running title: **QS molecule induces plant immunity**

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\textit{N}-acyl-homoserine lactone confers resistance towards biotrophic and hemibiotrophic pathogens \textit{via} altered activation of AtMPK6\$ \\
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Abstract

Pathogenic and symbiotic bacteria relay on quorum sensing (QS) in order to coordinate the collective behavior during the interactions with their eukaryotic hosts. Many Gram-negative bacteria use N-acyl-homoserine lactones (AHLs) as signals in such communication. Here we show that plants have evolved means to perceive AHLs and that the length of acyl moiety and the functional group at the $\gamma$ position specify the plants response. Root treatment with the $N$-3-oxo-tetradecanoyl-$L$-homoserine lactone (oxo-C14-HSL) reinforced the systemic resistance to the obligate biotrophic fungi Golovinomyces orontii in Arabidopsis, and to Blumeria graminis f. sp. hordei in barley plants. In addition, oxo-C14-HSL-treated Arabidopsis plants were more resistant towards the hemibiotrophic bacterial pathogen Pseudomonas syringae pv. tomato DC3000. o xo-C14-HSL promoted a stronger activation of mitogen-activated protein kinases AtMPK3 and AtMPK6 when challenged with flg22, followed by a higher expression of the defense-related transcription factors WRKY22 and WRKY29, as well as the pathogenesis related 1 (PR1) gene. In contrast to wild-type Arabidopsis and mpk3 mutant, the mpk6 mutant is compromised in the AHL effect, suggesting that AtMPK6 is required for AHL-induced resistance. Results of this study show that AHLs commonly produced in the rhizosphere are crucial factors in plant pathology and could be an agronomic issue whose full impact has to be elucidated in future analyses.
Introduction

Rhizosphere communication is based on a complex exchange and perception of molecules originating from interacting organisms. Many bacteria use N-acyl-homoserine lactones (AHLs) to coordinate the behavior of individual cells in a population. This communication phenomenon is called quorum sensing (QS), and was first described in *Vibrio fisheri* (Engelbrecht and Silverman, 1984). *V. fisheri* secretes N-3-oxo-hexanoyl-L-homoserine lactone (oxo-C6-HSL), which is synthesized by the enzyme LuxI from S-adenosyl methionine and an acyl chain carrier protein (ACP) (Schaefer et al., 1996) and sensed by a LuxR-type receptor (Hanzelka and Greenberg, 1995). In many Gram-negative bacteria, the control of the population density relies on production of diverse AHLs that influence gene transcription once detected by a companion. Numerous AHL derivatives varying in the length of the acyl chain (from 4 to 18 carbons) and in the substitution at the γ position of the chain with hydroxyl (OH) or oxo (O) groups have been so far identified, for review see (Williams, 2007). AHLs also have an impact on eukaryotic cells. N-3-oxo-dodecanoyl-L-homoserine lactone (oxo-C12-HSL), produced by the opportunistic plant and human pathogen *Pseudomonas aeruginosa*, induces distension of mitochondria and endoplasmic reticulum (Kravchenko et al., 2006). In addition, it increases phosphorylation of the mitogen-activated protein kinase (MAPK) p38 and the translation initiation factor eIF2α (Kravchenko et al., 2006). In human NCI-H292 cells, treatment with oxo-C12-HSL enhances the phosphorylation of the negative regulator of NF-κB, the I-κB, and the ERK1/2 MAP kinases (Imamura et al., 2004). Moreover, oxo-C12-HSL modulates the NFκB signaling and abolishes the TLR4-dependent immune response in mice (Kravchenko et al., 2008). It was suggested that mammalian cells perceive oxo-C12-HSL in a Toll-like receptor (TLR)-independent manner (Kravchenko et al., 2006). Jahoor et al., (2008) proposed the peroxisome proliferator-activated receptors PPARγ and PPARβ, members of the nuclear hormone receptor (NHR) family as potential candidates for AHLs receptors in animals. On the other hand, AHLs were shown to interact directly with
different cellular and artificial membranes, which suggest another possible membrane-located AHL-reception system (Davis et al., 2010).

In the plant-interacting Agrobacterium tumefaciens, N-3-oxo-octanoyl-L-homoserine lactone (oxo-C8-HSL) is produced by the TraI enzyme and perceived by the LuxR-homologous receptor TraR (Fuqua and Winans, 1994). Both, traI and traR genes are located on the Ti plasmid and are induced by the tumor-released octopine; TraR transcriptionally induces all genes required for conjugation and intrabacterial transfer of the Ti plasmid. oxo-C8-HSL also induces the repABC operon, obligatory for stable vegetative replication of the octopine-type Ti plasmid (Fuqua and Winans, 1994; Pappas and Winans, 2003). On the other hand, plants may interact with the Agrobacterium QS. Different plant signals induce the transcription of bacterial attM and aiiB genes coding for two lactonases, AttM and AiiB. They cleave the γ-butyrolactone ring of AHLs and might in this way modulate the QS-dependent virulence of Agrobacterium (Haudecoeur et al., 2009; Haudecoeur et al., 2009).

In recent years, several reports have shown that plants evolved means to perceive AHLs and respond to them with changes in gene expression or modifications in development (Mathesius et al., 2003; Schuhegger et al., 2006; Gotz et al., 2007; Ortiz-Castro et al., 2008; von Rad et al., 2008; Pang et al., 2009). Assessment of AHLs impact on root development revealed that C6-HSL promotes root growth (von Rad et al., 2008), C10-HSL alters root architecture similarly to auxin, but in an auxin-independent way, and C12-HSL strongly induces root hair formation (Ortiz-Castro et al., 2008). Several reports provided indirect evidences that AHLs play a role in plant immunity. Serratia liquefaciens MG1 increases systemic resistance of tomato plants against the fungal leaf pathogen Alternaria alternata. However, the AHL negative mutant S. liquefaciens MG44 is less effective (Schuhegger et al., 2006). Similarly, colonization with the Serratia plymuthica protects cucumber plants from the damping-off disease caused by Pythium aphanidermatum, as well as tomato and bean plants from infection with the grey mold fungus Botrytis cinerea (Pang et al., 2009). The splI mutant of S. plymuthica, impaired in the production of AHLs, could not provide this protection (Pang
et al., 2009). However, the molecular basis of AHL’s influence on the plant immune system is still unknown.

In this report we addressed the question how AHLs affect the plant immune system. We tested AHL-induced resistance towards different pathogens, using a monocotyledonous (barley) and a dicotyledonous (Arabidopsis) host. Our results show that even though the long-chained AHLs cannot be transported within the plant, their perception in roots enhances the systemic resistance towards biotrophic and hemibiotrophic pathogens. Furthermore, in Arabidopsis plants pretreated with oxo-C14-HSL, we observed prolonged activities of the MAPKs AtMPK3 and AtMPK6 after induction with flg22. The modified activities of MAPKs preceded enhanced transcription of the defense related WRKY22 and WRKR29 transcription factors, as well as the pathogenesis related 1 (PR1) gene. Taken together, results presented here suggest that perception of long-chained AHLs has a positive impact on plant resistance.

**Results**

**Long-chain AHLs have no impact on plant growth**

Several N-acyl-homoserine lactones (AHLs) have been reported to modify root development and promote plant growth (Ortiz-Castro et al., 2008; von Rad et al., 2008). Therefore, in order to study how N-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) modulates plant physiology, we first monitored the impact of oxo-C14-HSL on growth and development of Arabidopsis thaliana Col-0. Ten-day-old seedlings grown on standard MS/2 medium were transferred to medium supplied with 6 μM oxo-C14-HSL, and cultivated for additional 4 or 8 days. No alteration in the shoot or root development was observed (Fig. 1A). However, in accordance with previous reports (von Rad et al., 2008), the short-chained N-hexanoyl-L-homoserine lactone (C6-HSL), used here as a positive control, promoted shoot and root biomass accumulation (Fig. 1B), as well as the root length (Fig. 1A, C).

**Application of oxo-C14-HSL to roots does not result in AHL detection in leaves**

Next, we addressed the question whether AHLs can be transported within the plant. To this end, we recorded the presence of AHL exogenously applied to the root medium, in different
plant tissues. We used reporter bacteria carrying either the Green Fluorescent Protein (GFP) gene or the lux operon from V. fisheri under AHL-inducible promoters (Supplementary Fig. S1A, D). Cleared acetone extracts from either roots or shoots were prepared and applied to lawns of reporter bacteria (Supplementary Fig. S1B). GFP or luminescence signals were recorded 2 h after application. The most sensitive reporter bacterium was Pseudomonas putida F117 pKR C12 GFP (Steidle et al., 2001) (carrying the lasR+lasB::gfp construct) with a detection limit of approximately 60 nM for oxo-C14-HSL (Supplementary Fig. S1A). In accord with previous reports (von Rad et al., 2008), if Arabidopsis roots were treated with C6-HSL, the AHL was detectable in extracts from roots and shoots of the treated plants (Supplementary Fig. S1B). In contrast, the long-chained oxo-C14-HSL was not detected in leaf extracts even though it was detectable in extracts from the roots of oxo-C14-HSL-treated plants. Neither the P. putida F117 pKR C12 GFP strain, nor the E. coli strain pSB403 (Winson et al., 1998) carrying the lux operon from V. fisheri, were able to detect oxo-C14-HSL in the leaf extracts (Fig. 1D and Supplementary Fig. S1B). These results support the notion that long-chain AHLs are not transported systemically throughout the plant organism.

**AHLs confer resistance against hemibiotrophic and biotrophic pathogens**

Based on the strong effect of AHLs on the mammalian immune system (Kravchenko et al., 2008), we followed the working hypothesis that AHLs may induce plant resistance to microbial pathogens. Arabidopsis plants were grown for 5 weeks in sterile hydroponics culture, and then the growing medium was supplied with 6 μM oxo-C14-HSL for 3 days. Subsequently plants were spray-inoculated with Pseudomonas syringae pv. tomato DC3000 (Pst). Bacterial population on leaves was monitored during 96 hours post inoculation (hpi). Plants pretreated with oxo-C14-HSL developed significantly smaller populations of Pst bacteria then the control plants (Fig. 2A).

To exclude the possibility that oxo-C14-HSL has a direct effect on bacterial fitness, we cultivated bacteria in the presence of 6 μM oxo-C14-HSL and monitored population density over 48 h. No change in bacterial duplication time was observed (Supplementary Fig. S2A).

In addition, we examined the virulence of bacteria grown in the presence of oxo-C14-HSL
and subsequently sprayed onto Arabidopsis leaves (Supplementary Fig. S2B), or grown in standard Kings B medium and sprayed onto leaves together with the AHL (Supplementary Fig. S2C). None of these tests showed that oxo-C14-HSL lowers bacterial fitness or the virulence. Hence, results presented above suggest that oxo-C14-HSL enhances resistance of Arabidopsis towards Pst bacteria. Interestingly, not only oxo-C14-HSL, but also N-3-OH-tetradecanoyl-L-homoserine lactone (OH-C14-HSL) and N-3-oxo-dodecanoyl-L-homoserine lactone (oxo-C12-HSL) reduce Pst proliferation, though to lesser extent than oxo-C14-HSL (Fig. 2B and Supplementary Fig. S7A).

In order to expand our observations to other pathogen, we analyzed the impact of oxo-C14-HSL on the development of the fungus Golovinomyces orontii, the causal agent of powdery mildew in Arabidopsis. Hydroponically grown Arabidopsis plants were pretreated with 6 μM oxo-C14-HSL and leaves were subsequently inoculated with G. orontii (560 conidia/cm² leaf). The number of mycelia per leaf was calculated 5 days after inoculation. We found that the number of mycelia developing on leaves, was significantly reduced in plants pretreated with oxo-C14-HSL (Fig. 3A). On the other hand, the morphology of G. orontii mycelium was not affected by the AHL treatment (Fig. 3B-D). These results support the concept that long-chained AHL exhibits a positive effect on the resistance towards hemibiotrophic and biotrophic pathogens.

oxo-C14-HSL induces resistance against the biotrophic powdery mildew fungus in barley plants

In addition to experiments with Arabidopsis, we assessed oxo-C14-HSL-induced resistance in the monocotyledonous crop plant Hordeum vulgare (barley). Barley seedlings were grown for 5 days and then pretreated with 6 μM oxo-C14-HSL for 3 days in hydroponics culture. Subsequently first leaves were inoculated with the casual agent of barley powdery mildew Blumeria graminis f.sp. hordei (450 conidia/cm² of leaf). As shown by the increased rate of fungal penetration failure (Fig. 3E) the effect of oxo-C14-HSL on barley is similar to that on Arabidopsis. The frequency of elongating secondary hyphae (ESH) shown in Figure 3F, indicative of successful infection, decreased from 43% of all interactions in control plants to
14% in oxo-C14-HSL-treated plants, while the frequency of papillae (Fig. 3G), indicative of reduced host cell accessibility, increased from about 54% in control to over 77% of all interactions in AHL-treated plants (Fig. 3E). The third possibility to respond is the hypersensitivity response (HR) (Fig. 3H), also this resistance mechanism was enhanced in AHL-treated plants (Fig. 3E). In conclusion, oxo-C14-HSL affects the basal immune system of Arabidopsis and barley.

oxo-C14-HSL does not induce resistance against necrotrophic pathogens

To examine the hypothesis that oxo-C14-HSL induces resistance particularly against hemibiotrophic and biotrophic pathogens, we performed a series of tests with necrotrophic fungal pathogens. Arabidopsis plants were pretreated as above for 3 days with 6 μM oxo-C14-HSL, and detached leaves were subsequently inoculated with Botrytis cinerea or Plectosphaerella cucumerina BMM. Disease symptoms were analyzed after 5 days. Both pathogens caused maceration of leaf tissue, which can be classified into different symptom categories (B. cinerea, Fig. 4A-D) or measured by lesion diameter (P. cucumerina BMM, Fig. 4E-H). No significant differences in symptoms caused by B. cinerea or P. cucumerina BMM, as compared with H₂O and acetone controls were observed (Fig. 4). To evaluate the possibility that resistance against necrotrophic fungi may be induced by different AHL concentration, we conducted a dose-response experiment with B. cinerea, using various concentrations of oxo-C14-HSL (0.6 to 12 μM). Yet, none of the tested concentration significantly enhanced resistance towards this fungus (Supplementary Fig. S3).

AHL modulates the response to the bacterial PAMP flg22

To study the mechanism by which AHL induces resistance in more detail, we examined the response of AHL-treated Arabidopsis plants to the bacterial pathogen associated molecular pattern (PAMP) flg22. First, we monitored the accumulation of reactive oxygen species (ROS) after solely treatment with oxo-C14-HSL. For this, leaves of soil-grown plants were treated for 3 days with 6 μM oxo-C14-HSL in floating conditions and stained with diaminobenzidine (DAB). DAB staining revealed no changes in the steady state level of H₂O₂.
Next, we analyzed the production of ROS (oxidative burst) in those leaves after elicitation with 100 nM flg22. Likewise, ROS production induced by flg22 in Arabidopsis leaves was neither enhanced nor inhibited by pretreatment with AHL (Fig. 5A).

In following step, we analyzed the activation of the mitogen-activated protein kinases (MAPKs) AtMPK3 and AtMPK6. Both kinases have a high degree of functional redundancy and are involved in plant defense mechanisms. Total protein extract was prepared from 2-week-old seedlings grown on agar plates and pretreated with oxo-C14-HSL in liquid MS/2 medium for additional 3 days. MAPKs were activated by treatment with 100 nM flg22. Phosphorylated forms of AtMPK3 and AtMPK6 were visualized using the αpERK1/2 antibody (Hamel et al., 2005). Consistent with earlier reports, flg22 triggered a short transient activation of AtMPK3 and AtMPK6 at 15 min after application (Fig. 5B). Intriguingly, AHL-pretreated seedlings showed stronger activation of both kinases, which lasted until 60 min after treatment (Fig. 5B). Immunoblots with respective αAtMPK3 and αAtMPK6 antibodies showed no changes in AtMPK3 and AtMPK6 quantities during 1h after treatment (Fig. 5B). Similarly, quantitative RT-PCR analysis detected no changes in AtMPK3 and AtMPK6 transcript levels after pretreatment with AHLs (Supplementary Fig. S5A), and only slightly changes after the subsequent treatment with flg22 (Supplementary Fig. S5B and C). Although we cannot exclude a short transcriptional induction of AtMPK3/6 between 1 and 2 h after flg22 treatment (those intermediate time points were not examined), the results obtained in our tests indicate rather that oxo-C14-HSL modulates the flg22-induced phosphorylation status of AtMPK3 and AtMPK6 and not the amount of the kinases.

Owing to the fact that numerous WRKY transcription factors are induced upon pathogen attack and some WRKY are direct targets of activated MAPKs, we assessed whether oxo-C14-HSL-mediated enhanced activation of MAPKs influences the expression levels of defense-related WRKYs. We examined the relative expression levels of WRKY18, WRKY22, and WRKY29 whose activation is pathogenesis-associated or induced by flg22 (Asai et al., 2002; Xu et al., 2006; Zheng et al., 2006). Two-week-old Arabidopsis seedlings were pretreated with 6 μM oxo-C14-HSL as described above and subsequently elicited with 100
nM flg22. Total RNA was extracted and transcript levels of WRKYs normalized to the expression of UBQ (At5g25760). Pretreatment with the AHL alone did not induce the expression of either WRKY (Supplementary Fig. S6A-C). However, a subsequent treatment with 100 nM flg22 resulted in different expression patterns. Both, WRKY22 and WRKY29 show induction already 30 min after treatment with elicitor (Libault et al., 2007). Here we show a prolonged and stronger transcriptional activation of WRKY22 and WRKY29 in plants pretreated with oxo-C14-HSL even at 2, 6 and 12 h after flg22 treatment (Fig. 5C-D). WRKY18 expression did not change after the subsequent flg22 treatment (Fig. 5E). Pathogenesis related (PR) genes such as PR1, are prominent example of defense-associated gene in Arabidopsis. Consequently, we examined the expression level of PR1 after treatment with oxo-C14-HSL. Similar to the expression of WRKY22 and WRKY29, AHL treatment alone does not induce the expression of PR1 (Supplementary Fig. S6D). However, expression of PR1 is induced after treatment with 100 nM flg22, and pretreatment with oxo-C14-HSL enhances it even more (Fig. 5F).

Active kinases are necessary for AHL-induced resistance

To test the assumption that AtMPK3 and/or AtMPK6 activations are required for AHL-induced resistance we analyzed the mpk3 and mpk6 mutants. First we monitored the phosphorylation status of the kinases in both mutants. Two-week-old Col-0 wild type and mutants seedlings were pretreated for 3 days with 6 μM oxo-C14-HSL in liquid medium and MAPKs activation was triggered by addition of 100 nM flg22. In the mpk3 mutant the phosphorylation pattern of AtMPK6 is similar to that in Col-0 plants: the 45 kDa band representing the double-phosphorylated form of AtMPK6 is stronger in AHL-treated mpk3 plants (Fig. 6A). Interestingly, in the mpk6 mutant, the pAtMPK3 signal is much weaker if compare to pAtMPK3 in the Col-0 plants (Fig. 6A). In order to investigate this in further details, we tested AHL-induced resistance towards Pst in the mpk3 and the mpk6 mutants. Mutant plants were grown for 5 weeks in hydroponics culture and pretreated for 3 days with 6 μM oxo-C14-HSL prior to a subsequent inoculation with Pst. In clear contrast to Col-0 wild type and mpk3 mutant plants, the mpk6 mutant was compromised in the induced resistance
towards \textit{Pst}, suggesting that AtMPK6 plays a critical role in the AHL-induced resistance (Fig. 6B).

Next, we analyzed the expression pattern of \textit{WRKY22} and \textit{WRKY29} in the \textit{mpk3} and \textit{mpk6} mutants. In wild type \textit{Col}-0 plants, expression of both \textit{WRKYs} is upregulated upon treatment with flg22, and could be even more induced by pretreatment with AHL (Fig. 5C, D). \textit{mpk3} and \textit{mpk6} mutants were grown for 2 weeks on standard medium and transferred to liquid MS/2 medium supplied with AHL 3 days before the treatment with flg22. In both, the \textit{mpk3} and the \textit{mpk6} mutant, expression of \textit{WRKYs} is induced after treatment with flg22, yet it is not further modified by the pretreatment with oxo-C14-HSL (Fig. 6C-F).

**AHL pretreatment enhances ETI-based defense mechanism in \textit{Arabidopsis}**

In plants, recognition of a pathogen occurs at two levels: the pattern recognition receptors (PRRs) detect PAMPs and induce the PAMP-triggered immunity (PTI); the host encoded R-proteins, on the other hand, recognize (directly or indirectly) microbial effectors and initiate the effector-triggered immunity (ETI). We wondered whether AHL might influence also the second level of defense: the ETI. An increased frequency of hypersensitivity response (HR) is often associated with ETI as the consequence of the recognition of bacterial effectors via the R-proteins. Therefore, we used the \textit{Pseudomonas syringae pv. tomato} DC3000 strain expressing the \textit{AvrRpt2} gene, whose product is recognized in \textit{Arabidopsis} by the RPS2 receptor (Kunkel et al., 1993; Yu et al., 1993). \textit{Arabidopsis Col}-0 plants were grown in soil for 4 weeks and detached leaves were pretreated for 3 days with 6 \textmu{}M oxo-C14-HSL in floating conditions. Leaves were then spray-inoculated with the \textit{Pst AvrRpt2} strain and the accumulation of H\textsubscript{2}O\textsubscript{2}, as well as the cell death rate were analyzed during 48 h. Pretreatment with oxo-C14-HSL resulted in a strong accumulation of H\textsubscript{2}O\textsubscript{2} after \textit{Pst AvrRpt2} inoculation, as visualized by the DAB staining (Fig. 7A-C), and evidenced by the increased number of DAB-positive clusters per leaf area (Fig. 7D). Cell death was visualized with trypan blue (TB) stain. Similar to the DAB stain, the positively stained clusters were counted in the 48 h time range following infection (Fig. 7H). Alike H\textsubscript{2}O\textsubscript{2} production, the number of dead cell clusters was enhanced in leaves pretreated with AHL as indicated by the increased numbers of blue-
stained clusters in those leaves (Fig. 7I-N). In conclusion, this results support the notion that the AHL affects defense mechanisms on different levels. Moreover, the systemic action (Fig. 2 and Fig. 3) might be accompanied with local effects (Fig. 7).

**Discussion**

In this report we show that $N$-acyl-homoserine lactone (AHL) modulates plant-pathogen interactions. Until now AHLs were identified in over 70 species of Gram-negative bacteria. Among the numerous quorum sensing systems, the AHL-based mechanism is best understood (Whitehead et al., 2001; Zhang and Dong, 2004; Waters and Bassler, 2005; Dong et al., 2007). Several studies show that also eukaryotes (plants as well as animals) respond to the presence of AHLs (Mathesius et al., 2003; Jahoor et al., 2008; Kravchenko et al., 2008; Ortiz-Castro et al., 2008; von Rad et al., 2008). Nevertheless, a direct evidence for their involvement in plant defense reactions has been missing. In this report we show that perception of oxo-C14-HSL significantly increases the resistance towards hemibiotrophic bacteria and biotrophic fungi, while it appears rather ineffective to microbes with a necrotrophic life style. Furthermore, our data suggest that activation of MAPKs, especially AtMPK6, is crucial for AHL-induced resistance.

**Plants react to bacterial quorum sensing molecules**

AHLs trigger diverse plant reactions: C6-HSL promotes growth (von Rad et al., 2008), while C10-HSL and C12-HSL modulate root development and root hair formation, respectively (Ortiz-Castro et al., 2008). Here we show that the long-chained AHL (oxo-C14-HSL) increased *Arabidopsis* resistance towards *Pseudomonas syringae* pv. *tomato* (*Pst*) and powdery mildew fungi in *Arabidopsis* and barley (Fig. 2, 3). Diverse bacteria such as *Acidithiobacillus ferrooxidans*, *Burkholderia pseudomallei*, *Rhodobacter spheroids*, *Sinorhizobium meliloti*, and *Yersinia enterocolitica* produce C14-HSL derivatives (Williams, 2007). In biofilms of the opportunistic plant and human pathogen *Pseudomonas aeruginosa*, oxo-C14-HSL can reach concentration of 40 μM (Charlton et al., 2000). Interestingly, the length of the fatty acid chain is not the only determinant for induced resistance. Modifications
at the \( \gamma \) positions in the fatty acid chain are also critical. Substitution by a hydroxyl- or oxo-group at this position has impact on the plant response; in our tests the oxo-group appeared to be more efficient in resistance induction (Fig. 2B). The strongest effect on resistance was observed in plants pretreated with C14 or C12 derivatives of AHLs (Fig. 2, 3 and Supplementary Fig. S7A). In contrast, similarly to the previous report by (von Rad et al., 2008), the growth promoting effect was present only in plants pretreated with the short-chained C6-HSL (Fig. 1 and Supplementary Fig. S7B).

**Homoserine lactones induce systemic reaction**

The transport of AHLs within plants has been addressed in several previous reports. Götz et al. (2007) demonstrated move of both C6-HSL and C8-HSL from roots into barley shoots, whereas C10-HSL was not transported. Similarly, C6-HSL but not the C10-HSL was translocated from roots to shoots in *Arabidopsis* plants (von Rad et al., 2008). Using different marker bacteria with sensitivity as low as 60 nM AHL, we confirmed that C6-HSL is systemically transported in *Arabidopsis* plants from root to shoot. However, we were unable to detect oxo-C14-HSL in shoots after root treatment (Fig. 1 and Supplementary Fig. 1B), showing that increased chain length reduces the motility of AHLs within the plant. Accordingly, when roots were treated with long-chained AHL, the resulting change of the resistance status in leaves towards leaf pathogens is consistent with an inducible, systemic disease resistance.

**Active AtMPK6 is required for AHL-induced resistance**

Induction of different MAPK cascades is one of the first steps in pathogen perception. The involvement of AtMPK6 and upstream compounds of the MAPK cascade in prompt response to pathogen attack is well documented (Asai et al., 2002). AtMPK6 together with its closest homologue AtMPK3, plays an important role in signaling cascades downstream of several PRRs being crucial in induction of defense mechanisms, reviewed by (Pitzschke et al., 2009). Nonetheless, little is known about the role of MAPK cascades in systemic resistance mechanisms such as systemic acquired resistance (SAR) or ISR (Vlot et al., 2008; Shah,
Activation of the MAP kinase kinase 7 (M KK7) is required for SAR against *Pseudomonas syringae* pv. *maculicola* (Psm) ES4326 and *Hyaloperonospora parasitica* Noco2 (now *H. arabidopsidis*) in *Arabidopsis* (Zhang et al., 2007). The authors demonstrated that ectopic expression of MKK7 is sufficient to generate a SAR-inducing signal. By showing that the *edr1* mutant constitutively expresses SAR, the EDR1, a CRT1-like MAPK kinase kinase, was suggested to be a negative regulator of SAR (Frye and Innes, 1998; Frye et al., 2001). Here we show that already local pretreatment of *Arabidopsis* with AHL modulates the inductions of AtMPK6 and AtMPK3. Moreover, the AHL-induced systemic resistance to *Pst* is compromised in *mpk6* but not in the *mpk3*, supporting the assumption that the AtMPK6 is predominantly required for resistance reinforcement caused by bacterial quorum sensing molecules. This is further supported by the transcriptional induction of *PR1* gene in oxo-C14-HSL pretreated *Col-0* and *mpk3*, but not in *mpk6* mutant, after subsequent flg22 induction (Supplementary Fig. S8).

Interestingly, in direct response to oxo-C14-HSL, *Arabidopsis* plants did not accumulate ROS, nor was SAR-associated *PR1* gene induced (Supplementary Fig. S4 and S6). Nevertheless, if oxo-C14-HSL-pretreated plants are challenged with biotrophic or hemibiotrophic pathogens they show higher resistance if compared to control plants. This phenomenon is comparable to the priming response induced in many plants by chemical resistance-inducers such as benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH); (Conrath et al., 2006). Recently it was suggested that AtMPK3 is the molecular base of priming responses (Beckers et al., 2009). The authors showed that AtMPK3 initially accumulates in an inactive form in the response to BTH, and that, upon inoculation with *Pst* DC3000, the kinase is activated and shows a prolonged activity (Beckers et al., 2009). Results presented in this report are very similar to those presented by Beckers et al., which suggest that also AHL may induce priming-like state in *Arabidopsis* or barley. However, we did observe some differences. The lack of enhanced accumulation of transcripts or inactive forms of either AtMPK3 or AtMPK6 upon AHL-treatments or the fact that AtMPK6 seems to play the major role in AHL-induced resistance, whereas AtMPK3 appears to be the major player in BTH-priming (Beckers et al.,
2009) suggest that AHL-induced resistance may differ from the BTH-induced priming mechanism.

An interesting possibility to explain the mechanism of AHL-enforced activation of MAPKs may be the inhibition of phosphatases. In Arabidopsis, several dual-specificity phosphatases and at least one of Protein Phosphatases 2C (PP2C) interact with AtMPK6 or AtMPK3 (Schweighofer et al., 2007; Bartels et al., 2009; Lumbreras et al., 2010). The Arabidopsis mkp2 mutant exhibits delayed wilting symptoms after infection with the biotrophic bacterium Ralstonia solanacearum, albeit stronger disease symptoms were observed after infection with the necrotrophic B. cinerea (Lumbreras et al., 2010).

Conclusion

Together with other reports, the results presented here provide strong evidence that plants evolved potent ways to take advantage of the bacterial quorum sensing. The data show that the specificity of the plant's response to N-acetyl-homoserine lactones depends on the length of the acyl moiety and on the functional group at the \( \gamma \) position. We suggest that long-chained AHLs have the ability to induce resistance against microbial pathogens. Finally we demonstrate that the mechanism of systemic resistance induced by oxo-C14-HSL relies on the presence of AtMPK6, and is most probably different from other chemical-induced priming effects.

Materials and methods

Plant Growth

For pathogenicity assays Arabidopsis thaliana Col-0, mpk3 and mpk6 plants were grown in a sterile hydroponics system. Surface-sterilized seeds (3 min with 50% ethanol / 0.5% Triton X-100 mix and briefly rinsed with 95% ethanol), were germinated and grown for 5 weeks at 22°C with 150 \( \mu \text{mol/m}^2/\text{s} \) light in 8/16h day/night photoperiod. Seeds were directly planted into 96-well plates with half MS (MS/2) medium (Murashige and Skoog, 1962) supplemented with vitamins, 0.7% agar and 1% sucrose. Plates were placed on 200 ml liquid MS/2 in a
sterile box. Medium was exchanged every second week. AHLs were added directly into the medium.

For transcriptional and biochemical analyses, *Arabidopsis* seeds were surface sterilized and germinated on sterile MS/2 with 0.4% gelrite and 1% sucrose for 2 weeks. Seedlings were then transferred into 6-well plates with 5 ml liquid MS/2 medium, prior to the pretreatment with AHLs.

For oxidative burst, ROS accumulation, and HR rate assays *Arabidopsis* plants were grown on soil in short day condition (8/16h day/night photoperiod) at 21°C for 4 weeks. Detached leaves were floated on sterile medium (10 mM phosphate buffer pH 8.0), supplied with AHLs for 3 days, then treated with flg22 or spray-inoculated with *Pst* bacteria.

*Hordeum vulgare* seeds (cv. Golden Promise) were surface sterilized with 70% ethanol and 6% NaClO and germinated for 2 days in the dark. Seedlings were transferred for 5 days on PNM medium (Schafer et al., 2009) supplied with 0.4% gelrite, and then for 3 additional days to liquid PNM medium for AHL treatment.

**AHL Treatment**

*N*-acyl-homoserine lactones AHLs (Sigma-Aldrich, Deisenhofen, Germany) were solved in acetone as 60 mM stock. AHL pretreatment occurred either directly in the hydroponics system, or AHLs were added into liquid or solid (0.4% gelrite) MS/2 medium. Plants were pretreated for 3 days. All experiments were performed with two controls: untreated plants (control) and solvent control (acetone).

**AHL Detection**

The detection of AHLs was done using transgenic bacterial strains: *Pseudomonas putida* (F117 pKR C12 GFP) (Steidle et al., 2001), *Serratia liquefaciens* (MG44 pBAH9 GFP) (Li, 2010), *Escherichia coli* (MT102 GFP pJBA89) (Andersen et al., 2001), and the *E. coli* strain (Top10 pSB403) (Winson et al., 1998) expressing luxR’luxI::luxCDABE from *Vibrio fisheri*, detecting a range of AHL from C6-HSL to oxo-C14-HSL (see Supplement Fig. S2). Reporter bacteria were grown on LB medium with specific antibiotics. Leaves (70 mg) or roots (30 mg)
were homogenized in acetone. Ten µl of cleared acetone extract was dropped on the bacteria lawn. Fluorescence was observed 2 hours after incubation using an ex: 480/40 nm, em: 510 nm filter.

Pathogenicity tests

*Pseudomonas syringae* DC3000 pv. *tomato* (*Pst*) and *Pst* expressing *AvrRpt2*, were cultured over night in Kings B medium, washed in 10 mM MgSO₄. Inoculation solution was adjusted to OD₆₀₀ = 0.1. Bacterial solution with 0.02% Silwet was sprayed uniformly over the plants. After: 1, 24, 48 and 96 hours 100 mg leaves were homogenized in 10 mM MgSO₄. Samples were diluted and plated for colony forming units (cfu) counting.

*Golovinomyces orontii* spore suspension (40,000 spores/ml) was sprayed uniformly onto leaves from plants grown in hydroponics culture and incubated for 5 days. Infected leaves were stained with calcofluor, and mycelia formation was counted in fluorescence Axioplan2, (Zeiss, Germany) microscope using an ex: 360/40 nm, em: 460/50 nm filter.

Five µl of spore suspension of *Plectosphaerella cucumerina* BMM (20,000 spores/ml) or *Botrytis cinerea* (20,000 spores/ml) were dropped directly onto the leaf surface. Conidia of *Blumeria graminis* f.sp. *hordei* race A6 (450 conidia/cm²) were directly sprayed onto barley leaves.

Oxidative burst assay

Oxidative burst was monitored after treatment with 100 nM flg22 using luminol-based assay. Leaves from soil-grown, 4-week-old plants were floated for 3 days on AHL-supplied medium and used to cutout segments, which were incubated over night in sterile water in 96 well plates. H₂O was then replaced with 180 µl of luminol working solution (1 ml luminol stock, 50 µl peroxidase, 50 ml distilled H₂O), and 5 µl PSB pH 8.0. Luminol stock; 1.77 mg luminol (Sigma-Aldich, Deisenhofen, Germany) dissolved in 1 ml 10 mM NaOH and 9 ml H₂O. The background was measured for 10 min, and then 20 µl of 0.25 µM flg22 were added into each well and measured for additional 60 min.
Immuno detection of phosphorylated MAPKs.

Arabidopsis seedlings pretreated with AHLs (Col-0, mpk3 and mpk6) were treated as indicated with 100 nM flg22. Total proteins were extracted and separated on 12% SDS-polyacrylamide gel. Western blot analyses were done using αMPK3, αMPK6, αMPK4 (Sigma-Aldrich, Deisenhofen, Germany) and the αpERK1/2 (Cell Signaling, Danvers, MA, USA) antibodies.

Transcriptional analysis

Seedlings were harvested at h as indicated after treatment with 100 nM flg22. 50-100 mg of plant material was homogenized and RNA extracted using the Trizol system. Two µg of total RNA was used for DNase digestion. cDNA synthesis was done according to the qScript cDNA Synthesis Kit from Quanta BioScience Inc. Reverse transcription efficiency was verified with semi-quantitative amplification of the actin 2 transcript. qRT-PCR was done using primers listed in Supplementary Table S1. All expression values were normalized to expression of UBO4 (At5g25760). The fold induction was normalized to 0 h after flg22 treatment, note that mpk3 mutant has approximately 4 and 144 times lower basal level of WRKY22 and WRKY29 then Col-0 plants, respectively. The basal level of WRKY22 expression in mpk6 plants is slightly higher; the WRKY29 is 3 times lower then in Col-0 plants.

Trypan blue and DAB staining

Leaves of 4-week-old plants were floated for 3 days on AHL-supplied medium and afterwards spray-inoculated with Pst AvrRpt2. Samples were harvested after 0, 24, and 48 (hpi). Leaves were either; (i) incubated in trypan blue solution for 1 min at 60°C followed by 30 min at 22°C and destained in 25 mM chloral hydrate; or (ii) incubated in DAB solution (1 mg/ml 3,3'-diaminobezidine in H2O) over night at 22°C and destained in ethanol/chloroform/trichloroacetic acid (4:1:0.15) for 24 h.
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Supplementary Figures and Tables

Supplementary Figure S1. Detection of AHLs using reporter bacteria

Supplementary Figure S2. AHL has no impact on bacterial virulence

Supplementary Figure S3. Different concentrations of oxo-C14-HSL have no effect on the resistance against Botrytis cinerea

Supplementary Figure S4. oxo-C14-HSL has no effect on the accumulation of ROS or the spontaneous cell death

Supplementary Figure S5. Transcriptional regulation of the MAP kinases AtMPK6 and AtMPK3 after pretreatment with oxo-C14-HSL

Supplementary Figure S6. Pretreatment with oxo-C14-HSL is not sufficient to induce the transcription of WRKY or PR1 genes

Supplementary Figure S7. Long-chained AHLs impact on plants resistance and development

Supplementary Figure S8. Transcriptional analysis of PR1 gene in mpk3/6 mutants

Supplementary Table S1. List of primers used in quantitative RT-PCR
References


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Figures

Figure 1. Long-chained oxo-C14-HSL has no impact on plant growth and is not transport within the plant

A-C: Col-0 plants were grown for 10 days on MS/2 medium and afterwards transferred to MS/2 medium supplied with 6 μM oxo-C14-HSL, or 6 μM C6-HSL. A: Col-0 plants grown for 4 days on C6-HSL or oxo-C14-HSL, bar = 1 cm, B: Fresh weights (mg/plant) of plants treated with different AHLs, n≥80, C: Root length of Arabidopsis plants on media supplied with different AHLs for 4 days. n ≥ 40, * p ≤ 0.05.

D: oxo-C14-HSL is not transported systemically throughout the plant. Six μM oxo-C14-HSL was added to the root medium for 3 days. Subsequently, fresh plant material was extracted in acetone and detection assays were performed by using the reporter bacterium E. coli Top10 pSB403 luxR/luxI::luxCDABE.

Figure 2. C14-HSL derivatives induce resistance against Pseudomonas syringae

A: Proliferation of Pseudomonas syringae pv. tomato DC3000 (Pst) on 5-week-old Arabidopsis was analyzed on hours post inoculation (hpi) as indicated. Plants were untreated (control) or pretreated with acetone or 6 μM oxo-C14-HSL, 3 days prior to infection with Pst bacteria. ** p ≤ 0.005; *** p ≤ 0.0005.

B: Colony forming units (cfu) of Pst harvested from 5-week-old Arabidopsis plants pretreated for 3 days with acetone, 6 μM oxo-C14-HSL or 6 μM OH-C14-HSL, and subsequently inoculated with Pst bacteria for 96h. a = p< 5.2E-6, b = p< 9.7E-11

Figure 3. oxo-C14-HSL induces resistance against Golovinomyces orontii and Blumeria graminis

Five-week-old Arabidopsis or 5-day-old barley plants were pretreated for 3 days with 6 μM oxo-C14-HSL in hydroponics cultures and then fungal conidia suspension was sprayed on their leaves.
A-D: Proliferation of G. orontii on Arabidopsis leaves, A: Number of mycelia per leaf at 5 days post inoculation (dpi). Student’s t-test performed on the raw data resulted in p = 0.33 (control vs. acetone); p = 0.04 (control vs. oxo) and p = 0.01 (acetone vs. oxo). B-D: Mycelia formation on untreated Arabidopsis leaves (control) (B); pretreated with acetone (C); or oxo-C14-HSL (D), shows no alternation in fungal development.

E-H: Proliferation of B. graminis on barley, E: Percent of interaction sites with elongated secondary hyphae (ESH), papillae, and hypersensitive reaction (HR), on first leaves at 5 dpi, F: ESH formation, haustorium (arrow), G: papillae formation (arrowhead), H: HR. * p ≤ 0.05; ** p ≤ 0.005; *** p ≤ 0.0005.

Figure 4. oxo-C14-HSL does not induce resistance against Botrytis cinerea or Plectosphaerella cucumerina BMM

Five-week-old Arabidopsis plants were untreated (A, E), pretreated with acetone (B, F), or 6 μM oxo-C14-HSL (C, G) for 3 days. Conidial suspensions were dropped onto detached leaves. Necrotic symptoms or lesion diameters were analyzed at 5 dpi. Classification of symptoms: I: healthy leaf, II: leaf necrotic at 25%, III: 50% of leaf surface show necrosis, IV: 75% necrotic surface, V: 100% of the leaf surface is necrotic.

D: Necrotic symptoms caused by B. cinerea.

H: Lesions caused by P. cucumerina BMM.

Figure 5. Response to flg22 in plants pretreated with AHL

A: Oxidative burst in response to 100 nM flg22. Production of H2O2 was measured using the luminol-based assays in detached leaf discs from soil-grown plants. Leaf disc were untreated (control), or pretreated with acetone or oxo-C14-HSL for 3 days before treatment with flg22. Arrow shows the time of flg22 addition.

B: Time course of phosphorylation status of AtMPK6 and AtMPK3. Whole 2-week-old Arabidopsis Col-0 seedlings were pretreated with 6 μM oxo-C14-HSL for 3 days. Responses were analyzed in seedlings during minutes after treatment with 100 nM flg22. Upper panel
shows an immunoblot with αpERK1/2 antibody, the lower panel show coomasie stain of the membrane. Panels below show immunoblots with the specific αMAPKs antibodies.

C-F: Transcriptional regulation of *WRKY* and *PR1* genes after pretreatment with oxo-C14-HSL. Total mRNA was extracted from 2-week-old *Arabidopsis Col*-0 seedlings, pretreated for 3 days with 6 μM AHL plants and subsequent treatment with 100 nM flg22 at h as indicated. RT-qPCR analysis was performed using *WRKY* and *PR1* specific primers (Supplementary Tab. S1).

**Figure 6. AHL fails to modulate the response to flg22 in MAPK mutants**

A: Time course of phosphorylation status of AtMPK3 and AtMPK6 in *Col*-0, *mpk3* and *mpk6*. Whole 2-week-old *mpk3*, *mpk6* or *Col*-0 seedlings were pretreated with 6 μM oxo-C14-HSL for 3 days. Responses were analyzed during minutes after treatment with 100 nM flg22. Upper panels show an immunoblot with αpERK1/2 antibody, lower panels show coomasie stain of the membrane.

B: *Pst* proliferation on *mpk3* and *mpk6* plants. Plants were pretreated as indicated for 3 days and spray-inoculated with bacteria. Cfu numbers were analyzed after 48 h. *Col*-0 plants were used as a control.

C-F: Transcriptional regulation of *WRKY* transcription factors after pretreatment with oxo-C14-HSL. Total mRNA was extracted from 2-week-old *Arabidopsis mpk3* or *mpk6* seedlings, pretreated for 3 days with 6 μM AHL plants and subsequent treatment with 100 nM flg22 at h as indicated. RT-qPCR analysis was performed using *WRKY* specific primers (Supplementary Tab. S1). The fold induction was normalized to 0 h after flg22 treatment, note that *mpk3* mutant has approximately 4 and 144 times lower basal level of *WRKY22* and *WRKY29* then *Col*-0 plants, respectively. The basal level of *WRKY22* expression in *mpk6* plants is slightly higher; the *WRKY29* is 3 times lower then in *Col*-0 plants.
Figure 7. oxo-C14-HSL promotes accumulation of ROS and induces HR rate after challenge with *Pst* in *Arabidopsis* plant

*Arabidopsis* plants were grown on soil and detached leaves were pretreated for 3 days with H$_2$O (control) (A, E, I, L), acetone (B, F, J, M) or 6μM oxo-C14-HSL (C, G, K, N). Pretreated leaves were inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 strain *AvrRpt2* (avirulent) by spraying. Leaves were stained with diaminobenzidine (DAB) in order to visualize the accumulation of H$_2$O$_2$ (A-G), or with trypan blue (TB) in order to visualize the dead cells (H-N).

**A-C**: DAB stain of *Arabidopsis* leaves inoculated with *Pst AvrRpt2* for 48h. E-G show magnifications of respective A-C. Bar = 0.1 cm

**D**: Representation of DAB-positive stained clusters in *Pst AvrRpt2*-inoculated leaves. Pretreated leaves were infected with bacteria and stained with DAB at hpi as indicated. Number of stained clusters was calculated per cm$^2$, 60 clusters were analyzed in 3 independent experiments.

**H**: Numbers of TB-positive stained clusters in *Pst AvrRpt2*-inoculated leaves. Pretreated leaves were inoculated with bacteria and stained with TB at hpi as indicated. Clusters presence was calculated per cm$^2$, 60 clusters were analyzed in 3 independent experiments.

**I-K**: TB stain of *Arabidopsis* leaves inoculated with *Pst AvrRpt2* for 48h. L-N show respective magnifications of I-K.
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D: Representation of DAB-positive stained clusters in Pst AvrRpt2 inoculated leaves. Pretreated leaves were infected with bacteria and stained with DAB at hpi as indicated. Number of stained clusters was calculated per cm², 60 clusters were analyzed in 3 independent experiments.

H: Numbers of TB-positive stained clusters in Pst AvrRpt2 inoculated leaves. Pretreated leaves were inoculated with bacteria and stained with TB at hpi as indicated. Clusters presence was calculated per cm², 60 clusters were analyzed in 3 independent experiments.