N-acyl-homoserine lactones-producing bacteria protect plants against plant and human pathogens

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Summary
The implementation of beneficial microorganisms for plant protection has a long history. Many rhizobia bacteria are able to influence the immune system of host plants by inducing resistance towards pathogenic microorganisms. In this report, we present a translational approach in which we demonstrate the resistance-inducing effect of *Ensifer melloti* (*Sinorhizobium melloti*) on crop plants that have a significant impact on the worldwide economy and on human nutrition. *Ensifer melloti* is usually associated with root nodulation in legumes and nitrogen fixation. Here, we suggest that the ability of *S. melloti* to induce resistance depends on the production of the quorum-sensing molecule, oxo-C14-HSL. The capacity to enhanced resistance provides a possibility to the use these beneficial bacteria in agriculture. Using the *Arabidopsis-Salmonella* model, we also demonstrate that the application of N-acyl-homoserine lactones-producing bacteria could be a successful strategy to prevent plant-originated infections with human pathogens.

Introduction
The best understood mechanism of systemic resistance induced by beneficial microorganisms is the induced systemic resistance (ISR), where plants have a potentiated defensive capacity against future biotic challenges. Its mechanism requires the presence of an operable non-expressor of PR1 (NPR1) and components from ethylene (ET) and jasmonic acid (JA) signalling cascades. Together with systemic acquired resistance (SAR), which is usually associated with a previous pathogen attack, ISR and SAR are under intense study (Van Wees et al., 2008; Dempsey and Klessig, 2012; Fu and Dong, 2013; Shah and Zeier, 2013). Nevertheless, the molecular basis of ISR is not completely understood because, for example, the beneficial *Pseudomonas fluorescens* strain 89B61 induces resistance in a JA- and ET-independent manner (Ryu et al., 2003).

The exchange of signals between plants and nearby rhizobacteria contributes to the activation of ISR. Small signalling molecules, for example N-acyl-homoserine lactones (AHLs) from many Gram-negative bacteria, are used for their intra-population communication called quorum sensing (QS) (Kaplan and Greenberg, 1985; Fuqua and Winans, 1994). Remarkably, plants are able to detect and respond to bacterial QS molecules (Mathesius et al., 2003). The detection of AHLs and systemic response is an essential aspect of the establishment of mutualistic relationships (Bauer et al., 2005). Studies of plant responses to AHLs were first done in the model plant *Medicago truncatula*, where these molecules were found to affect extensive functions including cytoskeletal elements, transcriptional regulation and responses to defence, stress and hormones (Bauer et al., 2005). Another study on the interaction between *Serratia liquefaciens* and tomato (*Solanum lycopersicum*) provided also indications that QS molecules of rhizosphere bacteria influence plant defence responses (Schuhegger et al., 2006). In this study, authors used the *S. liquefaciens* strain MG1, which produces C4- and C6-homoserine lactones when colonizing the root surface (Gantner et al., 2006). Colonization of the roots with *S. liquefaciens* MG1 protected tomato plants against the leaf-pathogenic fungus *Alternaria alternata*, in contrast to the AHL-negative *S. liquefaciens* mutant MG44 that was not able to provide such protection (Schuhegger et al., 2006). Similarly, colonization with the AHL-producing *Serratia plymuthica* strain HRO-C48 protected cucumber plants (*Cucumis sativus*) from the damping-off disease caused by *Pythium aphanidermatum*, as well as tomatoes and beans (*Phaseolus vulgaris*) from the infection with the grey mould-causing fungus *Botrytis cinerea* (Pang et al., 2009). Comparable with the previous study, the AHL-negative *spl* mutant of *S. plymuthica* could not confer
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Barley and wheat can be primed by S. meliloti expR+ for enhanced resistance

Based on the observation that the inoculation of plants with AHL-producing bacteria induce resistance in plants towards diverse pathogens (Schuhegger et al., 2006; Pang et al., 2009; Zarkani et al., 2013), we tested the hypothesis that the induced resistance caused by S. meliloti in crop plants depends on AHL production in a similar way as in A. thaliana (Zarkani et al., 2013). For this purpose, we used two S. meliloti strains, the expR+ strain carrying the pWBexpR plasmid (M. McIntosh, pers. commun.), which allows the production of the long-chain oxo-C14-HSL, and the S. meliloti attM strain that is unable to accumulate AHLs due to the expression of the Agrobacterium tumefaciens lactonase gene attM from the pBRR2-attM plasmid, (Zarkani et al., 2013). Barley cultivar Golden Promise plants were grown on soil and inoculated with S. meliloti by watering three times during 2 weeks before the challenge with the powdery mildew fungus Blumeria graminis f. sp. hordei. The cultivar Golden Promise is susceptible to B. graminis, i.e. 50% of its epidermal cells allow fungal penetration causing the formation of elongated secondary hyphae (ESH) and subsequent disease symptoms (Fig. 1A and B). However, plants inoculated with the oxo-C14-HSL-producing S. meliloti strain expR+ (Fig. 1C) showed enhanced resistance as a result of the augmentation of hypersensitivity response (HR) reactions at the sites of fungal penetration, thus diminishing the number of developing pustules (Fig. 1B). Correspondingly, the lack of this enhanced HR response in plants inoculated with the attM strain (Fig. 1A) suggests that the increased resistance depends on the production of oxo-C14-HSL by S. meliloti.

Previously, we observed enhanced formation of papillae in barley plants pretreated with oxo-C14-HSL, which play a crucial role in resistance against fungal pathogens like B. graminis (Schikora et al., 2011a). Papillae are a complex structure between the plasma membrane and the plant cell wall, and depending of the plant species, the composition of these defence structures can consist of phenolics, reactive oxygen species (ROS) and cell wall proteins and polymers (Voigt, 2014). Hydrogen peroxide, one form of ROS that accumulates in forming papillae, is used by peroxidases to cause the cross-linking of proteins and phenolics for cell wall reinforcement. (Fig. 2A) (Hückelhoven, 2007; Deepak et al., 2010). In order to test whether oxo-C14-HSL influences this defence mechanism, we assessed the expression of one of the key enzymes in ROS production in barley, the peroxidase HvPRX7. To this end, barley plants were grown under sterile cultures for 10 days and the roots were pretreated with 6 μM oxo-C14-HSL or with the solvent control
acetone for 3 days; subsequently, the first and second leaves were inoculated with *B. graminis*, and finally harvested for total RNA extraction after 24 and 48 h. Results from the quantitative reverse transcription polymerase chain reaction (RT-PCR) revealed that in contrast to control, plants pretreated with oxo-C14-HSL displayed a higher expression of *HvPRX7* in response to *B. graminis* at 24 hai (Fig. 2B). Similarly, the expression of the Pathogenicity Related1 (*HvPR1*) gene was higher in oxo-C14-HSL-pretreated plants, compared with the control (Fig. 2C). To substantiate our findings, we tested the impact of oxo-C14-HSL on ROS production by exploiting a different pathogen–host system. We used wheat plants cultivar Bobwhite grown and pretreated as described above before a challenge with the stem rust-causing fungus *Puccinia graminis f. sp. tritici*. Because *P. graminis* enters the interior of mesophyll tissues via stomata openings, the closure of stomatal pores is an effective...
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Inoculation with oxo-C14-HSL-producing S. meliloti protects tomato from late blight disease

We extend our analysis from monocots to a dicot crop plant with high agronomic interest. We tested the induced resistance in tomato against Phytophthora infestans, which is nowadays one of the principal pathogens causing the late blight disease and a worldwide damage of 6 billion US dollars each year (Nowicki et al., 2011). Similar to the experiments with barley, soil-grown tomato plants cultivar Moneymaker were inoculated four times with S. meliloti during 4 weeks prior to the challenge with P. infestans; control plants were pretreated with MgSO₄. Disease symptoms were assessed 4 and 7 days after the challenge with P. infestans and the efficacy of the pretreatment was calculated using Abbott’s formula (Abbott, 1925). In accordance to previous results, inoculation with S. meliloti strain expR+ induced resistance against P. infestans in tomato plants (Fig. 4A and B). Moreover, we observed differences between the inoculation with the oxo-C14-HSL-producing expR+ strain and the AHL-negative attM strain (Fig. 4C), implying that as in the case of fungal pathogens, resistance towards this Oomycete depends on the production of AHL.

AHL-producing bacteria can promote resistance towards human pathogens

Salmonella are Gram-negative bacteria that are able to colonize humans and plants. These bacteria are the causal agents of gastroenteritis and typhoid fever in humans due to the ingestion of contaminated food or water (Pang et al., 1995). Additionally, in recent years the proportion of raw-food related outbreaks in the USA reached 25% (Rangel et al., 2005). The increasing number of infections related to the consumption of fresh fruits and vegetables contaminated with these bacteria is very alarming and suggests that plants may be a substantial reservoir for Salmonella. Many reports proposed a complex interaction between Salmonella and the host plant because the plant immune system seems to play a key role in the outcome of the colonization (Schikora et al., 2011b; 2012; Shirron and
Yaron, 2011). For this reason, the induction of defence mechanisms by oxo-C14-HSL-producing *S. meliloti* was tested as a potential measure to reduce the risk of plant-related infections. According to the experiments above, soil-grown *A. thaliana* Col-0 plants were watered four times with *S. meliloti* or with the respective controls before defying the plants with *Salmonella enterica* serovar Typhimurium strain 14028s. The proliferation of *S. Typhimurium* was assessed during 6 days after syringe infiltration. Interestingly, *Arabidopsis* plants pretreated with the *S. meliloti expR*+ caused a lower *Salmonella* proliferation than plants pretreated with *S. meliloti attM* or MgCl₂ (Fig. 5A), which corresponds to the diminished disease symptoms in leaves from *S. meliloti expR*+ pretreated plants (Fig. 5B and C). This suggest that in line with the effects seen in barley and tomato, the production of AHLs allowed *S. meliloti* to prime *Arabidopsis* plants for an enhanced defence against *Salmonella*.

**Discussion**

In this report, we present the impact of oxo-C14-HSL-producing bacteria on the plant immune system. We demonstrated that the previously described AHL-priming
(Schenk et al., 2014) and the effect of oxo-C14-HSL-producing bacteria is not restricted to the commercial molecule, nor to the model plant A. thaliana (Zarkani et al., 2013). In a translational approach, we showed that the use of AHL-producing bacteria could be a potential method to improve plant resistance and to decrease the yield loss caused by many pathogens. Moreover, AHL-induced resistance may reduce the risk of plant-originated outbreaks of salmonellosis in addition to other possible related diseases.

AHLs used by Gram-negative bacteria may vary in the length of the lipid side chain and in the substitution of the C2-atom (O- or OH- group). The length of the lipid side chain is essential for the effect on plants; for example, C4-HSL, C6-HSL, oxo-C6-HSL and oxo-C8-HSL promoted growth in Arabidopsis (von Rad et al., 2008; Liu et al., 2012; Schenk et al., 2012), whereas oxo-C10-HSL induced the formation of adventitious roots in mung beans (Bai et al., 2012). On the other hand, only some AHL molecules were reported to have resistance-inducing attributes. A comparison of plant responses with different AHLs at the transcriptome and proteome levels revealed that just long-chain AHLs could induce resistance-related changes at the transcriptome and proteome levels (Mathiesius et al., 2003; Miao et al., 2012; Schenk et al., 2014). The molecule oxo-C14-HSL and to a lesser extend OH-C14-HSL induced resistance in Arabidopsis and barley plants towards biotrophic and hemibiotrophic pathogens (Schikora et al., 2011a,b). Sinorhizobium meliloti produces different long-chain AHLs, like oxo-C14-HSL (Teplitski et al., 2003; Zarkani et al., 2013), and therefore we decided to use this bacterium in this work to study the interaction between crop plants and AHL-producing rhizobacteria. Besides the acknowledged benefit that the interaction between S. meliloti and its native host M. truncatula results in nodulation and N2-fixation, the oxo-C14-HSL-producing S. meliloti strain induced resistance in the non-host plant Arabidopsis against Pseudomonas syringae pathovar tomato (Zarkani et al., 2013). For this reason, to ascertain our translational approach, we used economically important non-host crop plants of S. meliloti to study the impact of AHLs. The resistance induced by beneficial bacteria is referred as ISR, and it has been exhaustively studied employing P. fluorescens and Bacillus spp. bacteria; for review, see Pieterse and colleagues (2014). Today, the mechanism of ISR is relatively well understood; it requires NPR1 and components of the JA- and ET-signalling pathways. The transcription factor MYB72 was postulated to play a key role in ISR and link JA- and ET-signalling pathways (Van der Ent et al., 2008). However, the AHL-induced resistance, termed AHL priming, seems to depend on other mechanism. Recent findings indicated that instead of MYB72 and JA/ET pathway(s), the salicylic acid/oxylipin pathway influenced the AHL priming (Schenk et al., 2014). Moreover, the resistance-inducing effect of the long-chain AHLs in Arabidopsis was reflected in the reinforcement of the cell wall through the accumulation of callose, phenolic compounds and lignins, as well as to an intensified stomatal closure in response to bacterial attack. Likewise, we observed that the inoculation with oxo-C14-HSL-producing S. meliloti strain, as well as pretreatment with the pure oxo-C14-HSL molecule, primed barley and wheat plants for enhanced ROS production. Membrane-bound NADPH oxidase and apoplastic peroxidase proteins usually contribute to this transiently increased production of toxic ROS known as oxidative burst. In addition, ROS act as secondary messengers, which allocates them a central role in plant defence mechanisms (Marino et al., 2012).

Intriguingly, S. meliloti expR+ induced plant resistance towards Salmonella, which is generally considered an animal or human pathogens. Until now, the infection mechanism(s) used by Salmonella to successfully and simultaneously colonize diverse hosts like animals and plants are poorly understood. Stomata openings were identified as possible entry points of bacteria into the inner layers of the mesophyll (Kroupitski et al., 2009). Remarkably, although some plant species (e.g. arugula) allowed Salmonella to internalize, others (e.g. parsley) seemed to prevent internalization (Golberg et al., 2011). The plant immune system appears to play a central role during colonization of Salmonella as indicated by the induction of defence mechanisms after inoculation with these bacteria (Schikora et al., 2008; Meng et al., 2013; Garcia et al., 2014) and by the fact that Salmonella can actively suppress those mechanisms in tobacco (Nicotiana tabacum) and Arabidopsis plants (Schikora et al., 2011a,b; Shirron and Yaron, 2011). Accordingly, the use of beneficial bacteria with the ability to enhance defence mechanisms in crop plants that are susceptible to infection with human pathogens could be an alternative to lower the risk of disease outbreaks associated with contaminated fruits or vegetables.

**Experimental procedures**

**Plant growth**

Barley (Hordeum vulgare) cultivar Golden Promise, wheat (Triticum aestivum) cultivar Bobwhite and tomato (S. lycopersicum) cultivar Moneymaker were grown on soil (for pathogenesis assays) or under sterile conditions (for transcriptional analyses and oxo-C14-HSL treatments) in a long day photoperiod at 19°C (barley and wheat plants) or at 25°C, 80% humidity (tomato plants). For the sterile system, 1 l of jars were used and plants grew on partially solidified 1/10 strength plant nutrient medium (PNM) (0.5 mM KNO3, 2 mM MgSO4, 0.2 mM Ca(NO3)2, 0.43 mM NaCl, 0.14 mM K2HPO4, 2 ml l−1 Fe-EDTA [20 mM FeSO4, 20 mM K2HPO4, 2 ml l−1 Fe-EDTA [20 mM FeSO4, 20 mM
Arabidopsis thaliana Colombia-0 plants were grown on soil at 22°C with 150 μmol m\(^{-2}\)s\(^{-1}\) light in 8/16 h day/night photoperiod.

**Seed disinfection**

For sterile growth, barley (H. vulgare) cv. Golden Promise and wheat (T. aestivum) cv. Bobwhite seeds were soaked shortly in sterile water and then in 70% ethanol. Subsequently, the seeds were immersed for 90 min in 6% sodium hypochlorite with continuous stirring. Seeds were then rinsed two times with sterile water at pH 3.0 and several times with sterile water at pH 7.0 until no trace of sodium hypochlorite was detected. For germination, the seeds were placed on wet sterile filter paper for 3 days. Arabidopsis thaliana Col-0 seeds were surface-disinfected with 50% ethanol/0.5% Triton X-100 for 30 min and briefly rinsed with 95% ethanol. For germination, seed were placed for 10 days on sterile half-strength MS medium supplied with 0.4% gelrite and 1% sucrose.

**Oxo-C14-HSL treatment**

Sixty millimolar stock solution of oxo-C14-HSL (Sigma-Aldrich) was prepared by dissolving the molecule in acetone. Ten-day-old barley or wheat plants cultivated on 1/10 PNM medium under sterile conditions were treated with oxo-C14-HSL at final concentration of 6 μM. All experiments were performed with the solvent control acetone.

Sinorhizobium meliloti inoculation

Sinorhizobium meliloti (Ensifer meliloti) Rm2011 expR+ containing the pWBexpR plasmid (M. McIntosh, pers. comm.) and S. meliloti (pBBR2-attM) carrying the lactonase gene attM from Agrobacterium tumefaciens were used. The rhizosphere was inoculated with S. meliloti expR+, S. meliloti attM (both OD\(\text{OD}_{600}\) = 0.2) or watered with 10 mM MgSO\(_4\) as control. Extraction of AHLs originated from S. meliloti liquid cultures was performed by vortexing with CHCl\(_3\) and discarding the aqueous phase after centrifugation. The CHCl\(_3\) phase was then evaporated using an ultra-speed vacuum centrifuge. The remaining residue was dissolved in acetone.

Detection of oxo-C14-HSL was accomplished by dropping 10 μl of the extracted AHLs onto reporter bacteria: Escherichia coli strain MT102 carrying the pJBA89 plasmid [Ap\(^{\text{r}}\); pUC18Not-luxR-P\(_{\text{lac}}\)RBSII-gfp(ASV)-T\(_{\text{c}}\)-T\(_{\text{m}}\)] (Andersen et al., 2001). After 2 h, the fluorescence was observed using an ex: 480/40 nm and em: 510-nm filters.

**Blumeria graminis treatment**

Three days after the last treatment with S. meliloti strains or MgSO\(_4\), barley leaves (cv. Golden Promise) were inoculated with Blumeria graminis f. sp. hordei by blowing fresh spores originated from infected barley leaves (~ 100 conidia/cm\(^2\)). The inoculated leaves were kept on 1% water-agar plates at room temperature under low-light conditions for 2 days.

**Puccinia graminis treatment**

Urediniospores of Puccinia graminis f. sp. tritici were collected from infected plants (density of ~ 10\(^6\) spores ml\(^{-1}\)) and sprayed on 10-day-old wheat plants (cv. Bobwhite) that were previously pretreated with oxo-C14-HSL or control (acetone) for 3 days. The inoculated plants were placed for 12 h in the dark. Subsequently, inoculated plants were exposed to normal light condition and kept for 11 days in a growth chamber with an average of 19°C and 90% relative air humidity.

**Phytophthora infestans treatment**

The P. infestans isolate was originally obtained from infected potato foliage. To maintain its virulence, it was invigorated by monthly passage through potato tubers and the P. infestans cultures (16–22 days old) were maintained on solid V8 juice agar in the dark at 15°C. In order to obtain P. infestans spore solution, the culture was flooded with sterile, distilled water. The spore density was counted using a Fuchs–Rosenthal counting chamber. To improve the zoospore release, the sporangial suspension was placed at 5°C for 3 h and the final solution was adjusted to a density of about 80,000 spores ml\(^{-1}\). For the treatment, plants were drenched with the inoculation solution using a pneumatic spray gun and kept at 16°C in the dark with 100% relative air humidity. After 48 h, plants were exposed to a dark/light regime of 16/8 h and 65% relative air humidity. The disease severity was assessed by visual estimation of the infested leaf area and documented with digital pictures. The scale for rating was 1, 5, 10, 20, 30, 50, 60, 70, 80, 90 and 100%. The rating was done 4 and 7 days after inoculation. Each plant was rated separately and means were calculated of five replications per treatment. The formula adapted from Abbott (1925): \(EF = (Mtr - Mte)/(100 - Mte)\), in which EF is the percentage of treatment efficiency, Mtr is the percentage of treatment severity and Mte is the percentage of control severity, was used to calculate the efficiency of the treatment.

**Salmonella Typhimurium treatment**

In order to assess the Salmonella proliferation rate in plants, soil-grown 4-week-old A. thaliana Col-0 plants were pretreated as indicated before, and thereafter infiltrated using syringe infiltration with wild-type S. enterica serovar Typhimurium strain 14028s carrying the pEC75 plasmid confering resistance to ampicillin. Bacteria were grown until the early log phase in LB medium, washed and resuspended in 10 mM MgCl\(_2\). Infiltration solution was adjusted to OD\(\text{OD}_{600}\) = 0.1, \((1.7 \times 10^8\) bacteria ml\(^{-1}\)). Bacterial population was monitored during 6 days post infiltration using selective LB medium containing ampicillin, as described in (Schikora et al., 2008).

**DAB staining**

Leaves were partially submerged in DAB-staining solution (pH 3.8) at a concentration of 1 mg ml\(^{-1}\) for 6 h. Thereafter, leaves were distained with ethanol : chloroform : trichloroacetic acid (4:1.0:1.5%) solution for 2 days and transferred to 50% glycerol until cytological observations. Development of ESH, formation of papillae or production of

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ROS were evaluated using an Axioplan 2 (Zeiss, Germany) microscope.

Transcriptional analyses

Barley cv. Golden Promise leaves pretreated with o xo-C14-HSL or acetone, and subsequently inoculated with Blumeria graminis f. sp. hordei were harvested at 0, 24 and 48 h after inoculation (hai). Plant material was homogenized and the total RNA was extracted using the Trizol system. cDNA synthesis was performed using 2 µg of total RNA according to qScript cDNA Synthesis Kit (Quanta BioScience Inc.), quantitative RT-PCR was performed using primers listed in Table S1. All expression values were normalized to expression of HvUBQ60 (Genbank: M60175.1).

Conclusions

We showed that the resistance-inducing effect of S. melliloti in crop plants depends on the production of o xo-C14-HSL. In three different crop plants of worldwide economic importance and relevant for the food chain, o xo-C14-HSL-producing bacteria enhanced their resistance against specific pathogens. In addition, using the Arabidopsis–Salmonella model, we demonstrate that the same strategy could be a successful method to prevent outbreaks of food-borne diseases originated from plants.

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Conflict of interest

None declared.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1.** List of primers used in quantitative RT-PCR. Annealing temperature for all primers was set at 60°C.