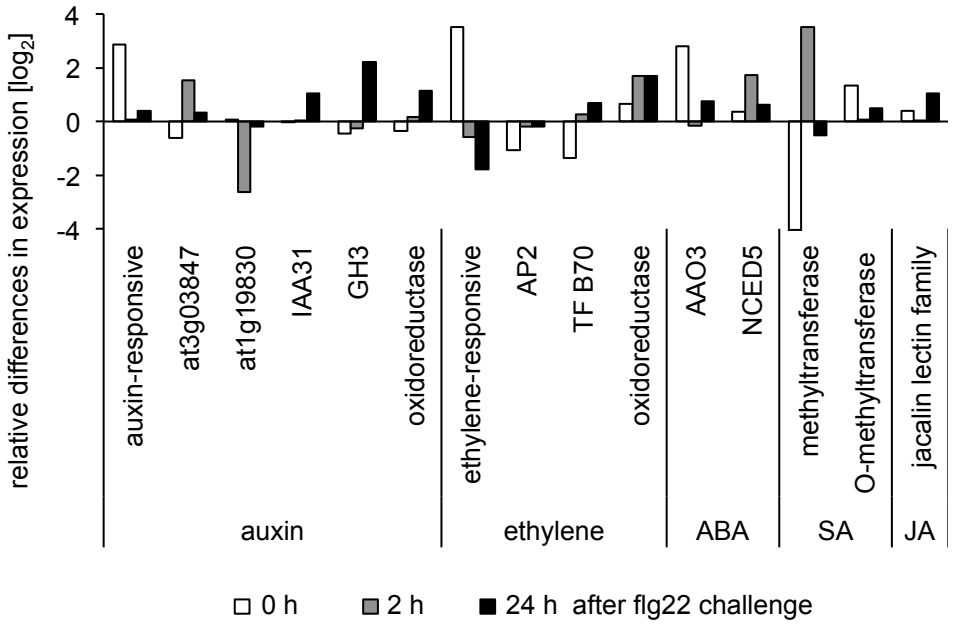
Supplemental Figure 2. Response to a Secondary Challenge in Oxo-C14-HSL-Pretreated Plants Varies from the Response in Non-Pretreated Plants

Two-week-old *Arabidopsis* seedlings were pretreated with 6 µM oxo-C14-HSL. After 3 days seedlings were inoculated with 100 nM flg22 in order to trigger the defense mechanisms. Response to biotic stress is the major functional category from which genes are differentially regulated in AHL-primed plants upon treatment with 100 nM flg22. The functional annotation was performed using the MapMan software (Thimm et al., 2004). The tables below the pie charts present a further analysis of the differentially expressed genes belonging to the “biotic stress” category, divided into subgroups, showing the number of genes and their percentage within the “biotic stress” functional category.



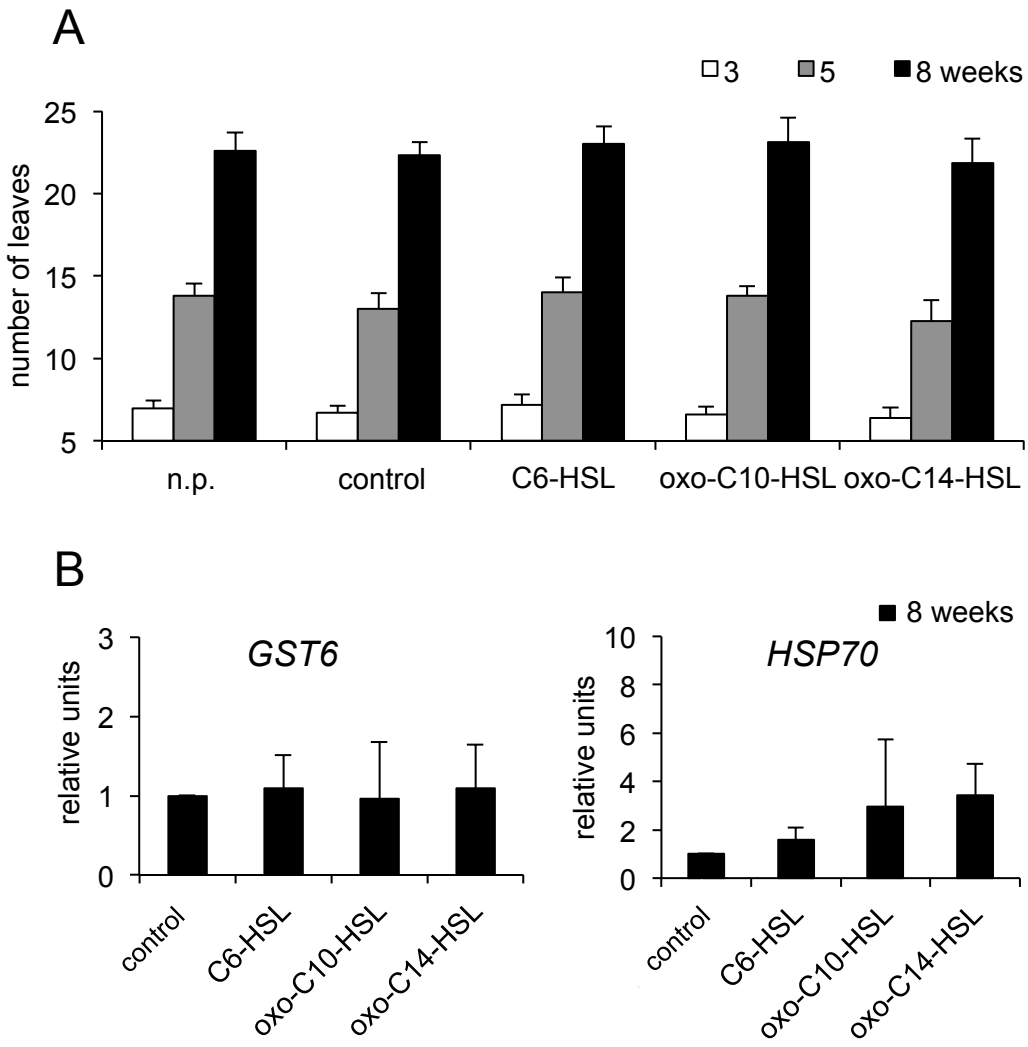
Supplemental Figure 3. Expression of Cell Wall-Related Genes, which Were Locally Regulated in Oxo-C14-HSL-Primed Plants after Flg22 Challenge, in Systemic Tissues of Oxo-C14-HSL-Pretreated Plants Challenged with *Pst*

The expression profiles of three cell wall-related genes: *xyloglucosyl transferase* (*XTR9*), *endo-xyloglucan transferase* (*XTH26*) and *expansin22* (*EXP22*) (**A**) and three genes related to secondary metabolism: *flavonoid 3'-monooxygenase*, *flavin-monooxygenase,* and *UDP-Glcosyltransferase* (**B**). Plants were grown in the sterile systemic hydroponic system, pretreated with 6 µM oxo-C14-HSL or acetone (control) for 3 days and subsequently challenged with *Pst* for the number of hours as indicated, hours after inoculation (hai). The level of respective mRNA is presented in relation to the expression of the ubiquitin ligase and the 0 hai time point. Graphs present a mean of three independent biological repetitions. Error bars represent standard error. * represents $p < 0.05$, ** $p < 0.005$ in Student's *t*-test.



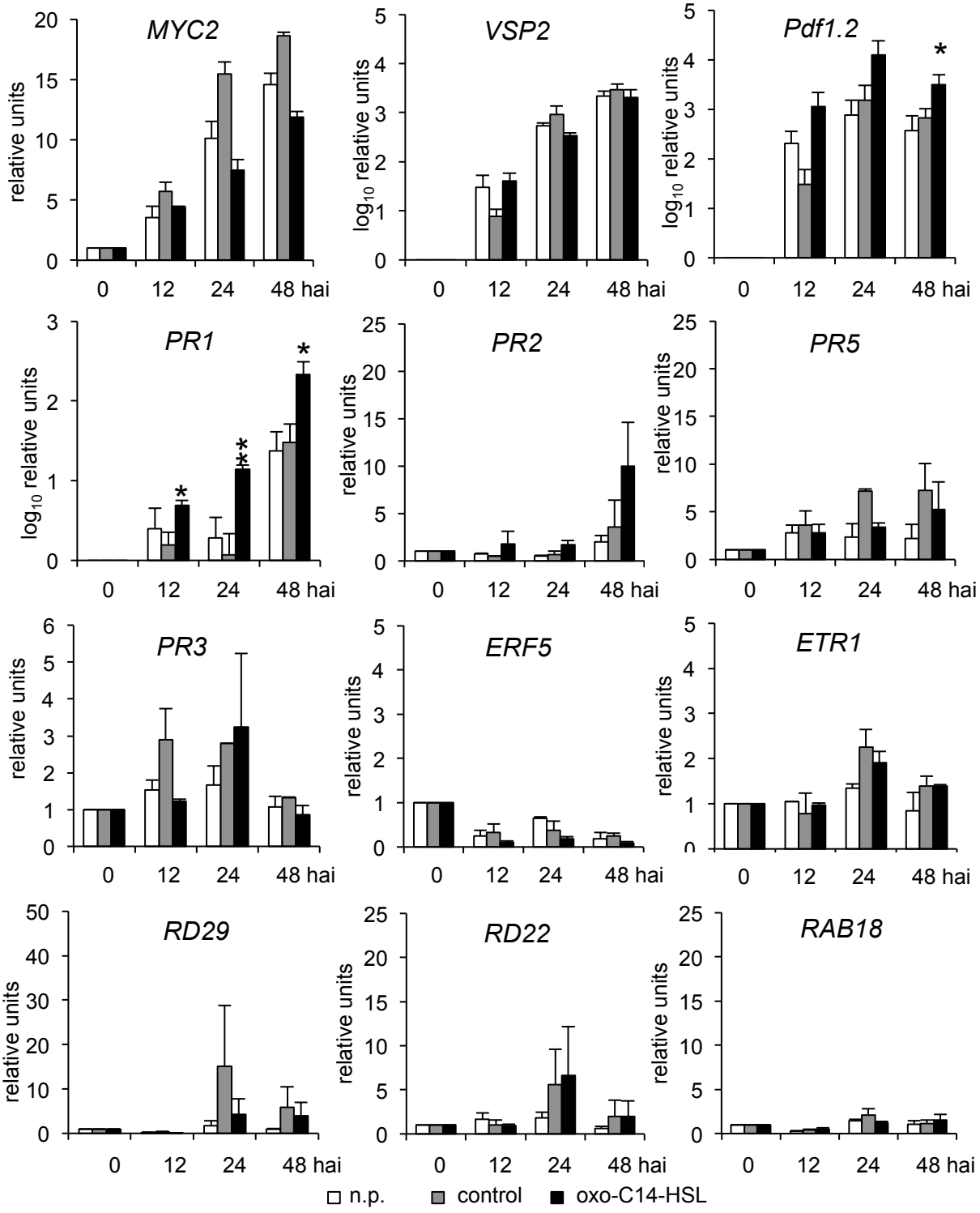
Supplemental Figure 4. Hormones-Associated Gene Ontology Terms Are not Enriched within the Differentially Expressed Gene Groups

Differentially regulated genes related to hormone signaling as revealed by the microarray experiment. Note: there is no enrichment in genes related to hormones upon pretreatment with the tested AHLs. The numbers represent the $\log_2$ fold change relative to the solvent control.



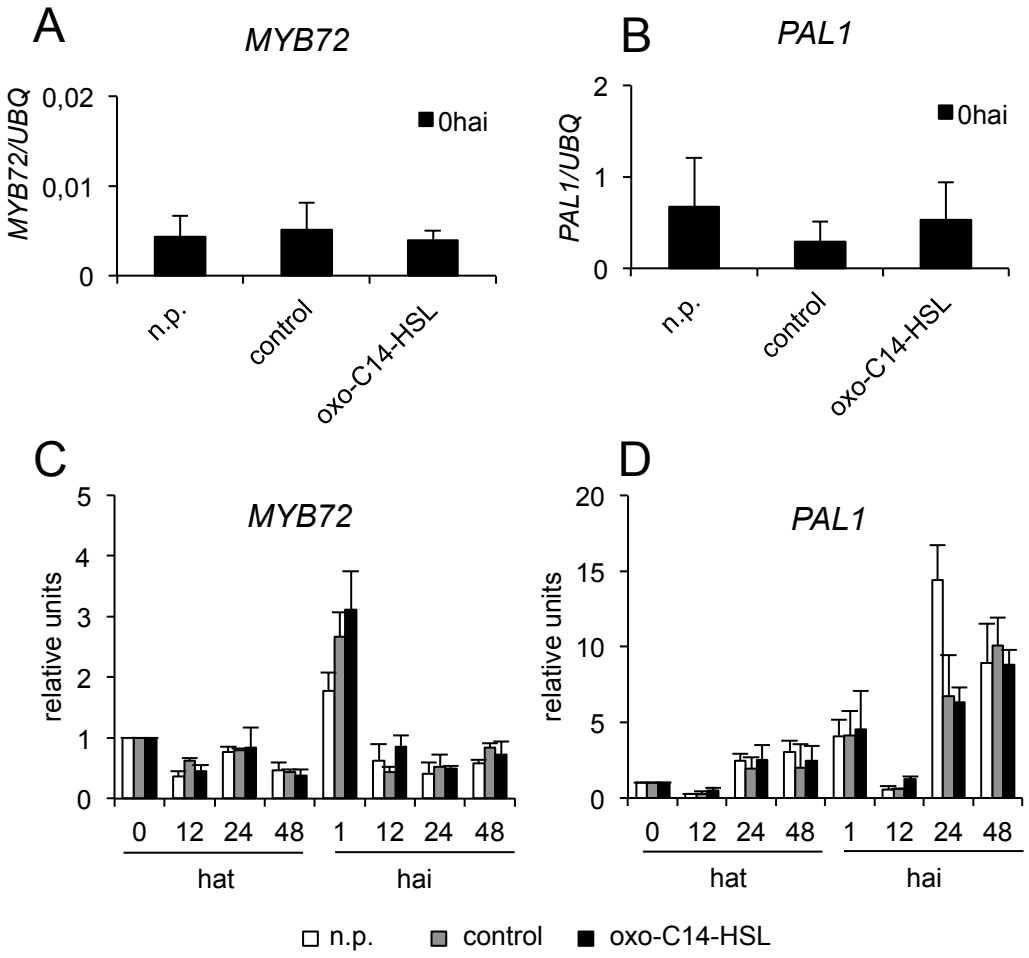
Supplemental Figure 5. Long-Term Observation of the Influence of AHLs on Plant Development and Plant's Defense System

A. *Arabidopsis* plants were cultivated for 8 weeks in the sterile systemic hydroponic system. The 1/2x MS medium was changed every week and supplied with 6 µM AHLs every fourth day. Number of developed leaves was assessed after 3, 5, and 8 weeks.
B. After 8 weeks plants were harvested for RNA extraction and transcriptional analysis of the OPDA-regulated genes (lower panels). The level of respective mRNA was determined *via* quantitative RT-PCR using the expression of ubiquitin ligase and the respective mRNA level in control plants for normalization. Error bars represent standard error, n = 30 per treatment.



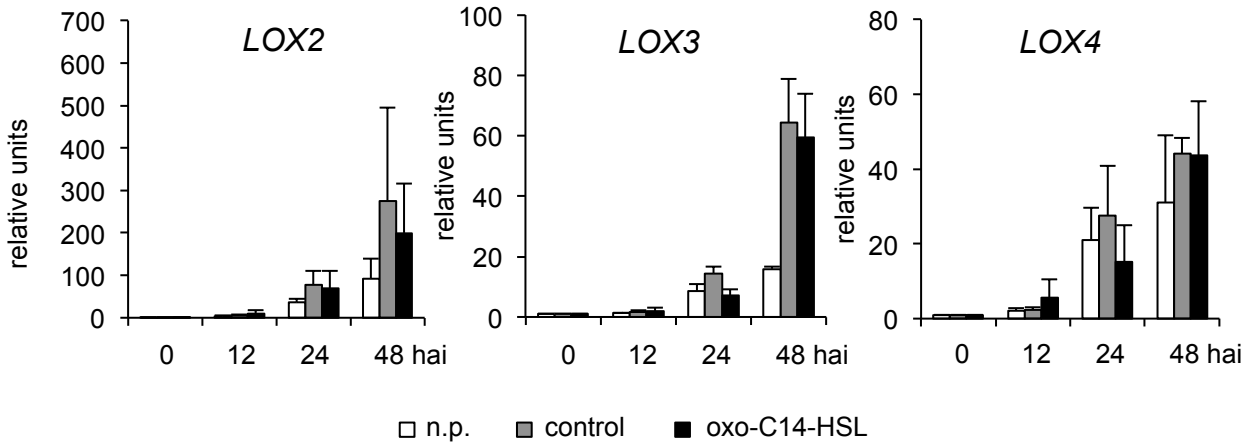
Supplemental Figure 6. Pretreatment with Oxo-C14-HSL Has No Impact on the Expression of JA-, SA-, ET-, and ABA-Regulated Genes

Relative expression of JA-regulated genes; *MYC2*, *VSP2,* and *Pdf1.2;* SA-regulated genes: *PR1*, *PR2, and PR5*; ET-regulated genes; *PR3*, *ERF5*, and *ETR1;* ABA-regulated genes: *RD29*, *RD22*, and *RAB18. Arabidopsis* plants were grown in the sterile systemic hydroponic system for 5 weeks, pretreated with 6 µM oxo-C14-HSL, its solvent acetone (control) for 3 days or not pretreated (n.p.), and challenged with *Pst* for the number of hours as indicated. The level of respective mRNA was determined *via* quantitative RT-PCR using the expression of ubiquitin ligase *At5g25760* for normalization. Graphs present a mean from three independent biological repetitions. Error bars represent standard error. hai; hours after inoculation. * represents $p < 0.05$, ** $p < 0.005$ in Student's *t*-test.



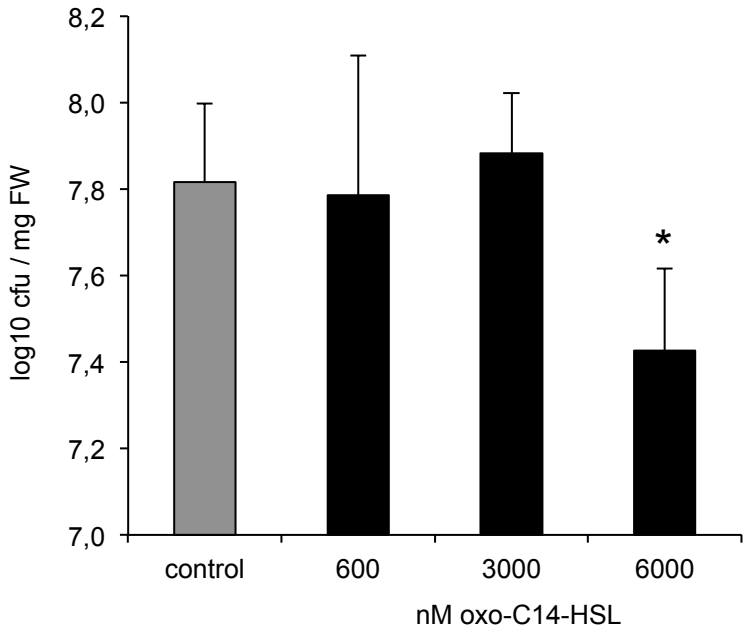
Supplemental Figure 7. Neither the *MYB72* Nor the *PAL1* Gene Was Induced upon AHL Priming

Expression profile of the *MYB72* transcription factor upregulated upon ISR (**A**, **C**) and the SAR-regulated *PAL* gene (**B**, **D**) encoding for phenylalanine-ammonia lyase in *Arabidopsis* plants grown in the sterile systemic hydroponic system and pretreated with 6 µM oxo-C14-HLS for 3 days (hat; hours after treatment) prior to challenge with *Pst* bacteria for the number of hours as indicated, hai (hours after inoculation). Quantitative RT-PCR was performed using the expression of the housekeeping gene ubiquitin ligase for normalization. Data represent a mean from three independent biological replications. Values in **C** and **D** were normalized to the 0 hat time point. Error bars represent standard error.



Supplemental Figure 8. Expression of *LOX* Genes

The expression profiles of three cytosolic lipoxygenases (*LOX2*, *LOX3*, and *LOX4*) in plants grown in the sterile systemic hydroponic system and subsequently challenged with *Pst* bacteria for the number of hours as indicated do not differ in plants pretreated with 6 µM oxo-C14-HSL, its solvent acetone (control) for 3 days or not pretreated (n.p.). Total RNA was extracted and the level of respective mRNA was determined *via* quantitative RT-PCR using the expression of ubiquitin ligase *At5g25760* for normalization. Graphs present a mean of three independent biological repetitions. Error bars represent standard error. hai; hours after inoculation.



Supplemental Figure 9. Effect of Different Concentrations of Oxo-C14-HSL on Induced Resistance

Pathogenicity assay with *Pseudomonas syringae* DC3000 pathovar *tomato* (*Pst*) on *Arabidopsis Col*-0 wild type plants grown for 5-weeks in the sterile systemic hydroponic system. Plant roots were pretreated with different concentrations of oxo-C14-HSL or its solvent acetone (control) for 3 days, and leaves were subsequently spray-inoculated with *Pst* ($OD_{600\ nm}$ = 0.1). The colony forming units (cfu) were counted after 48 hours. * represents $p < 0.05$ in Student's *t*-test.

Supplemental Table 1. List of Primers Used in Quantitative RT-PCR

Annealing temperature for all primers was set to 60°C

gene	primer sequence
PR1	TCG GAG CTA CGC AGA ACA ACT
	TCT CGC TAA CCC ACA TGT TCA
Pdf1.2	GTT TGC TTC CAT CAT CAC CC
	GGG ACG TAA CAG ATA CAC TTG
GST6	GCA TGT TCG GCA TGA CCA CTG
	GCA CCT TGG AGT CAG TAC CC
HSP70	CGC CAA CGA TCA AGG CAA CC
	GCT TCT CAC CTG GAC CGG AA
VSP2	CAA ACT AAA CAA TAA ACC ATA CCA TAA
	GCC AAG AGC AAG AGA AGT GA
MYC2	GGT TGG GAC GCA ATG ATT AGA GT
	CCA TCT TCA CCG TCG CTT GTT G
PR2	TCT TGA ACC CAC TTG TCG GC
	GGC TCT GAC ATC GAG CTC ATC
PR5	TCC TTG ACC GGC GAG AGT T
	AGG AAC AAT TGC CCT ACC ACC
RD29	GAG GAA GTG AAA GGA GGA GGA GG
	CAG TGG AGC CAA GTG ATT GTG G
RAB18	TCG GTC GTT GTA TTG TGC TTT TT
	CCA GAT GCT CAT TAC ACA CTC ATG
RD22	GCG AGC TAA AGC AGT TGC GGT ATG
	GGG AGG AAG TGG CAG ACC GGA AC
PR3	GAC GCC GAC CGT GCC GCC GGG
	CGG CGA CTC TCC CGT CTT GGC C
ERF5	GAC GAA GCA GCG TTT AGA CTA CGA GG
	GGA GAT AAC GGC GAC AGA AGC GG
ETR1	CGA GAA GCT CGG GTG GTA GT
	GCC GTG CAT CCT TTT CC
MYB72	TCA TGA TCT GCT TTT GTG CTT TG
	ACG AGA TCA AAA ACG TGT GGA AC

PAL1	AAC GGA GGA GGA GTG GAC G
	CTT TTC ATT TGC TCC GCT GC
LOX2	GTA GCC CCA GTT CTC ATT AAC AGG G
	CGG GTC TAG TTT GCT TAT TAA CGG C
LOX3	TAT GGA TTT GCG GCA GAG ATC GGA
	AGG CTC AGA ACT CGG AAC CAA CAA
LOX4	GGG ATC AAC CCG GTC AAC ATA GAA C
	GTC CAC CAT AAA CAA ACG GTT CGT C
XTR9	GGA GCA ACG CAC CAT TCA AGG
	CGT ACT GGG CAG GAT TGA GAG
XTH26	GGA AGG GAT CAG TCA GCA TCA AG
	GTG CTA TGA TTG CCG GAG CC
EXP22	TAA AGG AAA CAA GAC CGG GTG G
	CCT GAA CGA TAA ACC TTG GCC G
At5g07990	GCT GGG AGG AGA ATC TGT GC
	GGA TGT ACC ACC AAA GGA ACC G
At1g12140	AAC TTC GGA GAG AGC ATT CGT C
	GCT GTT GAA GAG GCA GAG AAG A
At3g29630	GCG GGT TCG GTT CAA TGT G
	GAC CGT ATC CCT CAA GCT CTC C
Actin 2	GGA AGG ATC TGT ACG GTA AC
	TGT GAA CGA TTC CTG GAC CT
At5g25760	GCT TGG AGT CCT GCT TGG ACG
	CGC AGT TAA GAG GAC TGT CCG GC

N-Acyl-Homoserine Lactone Primes Plants for Cell Wall Reinforcement and Induces Resistance to Bacterial Pathogens via the Salicylic Acid/Oxylipin Pathway

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