

**Systemic responses of barley to the growth promoting, endophytic bacterium
Acidovorax radialis N35 and role of 3-hydroxy-C10-homoserine lactone
production in root colonization and plant perception**

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To my family

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
Amp ^R	ampicillin resistance
BP	band pass (filter type for fluorescence microscope)
cDNA	complementary deoxyribonucleic acid
Cm ^R	chloramphenicol resistance
Cy3	cyanine dye 3
Cy5	cyanine dye 5
DAPG	2, 4-diacetylphloroglucinol
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Em ^R	erythromycin resistance
<i>et al.</i>	<i>et alii</i> , and others
EtOH _{abs}	absolute ethanol
Fig.	figure
Fluos	5(6)-carboxyfluorescein-N-hydroxysuccinimidester
Gm ^R	gentamycin resistance
i.e.	that is
Kb	kilo base pairs
Km ^R	Kanamycine resistance
LP	long pass (filter type for fluorescence microscope)
MCS	multi cloning site
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
rRNA	ribosomal ribonucleic acid

Abbreviations

Rp ^R	rifamycin resistance
Sm ^R	streptomycin resistance
Tell ^R	tellurite resistance
Tc ^R	tetracycline resistance
Tris	Tris (hydroxymethyl)-aminomethan
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 Introduction

1.1 Plant growth promoting rhizobacteria (PGPR)

The rhizosphere soil surrounding plant roots contains many times more microbes than the bulk soil (Lugtenberg and Kamilova 2009). Among the rhizosphere bacteria there is a category named PGPR characterized by their ability to promote plant growth and health. These PGPR can be classified into rhizospheric and endophytic bacteria based on the colonization behavior. The former ones only colonize the root surface (rhizoplane), such as some *Azospirilli* (Bloemberg and Lugtenberg 2001) while the latter ones can additionally penetrate into roots and grow inside of plants such as *Gluconacetobacter diazotrophicus* (Alqueres et al. 2012). Growth of various plants was shown to be affected by their root associated PGPR, for instance maize, rice, sugarcane, sorghum, wheat, lettuce, radish, pine, and rape. PGPR species are widely distributed across the phylogenetic tree, however, many of the isolates can be classified as *Pseudomonas* or *Bacillus* (Vessey 2003).

PGPR activity is influenced by or even dependent on plant root exudates. The roots provide soluble nutrients for their growth which includes mostly organic acids, making up 83% of the total amount of exudates, as well as photosynthates, sugars and the polyamine putrescine. Also a vast range of insoluble chemical compounds are released from roots (e.g. cellulose, lignin, proteins) (Liu et al. 2012a). Due to this high abundance of nutrients, PGPR can multiply in the rhizosphere and colonize the root surface. For example, *Pseudomonas putida* PCL1444 can reach a tenfold increase in cell numbers in the presence of grass seedling in soil (Liu et al. 2012a). On the other hand, the exudates can also include some toxic secondary metabolites that inhibit some microbes, providing a selection advantage to the resistant ones.

1.2 Microbial ecology of rhizobacteria and endophytes

Microbe-host interactions cover a wide spectrum, from pathogenic to beneficial and even symbiotic interactions in plant and animal/human hosts (Berg et al. 2005, Mendes et al. 2013). This extremely large scope of interaction types can be found even within a single bacterial genus, like *Burkholderia* (Angus et al. 2014) or *Herbaspirillum* (Balsanelli et al. 2012) and even within

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one species as recently reported for *Pantoea ananatis* (Sheibani-Tezerji et al. 2015). Plant growth promotion by rhizosphere-associated, root colonizing microbes is a well-documented phenomenon (Dessaux et al. 2010). It can be considered as a symbiotic and synergistic microbe-plant interaction, although no particular symbiotic organs exist. Benefits of these more or less loose associations can be observed particularly when the plant is challenged by limiting nutrient supply, by abiotic stresses like hypersaline conditions or lack of water, or when attacked by pathogens (Raaijmakers et al. 2009).

This beneficial effect of PGPR is achieved by several mechanisms: Some PGPR can help to dissolve and complex insoluble phosphate into orthophosphate which can be taken up by plants. Some siderophores released by PGPR help to transfer chelate insoluble polyhydroxy ferric complex to soluble Fe^{3+} complex which can be taken up by active transport mechanisms (Saharan 2011). A number of diazotrophic PGPR are known like *Herbaspirillum* spp. *Gluconacetobacter diazotrophicus* or *Azoarcus* spp. These bacteria contain *nif* genes which encode nitrogenase catalyzing atmospheric N_2 into ammonia and make it thus accessible to plants. These PGPR can also promote plant nitrate absorption (Mantelin and Touraine 2004). PGPR can also change the morphology and physiology of roots to enhance water and nutrients uptake mostly by phytohormonal interactions. For example *Azospirillum brasilense*, which is well documented to increase cereal yield by up to 30% (Song et al. 2011), produces phytohormones including gibberellins, cytokinins and auxin to stimulate plant development (Van Loon 2007). Several GAs were isolated from seven species of *Acetobacter*, *Azospirillum* and *Bacillus* PGPR (Bottini et al. 2004). Auxins are the most important plant hormone produced by *Azospirillum*, *Bacillus* and *Pseudomonas* spp. (López-Bucio et al. 2007). PGPR can ameliorate plant growth when inhibited by ethylene through a decrease in ACC (1-Aminocyclopropane-1-carboxylic acid) content. The decrease of ethylene through the degradation of the precursor ACC by ACC-deaminase activity is an efficient mechanism for well performing PGPR (Glick et al. 2007). Several volatiles with low molecular weight, such as 2, 3-butanediol and acetoin, are produced by *B. amyloliquefaciens* IN937a and GB03. They significantly promote *Arabidopsis* growth and enhance the area of leaves (Ryu et al. 2003).

Finally, PGPR exhibit also direct or indirect disease inhibition. The direct way is through antagonism with pathogens and nutrient competition. The indirect way includes induction of

plant systemic resistance which is called ISR (induced systemic resistance) and enhances the ability to suppress subsequent pathogen infection. For example, inoculation of the rhizosphere with *Pseudomonas* spp. enhanced the immunity of barley to the necrotrophic fungal pathogen *Gaeumanomyces graminis* causing “take-all-disease” and induced resistance to leaf pathogen *Rhynchosporium secalis* (Fröhlich et al., 2011).

1.3 Quorum sensing

Quorum sensing (QS) is a well-known universal communication mechanism in bacteria. In Gram negative bacteria, AHLs (N-acyl homoserine lactones) are used as signaling molecules known as auto-inducer (AI). In recent years several studies have shown that AHLs as pure substance can arouse similar beneficial effect in plants as the producing PGPR (Schikora, A et al. 2014).

1.3.1 QS in Gram-negative bacteria

Quorum sensing communication is based on a constitutive low synthesis rate of an AI signaling molecule. When the AI accumulates and surpasses a certain threshold concentration, the AI binds to the regulator protein R. The formed complex activates the synthesis gene I and other specific genes. The first QS system to be studied was the *lux* system in *Vibrio fischeri*. These bacteria are located inside of the light organ of fish and squid. When the density of bacteria increases towards a threshold level they start to luminesce. This density dependent luminescence is mediated by the autoinducing signaling compound N-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C6HL). AHLs are composed of two parts. One is the lactone ring which is the conserved structure among various AHLs; the second is the acyl side chain which determines the specificity of AHLs (Fuqua and Eberhard 1999). There are three main types of variation: The side chain can differ in length from 4 to 14 C-atoms, there can be either no substitution, a carbonyl or a hydroxyl group at the C3-atom, and the saturation of the acyl side chain can vary (fig. 1.1). This specification is determined by the acyl-binding pocket of AHL binding proteins, e.g. LuxR, which precisely fits a particular side-chain moiety. (Vannini et al. 2002, Zhang et al. 2002).

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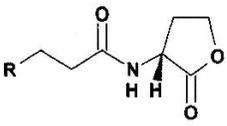
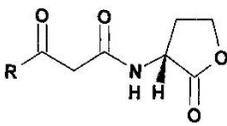
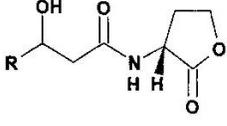
	R	Chemical name	Abbreviation
	CH ₃	<i>N</i> -Butanoyl-L-homoserine lactone	BHL
	CH ₃ (CH ₂) ₂	<i>N</i> -Hexanoyl-L-homoserine lactone	HHL
	CH ₃ (CH ₂) ₄	<i>N</i> -Octanoyl-L-homoserine lactone	OHL
	CH ₃ (CH ₂) ₆	<i>N</i> -Decanoyl-L-homoserine lactone	DHL
	CH ₃ (CH ₂) ₅ CHCH(CH ₂) ₃	7,8- <i>cis</i> - <i>N</i> -(Tetradecenyl)-L-homoserine lactone	7,8- <i>cis</i> -tDHL
	CH ₃ (CH ₂) ₂	<i>N</i> -(3-Oxohexanoyl)-L-homoserine lactone	OHHL
	CH ₃ (CH ₂) ₄	<i>N</i> -(3-Oxoctanoyl)-L-homoserine lactone	OOHL
	CH ₃ (CH ₂) ₆	<i>N</i> -(3-Oxodecanoyl)-L-homoserine lactone	ODHL
	CH ₃ (CH ₂) ₈	<i>N</i> -(3-Oxododecanoyl)-L-homoserine lactone	OdDHL
	CH ₃	<i>N</i> -(3-Hydroxybutanoyl)-L-homoserine lactone	HBHL
	CH ₃ (CH ₂) ₅ CHCH(CH ₂) ₃	7,8- <i>cis</i> - <i>N</i> -(3-Hydroxytetradecenyl)-L-homoserine lactone	7,8- <i>cis</i> -HtDHL

Fig 1.1: Structures of different AHLs (Eberl, L. 1999).

1.3.2 QS in pathogenic bacteria

Various QS systems are found important for several phytopathogenic bacteria to gain virulence. In *Rhizobium radiobacter* C58 the conjugation and transfer of the tumor inducer (Ti) plasmid is controlled by QS to cause crown gall in host plants (White and Winans 2006). The QS molecule 3-oxo-C8 HSL synthesized by *qsI* in *Pantoea stewartii*, controls its EPS synthesis and virulence (Von Bodman and Farrand 1995). 3-oxo-C6 HSL is synthesized by *carI* in *Erwinia carotovora*, and essential for its pathogenicity via control of the production of carbapenem, pectolytic enzymes, endoglucanases, proteases and secretion of harpin (Pirhonen et al. 1993). *Pseudomonas aeruginosa* is an opportunistic pathogen in cystic fibrosis patients. Its pathogenicity is quorum sensing dependent (O'Loughlin et al. 2013). In *Xanthomonas oryzae* pv. *oryzae* the quorum sensing regulator gene *oryR* is a global regulator controlling motility and chemotaxis (Gonzalez JF 2013). QS Controls the synthesis of the virulence factor EPS which protects *P. stewartii* subsp. *stewartii* from being recognized by the host plant (Koutsoudis et al. 2006).

3-OH palmitic acid methyl ester (3-OH PAME) plays an important role as QS signal molecule in *Ralstonia solanacearum*. 3-OH-PAME controls the production of EPS and some exoenzyme. 3-OH PAME enhanced the expression of EPS and exoenzymes and decreases motility and siderophore synthesis (Flavier et al. 1997). The diffusible signal factor DSF is a fatty acid derivative with similar structure as AHLs in *Xanthomonas campestris*. DSF regulates the

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expression of exoenzymes and synthesis of cyclic glucans as well as pigment formation in strain 8004 (Vojnov et al. 2001).

In summary, quorum sensing systems are important mediators for bacterial virulence. QS manipulation could be a potentially effective way to control diseases caused by bacteria (Helman and Chernin 2015).

1.3.3 QS in PGPR

QS plays not only an important role in plant pathogenic bacteria, but also in a wide range of PGPR functions including their beneficial phenotypes (Robson et al. 1997). QS can enhance the resistance of bacteria to oxidative, osmotic, thermal and heavy metal stress (García-Contreras et al. 2015). The QS molecules in *Azospirillum lipoferum* are associated with rhizosphere competence and adaptation to plant roots (Boyer et al. 2008b). In *P. fluorescens* 2p24 QS is involved in its biocontrol and colonization ability on wheat roots (Wei and Zhang 2006). AHL is important for *Burkholderia phytofirmans* PsJN to efficiently colonize roots of *Arabidopsis thaliana* plants and for its beneficial interactions (Zuniga et al. 2013). QS is involved to regulate functions linked to rhizosphere competence and adaptation to plant roots in *A. lipoferum* B518 (Wisniewski-Dyé and Vial 2015). In *P. aeruginosa* pupa3 isolated from a rice rhizosphere, QS is involved in the regulation of plant growth-promoting traits (Steindler et al. 2009). *A. brasilense* biofilm formation which is controlled by QS can be promoted by soil born bacteria *P. putida* X236 and root exudates of maize. The soil born bacterium *P. putida* x236 promoted biofilm formation of *A. brasilense* indicating the potential to create a co-inoculum with *A. brasilense*. Furthermore, root exudates of maize plants inoculated with *A. brasilense* provoke a raise in its biofilm formation activity (Cerqueira 2015). In addition, QS was also shown to be important to gain biocontrol ability in the colonized host plant. For example, colonization of tomato roots by QS deficient mutants of *Serratia liquefaciens* MG1 and *P. putida* IsoF showed a reduced induction of systemic resistance in tomato to leaf pathogen *Alternaria alternata* (Schuhegger et al. 2006).

1.4 AHL-mediated interkingdom signaling

AHLs produced by plant root-associated rhizobacteria are synthesized at the root surface (Gantner et al. 2006) causing plant responses which specifically depend on certain AHL species. AHL-compounds with a short to medium C-side chain such as C6-HSL, 3-oxo-C6-HSL, and 3-oxo-C8-HSL can be transported into the shoot (Götz et al. 2007, von Rad et al. 2008). Thus, short side chain AHLs can significantly promote root growth in *Arabidopsis*, while long side chain AHL like N-dodecanoyl-DL-homoserine lactone (C12-HSL) and N-DL-tetradecanoyl-homoserine lactone (C14-HSL) failed to promote root growth but stimulate systemic pathogen resistance depending on the ethylene and jasmonic acid pathways (Liu et al. 2012b, van Rad et al. 2008, schikora et al. 2011). For instance, the C14-HSL can reinforce the systemic resistance to the obligate biotrophic fungi *Golovinomyces orontii* and towards the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis* (schikora et al. 2011). This resistance is achieved by cell wall reinforcement such as callous deposition and lignification of cell walls and increasing the accumulation of phenolic compounds which depend on the salicylic acid or oxylipin pathway (Schenk et al. 2014). Other genes including auxin responsive promoter GH3 and chalcone synthase genes CHS1, CHS2 and CHS3 are activated in *M. truncatula* via root treatment by 50uM 3-oxo-C12-HSL (Mathesius et al. 2003). In addition to the length of side chains, the functional groups at the C3 position of AHLs are relevant for AHLs to exhibit specific functions in stimulating the formation of adventitious roots and expression of auxin response in mung bean- seedlings (Bai et al. 2012).

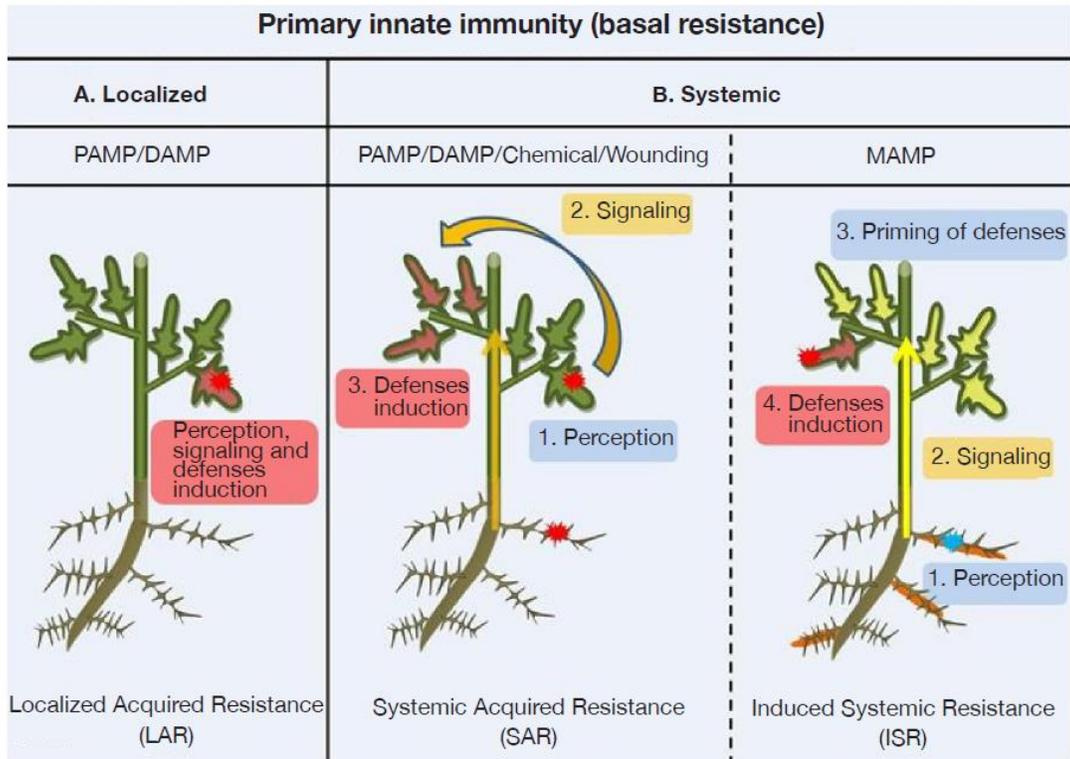


Fig. 1.2: The primary innate immunity can be localized (A) or systemic (B) (Henry et al. 2012).

1.5 Pathogen resistance in plants

1.5.1 PAMP/MAMP triggered immunity

Plant cell membrane located pattern recognition receptor (PRR) proteins sense pathogenic and beneficial bacteria via their pathogen/microbe associated molecular patterns (PAMP/MAMP) which trigger a serial resistance response. This process taking place locally at the infection site is referred to PTI (PAMP- triggered immunity) (Nicaise et al. 2009). Ligands which can cause plant PTI include various molecular patterns, such as the flagellin, EF-Tu factor, peptidoglycans (PGNs), lipopolysaccharides (LPS), RNP-1, bacterial siderophore pseudobactin and chitin (Gust et al. 2007, Erbs et al. 2008). Plant PRRs are plasma membrane-localized receptor-like kinases (RLK) or receptor-like proteins (RLPs) with an extracellular domain for the recognition of PAMPs like flagellin, EF-Tu, PGN, or lipopolysaccharides (LPS) (Zipfel 2014).

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After binding the ligands the activated receptor kinases cause immediate reaction including the plasma membrane receptor endocytosis, which involves phosphorylation and ubiquitination as well as activation of heterotrimeric G protein (Salomon and Robatzek 2006). These reactions are followed by plant downstream signaling including ion fluxes of H^+ , K^+ , Cl^- and Ca^{2+} , transcriptional reprogramming and an oxidative burst. Cascade activation of microtubule associated protein kinases (MAPKs) leads to the activation of key WRKY-type transcription factors. This cascade finally activates the expression of resistance genes such as PR-1 and PR-5 which inhibit growth of pathogens (Gómez-Gómez and Boller 2000).

However, pathogenic bacteria evolved strategies to bypass the host immunity response. As corresponding response, the plant evolved R proteins to recognize the pathogen effectors and prime a longer lasting and more potent resistance. This process is called effector triggered immunity (ETI), which often results in apoptotic hypersensitive reaction (HR) (Dangl and Jones, 2001).

1.5.2 ISR caused by PGPRs and their derivatives

There is also a systemic immune response of plants to an initial pathogen attack which is called systemic acquired resistance (SAR), mostly mediated by salicylic acid (SA). Quite different from this pathogen triggered response is however the response of plants to beneficial rhizobacteria associated with plant roots. These bacteria can also induce or prime a systemic resistance response termed induced systemic resistance (ISR). In most cases ISR involves jasmonic acid (JA) and ethylene (ET) rather than SA as signal mediators (fig. 1.2). Numerous studies have reported the ability of PGPR to promote plant health via ISR include *Pseudomonas*, *Serratia*, *Bacillus*, and *Azospillum*, but there are also some nonpathogenic plant growth promoting fungi (PGPF) e.g. *Fusarium oxysporum*, *Trichoderma*, and *Piriformospora indica* (tab. 1), which exhibit a similar effect. Various PGPR components from *Pseudomonas*, *Bacillus* and *Serratia* are able to cause ISR in many host plants which mainly include membrane pattern molecules such as LPS and flagella. Furthermore, some antibiotics, for instance DAPG, pyocyanin, the siderophore pyoverdine, plant hormones like salicylic acid, AHL quorum sensing molecules, cyclic lipopeptides as massetolid A, fengycins, and volatiles such as 2R, 3R-butanediol (tab. 2) can be causal agents (Ryu et al. 2004).

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Table 1.1: PGPR or PGPF caused ISR in different plants.

PGPR	Plants	Pathogen	References
<i>Pseudomonas fluorescens</i> WCS417r	carnation	<i>Fusarium oxysporum</i>	Schippers 1992
<i>Pseudomonas</i> and <i>serratia</i>	cucumber	<i>Colletotrichum orbiculare</i>	Wei et al. 1991
<i>Serratia marcescens</i> 90-166	crop plants and <i>Arabidopsis</i>	<i>Colletotrichum orbiculare</i>	van Loon et al. 1998
<i>Pseudomonas fluorescens</i> CHA0	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	Iavicoli et al. 2003
<i>P. aeruginosa</i> 7NSK2 and <i>Serratia plymuthica</i> IC1270	rice	<i>Magnaporthe oryzae</i>	De Vleesschauwer et al. 2008
<i>Pseudomonas fluorescens</i> WCS374r	rice	<i>M. oryzae</i>	De Vleesschauwer et al. 2008
<i>Pseudomonas putida</i> LSW17S	<i>Arabidopsis</i>	<i>Fusarium oxysporum</i> f. sp. <i>Lycopersici</i> .	Ahn et al. 2007
<i>Pseudomonas fluorescens</i> strain MKB158	barley	<i>Fusarium fungi</i>	Petti et al. 2010
<i>Pseudomonas fluorescens</i> S97	bean	<i>P.syringae</i> pv. tomato DC3000	Pieterse et al. 2014
<i>Pseudomonas fluorescens</i>	tomato	<i>Ralstonia solanacearum</i>	Murthy et al. 2014
<i>Rhizobium radiobacter</i>	barley	powdery mildew	Sharma et al. 2008
<i>Bacillus amyloliquefaciens</i> and <i>B. subtiliscan</i>	<i>Arabidopsis</i>	<i>Erwinia carotovora</i>	Ryu et al. 2004
<i>B. pumilus</i>	cucumber tomato	<i>Erwinia tracheiphila</i> and <i>cucumber mosaic virus</i>	Zehnder et al. 2000
<i>B. mycoides</i> resistance	sugar beet	<i>Cercospora beticola</i>	Bargabus et al. 2002
<i>B. sphaericus</i>	loblolly pine	<i>Cronartium quercuum</i>	Choudhary and Johri 2009
<i>Azospirillum brasilense</i> REC2 and REC3	strawberry	<i>Colletotrichum acutatum</i> M11	Tortora et al. 2011
<i>Penicillium</i> sp. GP16-2	<i>Arabidopsis</i>	<i>Pseudomonas syringae</i> pv. <i>Tomato</i> DC3000	Hossain et al. 2008
<i>T. barzianum</i> T39, and <i>Piriformospora indica</i>	<i>Arabidopsis</i>	abiotic and biotic stress	Waller et al. 2005
<i>Penicillium</i> sp. GP 16-2	<i>Arabidopsis</i>	<i>P. syringae</i>	Hossain et al. 2008
<i>Glomus mossae</i>	tomato	phytophthora	Pozo et al. 2002
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycoperisici</i> (FORL)	barley	<i>Blumeria graminis</i> f. sp. <i>hordei</i> (BGH)	Nelson 2005
<i>Fusarium</i> isolate AHD	palm seedlings	<i>Fusarium</i>	El Hassni et al. 2004

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Table 1.2: PGPR components causing ISR in different plants

ISR elicitors	PGPR	Pathogens resistance	Plants	References
lipopolysaccharide	<i>P. fluorescens</i> strain WCS417	<i>P. syringae</i> pv. <i>tomato</i>	<i>Arabidopsis</i>	Van Wees et al. 1997
flagella	<i>P. putida</i> WCS358	<i>Botrytis cinerea</i> , <i>Collectotrichum lindemuthianum</i>	bean and tomato	Meziane et al. 2005
pyoverdine	<i>P. fluorescens</i> CHA0	TNV	tobacco	Maurhofer et al. 1994
Salicylic acid	<i>P. aeruginosa</i> 7NSK2	Tobacco mosaic virus TMV	tobacco	De Meyer et al. 1999
DAPG	<i>P. fluorescens</i> CHA0	<i>Peronospora parasitica</i>	tomato	Audenaert et al. 2002
pyocyanin	<i>P. aeruginosa</i> 7NSK2	<i>B.cinerea</i>	<i>Arabidopsis</i>	Audenaert et al. 2002
AHLs	<i>Serratia liquefaciens</i> MG1	<i>Alternaria alternata</i>	potato	Schuhegger et al. 2006
Massetolid A	<i>P. fluorescens</i> SS101	<i>Phytophthora infestans</i>	tomato	Tran et al. 2007
2R,3R-butanediol	<i>B. subtilis</i> GB03	<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	<i>Arabidopsis</i>	Ryu et al. 2004
fengycins	<i>B. subtilis</i> S499	<i>Botrytis cinerea</i>	Bean and tomato	Ongena et al. 2007
lipopolypeptides and polyketides	<i>B.amyloliquefaciens</i> FZB42	<i>Rhizoctonia solani</i>	<i>Arabidopsis</i> , Lettuce, and tobacco	Chowdhury et al. 2015

1.5.3 Mechanism of ISR priming process

Until now the mechanism of the ISR-eliciting root colonizing microbes and the signaling pathways triggering specific plant defense in leave tissues is not completely clear. One described phenomenon named “priming” involves a substantial transcriptional reprogramming in the host plant leading to the upregulation of the transcription of defense-related genes like PR genes. In consequence, host resistance capacity is enhanced once the infection process is started. This process is JA-dependent (Memelink 2009). Plants which are in priming state have faster and stronger defense responses upon pathogen invasion. One root-specific R2R3-type MYB transcription factor (MYB72) is essential for the onset of ISR caused by *P. fluorescens* WCS417r and *P. putida* WCS358r as well as PGPF *Trichoderma* (Segarra et al. 2009). All the results indicate that MYB72 plays an important role in the node of convergence in the ISR signaling pathway triggered by various beneficial microbes. It was found that compared to the SAR response the priming status requires less cost to the plants (Walters et al. 2008). The

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ability to activate an SA-independent ISR pathway is common for beneficial microbes, involves JA and ET and occurs in a broad range of plant species (Van Loon and Bakker 2005). For example *Serratia marcescens* 90-166, *P. protegens* CHA0, and *P. fluorescens* Q2-87, and PGPF *Penicillium* sp. GP16-2, *Trichoderma barzianum* T39, and *P. indica* caused ISR in *Arabidopsis thaliana* (Pieterse et al. 2014). *B. amyloliquefaciens* FZB42 produces secondary metabolites such as lipopolypeptides and polyketides, essential for causing plant ISR via JA /ET pathway, which contributes to the disease suppression towards the pathogen *Rhizoctonia solani* (Chowdhury et al. 2015). *Pseudomonas fluorescens* WCS374r triggers ISR in rice against *M. oryzae* depending on the JA or ET-modulated signaling pathway (Djonović et al. 2007). Only few studies suggested that ISR may also depend on the SA-signaling pathway, such as in *Rhizobacter* (Audenaert et al. 2002). Also in *Bacillus thuringiensis* induced resistance to *Ralstonia solanacearum* was found via the SA-dependent signaling pathway (Takahashi et al. 2014). Moreover, in some cases, like in mycorrhized maize plants, the activation of ISR defense-related genes involved the activation of SA- and JA-dependent pathways. However, ISR required the NPR1 protein like in SAR (Stein et al. 2008).

Several enzymes are involved in ISR-triggered defense response by *P. fluorescens*, *B. pumilus* and *B. subtilis* including peroxidase (POX), phenol oxidase (Chen et al. 2009), phenylalanine ammonia lyase (PAL) and beta-1, 3-glucanase during ISR (Udayashankar et al. 2011, Vanitha and Umesha 2011). Additionally, proteinaceous elicitors have been shown to facilitate the ISR-effect caused by *Piriformospora indica* in barley (Molitor and Kogel 2009). *Trichoderma virens*, an endophytic beneficial fungus in maize activated PR1, PR2, PR5 genes and the heat-shock protein 70 (hsp70) (Djonović et al. 2007). Moreover, the metabolite fengycins of *Bacillus subtilis* caused ISR in potato, leading to higher accumulation of plant phenolics derived from the phenylpropanoid metabolism (Ongena et al. 2005). This pathway is also well known to be stimulated concomitantly with the activation of plant defense reactions (Dixon et al. 2002). Most interestingly, also AHLs can be an important elicitor in ISR. *Serratia liquefaciens* MG1 producing C6 and C8-AHLs was shown to induce systemic resistance in tomato plants (Schuhegger et al. 2006). C4-HSL, C8-HSL, and 3-oxo-C8-HSL produced by *Serratia marcescens* strain 90-166 is important for ISR induction in tobacco (Ryu et al. 2013). QS-dependent ISR is elicited by *S. marcescens* 90-166 in a pathogen dependent manner leading to

resistance against *Pectobacterium carotovorum* subsp. *carotovorum* and *Pseudomonas syringae* pv. *tabaci*.

1.6 *Acidovorax radialis* N35

Acidovorax radialis N35 is a wheat endophytic PGPR belonging to the β -*Proteobacteria* (Willems and Gillis 2005, Li et al. 2011). *A. radialis* N35 can colonize barley roots on the surface as well as endophytically and promote plant growth. However, its growth promoting ability was not detectable anymore after the bacterium has undergone a spontaneous genetic modification which is termed phase variation. In this phase or phenotypic variants, named N35v, the only detectable conserved genetic difference was a deletion in the mismatch repair gene *mutL*, which might result in changes in the transcriptional regulation patterns of the variant strain (Li et al 2012). Thus, the variant strain shows significant differences to the wild type. The v-strain is turbid in liquid medium and forms smooth colonies on agar plates while the wild type sediments in liquid medium and forms rough colonies on solid medium. This phenotype variation is irreversible. When plants were inoculated with wild type and phenotypic variants individually, no apparent differences in colonization behavior were observed. However, when *A. radialis* wild type and phenotypic variants were co-inoculated in a 1:1 mixture, N35 was more successful in colonization (Li et al. 2012). Therefore, the phenotypic variants possess reduced fitness in plant root colonization relative to the parental strain. In addition to its efficient root colonization ability, *A. radialis* stimulated growth of barley plants in nutrient poor soil (Li et al. 2012). After 4 months of growth, dry weight of *A. radialis* N35 inoculated barley plants was significantly increased by 40% relative to non-inoculated control plants. Plants inoculated with the phenotypic variant N35v, showed no significant increase (13%) compared to the non-inoculated control plants. A significant increase of 20% in shoot biomass was observed in N35 inoculated plants over the non-inoculated control, while the plants inoculated with the phenotypic variant showed no significant increase (Li et al. 2012). Thus, *A. radialis* N35 is able to promote plant growth under nutrient limiting conditions. While swarming and colonization were diminished, siderophore and lipase production were unaffected by phenotypic variation. Phenotypic variation had also no influence on AHL production.

One *luxI/R* type QS system exists in *A. radicans* N35 and its dominant QS-auto inducer molecule is 3-OH-C10-HSL. The corresponding AHL synthase gene is the luxI type *araI* (Li 2010). Based on the *A. radicans* N35 genome sequence, a 555 bp fragment was identified with high homology to the I-type biosynthesis gene, *araI*. The *araI* homologue was deleted by directed insertion mutagenesis in strain N35 (Li 2010) leading to an AHL-deficient mutant phenotype. The AHL-deficient mutant was unaffected in swarming motility and in siderophore and lipase production, traits that are often regulated by QS. In contrast, the AHL-deficient mutant was a less efficient root colonizer than the wild-type strain as tentatively shown by co-inoculation of the wild type and the *araI::tet* mutant (Li 2010).

1.7 Flavonoid biosynthesis

Flavonoids are phenylpropanoid metabolites in plants. Several thousand flavonoids have been identified. The diversity comes from the combination of a number of skeleton structures with various modifications including glycosylation, acylation or polymerization (Hassan and Mathesius 2012).

The main two flavonoids in barley are saponarin and lutanarin in an approximate ratio 4.5:1. They are beneficial agents for barley health in case of diseases caused by oxidative damage (Kamiyama and Shibamoto 2012). Saponarin isolated from barley sprouts exhibits anti-oxidant (Vitcheva et al. 2011), antimicrobial (Basile et al. 1999), hepatoprotective activities and anti-inflammatory effects (Seo et al. 2014). In barley, saponarin and lutanarin synthesis starts from the general phenylalanine metabolism catalyzed by an endoplasmic reticulum cytoplasmic surface-located multi-enzyme complex (CHS, CHI). The first committed step is catalyzed by a chalcone synthase (CHS) to form naringenin by the condensation of three molecules of malonyl-CoA and one molecule of 4-coumaroyl-CoA followed by chalcone isomerase (CHI) to catalyze ring closure (Hassett et al. 1999). Then naringenin is catalyzed into isovitexin via an unclear process. Only trace amounts of isovitexin accumulates in barley, since most of it is immediately processed to saponarin via an UDP-Glc flavone glucosyltransferase (OGT), which is soluble and cytosolic (Fig. 1.3). Saponarin is transported into the vacuoles through accumulation via H⁺ antiporters which are energized by a pH gradient (Wink 1997). Saponarin and lutanarin accumulate in the primary leaves of barley to protect its DNA from UV-B damage. Like other flavonoids, saponarin

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was speculated to be transported to sink organs by long distance transport via ABC-transporter or MATE-transporter (Zhao and Dixon 2010).

Other flavonoids like proanthocyanidin and dihydroquercetin are involved in barley defense response against *Fusarium* species via inaction of several enzymes which includes microbial cellulases, xylanases, and pectinases, chelation of metals necessary for enzyme activity, and/or formation of special physical barrier to hinder pathogen attack (Skadhauge et al. 1997).

Flavonoids support plant abiotic (UV light) and biotic resistance due to their antioxidant, fungicide, bactericide and anti-pest properties (Treutter 2005, Cushnie and Lamb 2011, Hassan and Mathesius 2012). Specific flavonoids secreted by legumes can also induce Nod-gene expression in *Rhizobium* leading to the nodule formation in the host plant. Nod gene-inducing flavonoids also increased AHL-synthesis in *Rhizobium* strains (Perez-Montano et al. 2011).

Secreted flavonoids by leguminous plants can function as signal molecules for *Rhizobium*. The flavonone naringenin can stimulate *Azorhizobium* colonization of lateral roots via crack invasion leading to an increased number of lateral roots per plant (Webster et al., 1998). The occurrence of flavonoids such as anthocyanin accumulation in cotton leaves is taken as an indicator of resistance to the bacterial blight pathogen *Xanthomonas campestris* pv. *malvacearum* (Kangatharalingam et al. 2002).

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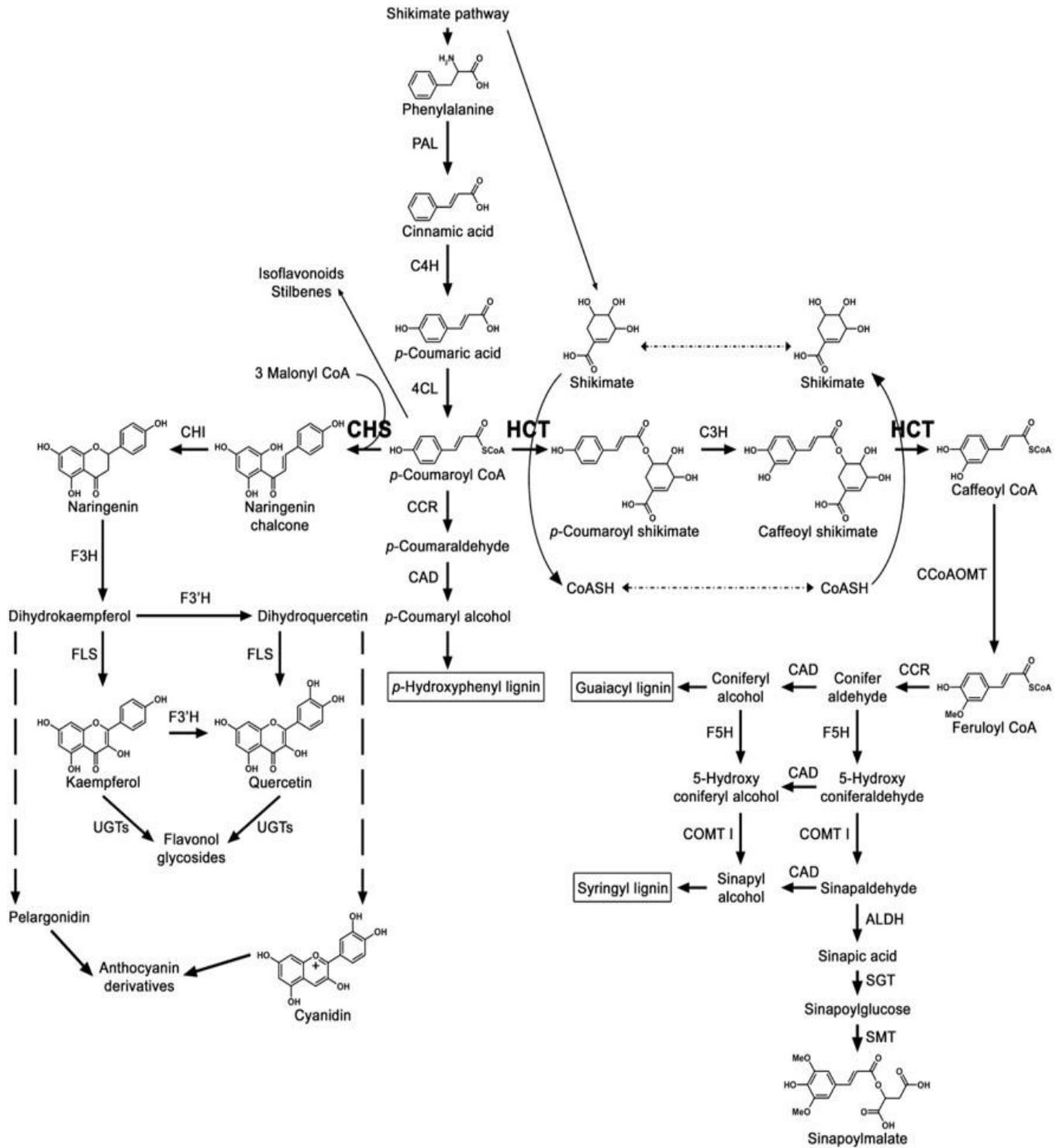


Fig. 1.3: The phenylpropanoid pathway (Besseau et al. 2007).

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Flavonoid synthesis was found to be affected by various biotic and abiotic factors. For example, the colonization by the mycorrhizal fungus *Glomus versiforme* elevated the transcription of flavonoid synthesis important genes (PAL) and chalcone synthase (Hassan and Mathesius 2012). The PGPR *Chryseobacterium* or *Azospirillum* were found to be able to elicit plant flavonoid exudation when colonizing soybean roots (Dardanelli et al. 2010). Colonization of barley leaves by the pathogenic fungus *Blumeria graminis* f. sp. *Hordei* (Bgh) stimulated the expression of HvCHS2, a chalcone synthase in barley leaves. Drought stress and UV light caused flavonoid accumulation in leaves of two different cultivars of wheat and barley (Christensen et al. 1998). AHLs trigger flavonoid biosynthesis and the transcription of different flavonoid metabolite-related genes. Exogenous 3-oxo-C12-HSL can stimulate chalcone synthase gene expression at root sites of AHL-treated white clover roots. 3-oxo-C14-HSL could upregulate flavonoid metabolite genes including chalcone isomerase, glycosyltransferase, and flavonoid 3'-monooxygenase in *Arabidopsis* (Mathesius et al 2003). C12 and C16-HSL were used to treat roots of *M. truncatula*, their effect on flavonoid metabolism was found to be dependent on AHL concentration and length of treatment (Mathesius et al. 2003, Schenk et al. 2014).

1.8 Objectives

PGPR exhibit many beneficial effects to colonized plant roots, which include promotion of plant growth, enhancing plant resistance via direct antibiotic production or indirect ISR stimulation. QS system using AHLs as autoinducer were found to be important for PGPR to establish these beneficial effects. Flavonoids are secondary metabolites which are also involved in plant resistance to biotic and abiotic stress and their synthesis were found to be affected by QS signals. Based on this information, several questions were proposed to be studied in the *A. radialis* N35 barley interaction:

1. Does QS influence plant growth promotion in *A. radialis* N35?
2. Does QS influence root colonization in N35?
3. Does QS of N35 contribute to plant response and specificity to flavone biosynthesis?

2 Material and methods

2.1 Cultivation of bacteria

2.1.1 Bacterial strains and plasmids

Table 2.1 Strains specifications

Organism	Relevant characters	Reference
<i>Acidovorax radicans</i> N35	Wild type isolated from surface sterilized wheat roots, rough colony surface, flocculation in liquid medium	Klein, 2003
<i>A. radicans</i> N35v	Phenotype variant of N35, smooth colony surface, no flocculation in liquid medium	Li, 2010
<i>A. radicans</i> N35 <i>araI::tet</i>	AHL negative mutant, Tc ^R	Li, 2010
<i>A. radicans</i> N35 GFP & <i>A. radicans</i> N35v GFP	Km ^R , chromosomally labeled with GFP (green fluorescent protein)	Li, 2010
<i>A. radicans</i> N35 YFP & <i>A. radicans</i> N35v YFP	Km ^R , labeled with YFP	Li, 2010
<i>A. radicans</i> N35 <i>araI::tet</i> GFP	Km ^R labeled with GFP	This study
<i>A. radicans</i> N35 <i>araI::tet</i> YFP	Km ^R labeled with YFP	This study
<i>A. radicans</i> N35 <i>araI::tet</i> C	Km ^R , complemented <i>araI::tet</i> mutant, labeled with GFP or YFP	This study
<i>Serratia liquefaciens</i> MG44	AHL negative mutant, host for AHL biosensor pBAH9, Amp, Tc ^R & Sm ^R	Eberl et al., 1996
<i>Agrobacterium tumefaciens</i> A136	pCF218, pCF372	Stickler et al. 1998

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Table 2.2: Plasmid

Plasmids	Relevant characters	Reference
pCR2.1-TOPO	Amp ^R , Km ^R ; <i>lacZ</i> _α ,	Invitrogen, Carlsbad, USA
pEYFP	Amp ^R , <i>lacZ</i> , <i>eYFP</i> carrier vector	Clontech, CA, USA
pJBA28	Amp ^R , Km ^R ; carrier plasmid for mini-Tn5-Km-PA1/04/03-RBSII- <i>gfp</i> mut3*-T0-T1	Andersen <i>et al.</i> 1998
pBBR1MCS-2	Km ^R ; <i>lacZ</i> _α , cloning vector	Kovach <i>et al.</i> 1995
pBAH9	Km ^R , green fluorescent AHL sensor plasmid for C4-C14-HSL	Huber, unpublished
pEX18Gm	Gm ^R , <i>oriT</i> ⁺ , <i>sacB</i> ⁺ , gene replacement vector with MCS from pUC18	Hoang <i>et al.</i> 1998
pEX18Tc	Tc ^R , <i>oriT</i> ⁺ , <i>sacB</i> ⁺ , gene replacement vector with MCS from pUC18	Hoang <i>et al.</i> 1998
pRK600	Cm ^R , ColE1 <i>oriV</i> RP4 <i>tra</i> ⁺ RP4 <i>oriT</i> , helper strain for conjugation mating	Figurski and Helinski, 1979
pMLBAD- <i>aiiA</i>	Tell ^R , <i>aiiA</i> lactonase gene in expression vector, lactonase activity induced by 0.2% arabinose	Wopperer <i>et al.</i> 2006

2.1.2 Media and buffers

Unless otherwise noted all solid media were prepared with 15 g agar per liter. pH was adjusted with 0.1 M NaOH or 0.1 M HCl.

2.1.2.1 Bacteria culture medium

NB (Nutrient Broth) medium (No. 4, Fluka, Steinheim, Germany):

Meat peptone	5 g
Meat extract	3 g
ad H ₂ O _{dem.}	1.0L
Adjust to	pH 7.0

LB (Luria-Bertani) medium (Bertani, 1951, modified):

Peptone from casein	10 g
Yeast extract	5 g
NaCl	5 g
ad H ₂ O _{dem.}	1000 ml

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Adjust to pH 7.0

Yeast Mannitol Broth M716:

Yeast Mannitol Broth is used for cultivation of *Rhizobium* species.

Composition

Ingredients g / L

Yeast extract 1.0

Mannitol 10.0

Dipotassium phosphate 0.5

Magnesium sulphate 0.2

Sodium chloride 0.1

Calcium carbonate 1.0

Final pH (at 25°C) 6.8±0.2

**Formula adjusted, standardized to suit performance parameters

2.1.2.2 Fungi culture medium

Composition:

Ingredients g/L

Potato extract 4.0

Dextrose 20.0

Agar 15.0

Final pH 5.6 +/- 0.2 at 25°C

Store prepared media below 8°C, protected from direct light. Store dehydrated powder, in a dry place, in tightly-sealed

Containers at 2-25°C.

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2.1.2.3 Barley culture medium

Table 2.3: Hoagland medium composition

	stock solution	molecular weight	100%		
Macronutrients	M	g/mol	mg/L	mM	ml/L
KNO ₃	1	101.11	606.6	6	6
Ca(NO ₃) ₂ .4H ₂ O	1	236.00	944.64	4	4
NH ₄ H ₂ PO ₄	1	115.03	230.2	2	2
MgSO ₄ .7H ₂ O	1	246.48	246.48	1	1
Micronutrients		g/mol	mg/L	μM	ml/L
KCl		74.55	3.73	50.03	1
H ₃ BO ₃		61.83	1.55	25.07	
MnSO ₄ .H ₂ O		169.01	0.34	2.01	
ZnSO ₄ .7H ₂ O		287.54	0.58	2.02	
CuSO ₄ .5H ₂ O		249.68	0.12	0.48	
H ₂ MoO ₄ (85% MoO ₃)		161.97	0.09	0.56	
CoCl ₂ .6H ₂ O		237.93	2.0	8.41	
Na ₂ SeO ₃		172.94	0.1	0.58	
NiSO ₄ .6H ₂ O		262.86	0.06	0.23	
Iron	g/l	g/mol	mg/L	μM	ml/L
C ₁₄ H ₁₈ N ₃ O ₁₀ FeHNa	10	468.15	10.0	21.36	1

Murashige & Skoog medium prod.No M0221.0050 (Duchefa Biochemie, RV Haarlem)

2.1.3 Selective agents

Table 2.4: Antibiotics and medium supplements

Medium supplements	Abbr.	Activity mechanisms	Solvent	Concentr.
Ampicillin	Amp	β -lactam antibiotic, inhibition of the synthesis of peptidoglycan	50% ethanol	100 mg/l
Kanamycin ultra-pure	Km	aminoglycoside, inhibition of protein synthesis by binding to 30S ribosomal subunit	Ultra-pure water	50 mg/l
Tetracycline	Tc	inhibition of protein synthesis by binding to 30S ribosomal subunit	methanol	20 mg/l
Rifampicin	Rp	inhibition of RNA synthesis by binding to the RNA polymerase	DMSO	100 mg/l
Chloramphenicol	Cm	inhibition of the formation of peptide bonds by binding to 50S ribosomal subunit	50% ethanol	10 mg/l
Trimethoprim	Tm	interference with the production of tetrahydrofolic acid	DMSO	100 mg/l
Gentamycin	Gm	inhibition of protein synthesis by binding to the 30S ribosomal subunit	ultra-pure water	20 mg/l
Streptomycin ultra-pure	Sm	inhibition of protein synthesis by binding to 30S ribosomal subunit	water	50 mg/l
Tellurite	Tell	oxidizing agent	ultra-pure water	100 mg/l
5-bromo-4-chloro-3-indolyl-beta-D-Galactopyranosid	X-gal	substrate for β -galactosidase	dimethylformamide	40 mg/l

2.1.4 Cultivation of microorganisms

Sterile wire loops and tooth picks were used for inoculation of bacterial strains in liquid and solid media. All microorganisms were re-cultivated every 3-4 weeks and stored at 4 °C. Glycerol stocks were used for long time preservation. For preparation of glycerol stocks microorganism were grown over night. The strains were stored at -80 °C.

2.2 Cultivation of barley

2.2.1 Barley in monoxenic system

2.2.1.1 Monoxenic system

The monoxenic system is suitable for the inoculation of defined bacterial strains on plant roots. Barley seedlings were grown in glass tubes (\varnothing 30 mm, Schott glass, Mainz, Germany) filled 6 cm

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in height with sterilized quartz sand (\varnothing 1.0-2.5 mm, Sakret, Ottobrunn). 10 ml MS medium was supplied for plant nutrition. Plants were grown at 16 °C/12 °C day/night cycle, 50% relative humidity and a photo period of 12 h. Barley plants were cultivated for a maximum of 3-4 weeks in this system.

2.2.1.2 Seed sterilization

Barley seeds (*Hordeum vulgare* var. Barke) were obtained from Saatzucht Josef Breun GmbH (Herzogenaurach, Germany). Seeds used in the monoxenic system were surface sterilized to eradicate fungi and bacteria. Barley seeds were shaken in 1% (v/v) Tween 20 for 1 min then in 70% ethanol for 2 mins and incubated in 2% NaOCl for 20 min. Then they were washed with H₂O_{dem.} 5 times and incubated in 600 mg/l penicillin and 250 mg/l streptomycin solution for 30 min. Finally, the seeds were incubated on NB plates at 30 °C, letting them germinate for 2 days. After this period they were inspected for contaminations and only uncontaminated seedlings were selected for inoculation.

2.3 Localization of bacteria on barley roots

2.3.1 Inoculation of barley roots with bacteria

An overnight culture of different bacterial strains was harvested at 5000 g (Eppendorf 5417R, Eppendorf, Hamburg, Germany) for 5 min at RT and the supernatant was discarded. The cells were washed twice with 10 ml of 1x PBS by centrifugation/ resuspension and thereafter suspended in 10 ml 1x PBS. The optical density (OD) of cells was measured at a wave length of 435 nm using a spectral photometer (CE3021, Cecil, Cambridge, England). The cell density was adjusted to an OD_{435nm} 1.5 for *A. radialis* N35 and *A. radialis* N35 *araI::tet*. This culture density corresponds to 10⁸cfu /ml (Li, 2010). For the inoculation of single bacterial strains, the seedlings were incubated in the bacterial suspension for 1 h at room temperature. Finally, the seedlings were transferred to a monoxenic or soil system.

2.3.2 Root harvest

Barley roots were harvested after 2 weeks growth in monoxenic system and 2 months in soil system. After removing the quartz sand or soil particles, roots were cut from the shoots and washed several times with 1x PBS to detach only loosely attached bacteria or particles. The harvested roots were divided in two groups. One was used for the microscopic detection of colonizing bacteria, the other for the measurement of the weight to study the growth promotion effect by inoculated bacteria.

2.3.3 Roots for microscopic detection of bacteria

For fluorescence in situ hybridization (FISH, see 3.7.1) washed roots were incubated in 4% PFA at 4 °C overnight then fixed with serial ethanol concentrations 50%, 80% and 100% to dehydrate the samples. At the last step, the roots were placed on a paper towel to soak the remaining ethanol. They were transferred to an objective slide for final drying. After hybridization in hybridization buffer, the roots were washed with washing buffer. Thereafter, roots were put on a microscope slide in a small droplet of citifluor. Since thin roots samples float during microscopy, a cover slip was placed on top. The sample was then observed with a CLSM.

2.4 Microscopy

2.4.1 Epifluorescence microscopy

For the visualization of bacterial pure cultures probe an epifluorescence microscope Axionplan 2 (Zeiss, Oberkochen, Germany) was used equipped with a water immersion objective (C-Apochromat, 40x1.2 Korr). The light source was a mercury short arc reflector lamp.

Table 2.5: Data of filter systems used in epifluorescence microscopy

Fluorophores	Excitation filter	Beam splitter	Emission filter
Green fluorescence, Fluorescein and GFP and YFP	BP 470/40	FT 495	BP 525/50
Orange red fluorescence: Cy3	BP 545/25	FT 570	BP 605/70
Cy5	BP 640/30	FT 660	BP 690/50

2.4.2 Confocal microscopy

For the visualization of the GFP- or YFP-tagged *A. radicans* N35 cells colonizing barley roots, freshly harvest roots of barley were embedded in Citifluor and placed on a glass slide. The fluorescence derived from fluorescent proteins was detected using a LSM 510 Meta (Zeiss, Oberkochen, Germany). In this CLSM there are two different excitation lasers: one is an argon ion laser which can excite GFP or fluorescein at 488nm, the other one is a helium neon lasers providing excitation wavelengths at 543nm and 633nm, which is specific for Cy3 and Cy5 respectively. The three resulting emission colors were combined and shown as red, blue and green (RGB) images. Root and plant material in general shows autofluorescence in all three fluorescence channels. In order to discriminate between specific fluorophore and autofluorescence usually not more than two different fluorophores were use, so that at least one fluorescence channel showed only autofluorescence.

Besides this standard mode there is also a so called lambda mode available in the used LSM system. Lambda mode allows the identification and separation of very similar emission spectra of fluorophores, such as GFP with a maximum emission wavelength of 510 nm versus YFP with 530 nm. In this study this lambda mode was used for GFP and YFP separation when both fluorescence proteins were used for labeling of *A. radicans* N35 and its *araI::tet* mutant strain in colonization studies. The image analysis was performing using Zeiss software LSM Image Browser Version 3.5. Further specifications of the CLSM are shown in the following table.

Table 2.6: Characteristics of fluorophores, as well as filter and laser system

Fluorophores	Excitation maximum (nm)	Emission maximum (nm)	Laser Type (nm)	Beam splitter	Filter
GFP	498	509	Argon488	NFT490 HFT488/543	BP 500-550
YFP	514	527	Argon 488	NFT490 HFT488/543	BP 500-550
Fluos Argon	490, 494	520, 525	488 NFT490	HFT488/543	BP 500-550
Cy3	514, 552, 554	566, 570	Helium-Neon 543	HFT 488/543	LP 560
Cy5	649	666, 670	Helium-Neon 633	HFT UV/488/543/633	LP 650

2.5 Plant growth promotion measurement

2.5.1 Plant growth in phytochamber at axenic conditions

After surface sterilization and germination of barley seeds on NB medium for two days, they were placed into sterilized glass tubes containing 50 g glass beads and 10 ml MS liquid medium. Barley seedlings were grown under phytochamber condition with light time and dark time for 12 hours respectively. A day temperature of 23°C and night temperature 18°C were used.

After two weeks barley seedlings were harvested and the total, root and shoot weights were measured.

2.5.2 Plant growth in greenhouse at unsterile conditions

After germination of barley seeds in wet paper towel for 2 days the seedlings were inoculated with *A. radialis* N35 and *araI::tet* mutant respectively for one hour in a 10^8 cells per ml bacterial suspension. Inoculated seedlings were placed into pots filled with commercial “Graberde” (nutrient limited substrate, Alpenflor, Weilheim, Germany) mixed with sand (v/v 1:1). Each pot (10cm height, 8cm diameter) was filled with the same volume of soil substrate. 1l tap water was added to initially water the pots. For each treatment 15 pots with only one plant per pot were cultivated for two weeks or 2 months. The plants were watered twice a week. Throughout the experiment, the plants were fertilized once each week with Hoagland solution (10ml 50x stock, diluted in 1l water). Barley plants were grown under greenhouse conditions at temperatures of 15-25°C during the day and 10-15°C during the night.

2.6 AHL detection methods

2.6.1 Biosensor analysis

AHL-production of *A. radialis* N35 wild type, as well as the AHL-negative and *araI* complemented mutants of *A. radialis* N35 were examined via sensor plasmids pCF218 and pCF372 in *A. tumefaciens* A136. These two plasmids bear *traR*- and *traI-lacZ* fusion genes, respectively. The system shows highly efficient detection of AHLs (Stickler et al. 1998). The

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biosensor strain was streaked onto the center of LB or NB agar plate containing 40µg/ml X-gal, and the tested bacterial strains were cross-streaked near the biosensor. The plates were incubated at 30 °C in the dark for 24-48 hours. The detection of AHL production was observed based on the blue color development near the cross-over place between the AHL-biosensor A136 and *A. radicis* N35 or its *araI::tet* mutant.

2.7 Molecular genetic methods

2.7.1 Fluorescence *in situ* hybridization

2.7.1.1 Preparation of 5% paraformaldehyde solution

2.5 g PFA was dissolved in 45 ml H₂O_{dem} which was warmed to 60-65 °C applying dropwise 10N NaOH until the solution became clear. Afterwards 5 ml 10x PBS was added. At last, pH=7.2-7.4 was adjusted when the solution had cooled to room temperature. The 5% PFA solution was sterile filtered with 0.45 µm filter (Millipore) and stored at 4 °C for one day and in -20 maximum for one week.

2.7.1.2 Oligonucleotide probes

Oligonucleotide probes were labeled with different dyes which include fluorescein (Fluos), Cy3 and Cy5. Cy3 and Cy5 were obtained from Thermo Electron (Ulm, Germany). The working solution was prepared in nuclease free water and the concentration for Cy3, Cy5 was 30µg/ml and for Fluos 50µg/ml, which was measured with NanoDrop ND-1000 (NanoDrop, Wilmington, USA; 2.7.4).

Material and methods

Table 2.7: Oligonucleotide probes

Probe	Specificity	Binding position ¹	Probe sequence 5'-3'	FA ² %	Reference
EUB 338I ³	Bacteria without Planctomycetales, Verrucomicrobiales	16S, 338-355	GCTGCCTCCCGTAGGAGT	var.	Amann et al.,1990
EUB 338II ³	Planctomycetales	16S, 338-355	GCAGCCACCCGTAGGTGT	var.	Daims et al.,1999
EUB 338III ³	Verrucomicrobiales	16S,338-355	GCTGCCACCCGTAGGTGT	var.	Manz et al.,1992
Rhi1247	<i>Rhizobium</i> sp., <i>Agrobacterium</i> sp., <i>Ochrobacterium</i> sp., some <i>Azospirillum</i> sp., few <i>Sphingomonas</i> sp.	16S,1247-1252	TCGCTGCCCACTGTG	35	Sharma et al., 2008
ACISP145	<i>Acidovorax radialis</i> N35 and N35v, <i>Acidovorax defluvii</i> , <i>Acidovorax facilis</i>	16S, 145-162	TTTCGCTCCGTTATCCCC	35	Rothballer, unpublished

1, position in ribosomal nucleotides of *E.coli* (Brosius et al., 1981)

2, % formamide in hybridization buffer

3, probes, EUB338I, II, and III were mixed in equal molar

2.7.1.3 Fixation of bacterial cultures and roots

For fixation of bacterial cell cultures 2 ml mid logarithmic liquid culture was harvested by centrifugation at 5000x g for 3 min at RT. Then, these cells were re-suspended in 200 µl 1x PBS, 600 µl of 5% PFA was added (resulting in a 4% PBS/PFA mixture) and incubated at 4 °C for 1.5 h. Roots were fixed by adding 4% PFA directly and incubation at room temperature for 2 hours. After fixation, cells as well as roots were washed twice with 1x PBS and stored in 50% PBS/EtOH_{abs} (v/v) at -20 °C.

2.7.1.4 Hybridization with oligonucleotide probes

1-5 µl bacterial pure culture was placed on epoxy coated slides (Roth, Karlsruhe, Germany) after dehydration in 50, 80 and 100% ethanol for 3 minutes successively. After the last dehydration step in ethanol, the slices were placed into the clean bench to dry for 10 mins to guarantee there is no ethanol left on the sample slice. Then, 8 µl hybridization buffer containing 35% formamide plus 1 µl of each oligonucleotide probe were mixed and dripped on the wells containing the

Material and methods

sample. The slices were placed into 50ml falcon tubes and transferred into 46°C for 1.5 hours for hybridization. The slides were washed with washing buffer at 48 °C in a water bath for 20 minutes. After drying the slide, a small droplet of Citifluor was added to cover the slide for microscopy.

Washed roots were cut into about 2 cm long pieces and put into 2 ml Eppendorf tubes for dehydration using 50, 80 and 100% ethanol for 3 minutes successively. After the last step in ethanol, the roots were placed on paper towel for drying. Then the roots were transferred into 40µl hybridization buffer containing 5µl of each hybridization probe. The tubes were incubated at 46°C for 2 hours for hybridization. Afterwards, the roots were washed with washing buffer for 20 minutes. As the CLSM system is based on an inverse microscope, the roots were placed directly on a cover slip in a small droplet of Citifluor without using an objective slide. To prevent floating, small root pieces were covered with a second cover slip.

The composition of hybridization and washing buffers:

Hybridization buffer:

NaCl (5M) 360 µl

Tris/HCl (1M, pH 8) 40 µl

Formamide 700µl

Ultra pure H₂O 900µl

SDS (10%w/v) 2 µl

Washing buffer:

Tris/HCl (1M, pH8.0) 1 ml

Na-EDTA (0.5M, pH 8.0) 500 µl

NaCl (5M) table 700µl

add ultra pure water 50 ml

SDS (10% w/v) 50 µl

2.7.2 DNA isolation

2.7.2.1 Plasmid isolation

Briefly, bacterial cultures were centrifuged and the pellets were processed with NucleoSpin Plasmid Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's protocol. Lysis was performed in alkaline buffer neutralized with A3 buffer. After centrifugation, the supernatant was loaded to a silica matrix column, which binds DNA. Then, the columns were washed and eluted with elution buffer AE and quantification of DNA was performed with NanoDrop 1000. Plasmid samples were stored at -20°C.

2.7.2.2 Chromosomal DNA isolation

Chromosomal DNA isolation of a bacterial pure culture was carried out with FastDNA SPIN Kit for soil (MP biomedical, Illkirch, France). Briefly, DNA was liberated from the cells via mechanical shaking by lysing matrix E containing a mixture of ceramic and silica particles, homogenization and protein solubilization. The released DNA was loaded to a silica matrix column which was eluted with DNA elution buffer. DNA samples were stored at -20 °C.

2.7.3 DNA purification

DNA after enzymatic manipulation, PCR products or DNA extracted from TAE agarose gels was purified with NucleoSpin Extract II kit (Machery-Nagel). Then DNA was bound in the presence of chaotropic salts to a silica membrane and was released with elution buffer.

2.7.4 Quantification of DNA concentration

DNA concentrations were measured by NanoDrop ND-1000 (NanoDrop, Wilmington, USA). This spectrophotometer has a light source spectrum of 220-750 nm which can be used to measure the absorbance of DNA, RNA, proteins and dyes. A 2µl DNA droplet was measured at 230 nm, 260 nm and 280 nm, respectively. The data were analyzed by software ND-1000 V3.1. In particular, the ratios 260/230 and 260/280 were calculated for evaluating the purity of DNA.

Oligonucleotide probes after 200 times dilution were also determined using NanoDrop ND-1000 by measuring the absorbance at 495 nm for Fluos, 550 nm for Cy3 and 650 nm for Cy5, respectively.

2.7.5 Enzymatic DNA modification

2.7.5.1 Digestion of DNA with restriction endonuclease

The restriction endonucleases from Fermentas (St. Leon-Rot, Germany) or New England BioLabs (Frankfurt am Main, Germany) were used to digest DNA following the protocol supplied from these manufacturers. For analysis, 200 ng DNA was digested with 2 to 5 U restriction endonuclease in 10 μ l volume and the buffer was incubated at specific temperature (mostly 37 °C) for 16 h. For cloning construction, 1-5 μ g DNA in 20-50 μ l volume with 10 U restriction endonuclease were incubated for 16 h. After digestion, the enzyme was inactivated through heat and DNA was purified using PCR purification kit.

2.7.5.2 Dephosphorylation of linear DNA

1 U alkaline phosphatase (Fermentas) and 5 μ l 10 x reaction buffer, in total 50 μ l reaction mixture, were used to catalyze the release of 5'-phosphate terminal groups from DNA to inhibit re-ligation of linear DNA at 37 °C for 30 min. Afterwards, alkaline phosphatase was inactivated at 85 °C for 15 min.

2.7.5.3 Ligation of DNA

5U T4 DNA ligase (Fermentas) and 5 μ l 10 x ligation buffer in 50 μ l volume was used to catalyze the formation of a phosphodiester bond between 5'-phosphate and 3'-hydroxyl terminal groups of a vector and an insert. Three times in molar amounts of insert DNA fragments in total less than 10 μ g/ml DNA was applied. This reaction was performed at 16°C overnight for 16 hours. Afterwards, the ligase was inactivated at 65 °C for 10 min.

2.7.6 Gel electrophoresis

DNA samples were mixed with 6x loading dye solution (Fermentas) and loaded into the wells of the horizontal electrophoresis system of Peqlab (VWR, Erlangen, Germany) using 1% or 2% gels

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with 0.5 µg/ml ethidium bromide (Roth, Karlsruhe, Germany) and 1x TAE buffer (made from 50x TAE buffer, AppliChem). DNA samples were separated at 120 mA and detected under UV light ($\lambda=312$ nm) in a trans-illuminator (Biostep, Johnsdorf, Germany). The performance of the PCR was documented and edited with the Argus X1 documentation system (Biostep, Johnsdorf, Germany). The desired DNA fragments were cut using an xtracta (Biozym, Oldendorf, Germany) and purified using a PCR and gel purification kit (see 3.7.3).

2.7.7 Amplification of specific DNA fragments by PCR

2.7.7.1 PCR primer

The primers used in this work were synthesized by Sigma Genosys (Steinheim, Germany). Some of the primers contained a recognition site of a restriction enzyme with two additional protective bases at the 5' end. Stock primer concentration was 100 pmol/µl and the final working solution in the PCR mix was 0.2 pmol/µl.

Table 2.8: PCR and sequencing primers¹

Name	Sequence(5'-3')	Application	Annealing temperature (°C)
M13F	GTAAAACGACGGCCAG	For insertion fragment in pCR2.1-TOPO vector	50
M13R	CAGGAAACAGCTATGAC		
AHLsyn-s2	GCCAGCTTGTCATAGGACTC	AHL synthase <i>araI</i> gene of <i>A. radialis</i> N35	55
AHLsyn-as2	ATGCACCTCCAGAAAACG		
eYFP-for	CGCCAATACGCAAACC	eYFP	50
eYFP-rev	GTTGGAATTCTAGAGTCG		
AraIF	CGGGATCCTCACTGGCACCGGAT	Insert into pscA-amp/kan	57
AraIR	CGGAATTCATGCGCATCACCTCCG		

2.7.7.2 Standard PCR

Standard PCR was performed in 50µl volume mix with 10x PCR buffer 5 µl, Q buffer 10 µl, 10x coralload 5 µl, dNTP 200 µM, specific primer 0.2 pM each, and 2.5 U top *Taq* DNA polymerase. The templates were either bacterial cells or 100 ng DNA sample. Nuclease free water was added to fill the sample up to 50 µl. For colony PCR, toothpicks were used to transfer bacterial cells from a colony on a plate to 100 µl 1xPBS suspension. 1µl of this suspension was used as template.

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The thermocycler PeqStar 96X (VWR, Erlangen, Germany) was applied for the standard PCR. The cycle program included initial denaturation at 94°C for 3 min followed by 35 cycles at 94 °C for 30 sec, annealing at temperatures based on specific primer pairs for 1 minute and elongation at 72 °C for 1-2 minutes. With a further elongation step at 72 °C for 10 min the program was finished.

2.7.8 Cloning of PCR amplicons

PCR amplicons were ligated into the TOPO TA cloning vector using T4 ligase after digestion with the same restriction endonuclease (Invitrogen, Carlsbad, USA). Then, the recombinant vector was transformed into *E.coli* competent cells using the Invitrogen kit. 100 µl transformed cultures were spread on LB agar plates containing 50 mg/l kanamycin and 40 mg/l X-gal and incubated overnight at 37 °C. The colonies containing plasmid inserts with PCR amplicons in the multi-cloning site appeared white on selective antibiotic plates. Colonies containing the empty vector showed blue color. Several white colonies were picked and the confirmation of correct cloning included colony PCR, plasmid isolation and digestion.

2.7.9 DNA sequence analysis

2.7.9.1 DNA sequencing using ABI 3730 Analyzer

Sequencing of PCR products and plasmid DNA was performed using the BigDye Terminator Kit v3.1 (Applied Biosystems, Foster City, USA) applying specific sequencing primers and annealing temperatures. Sequencing reactions for the ABI 3730 analyzer (Applied Biosystems) were prepared and purified by ethanol precipitation according to the manufacturer's instructions.

2.7.9.2 Sequence data analysis

The sequencing data of the ABI 3730 sequencer were analyzed using the freeware Bioedit Sequence Alignment Editor. Homologous sequences were searched for by BLAST (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al., 1997). All open reading frames were identified by the ORF Finder program (open reading frame finder,

<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). For translation of DNA sequences to protein sequences, ExPASy, <http://www.expasy.ch/tools/dna.html> was used.

2.7.10 DNA transfer into gram-negative bacteria

2.7.10.1 Preparation of electro-competent cells

1 l culture of the recipient strains (e.g. isolate N35) was grown overnight at 37 °C and shaking until OD₆₀₀ reached 0.5. The bacterial culture was cooled on ice for 15 min and centrifuged at 5000x g and 4 °C for 15 min. The bacterial pellet was washed twice with ice cold ultra-pure water and one time with 10% ice cold glycerol. At last, the cells were suspended in 2 ml 10% ice cold glycerol, aliquoted in small volumes (e.g. 50 µl) and stored at -80 °C.

2.7.10.2 Electroporation procedure

Plasmid DNA can be transferred into competent bacterial cells through local perforations of the cell wall caused by an externally applied electrical field. High salt contents in DNA samples (e.g. ligation products) were reduced by drop dialysis against ultra-pure water for 30-60 min.

Plasmid DNA was purified using nitrocellulose membranes, pore size 0.025µm (Merck Millipore, Darmstadt, Germany) to remove the salt. 300-500 ng DNA was added to the competent cells suspension, which was chilled on ice for 30 min. The mixture was pipetted into a cold electroporation cuvette (electrode distance 2 mm, VWR Peqlab, Erlangen, Germany). The electroporation was accomplished using a Gene Pulser (Bio-Rad, Munich, Germany) with a voltage of 2.5 kV for 4.5-5.5 msec. Immediately after electroporation, 1 ml SOC or NB medium was added to the bacterial suspension, followed by incubation at optimal growth temperature for 1 h or more to allow the recovery of the cells and expression of antibiotic resistance markers. Finally, the cells were spread on selective agar plates.

2.7.11 Plasmid construction

To determine the endonuclease restriction sites, Clone Manager Version 5.02 (Scientific & Education software, Durham, USA) was used to analyze multi-cloning sites sequences of plasmids. DNA was isolated and digested with restriction enzymes. After digestion and

purification using NucleoSpin Plasmid kit for the vector and insert DNA were ligated. The ligation product was transferred into competent cells via electroporation and spread onto selective agar plates. After incubation at 37 °C for 16 hours the resulting colonies were picked and grown in liquid medium. The DNA inserts were confirmed by restriction analysis or colony PCR.

2.7.12 GFP and YFP labelling

2.7.12.1 GFP labelling of N35 and N35v

The vector pJBA28 (a mini-Tn5 derivative located on a pUT vector) was used for chromosomal GFP labeling of *A. radialis* N35. This plasmid contains a transposon cassette with kanamycin as selective marker and a constitutively expressed GFP reporter gene. Its replication depends on the *pir* origin and therefore the plasmid cannot replicate after transferring into strain N35. pJBA28 was transferred into electrocompetent cells of *A. radialis* N35 via electroporation. GFP labeled bacteria were selected by kanamycin resistance and its GFP fluorescence was verified by a binocular microscope (Zeiss, Lumar V12).

2.7.12.2 YFP labeling by plasmid transformation

The enhanced *yfp* gene (eYFP) was cloned and ligated with broad host range vector pBBR1MCS-2 to form pBBR1MCS-2-eYFP which was transferred into electrocompetent cells of *A. radialis* N35 *araI::tet* mutant via electroporation. The eYFP labeled bacteria were selected by kanamycin resistance and YFP specific fluorescence was verified via the λ - mode of the CLSM.

2.7.13 Knockout mutagenesis via a gene replacement vector

The target gene *araI* was ligated into the pEX18 suicide vector, which was also used for mutant construction. The plasmid carrying the mutant gene cassette was transformed into *A. radialis* N35. After plating on selective agar, several colonies grew on selective plates. After inoculation at 37°C for 24 hours colonies were picked and grown in NB medium and then spread on to 10% sucrose and tetracycline antibiotic plates for the second crossover between vector and chromosome. This resulted in the replacement of wild type gene with mutant gene while the vector was discarded. For details see Li (2010).

2.8 Statistics

The length and weight data from the barley growth promotion experiment were analyzed with Microsoft Office Excel 2010 and sigma plot software. The difference analysis (T-test) was performed with sigma plot 10.0 (Systat, Erkrath, Germany).

2.9 RNA-seq

For plant cultivation see 3.5.2. The barley seedlings were harvested 1 day and 10 days after inoculation with the bacteria. Shoot parts were frozen in liquid nitrogen and stored at -80°C freezer. RNA was isolated using RNeasy Mini kit (Qiagen) and quantified using NanoDrop1000. Requirements for RNA quality were that OD260/280 and OD260/230 were higher than 2.0 and the amount for RNA more than 500ng. The RNA integrity was measured by bioanalyzer 2100 RNA 6000 nano kit from agilent technologies. cDNA was generated using the high capacity cDNA reverse transcription kit by Applied Biosystems. cDNA-libraries were sequenced using HiSeq 2500 (Illumina) in single read mode and running 100 cycles. The library construction and sequencing was done as a service provided by the “Kompetenzzentrum Fluoreszente Bioanalytik (KFB)” of the University of Regensburg. The bioinformatics analysis was performed as described in Dugar et al. (2013). The alignment of reads, coverage calculation, gene-wise read quantification, and differential gene expression were performed in cooperation with Klaus Meyer and Eva Trost from the Research Unit Plant Genome and Systems Biology (PGSB) at the Helmholtz Center Munich using READemption which was relying on segemehl version X (Hoffmann et al. 2009), DEseq version V. Visual inspection of the coverage was performed using the integrated genome browser (IGB) developed by the PGSB.

2.10 RT-qPCR

Total RNA was isolated using RNeasy plant mini kit (QIAGEN) according to the manufacturer's instruction. cDNA was generated using high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR (RT-qPCR) was performed using the primers of table 2.9 applying the KAPA SYBR FAST RT-qPCR Kit (VWR Peqlab, Erlangen, Germany) on a Real-time RT-qPCR system (peqSTAR 96Q). All primer pairs were verified by melting curves

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showing only one peak and a slope value close to -3.33. Transcript accumulation was analyzed using relative quantification with the software sigma plot. The q-PCR results are the average of three technical repetitions per sample and five independent plant inoculation experiments.

Table 2.9: Primer used in RT-qPCR analysis

Genes	Primer sequence	Gene names
MLOC_67149	AAGGCATGGGAGATGGTTGG TATCATGGCGTCCCACACG	F-Box family-3(fb-3)
MLOC_10956	GCCAGAAGCCATATCTGCAC GCAGAAAACTCACCGGAGC	UDP-glycosyltransferase-like protein (UGT)
MLOC_58764	TGACACCCCTGCTTCGTTAG ACGACAGCGACCTGTGTTAG	4-coumarate:CoA ligase (4-CL)
MLOC_5324	CTTCGACGCACTTGTCTCGG ACTGCGACCCCTTGATCTCC	Chalcone-flavonone isomerase (CFI)
MLOC_74116	CCGACTACCCGGACTACTAC TGTACCTCTTCCTGATCTGCG	Chalcone synthase (CHS)
MLOC_72837	TGCTGCACAACCTTTCACTCC ACTGAAACTCCCATCCCAGC	Chaperone protein (DnaJ)
MLOC_59602	ACTGAAACTCCCATCCCAGC TAGACCCTCCGCTGGTATCC	E3 ubiquitin-protein ligase (PRT1)
MLOC_5618	TTCATCAGCTACCCATCTACC CTCCTTCTTGTCGAGGCAG	Heat shock protein 90 (HSP90)

2.11 Measurement of flavonol glycosides in barley

Barley leaves or roots were cut from the plant and placed immediately in liquid nitrogen. About 150 mg of frozen plant material were homogenized in liquid nitrogen in a small mortar and about 50 mg of this homogenate was transferred to a 2 ml Eppendorf tube pre-cooled with liquid nitrogen. All the samples were kept in liquid nitrogen to prevent from thawing. 10 µl of methanol (HPLC-grade) was added for every mg of sample material. The samples were then thoroughly vortexed for 1 h on a lab shaker at 700 rpm in the dark. Before centrifugation for 10 min at 11000 rpm, the samples were vortexed again. The supernatants were transferred to a new cap and stored at -80°C until HPLC measurement. For HPLC analysis, a reversed-phase HPLC system was applied. A linear gradient over 45 min was applied with 100% solution A (2% formic acid containing 0.1% ammonium formate) to 100% solution B (0.1% ammonium formate in 88% methanol) and maintained for another 5 min. Finally, the absorbance of the eluent was measured at 280 nm (Yin et al., 2012)

3 Results

3.1 *AraI* gene determines 3-OH-C10-HSL production in *A. radialis* N35

The homologous AHL synthase gene *araI* could be identified in the genome sequence of *A. radialis* N35 by homologous sequence blast using known lux I type genes. To investigate the function of QS in *A. radialis* N35, an AHL-deficient mutant has been constructed using the recombination method by Dan Li in her PhD thesis by introducing the tetracycline resistance gene *tet* (1.5 Kb) into the *araI* gene. (Li 2010). A complemented *araI* strain was produced in this thesis by expressing the wild type *araI* gene using the broad host range plasmid pBBR1MCS2 in the *araI::tet* mutant strain. The successful construction of the *araI::tet* mutant and complemented strains was confirmed using PCR and sequencing (Fig. 3.1A). To characterize the AHL production abilities, the AHL biosensor *Agrobacterium tumefaciens* A136 (carrying pCF218 or pCF372) was applied. This strain has been used successfully to detect various types of AHLs, especially C10-HSL including the hydroxyl- or oxy-derivates at the C3 position (Stickler et al. 1998). Fig. 3.1B shows the result of an indicator plate with the biosensor *A. tumefaciens* A136 in the center and the wild type *A. radialis* N35, the *araI::tet* mutant and its complementary strain. AHL production is indicated by the blue color development only with the wild type and the complemented *araI::tet* mutant. The correct phylogenetic authenticity of these bacterial strains was confirmed using FISH with the probes shown in material and methods (M&M).

To further analyze the quantity of AHL production in wild type, *araI::tet* mutant and complemented *araI::tet* mutant, these bacteria were introduced to barley seedlings. Aliquots of the root MS medium were analyzed using biosensor A136. The complemented *araI::tet* mutant produced very high amounts of AHL when colonizing barley roots (data not shown). This indicated that probably due to the construction of the complementation, an overproduction of AHL was the consequence. This may cause erotic responses of the plants, since the AHL may reach subtoxic or toxic levels.

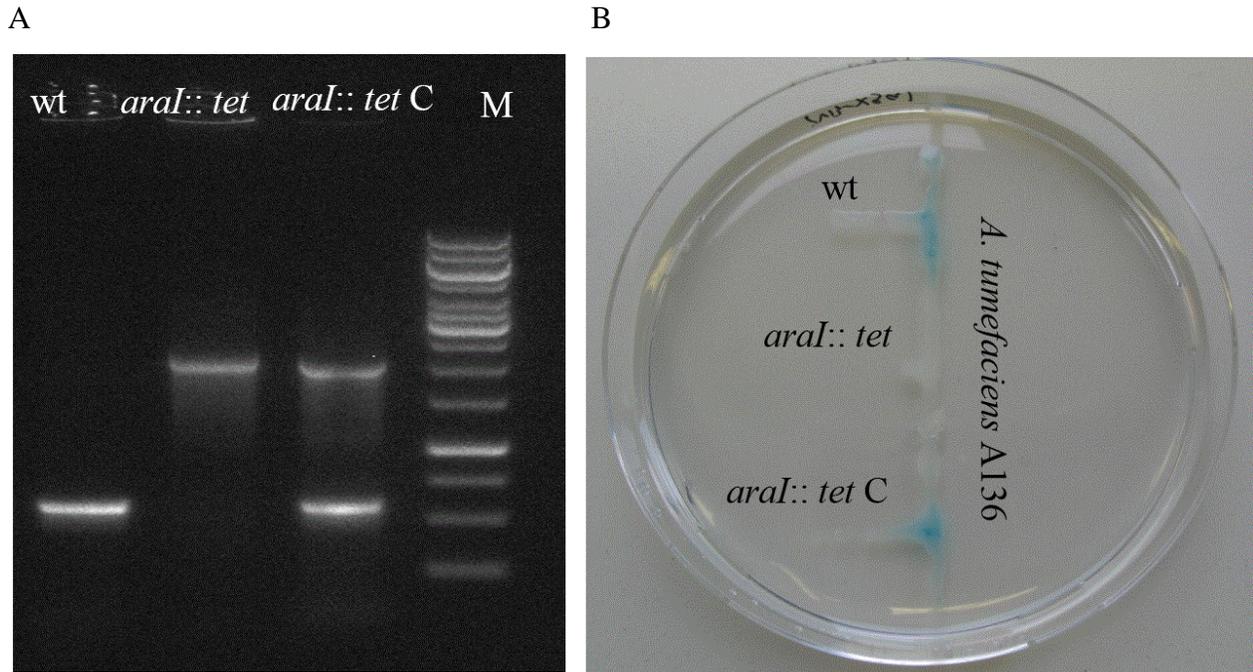


Fig. 3.1: AHL production by *A. radialis* N35.

A, PCR-assay using an *araI* specific primer to detect the *araI* gene in *A. radialis* N35 wt, *A. radialis* N35 *araI::tet* mutant, and *A. radialis* N35 *araI::tet C* (complemented strain). B, Application of AHL biosensor strain *A. tumefaciens* A136 (harbouring *traI-lacZ* fusion plasmid) to detect AHL production by these three strains. The blue color represents AHL production.

3.2 Competitive colonization

To be able to analyze, if AHL production and QS of *A. radialis* N35 has an influence on the ability to colonize barley roots, differentially GFP/YFP-labeled wild type and *araI::tet* mutant strains (construction see M&M) were applied. Single strains and a mixture of equal amounts of these differentially labeled wild type and *araI::tet* mutant cells were applied to barley roots, and the barley seedlings were cultivated under axenic conditions for one week. After harvesting and washing the roots, the colonization behavior of wild type and *araI::tet* mutant on the roots was examined using confocal microscopy in the lambda mode, which can distinguish GFP from YFP emitted fluorescence based on the specific spectrum character. The YFP-fluorescence of the wild type strain is shown in red while the GFP fluorescence of the *araI::tet* mutant is shown in green color. Both wild type and *araI::tet* mutant colonized barley roots well when applied alone,

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although the *araI::tet* mutant showed more a single cell colonization pattern and less biofilm formation. When the *A. radicis* N35 wild type and *araI::tet* mutant were applied as a 1:1 mixed inoculum, the wild type clearly predominated colonization over the QS mutant strains (Fig. 3.2). This indicates that AHL- production by *A. radicis* N35 is important for its competitive colonization ability on barley roots.

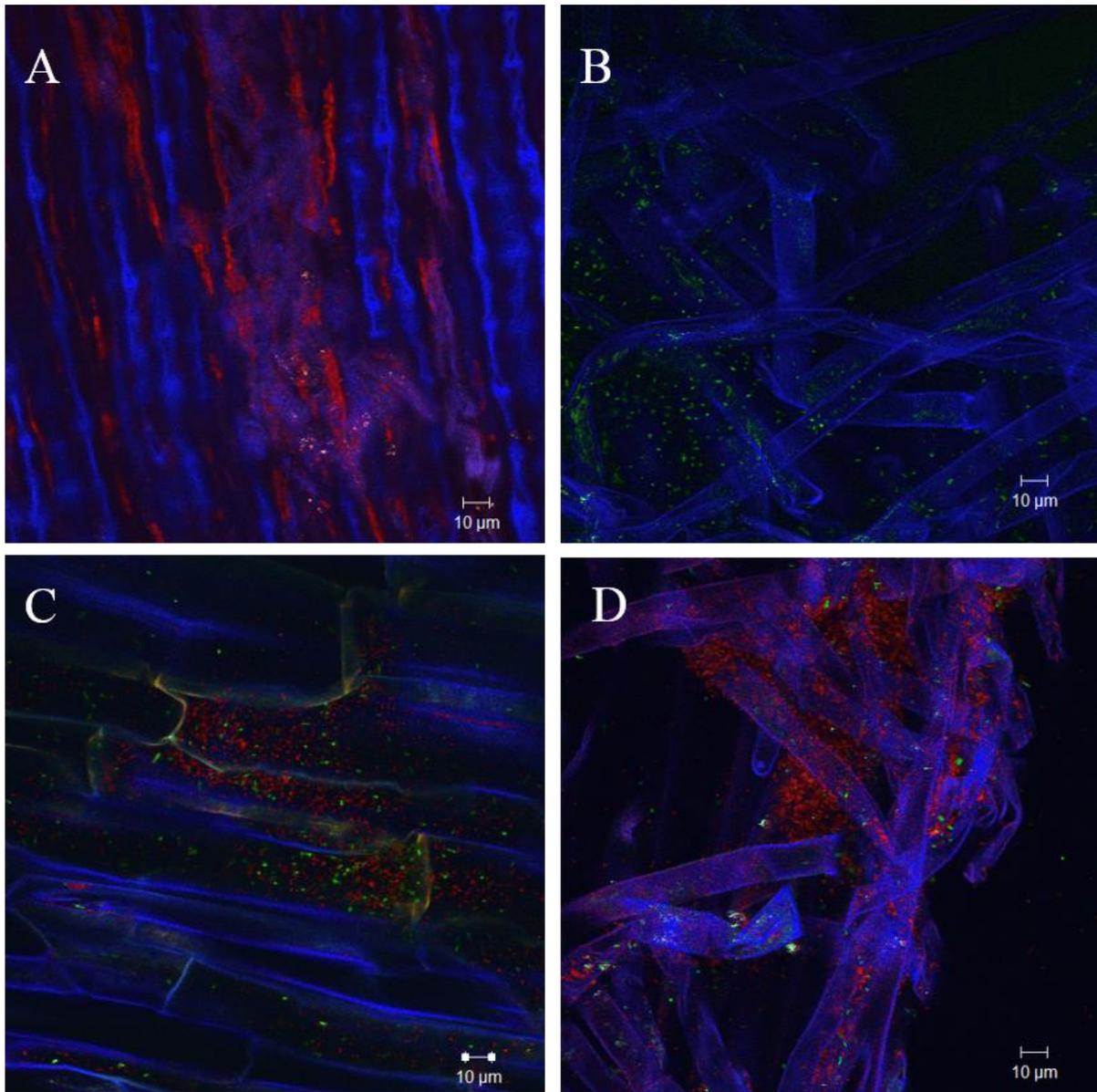


Fig. 3.2: Colonization of *A. radicis* N35 wt and *araI::tet* mutant on barley roots, detected by CLSM lambda mode. A: YFP-labeled *A. radicis* N35 form biofilm structure in the main root part and root hair part. B: The GFP-labeled *A. radicis* N35 *araI::tet* mutant. C and D: 1:1 mixture of YFP-labeled *A. radicis*

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N35 wt and GFP- labeled *A. radialis* N35 *araI*::tet mutant. In these images blue color represents autofluorescence of root cell walls, the red color represents YFP labeled *A. radialis* N35 wild type and the green color indicates the GFP labeled *A. radialis* N35 *araI*::tet mutant. Cell suspensions of *A. radialis* N35 and its *araI*::tet mutant strain with OD₆₀₀=1.5 were mixed in 1:1 ratio. 2 days old germinated barley seedlings were inoculated for one hour and then grown for one week under axenic conditions as described in M&M. CLSM-analysis was performed under the lambda mode to visualize GFP- and YFP-fluorescence simultaneously.

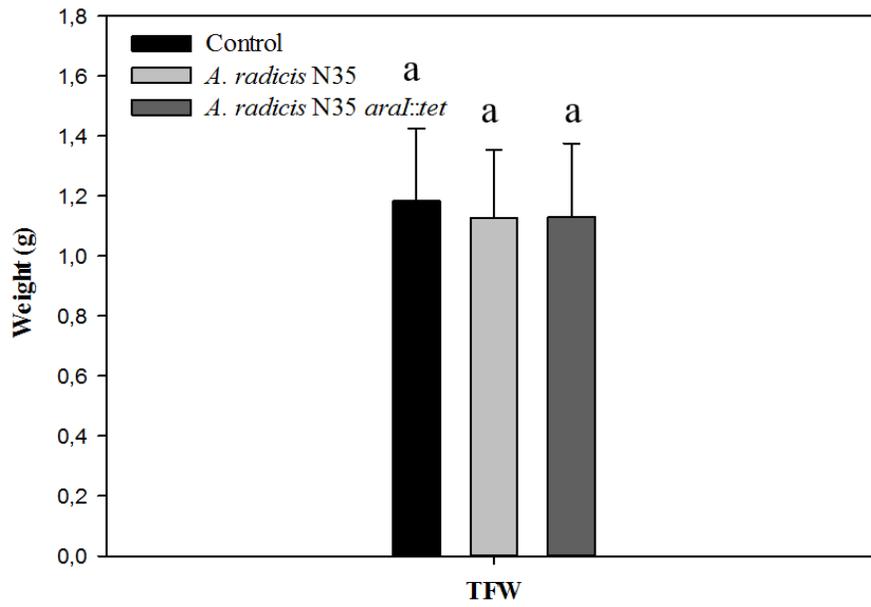
3.3 Plant Growth Promotion

3.3.1 Plant growth promoting effect

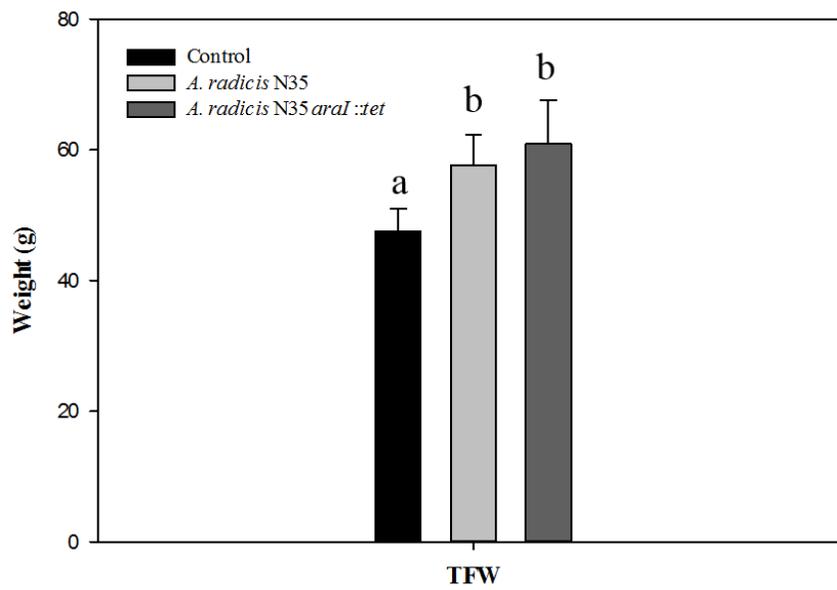
To assess an AHL dependent growth promoting effect on barley, seedlings were inoculated with *A. radialis* N35 wild type or the *araI*::tet mutant strain or not inoculated as control. Barley seedlings were grown under axenic conditions in the growth chamber or under un-sterile soil conditions in the greenhouse (see M&M). After two weeks / two months in the soil system or two weeks in the axenic system, barley plants were harvested and total plant fresh weight as well as shoot and root length were measured (Fig.3.3). In the soil system, a significant growth promotion effect on total plant fresh weight was found after inoculation with *A. radialis* N35 and *araI*::tet mutant only after two months. In the axenic growth system, no significant stimulation of fresh weight could be observed after inoculation with the wild type strain after two weeks. When the colonization of roots was analyzed using the FISH method, *A. radialis* N35 cells could not be detected after two months in the soil system (Fig. S1). In the axenic system, the colonization by *A. radialis* N35 was very well detectable using the FISH method

Results

A



B



Results

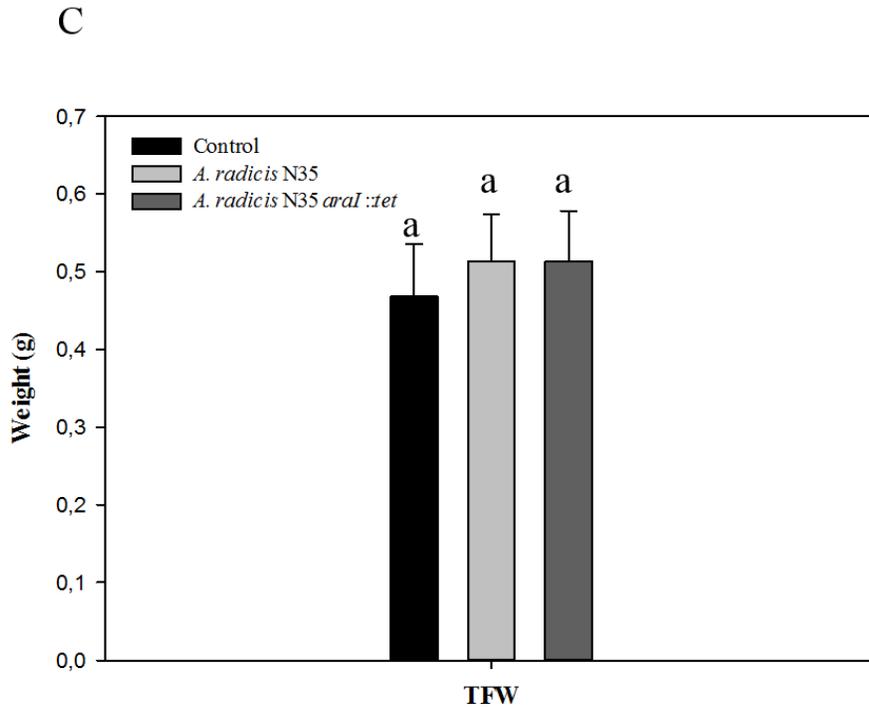


Fig. 3.3: Total fresh weight (TFW) of barley obtained after two weeks (A) and two months (B) grown in soil and total fresh weight of barley obtained after two weeks growth under axenic condition with or without inoculations (C). a: no significant difference, b: significant difference at level $p < 0.05$.

3.4 Barley transcriptome analysis

To be able to investigate, which plant genes were differentially regulated in barley leaves after inoculation with the AHL producing *A. radialis* N35 wild type strain in comparison with uninoculated control plants and plants inoculated with the AHL deficient *araI::tet* mutant strain, a RNA-seq experiment and a series of specific RT-qPCR tests were performed. In the barley leaf transcriptome a number of gene transcripts were significantly enhanced or suppressed by *A. radialis* N35 or the *araI::tet* mutant at 10 days post inoculation (dpi) compared to the uninoculated control plant.

3.4.1 RNA-sequencing pre-experiment

To determine the right time points for RNA isolation, GFP-labeled *A. radialis* N35 was used to analyze the colonization behavior at different time points in the axenic growth system (M&M). As shown in fig 3.4, one day after inoculation, *A. radialis* N35 was only visible at the surface of

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the roots in a scattered colonization mode. After one week, the bacteria formed sessile microcolony- or biofilm-like structures, most of which were found especially in the region of root hairs. Similar sessile structures were found in the apoplast region. After 10 days only few bacteria were found on the surface and many bacteria penetrated into the roots. In order to analyze the response of barley at different time points after inoculation of *A. radialis* N35, 1 and 10 dpi were selected as sampling time points.

Results

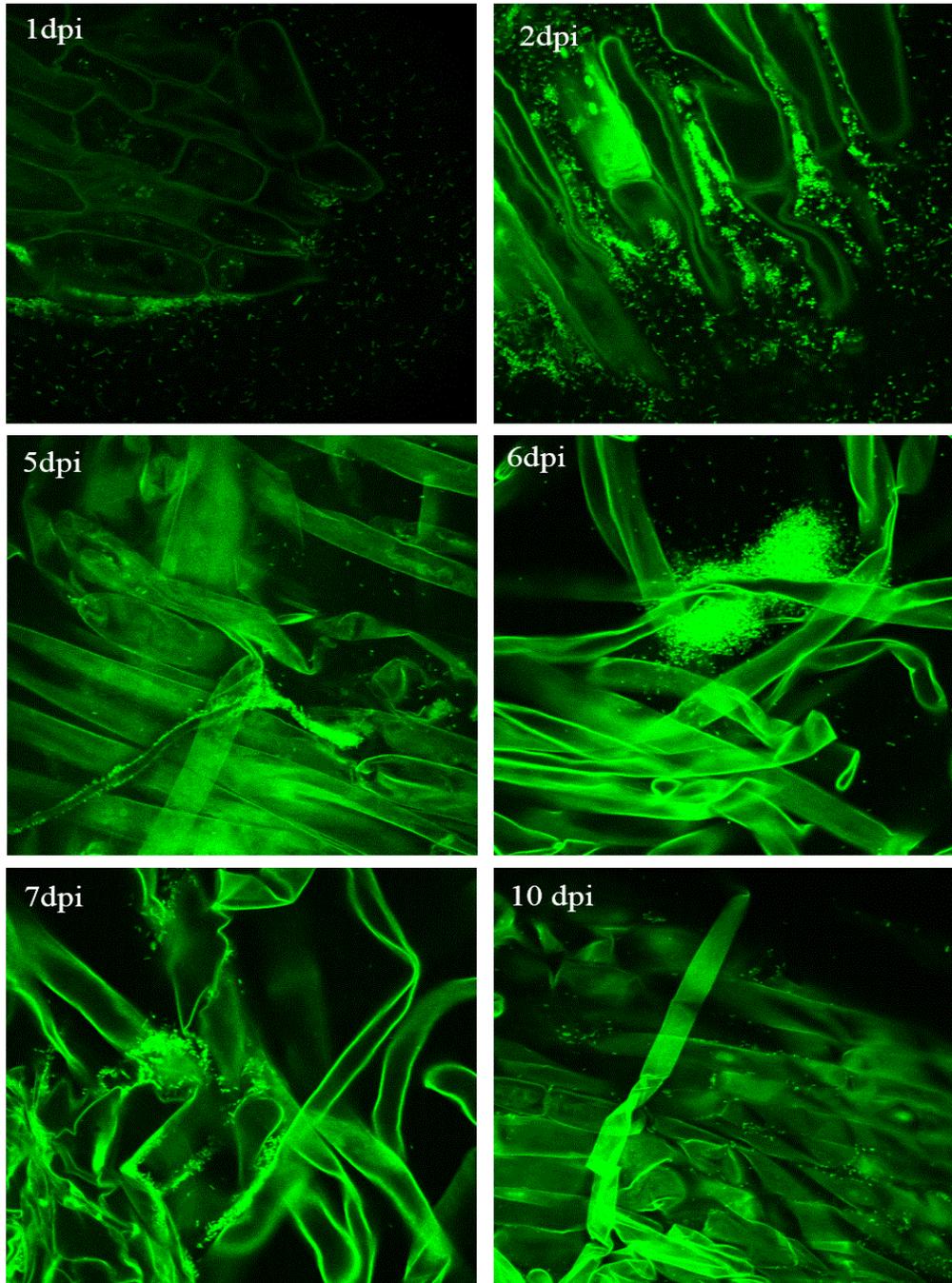


Fig. 3.4: Colonization of barley roots by GFP-labeled *A. radialis* N35 in a monoxenic system at different time points after inoculation (1 to 10 dpi as indicated in the pictures).

3.4.2 RNA-sequencing (RNA samples)

1 day and 10 days after bacterial inoculation and incubation in axenic system, the second youngest leaves of barely seedlings were harvested for RNA isolation using RNeasy Mini kit (Qiagen) following the protocol as described in (1.9). Barley seedlings without any inoculation

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were used as control. RNA samples were quantified and their integrity was verified as described in 3.9. Measured by bioanalyzer 2100 RNA 6000 nano kit from agilent technologies.

Table 3.1: Characterization of RNA samples for sequencing was isolated from barley leaf samples.

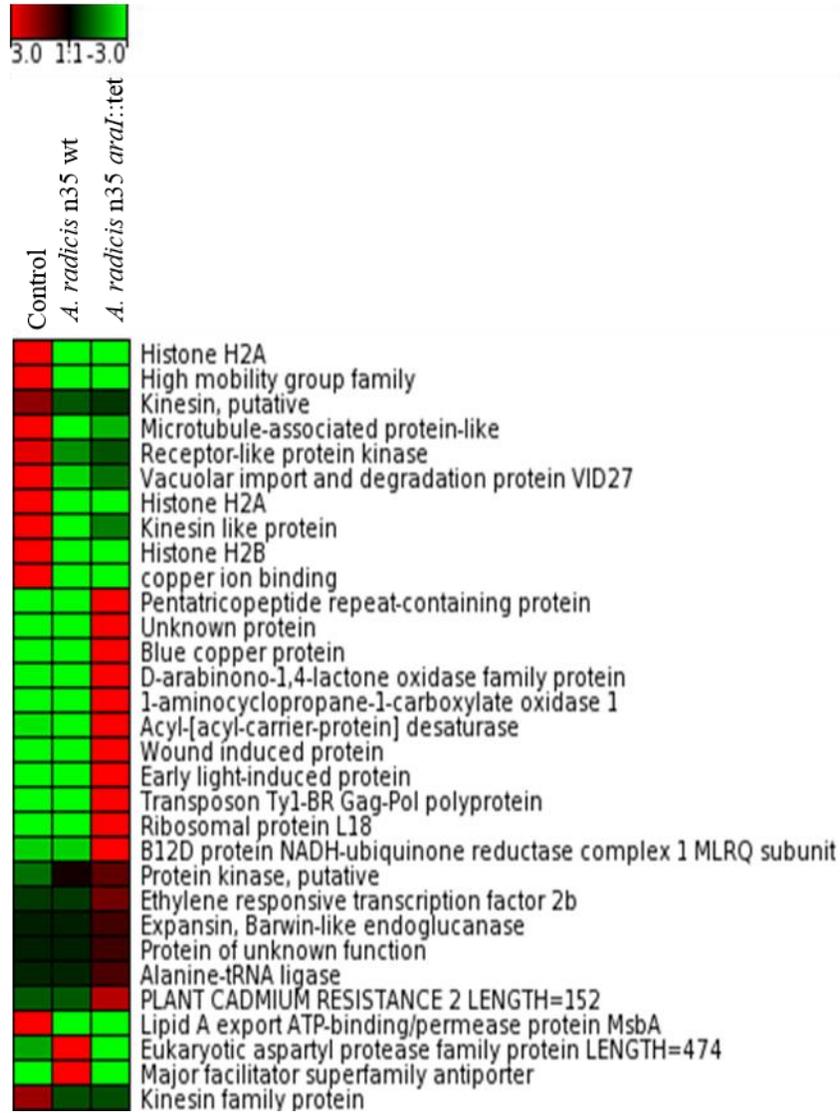
Sampling time (dpi)	treatments	RNA		
		ng/ul	OD260/OD280	OD260/OD230
1-1	Ck	508	2.05	2.39
	<i>A. radialis</i> N35	427	1.91	2.42
	<i>A. radialis</i> N35 <i>araI::tet</i>	126	1.92	2.25
1-2	Ck	449	1.87	2.43
	<i>A. radialis</i> N35	535	2.01	2.42
	<i>A. radialis</i> N35 <i>araI::tet</i>	426	2.01	2.32
10-1	Ck	376	1.96	2.35
	<i>A. radialis</i> N35	510	1.99	2.40
	<i>A. radialis</i> N35 <i>araI::tet</i>	384	1.96	2.36
10-2	Ck	453	2.04	2.32
	<i>A. radialis</i> N35	875	2.05	2.40
	<i>A. radialis</i> N35 <i>araI::tet</i>	525	2.03	2.37

3.4.3 RNA-seq results (genes category)

Dozens of gene transcripts of barley leaves were enhanced or suppressed by *A. radialis* N35 wt or *araI::tet* mutant inoculation at 1dpi and 10dpi (Fig.3.5 A and B). As compared to the uninoculated control plants, these genes were divided into three groups: (i) **QS-independent, general MAMP-triggered responses** (Tables 3.2 and 3.3), when gene expression was affected by inoculation with both *A. radialis* N35 and *araI* inoculation in the same manner; (ii) **QS-dependent regulation** (Table 3.4 and 3.5), when specific gene transcription occurred only after inoculation with *A. radialis* N35 wild type and not with the *araI::tet* mutant; and (iii) most interestingly, a **QS-deficient regulation** (Tables 3.6 and 3.7), when specific genes were upregulated, which were not detected in the control plants and the Wt-inoculated plants. There was also a clear plant development dependent effect apparent, because almost no identical gene transcripts were found at 1dpi and 10dpi. In this study, seven genes were further selected for RT-PCR verification, since their functions are well known in plant resistance pathways.

Results

A



Results

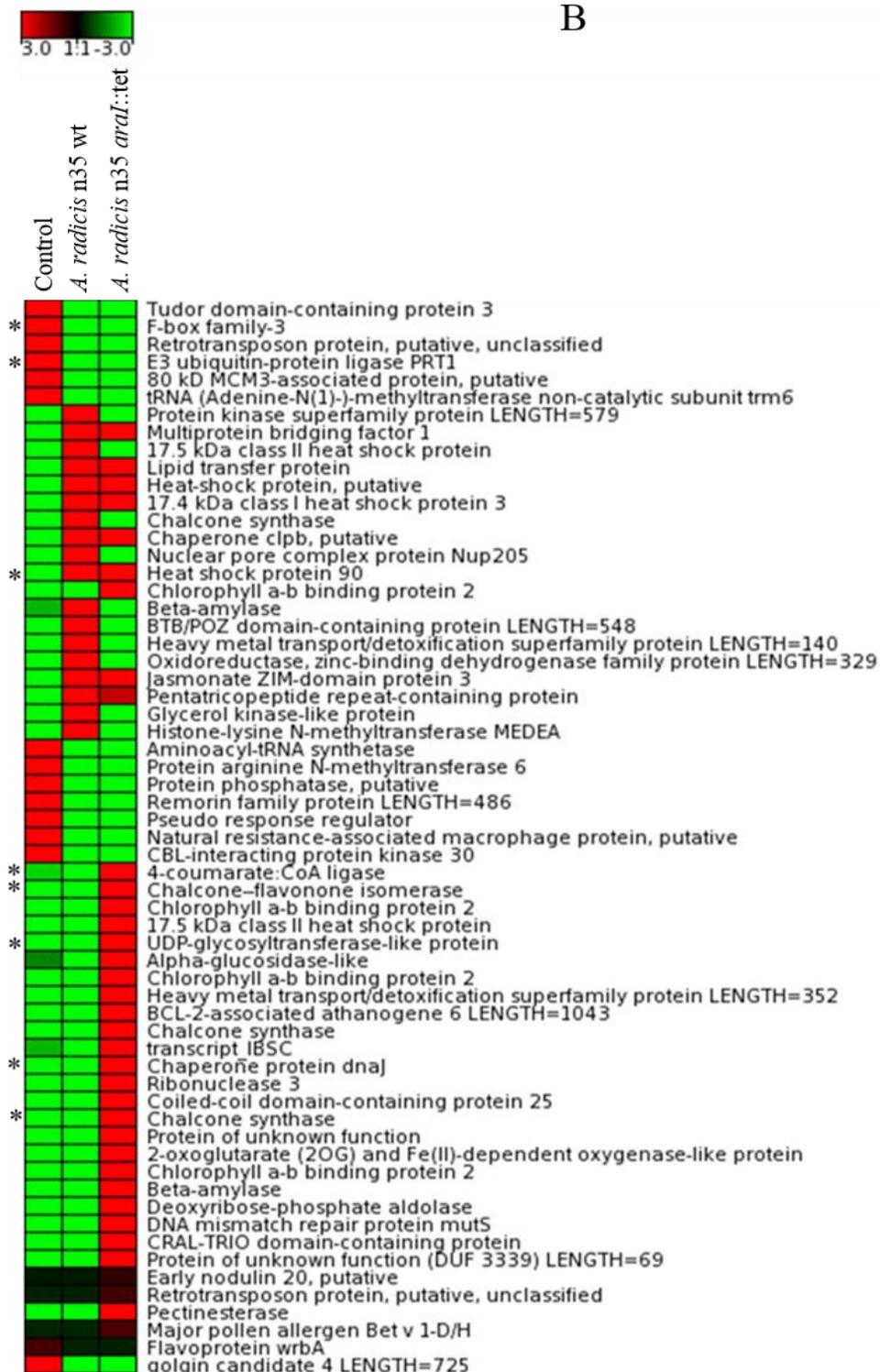


Fig. 3.5: Transcriptome analysis in barley leaves by RNA-sequencing. mRNA was isolated from barley leaves 1 day (A) and 10 days (B) after root inoculation with *A. radialis* N35, the *araI::tet* mutant, and from not inoculated plants (ck) respectively. For the heatmap CARMAwdb 1.5 software

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(<https://carmaweb.genome.tugraz.at/carma/>) was used. Red (upregulated) and green (downregulated) colors represent an at least 3-fold difference in the amount of detected gene transcripts for the respective gene between the analyzed samples.

3.4.3.1 QS-independent general MAMP-triggered responses

Several plant responses towards bacterial inoculation at 10 dpi were directed to different posttranscriptional reactions (tab. 3.2). E.g. a DNA methylation related gene (the Tudor domain-containing protein) assists in H3K9me3 localization and DNA methylation (Cheng, J.C. et al. 2012). tRNA methylation related genes (Trm6) were found in mammalian cells and are known to methylate the adenosine 58 of the initiator methionine tRNA (tRNA_i^{met}). This helps to stabilize its structure which is important for its function in tumor genesis (Macari, F et al. 2015).

Another category of genes was coding for chaperones including the 17.5 kd heat shock protein, HSP90, and chaperone clbp. The pollen allergen bet V 1-D/H gene was induced only at 10dpi, while common protein kinase expression was upregulated already at 1 dpi (tab. 3.3).

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Table 3.2: List of **QS-independent** regulated plant genes expressed differently at **10 dpi** in plants inoculated with *A. radicis* N35 and its *araI* ::tet mutant as compared to the uninoculated control plants (ck). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr. Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to CK	
		N35	<i>araI</i> ::tet mutant
MLOC_54481	Tudor domain-containing protein 3	-7.34	-6.93
MLOC_59602	E3 ubiquitin-protein ligase PRT1	-3.99	-3.71
MLOC_68945	tRNA (Adenine-N(1)-)-methyltransferase non-catalytic subunit (trm6)	-3.54	-3.40
MLOC_63473	Nuclear pore complex protein Nup205	2,14	-2,68
MLOC_72290	Chlorophyll a-b binding protein 2	2.39	1.59
MLOC_32229	17.5 kDa class II heat shock protein	1.80	1.61
MLOC_13045	Multiprotein bridging factor 1	1.58	1.73
MLOC_6787	17.4 kDa class I heat shock protein 3	1.93	1.75
MLOC_58758	Chlorophyll a-b binding protein 2	2.39	1.78
MLOC_54379	Heavy metal transport/detoxification superfamily protein LENGTH=352	2.67	1.86
MLOC_75175	Heat-shock protein, putative	1.90	1.97
MLOC_74116	Chalcone synthase	2.04	2.43
MLOC_20041	Lipid transfer protein	1.82	2.18
MLOC_50979	Chaperone clpb, putative	2.08	2.23
MLOC_5618	Heat shock protein 90	2.30	2.25
MLOC_72040	Pentatricopeptide repeat-containing protein	3.71	2.97
MLOC_56051	Chlorophyll a-b binding protein 2	2.39	2.98
MLOC_5168	Beta-amylase	2.39	3.17
MLOC_9995	Jasmonate ZIM-domain protein 3	3.49	3.83
MLOC_33369	Retrotransposon protein, putative, unclassified	-4.30	Infinite
MLOC_57345	Major pollen allergen Bet v 1-D/H	Infinite	Infinite

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Table 3.3: List of plant genes expressed at **1 dpi** differently in plants inoculated with *A. radialis* N35 as well as its *araI::tet* mutant (**QS-independent**). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr.Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to CK	
		N35e	<i>araI::tet</i> mutant
MLOC_44415	Protein kinase, putative	Infinite	Infinite
MLOC_75843	Kinesin, putative	Infinite	-2.82

3.4.3.2 QS-dependent regulation

Changes in expression level only visible after inoculation with the *A. radialis* wild type were scarce. One example is the upregulation of histone lysing methyltransferase MEDEA at 10 dpi (table 3.4).

Table 3.4: List of plant genes expressed at **10 dpi** differently only in plants inoculated with *A. radialis* N35 (**QS-regulated**). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr.Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to Ck
MLOC_67149	F-box family-3	-7.34
MLOC_58431	80 kD MCM3-associated protein, putative	-3.91
MLOC_11419	Protein kinase superfamily protein LENGTH=579	1.37
MLOC_59476	BTB/POZ domain-containing protein LENGTH=548	2.57
MLOC_66526	Oxidoreductase, zinc-binding dehydrogenase family protein LENGTH=329	3.36
MLOC_20784	Glycerol kinase-like protein	6.68
MLOC_1303	Histone-lysine N-methyltransferase MEDEA	8.34

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Table 3.5: List of plant genes expressed at **1 dpi** differently only in plants inoculated with *A. radialis* N35 (**QS-regulated**). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr.Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to CK
MLOC_10398	Lipid A export ATP-binding/permease protein MsbA	-2.51
MLOC_64967	Eukaryotic aspartyl protease family protein LENGTH=474	2.75
MLOC_44183	Major facilitator superfamily antiporter	6.68

3.4.3.3 QS-deficient regulation

Inoculation with the *araI::tet* mutant caused specific changes in the transcription of many genes, which were not visible in response to inoculation with the wildtype or in the control samples. This included arginine N-methyltransferase 6, which was downregulated at 10 dpi, while pennelpropanoid metabolism pathway genes were upregulated (tab. 3.6). At 1 dpi inoculation of the mutant caused different expression changes, including the upregulation of 1-aminocyclopropane-1-carboxylate oxidase 1, which can catalyze the synthesis of ethylene and ethylene transcription regulator (tab. 3.7). This result indicates that the *araI::tet* mutant may cause responses in the ethylen-dependent pathway.

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Table 3.6: List of plant genes expressed at **10 dpi** differently only in plants inoculated with *A. radialis* N35 *araI::tet* mutant (**QS-deficient**). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr. Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to CK
MLOC_36614	Aminoacyl-tRNA synthetase	-7.66
MLOC_19129	Protein arginine N-methyltransferase 6	-6.95
MLOC_53416	Protein phosphatase, putative	-6.39
MLOC_53511	Remorin family protein LENGTH=486	-5.87
MLOC_62596	Pseudo response regulator	-5.43
MLOC_63231	Natural resistance-associated macrophage protein, putative	-2.68
MLOC_52019	CBL-interacting protein kinase 30	-1.81
MLOC_58764	4-coumarate:CoA ligase	1.51
MLOC_5324	Chalcone--flavonone isomerase	1.57
MLOC_10956	UDP-glycosyltransferase-like protein	1.69
MLOC_822	Alpha-glucosidase-like	1.75
MLOC_13672	EST D48432(S14625) corresponds to a region of the predicted gene	1.94
MLOC_12681	BCL-2-associated athanogene 6 LENGTH=1043	2.05
MLOC_7936	transcript_IBSC	2.18
MLOC_72837	Chaperone protein dnaJ	2.20
MLOC_66363	Ribonuclease 3	2.30
MLOC_64658	Coiled-coil domain-containing protein 25	2.41
MLOC_64305	Chalcone synthase	2.43
MLOC_18785	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein	2.93
MLOC_55585	Deoxyribose-phosphate aldolase	4.29
MLOC_76334	DNA mismatch repair protein mutS	4.54
MLOC_7481	CRAL-TRIO domain-containing protein	4.55
MLOC_47898	Protein of unknown function (DUF 3339)	14.16

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	LENGTH=69	
MLOC_30326	Early nodulin 20, putative	infinite
MLOC_54267	Pectinesterase	infinite
MLOC_38677	Flavoprotein wrbA	infinite
MLOC_67517	golgin candidate 4 LENGTH=725	infinite

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Table 3.7: List of plant genes expressed at **1 dpi** differently only in plants inoculated with *A. radialis* N35 *araI::tet* mutant (**QS-deficient**). The analysis of RNA seq reads was performed in cooperation with Dr. Eva Trost and Dr. Klaus Meyer from HMGU/PGSB.

Gene	Name	Log2 fold change to CK
MLOC_64906	Histone H2A	-3.11
MLOC_77667	High mobility group family	-3.08
MLOC_12156	Microtubule-associated protein-like	-2.76
MLOC_37098	Receptor-like protein kinase	-2.60
MLOC_45226	Vacuolar import and degradation protein VID27	-2.42
MLOC_35155	Histone H2A	-2.17
MLOC_64189	Kinesin like protein	-2.04
MLOC_76747	Histone H2B	-1.99
MLOC_7518	copper ion binding LENGTH=250	-1.75
MLOC_72040	Pentatricopeptide repeat-containing protein	2.01
MLOC_65674	Blue copper protein	2.86
MLOC_68610	D-arabinono-1,4-lactone oxidase family protein LENGTH=591	2.98
MLOC_70078	1-aminocyclopropane-1-carboxylate oxidase 1	3.06
MLOC_61465	Acyl-[acyl-carrier-protein] desaturase	3.23
MLOC_26534	Wound induced protein	3.68
MLOC_78997	Early light-induced protein	4.57
MLOC_10400	Ribosomal protein L18	infinite
MLOC_39185	B12D protein NADH-ubiquinone reductase complex 1 MLRQ subunit	infinite
MLOC_51143	Ethylene responsive transcription factor 2bpathway)	Infinite

Footnote: infinite ratios resulted from very low expression in the control.

3.4.3.4 Genes selected for RT-qPCR analysis

Interestingly, RNA-seq results from leaves after 10dpi indicated that the transcription of several flavonoid-synthesis pathway genes (Besseau et al. 2007) were upregulated only in the case of

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inoculation with the *araI::tet* mutant, including UDP-glycosyltransferase-like protein (UGT), chalcone-flavonone isomerase (CFI), chalcone synthase (Hassett et al. 1999), and 4-coumarate-CoA ligase (4-CL). Also the flavonoid response gene DnaJ was found to be upregulated only after inoculation with the *araI::tet* mutant. In addition, two ubiquitin E3 ligase, F-box family-3 gene and the E3 ubiquitin-protein ligase PRT1 were chosen due to their roles in plant immunity.

3.5 Transcript analysis of selected plant genes by RT-qPCR

3.5.1 Primers designed for RT-qPCR

Specific primers for RT-qPCR were developed to detect candidate genes (tab. 2.9, M&M). These primers were validated based on the slope of the standard curve (value is about -3.3) and the melting curve (only one clear peak), see supplementary fig. S2.

3.5.1.1 RT-qPCR of flavonoid synthase related genes

The expression of four flavonoid synthase genes UGT, 4-CL, CFI and CHS and the chaperone protein DnaJ were tested by q-PCR analysis with 5 replications. All these flavonoid synthase genes were significantly up-regulated in plants inoculated with *A. radialis* N35 *araI::tet* mutant, while they were unaffected or slightly down-regulated in plants after inoculation with *A. radialis* N35 wild type (Fig. 3.6 A). The two flavonoid synthesis inhibitors F-box protein 3 and E3 ubiquitin-protein ligase (PRT1) were down-regulated in leaves of wild type and mutant inoculated barley plants. This effect was more pronounced in plants inoculated with the *araI::tet* mutant (Fig. 3.6 B).

Two chaperone coding genes, HSP90 and *dnaJ*, were also tested using q-PCR. HSP90 was upregulated after inoculation with Wt and *araI::tet* mutant, while *dnaJ* was upregulated only after mutant treatment (Fig. 3.6 C).

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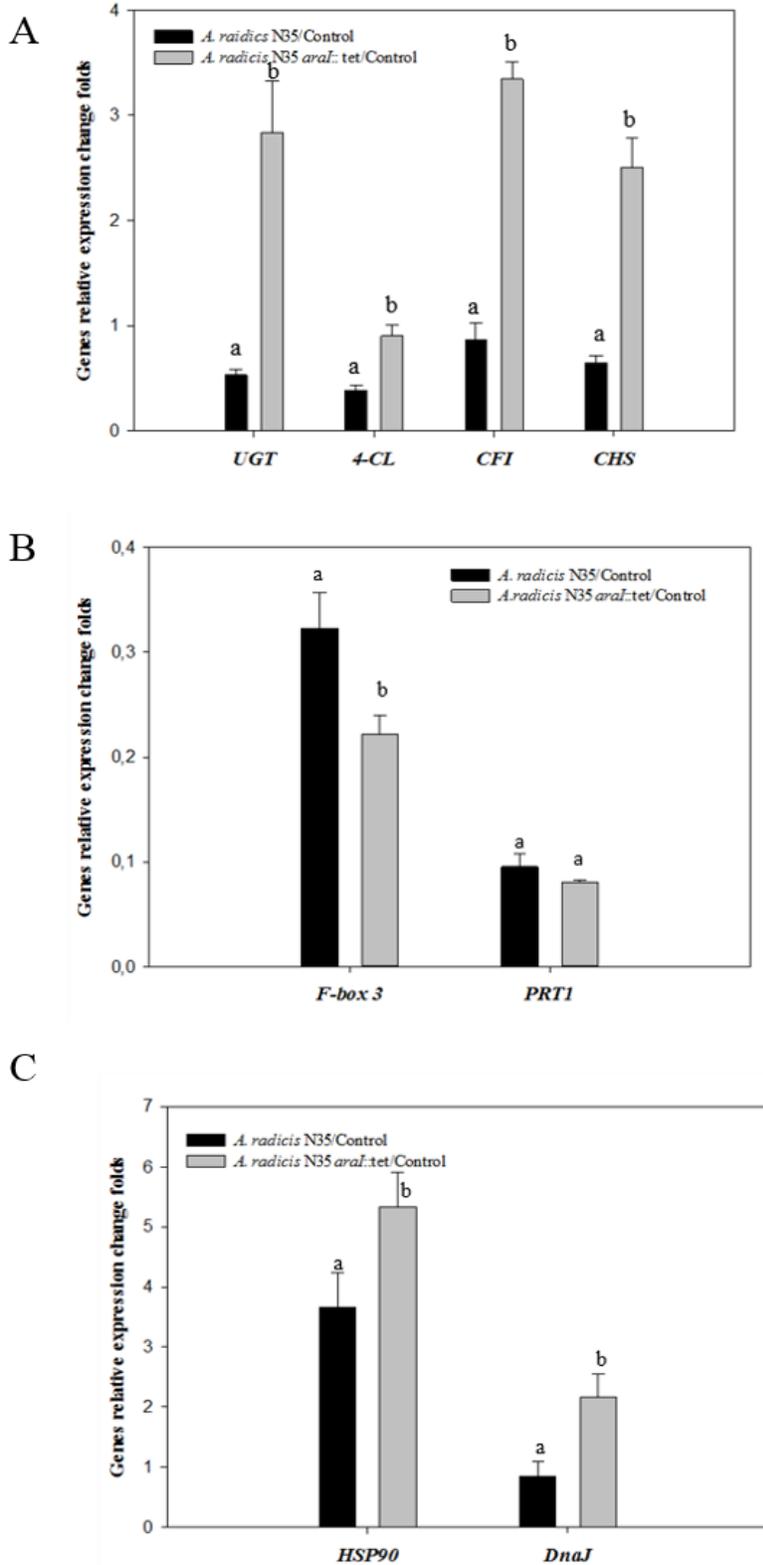


Fig. 3.6: Q-PCR analysis of the expression of genes under the influence of *A. radialis* N35 wild type and the *araI::tet* mutant. Barley seedlings were not inoculated (control, CK) or inoculated with *A. radialis* N35 wild type or the *araI::tet* mutant, respectively. Cultivation was performed monoxenically for 10 days (see M&M). Then one leave was taken in the three leaves stadium, RNA was isolated, and q-PCR was performed from transcribed cDNA. Statistical analysis was applied using one way ANNOVA. Same letter means no significant different, different letter means significant difference ($p < 0.05$). (A) Flavonoid biosynthesis pathway genes: 4-coumarate CoA ligase (4-CL), chalcone-flavonone isomerase (CFI), chalcone synthase (CHS), UDP-glycosyltransferase-like protein (UGT) (B) Fb-3, F-Box family-3 and E3 ubiquitin-protein ligase (e3ul) (C) Heat shock protein 90 and DnaJ (heat shock protein 40)

3.6 Flavonoid content measurement

The HPLC analysis of flavonoid contents revealed that in the leaves of the tested barley cultivar Barke the amount of saponarin was generally higher than of lutoarin. In plants inoculated with the *A. radialis* N35 *araI::tet* mutant, the contents of lutoarin and saponarin were increased more than twice as compared to plants inoculated with the wild type or un-inoculated controls (Fig. 3.7). Also lutoarin and saporanin methylether derivatives reached almost twice the levels in the *araI::tet* mutant as compared to wild type and un-inoculated control plants. These results corroborate the results of gene transcriptome profiling by RNA-seq and q-PCR, that the production of *N*-acylhomoserine lactones in *A. radialis* N35 plays an important role in controlling the flavonoid production in barley leaves during the process of colonization by *A. radialis*.

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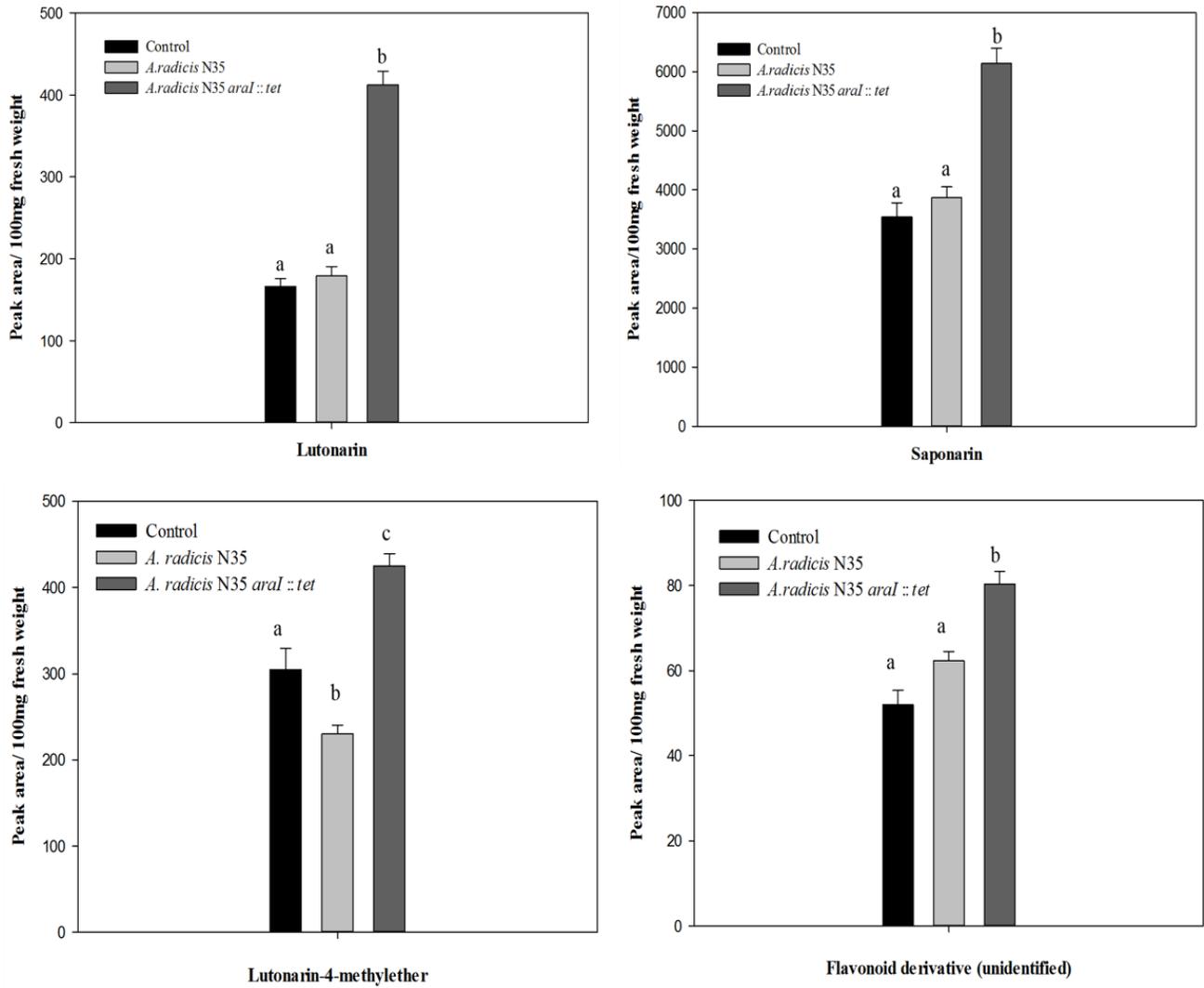


Fig. 3.7: Accumulation of flavonoids in barley leaves measured by HPLC. The flavonoid components are: (A) lutonarin, (B) saponarin, (C) lutonarin-4-methylester. (D) An unidentified flavonoid derivative. a: no significant difference, b: significant difference at level $p < 0.05$.

4 Discussion

4.1 The role of Quorum sensing in bacteria-plant interactions

4.1.1 Biofilm formation and root colonization ability determined by QS

The AHL-defective *A. radialis* mutant was found to be less successful in root colonization (Fig. 2), which indicates a role of QS for the colonization ability of this species. The QS-deficient *araI::tet* mutant strain showed colonization mostly by single cells spread randomly over the root surface, while the N35 wild type cells aggregated and formed biofilms at the root surface. This result corroborates the observation by Li (2010), who showed in a 1:1-mixture of GFP-labeled N35 wild type cells and the SYTO orange labeled *araI::tet* mutant that only a few mutant cells colonized the roots, while wild type cells showed dense colonization. In *A. radialis* N35, also phenotypic variants showed reduced root colonization. However, in contrast to *araI::tet* mutants, these variants had much reduced ability of plant growth promotion (Li et al. 2012). The reduced colonization of the *araI::tet* mutant could be caused by a reduced tolerance towards reactive oxygen species (ROS) released by barley roots upon first contact with microbes as has been found in the case of the endophyte *Gluconacetobacter diazotrophicus* during colonization of rice roots (Alquerque et al. 2013). In this case, ROS-quenching enzymes catalase and superoxide dismutase of the endophyte have a major role in the degradation of ROS released by the host plants during early host defense. In *P. aeruginosa*, QS was found to be involved in stress tolerance, and *luxI* type QS-deficient mutants (*lasI*, *rhlI*, and *lasR*, *rhlR*) have defective expression of catalase (CAT) and superoxide dismutase (SOD). These mutants were more sensitive to oxidative stress than the parental strain (Hassett et al. 1999). Therefore, the hypothesis is that in the *araI::tet* mutant of *A. radialis* N35, ROS-quenching enzymes were reduced or even lacking as compared to the wildtype. Another study showed that the QS based anti-oxidative tolerance may inhibit to quench quorum sensing activities and may contribute to prevent social cheating (Garcia-Contreras et al. 2015). In the co-inoculation experiment, the

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quorum sensing *araI::tet* mutant may behave even as a quorum sensing cheater, because it does not produce AHL.

The positive influence of AHL-mediated QS on biofilm formation was shown in several studies. For instance, in *P. fluorescence* 2p24, the *pcoI* coded AHL synthase mutant resulted in seriously decreased biofilm formation, leading to less root colonization ability (Wei and Zhang 2006). In *P. aeruginosa*, a quorum sensing *lasI* mutant formed flat undifferentiated biofilms which are more sensitive to the biocide sodium dodecyl sulfate than the wild type. These flat biofilm types of AHL-defective mutants could be restored by exogenous addition of AHLs (Davies et al. 1998). Also in *Sinorhizobium fredii* SMH12, micro-colony biofilm formation was found to be regulated by QS and in *Rhizobium* spp. the biofilm formation is dependent on the production of AHLs (Davies et al. 1998, Rinaudi and Giordano 2010, Perez-Montano et al. 2014). It could even be shown, that the exopolysaccharide production in *Sinorhizobium fredii* NGR234 was modified by AHL production (Krysciak et al. 2014). In the *Acidovorax* sp. strain MR-S7, exogenous addition of AHLs could promote biofilm formation (Kusada et al. 2014). The importance of QS for biofilm formation could be due to secretion of important compounds like extracellular DNA, the biosurfactant rhamnolipid and the secretion of the BapA protein as shown in *P. aeruginosa* (Tolker-Nielsen 2015). Furthermore, QS compounds play an important role in *Pseudomonas fluorescence* 2p24 for its colonization on wheat roots and development of biocontrol ability towards the take-all disease fungus (Wei and Zhang 2006). In *Burkholderia phytofirmans* PsJN, QS was also found to be important for its competitive biofilm formation and efficient colonization of *Arabidopsis thaliana* roots (Zuniga et al. 2013). Thus, there is an increasing knowledge about the important role of AHLs in plant beneficial rhizosphere bacteria and endophytes in diverse plant systems and their involvement in different mechanisms of plant growth promotion.

A colonization related plant gene (coding for the remorin protein) was found in transcriptome analysis of *Medicago truncatula* in response to inoculation with a QS mutant of *S. meliloti* (Lefebvre et al. 2010). Remorin protein is a membrane associated protein, which was found to interact with symbiotic receptors. It was shown to be specifically induced during root nodule symbiosis of *Medicago truncatula* and *Sinorhizobium meliloti*, possibly working as plant specific

scaffolding protein (Lefebvre et al. 2010). This gene may also be involved in *A. radialis* N35 colonization, because it was downregulated in barley by N35 *araI*::tet mutant inoculation (Table 3.6).

4.1.2 QS importance in plant growth promotion

According to our results, production of 3-OH-C10-HSL by *A. radialis* N35 is not a determinant factor of *A. radialis* N35 to promote barley growth, because both Wt and *araI*::tet mutant exhibit comparable PGPR activity, at least at the conditions tested (Fig.3.3). There was a similar conclusion in a study of *Azospirillum lipoferum*, since its AHL inactivation had no deleterious effect on the phytostimulation (Boyer et al. 2008a). However, in hydroponic axenic culture, addition of pure 3-oxo-C10-HSL could promote barley shoot length, root fresh weight and lateral roots formation (Götz-Rösch 2015). Furthermore, the addition of short carbon chain C6-HSL and C8-HSL caused alteration of the plant hormone auxin/cytokinin to promote roots elongation in *Arabidopsis* (von Rad et al. 2008, Liu et al. 2012). However, there is no information available if 3-OH-C10-HSL also shows the same influence on auxin regulation in barley or any interaction with other functions of PGPR, which were shown to be involved in promoting plant growth including N₂ fixation, phosphate solubilization, biocontrol activities and rhizosphere competence (Imran, A., 2014, Annals of Microbiology). Furthermore, *A. radialis* N35 is producing auxin itself which is a functional plant hormone supporting root development and plant growth under controlled conditions. Maybe its production is QS-independent. However, several QS independent activities may contribute to PGPR-phenotype.

4.2 Systemic transcription analysis of barley

The transcription analysis of barley plants towards inoculation with *A. radialis* N35 wt and *araI*::tet mutant revealed numerous interesting insights in the complex perception process of plants in response to the colonization by bacteria, in this case by a beneficial endophyte. According to the detailed colonization studies, 1 dpi and 10 dpi time points were selected, because they represent first association and advanced biofilm-like and endophytic colonization

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states, respectively. In general, at 10 dpi more and quite different plant responses were recorded during root colonization of Wt and *araI::tet* mutant.

In the 1dpi samples more plant genes were changed by inoculation with N35 *araI::tet* mutant than with the wild type. This may already indicate that the barley response to N35 *araI::tet* mutant was more pronounced than to the Wt producing AHL.

Several heat shock proteins were found to be upregulated in both Wt and N35 *araI::tet* mutant treatment. HSP90 and several small HSP are known to be important for plant resistance. The HSP90 is known to interact with SGT1 and RAR1 to form complexes with R-proteins to mediate the plant resistance to pathogens. For example in rice, the HSP90 binds with its co-chaperone Hop/sti1 leading to chitin response and anti-fungal immunity (Park and Seo 2015b). An HSP20 member is known to specifically interact with I-2, which confers resistance to *Fusarium oxysporum* by accumulation of I-2. Another HSP20 from *Nicotiana tabacum* (NtsHSP) was shown to be involved in disease resistance in plants. Disease symptoms caused by *Ralstonia solanacearum* are enhanced in NtsHSP-silenced plants (Park and Seo 2015a). This indicates that in both Wt and N35 *araI::tet* mutant, inoculation enhanced to some extent biotic and abiotic resistance in barley. In addition, another heat shock protein DnaJ, identical with heat shock protein 40, was shown to be upregulated only in N35 *araI::tet* mutant treatment together with flavonoid biosynthesis genes (Fig 6). It also plays a role in cell death and disease resistance in *Nicotiana benthamiana* leaves. HSP40 was demonstrated to function in plant immunity, as overexpression of HSP40 causes HR-like cell death and silencing of HSP40 enhances susceptibility to soybean mosaic virus in soybean (Liu and Whitham 2013).

Another plant physiological important gene, which was found induced after inoculation with both Wt and N35 *araI::tet* mutant is the pentatricopeptide repeat-containing protein (PPR). This gene was upregulated at 1dpi and 10 dpi by inoculation with the *araI::tet* mutant, but only upregulated at 1dpi with the Wt. This protein is involved in RNA-editing in plants and is important for general key plant physiological processes like photosynthesis, leaf development and pigmentation as well as response to abiotic stress (Barkan and Small 2014). Thus, increase of the expression of PPR-encoding genes is expected to improve the fitness and growth vigor of plants.

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Some genes were specifically expressed after inoculation with the *araI::tet* mutant. Flavonoid synthesis pathway genes were upregulated only after *araI::tet* mutant treatments, which included F3H, CHS, CHI, and UGT. The upregulation of all these genes was confirmed by Q-PCR. Another flavonoid synthesis related gene is 2-oxo-glutarate and Fe (II)-dependent oxygenase like protein (2-OG). 2-OGs include several enzymes: FNS1, (flavone synthase I); FLS, (flavonol synthase); LDOX, (leucoanthocyanidin synthase); F6H, (flavone-6-hydroxylase); ANS, (anthocyanidin synthase); FHT, (flavanone 3 beta-hydroxylase). These enzymes catalyze desaturation of naringenin and its derivate dehydrokaempferol as substrates or add hydroxyl group to form different flavonoids (Farrow and Facchini 2014).

MYB transcription factor (Stracke et al. 2007) and epigenetic regulation were generally found to be involved in systemic transcription control and especially in the flavonoid biosynthesis process (Sharma et al. 2016). In systemic transcriptom analysis, an epigenetic gene for ribonuclease III was upregulated in barley leaves at 10 dpi *araI::tet* mutant treatment. Ribonuclease III (dicer like protein, DIL) can cut double stranded RNA to form microRNA (miRNA) and siRNA. miRNAs have important regulation functions via posttranscription interference mechanism to regulate the leaves of gene transcripts. For instance, in *Arabidopsis* the antibacterial resistance is regulated through miR393 (Navarro et al. 2006). Moreover, miRNA control MYB transcription factors in *Arabidopsis* which contribute to promote flavonoid synthesis (Sharma et al. 2016). Thus, after inoculation with the *araI::tet* mutant, epigenetic regulation was affected.

Furthermore, the inoculation with the *araI::tet* mutant downregulated the expression of ubiquitin E3 ligase genes F-box protein 3 and E3 ubiquitin ligase PRT1, both belonging to the E3 ligase. The former one is a ring-finger E3 ligase, the latter one is playing an important role in the ubiquitination of proteins involved in the cell cycle and plant immunity. F-box protein contributes to the specificity of SCF target protein and mediates the SCF complex into proximity with functional E2 protein (Marino et al. 2012). PRT1 functions as an ubiquitin protein ligase in the heterologous host. In *Arabidopsis*, PRT1 functions encodes a 45 kD protein with two ring-finger domains and one ZZ domain, mediating the degradation of N-end rule substrates with aromatic termini such as F-dihydrofolate reductase instead of those with aliphatic or basic amino-termini (Stary et al. 2003).

4.3 Saponarin and lutanarin production

Compared with *A. radialis* N35 wild type, the colonization of roots by the 3-oxo-C10-HSL deficient *araI::tet* mutant caused an accumulation of saponarin and lutanarin in barley leaves (Fig. 6). This indicates that 3-oxo-C10-HSL itself or bacterial components induced by 3-oxo-C10-HSL are involved in the induction/expression of flavonoid biosynthesis in the host plant. A direct stimulatory effect of AHLs on the induction of flavonoid biosynthesis was found in *Medicago truncatula*. In this case, 3-oxo-C12-HSL was shown to activate the transcription of chalcone synthase genes in white clover roots (Mathesius et al. 2003). In the *A. radialis* N35 - barley interaction, a different AHL (3-OH-C10-HSL) is operating, which may have caused an inhibition of flavonoid biosynthesis. In *A. thaliana*, the influence of the length of the acyl chain and the substitution at the C3-atom were shown to cause different systemic responses (Schikora et al. 2011). The contrasting response of barley to 3-OH-C10 HSL may also be due to the fact that the monocotyledonous barley may respond differently to AHLs than the dicotyledonous white clover. On the other hand, since QS autoinducers are able to regulate bacterial surface exopolysaccharide production (Krysciak et al. 2014), the lack of AHLs in the *A. radialis araI::tet* mutant could also have resulted in considerable changes in the surface exopolysaccharide structure and this may have caused a different plant response.

Flavonoids can help plants to acquire resistance towards various biotic and abiotic stresses (Treutter 2005). The enhanced accumulation of the two flavonol glycosides saponarin and lutanarin in barley leaves caused by the colonization of the roots by the *A. radialis* N35 *araI::tet* mutant is an example for this kind of defense response. The expression of several flavonoid biosynthesis genes were upregulated due to inoculation with the *A. radialis* N35 *araI::tet* mutant (Fig.3.6A). This clearly indicated that the AHL-deficient mutant strain activated a defense response which was not activated in the 3-OH-C10-HSL producing N35 wild type. Three closely related R2R3-MYBs transcription factors (MYB11, MYB12 and MYB111) redundantly activate the transcription of early flavonoid biosynthesis genes (EBGs). The UDP-glycosyltransferases UGT91A1 and UGT84A1 together with CHS, CHI, and F3H, FLS1 are controlled by this R2R3MYB factors in *Arabidopsis* (Stracke et al. 2007). However, no data were obtained for the

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regulation at the transcriptional level. The flavonoid accumulation is not only regulated at the transcriptional, but also at the post-transcriptional level through PAL degradation mediated by Kelch domain containing F-box (KFB) complexes leading to the suppression of the phenylpropanoid pathway (Feder et al. 2015). In this study, it could be demonstrated by the transcriptomic sequencing results and confirmed by q-PCR, that the expression of F-box protein and E3 ubiquitin-protein ligase were downregulated in the presence of *A. radialis* N35 wild type and even more in the *araI::tet* mutant (Figure 3.6). F-box family proteins are components of the SCF-protein complex, which is involved in the proteome degradation pathway. This process is for example important for plant development and immunity response to various stress condition (Thines et al. 2007). In addition, also an upregulation of *dnaJ* expression after inoculation with the *araI::tet* mutant was shown to be mediated by transcription factor SG7 MYB. Its expression was found to correlate with flavonoid related genes and to be under the control of MYB transcription factors (Stracke et al. 2007). The upregulation of *dnaJ* expression also correlated with the upregulation of the flavonoid biosynthesis genes and flavonoid accumulation. DnaJ may also be involved in salt stress resistance and known to interact with HSP70 in the heat shock resistance process (Zhu et al. 1993). Since *dnaJ* expression was also found to be involved in regulation of saline tolerance, it is reasonable to test whether the higher expression of *dnaJ* will also result in increased salt stress tolerance by the plants.

As mentioned in the introduction flavonoids can be transported through the whole plant to help plant suffer biotic and abiotic stress. It is reasonable to test in further experiments whether the increased flavonoid levels are secreted outside of roots which inhibited the QS mutant growth and colonization because they are known as bactericides too.

4.4 Integrated role of AHLs by *A. radialis* in plant perception

Due to common evolutionary history of plants and microbes, an elaborate system of mutual detection, cooperation or deterrence has developed. In the first recognition step, the most important role plays the plant's innate immune system recognizing MAMPs and diverse microbial elicitors. On the microbial side the response to plant surface structures and exudates has a central role in recognition. The quorum sensing communication system of rhizobacteria

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based on AHL compounds may be considered as an integrated part in the invade perception process of bacteria by plants. In the plant growth beneficial endophyte *A. radialis* N35 3-OH-C10-HSL is the dominant AHL (Fekete et al. 2007). Many Gram-negative plant pathogenic rhizobacteria also synthesize AHLs, although with different chain lengths and other functional groups. Since the onset of virulence is regulated by these autoinducers, the plant needs to learn about the presence of AHLs in its vicinity as soon as possible. In the case of pathogens, the network of multiple interactions may conclude to initiate full expression of the defense cascade, while in the case of beneficial endophytes, which lack PAMP signals, the plants's defense response is dampened or completely suppressed allowing a cooperative interaction. There are several examples, that AHL compounds applied to rooting solutions of plants can exert diverse beneficial effects on plants, which include growth promotion as well as priming or induction of pathogen resistance in the host. This was shown in different plant species, such as *M. truncatula*, tomato, *Arabidopsis thaliana*, and barley (Mathesius et al. 2003, Schuegger et al. 2006; von Rad et al. 2008, Schenk et al. 2014, Hartmann and Schikora 2002, schikora et al. 2016). However, it is much less clear, what role AHLs of a beneficial root colonizing bacterium play in the concert of interaction with all the other compounds of plants' recognition and perception systems. In the current study, it could be shown, that the production of 3-OH-C10-HSL during the colonization process by the plant growth promoting, endophytic *A. radialis* N35 is able to efficiently influence the plant response and reduce or even prevent the onset of a defense cascade. Whether this is caused by a direct interaction of AHLs with plants or by an indirect effect through the induction of e.g. different surface structures of the bacteria in the presence of AHLs, which are not recognized as a pathogenic signal, is not known yet. Nevertheless, the response of barley plants to *A. radialis* N35 wild type is characterized by the absence of expression of several genes involved in flavonoid biosynthesis in the plant possibly leading to less or even no defense response. Even a priming effect for a defense response was deleted. Apparently, the 3-OH-C10-HSL production is playing a major role in this process. Future detailed studies need to focus on the role of the quorum sensing compound 3-OH-C10-HSL in the regulation of gene expression influencing fine structure modification of the cell surface lipo- and exopolysaccharides or of type III secretion systems or other transport systems potentially involved in *Acidovorax radialis* N35 and other PGPR interactions. As a first step in this direction, a study has been published recently

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showing that type III secretion system does not play an effective role in the ISR response caused by PGPR *P. fluorescence* 2p24 (Liu et al. 2015).

5 Summary and general outlook

In this study, barley and root endophytic *A. radialis* N35 were used as interaction model to investigate the role of AHL-mediated QS in root associated bacterial colonization, plant growth promotion, and plant systemic response. Results pointed out that the biofilm formation was only observed in *A. radialis* N35 wild type while the QS mutant was randomly scattered on the root surface. However, the plant growth promotion property was not attenuated in the QS mutant. QS dependent and QS independent plant transcripts related to the systemic response were identified using RNA-seq. HSP90 is a conserved protein which is involved in the systemic response to Wt and QS mutant, while the higher flavonoid components were only found when plants were inoculated with the QS-deficient *A. radialis* N35 *araI::tet* mutant. This different response pattern may be attributed to AHL-controlled surface components of the mutant bacteria or / and to direct AHL effects on the plants.

Further studies need to focus on a detailed comparative transcriptome study of *A. radialis* N35 wild type and *araI::tet* mutant in culture as well as in the association with roots. Furthermore comparison of the response of barley to a pathogen with the response to a PGPR like *A. radialis* N35 and its *araI::tet* mutant may help to further elucidate details of bacteria-plant interactions.

6 Zusammenfassung:

Acidovorax radialis N35 ist ein endophytisches Bakterium, das die Entwicklung von Weizen und Gerstenpflanzen optimiert. Die Wahrnehmung der Pflanzen konnte durch RNA seq, qPCR und ausgewählten Metabolitanalysen charakterisiert werden. Es zeigte sich, dass Gerstensämlinge spontan auf die Bakterienbesiedlung durch eine Umprogrammierung der Genexpression und Priming von Verteidigungsreaktionen reagieren. Insbesondere sollte die Rolle der Quorum-Sensing Auto-Induktoren vom N-Acyl-Homoserin-Lacton (AHL) -Typ bei der Pflanzenreaktion analysiert werden. *A. radialis* N35 produziert 3-Hydroxy-C10-homoserinlacton (3-OH-C10-HSL) als Haupt-AHL-Signalsubstanz. In dieser Arbeit wurde der Einfluss von 3-OH-C10-HSL, das *A. radialis* N35 produziert, auf Gerstensämlinge durch Vergleich zwischen Wildtyp und einer *araI*-Insertionsmutante untersucht, die keine AHL-Produktion zeigte. Der Vergleich der Inokulationseffekte zwischen *A. radialis* N35 Wildtyp und *araI::tet* mutante ergab bemerkenswerte Unterschiede: Während Pflanzen durch den N35 Wildtyp in biofilmähnlichen Strukturen besiedelt wurden, trat die *araI::tet* mutante an der Wurzeloberfläche als einzelne Zellen auf. Außerdem überwog der Wildtyp bei der Besiedlung nach einer gemischten Inokulation des Wildtyps und der *araI::tet* mutante. Trotzdem konnte ein deutlicher Pflanzenwachstumsförderungseffekt 2 Monate nach der Inokulation von Gerste mit dem Wildtyp und der *araI::tet* mutante im Boden nachgewiesen werden. Der *A. radialis* N35 Wildtyp zeigte weniger Induktion von frühen Abwehrreaktionen in der Pflanzen-RNA-Expressionsanalyse, allerdings verursachte die *araI::tet* mutante z.B. erhöhte Expression von Flavonoid-Biosynthesegenen, was durch die Akkumulation von mehreren Flavonoidverbindungen wie Saponarin und Lutonarin in Blättern von wurzelinokulierten Gerstensämlingen bestätigt wurde. So lässt sich schließen, dass die Synthese von 3-OH-C10-HSL durch *A. radialis* Auswirkungen auf die Kolonisierungseffizienz von Pflanzenwurzeln und die Wahrnehmung durch die Wirtspflanze hat.

Abstract:

Acidovorax radialis N35 is a plant growth promoting endophytic bacterium in wheat and barley. The perception by plants can be characterized using RNAseq, q-PCR and selected metabolite analyses. It could be shown, that barley seedlings are quickly responding to bacterial colonization by a reprogramming of gene expression and priming of defense responses. Especially, the role of quorum sensing auto-inducers of the N-acyl homoserine lactone (AHL) type in the perception by plants should be analyzed. *A. radialis* N35 produces 3-hydroxy-C10-homoserine lactone (3-OH-C10-HSL) as major AHL-compound. In this communication the influence of *A. radialis* N35-produced 3-OH-C10-HSL on barley seedlings was investigated by comparing wild type and an *araI* insertion mutant, lacking AHL-production. The comparison of inoculation effects of the *A. radialis* N35 wild type and the *araI::tet* mutant discovered remarkable differences. While the N35 wild type colonized plant roots effectively by forming biofilm-like structures on the root surface, the *araI::tet* mutant occurred at the root surface as single cells. In addition, in a mixed inoculum of wild type and *araI::tet* mutant, the wild type was predominant in colonization compared to the *araI::tet* mutant. Nevertheless, a significant plant growth promotion effect could be shown after inoculation of barley with the wild type and the *araI::tet* mutant in soil after 2 months. *A. radialis* N35 wild type showed less induction of early defense responses in plant RNA-expression analysis, whereas the *araI::tet* mutant caused e.g. increased expression of flavonoid biosynthesis genes, which was corroborated by the accumulation of several flavonoid compounds like saponarin and lutanarin in leaves of root inoculated barley seedlings. Thus, it can be concluded, that the synthesis of 3-OH-C10-HSL by *A. radialis* has implications on the colonization efficiency of plant roots and the perception by the host plant barley.

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8 Supplementary

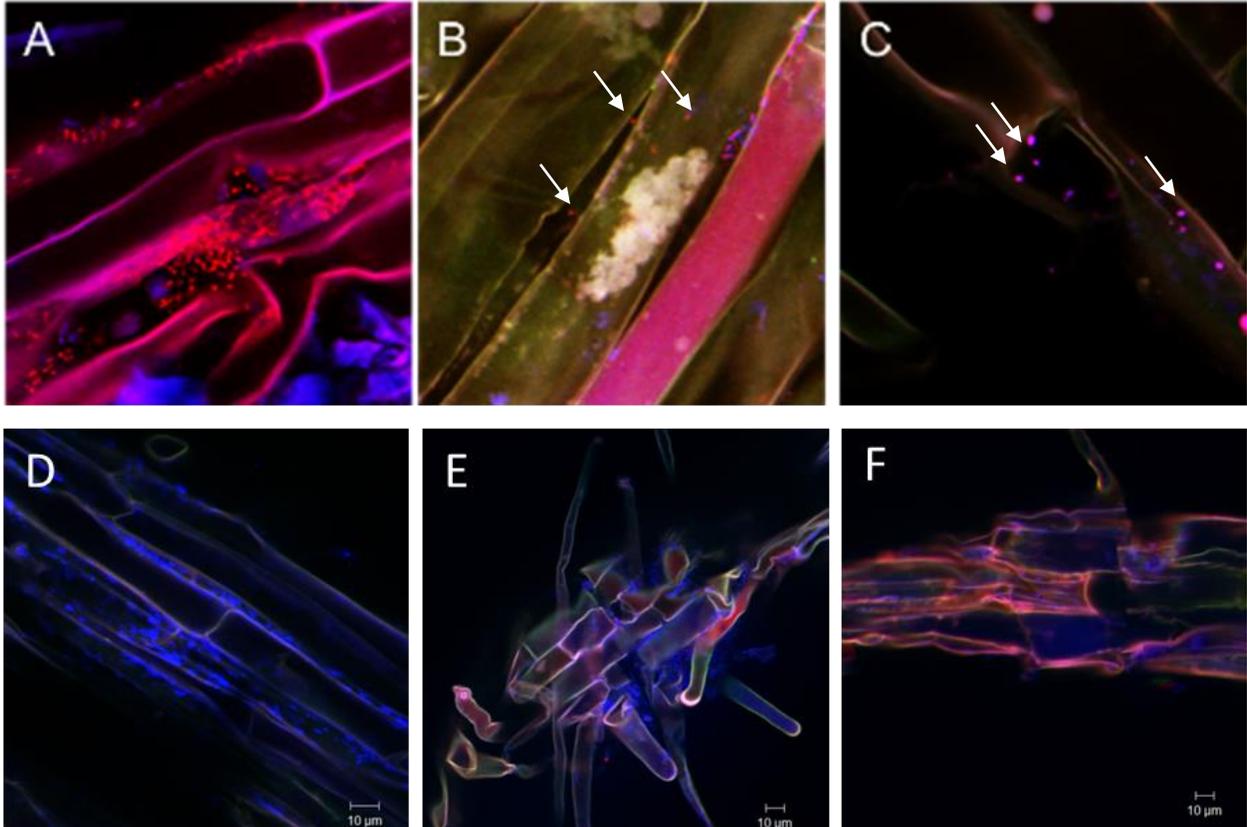
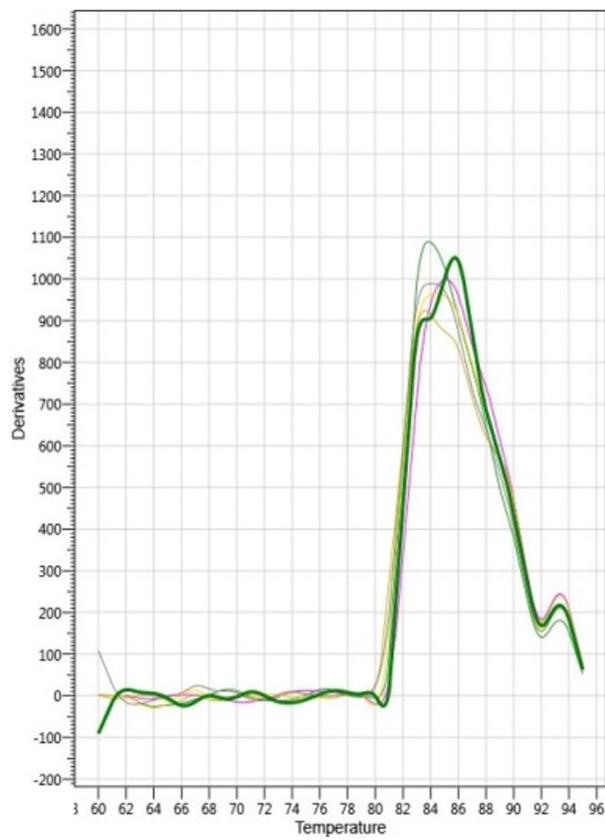


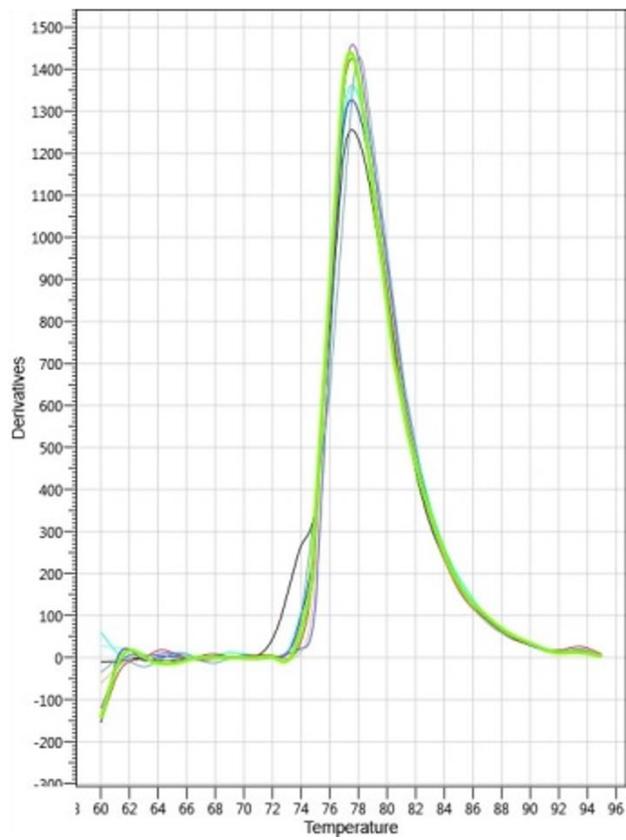
Fig S1: *A. radialis* N35 and *A. radialis aral::tet* mutant colonization of barley roots in soil for 2 weeks and 2 months detected using FISH method. *A. radialis* N35 is always shown in purple. In the top row (2 weeks samples) All bacteria can be detected by EUB 338 Cy3 probe which are depicted in red. *Acidovorax radialis* N35 detected by ACISP145 Cy5 which are depicted in blue. *Acidovorax radialis* n35 can be detected by both EUB and ACISP145 probe which are depicted in purple. In the bottom row (2 months samples) *Acidovorax radialis* N35 can be detected by both EUB338 Cy5 which is depicted in blue and also by ACISP Cy3 which is depicted in red so it was shown in purple color. . A, D: Control; B, E: *A. radialis* N35; C, F: *A. radialis aral::tet* mutant. The white arrow are labeled *A. radialis* N35 cells.

Supplementary

Q1

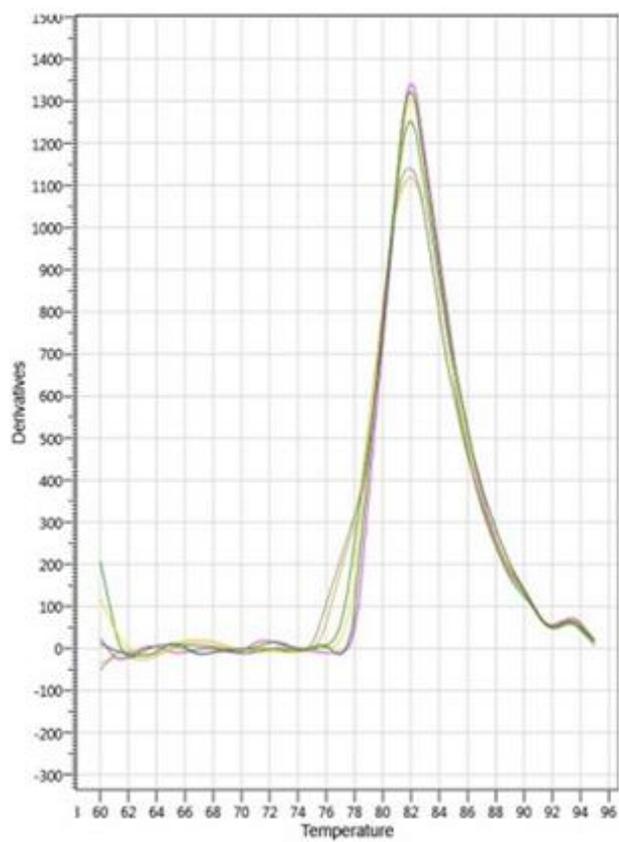


Q13

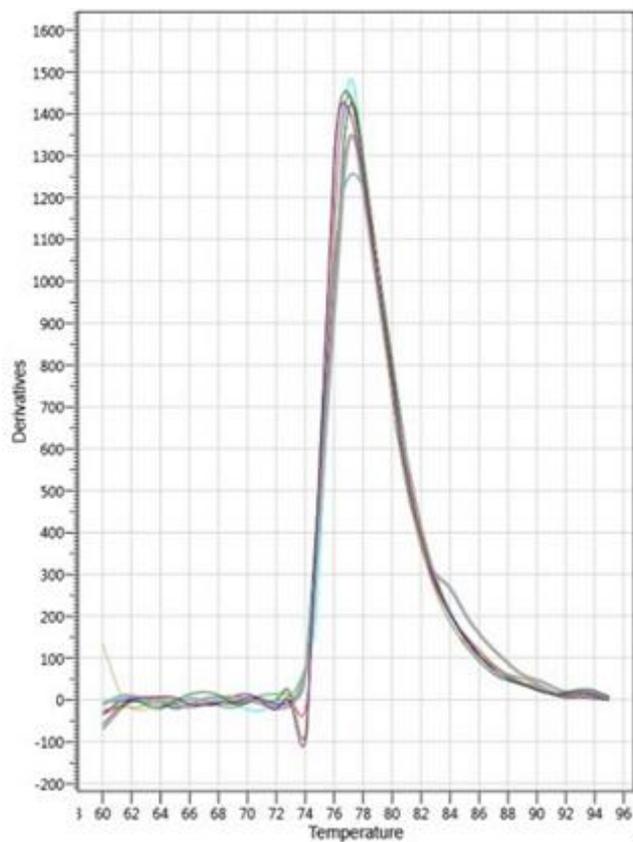


Supplementary

Q20

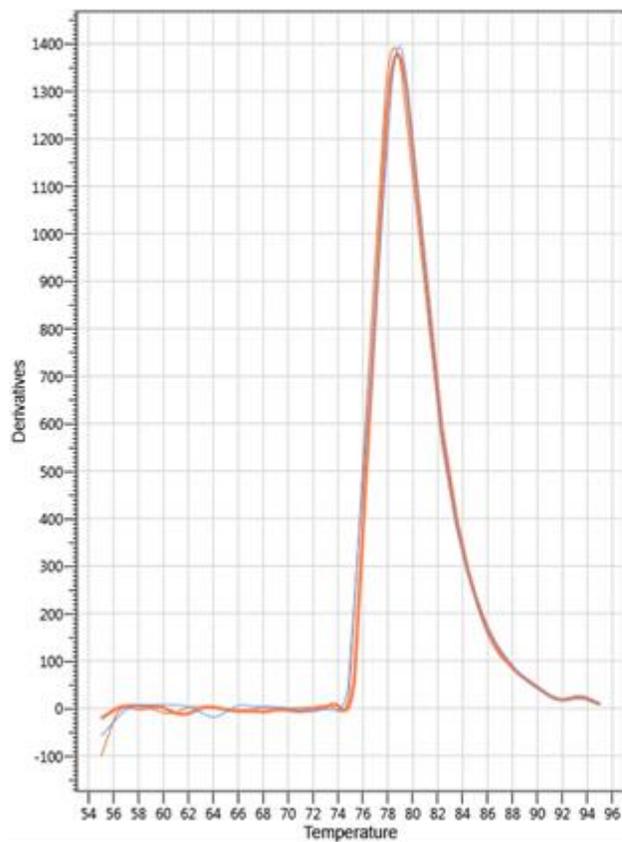


Q24

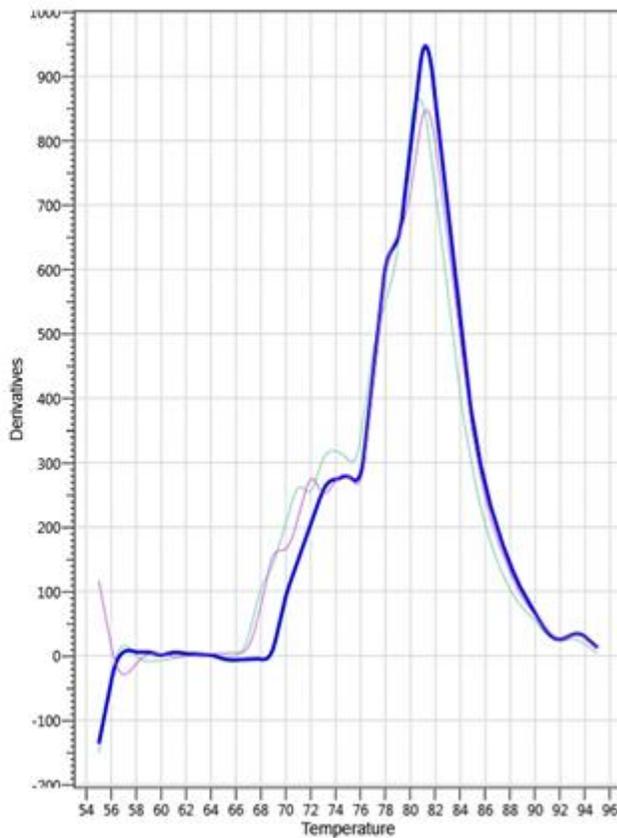


Supplementary

Q29



Q30



Supplementary

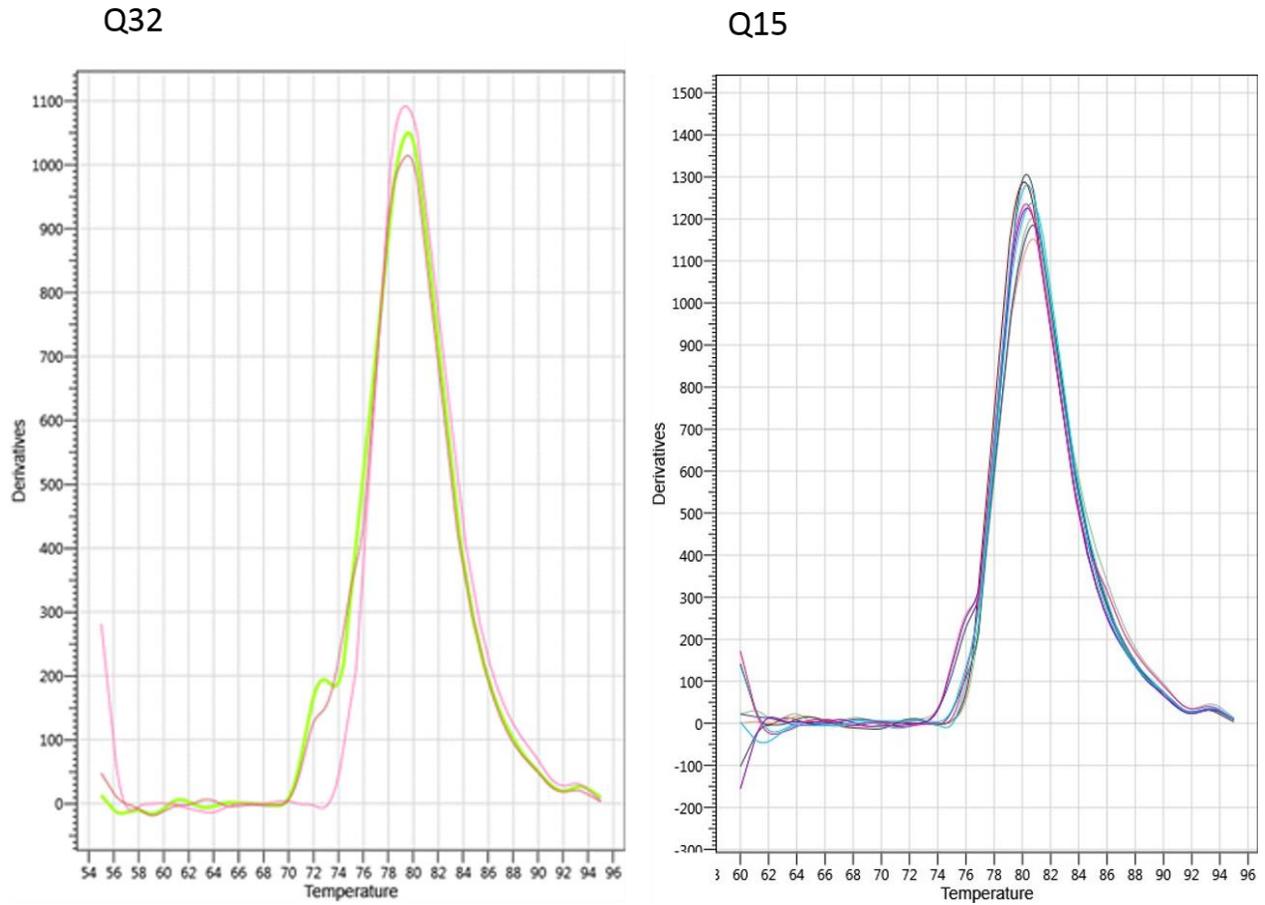


Fig S2: RT-qPCR primer melting curves. Q1: F-Box family 3, Q13: E3 ubiquitin-protein ligase PRT1, Q15: Heat shock protein 90, Q20: UDP-glycosyltransferase-like protein, Q24: Chaperone protein dnaJ common, Q29: 4-Coumarate: CoA ligase, Q30: chalcone-flavonoid isomerase, Q32: chalcone synthase.

9 List of publications:

Shengcai Han, Dan Li, Michael Rothballer, Michael Schmid, and Anton Hartmann. 2017.

Acidovorax radidis: A plant growth promoting endophytic bacterium in wheat and barley roots.

Chapter 10 in *Acidovorax*, Editor: Saul Burdman, Publisher: Wiley (in press).

Shengcai Han, Dan Li, Eva Trost, Corina Volt, Michael Schmid, Anton Hartmann, Michael

Rothballer. 2016. Systemic responses of barley seedlings to quorum sensing compound N-acyl-

homoserine lactone producing *Acidovorax radidis* N35. *Frontiers in Plant Science*. 7:1868.

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Eidesstattliche Erklärung

Eidesstattliche Erklärung:

Hiermit versichere ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und erlaubten Hilfsmittel verwendet, sowie Zitate kenntlich gemacht habe.

München,

(Shengcai Han)