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Colonisation and T3SS-Mediated Protein Secretion of *Pseudomonas fluorescens* etHAn on Roots of *Arabidopsis thaliana* Columbia-0

Master Thesis

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Colonisation and T3SS-Mediated Protein Secretion of *Pseudomonas fluorescens* etHAn on Roots of *Arabidopsis thaliana* Columbia-0

Master Thesis

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Thesis Abstract

This thesis provides a comprehensive investigation of the plant growth-promoting rhizobacterium *Pseudomonas fluorescens* etHAn on the roots of *Arabidopsis thaliana* Columbia-0. The study focuses on root colonisation patterns and protein secretion through the type 3 secretion system (T3SS). Employing a combination of plant science and microbial techniques, *P. fluorescens* etHAn, genetically modified to express GFP with a T3SS secretion signal, was applied to *A. thaliana* roots. Microscopic techniques were predominantly utilised to analyse both root colonisation and protein secretion.

The findings indicate a preference *for P. fluorescens* colonisation in the upper region of the root, with diminishing bacterial numbers in the root apical region. However, protein secretion via the T3SS into *A. thaliana* roots could not be confirmed. The implications of these results are discussed and compared to the results of fellow students of the iGEM competition 2023.

The methods employed in this study provide a valuable framework for future experiments aiming to deepen our understanding of the interaction between *P. fluorescens* and plant roots. This work contributes to the broader field of plant-microbe interactions, paving the way for further exploration and applications in sustainable agriculture.

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List of Abbreviations

Abbreviation	Full Name	Explanation
P. fluorescens	*Pseudomonas fluorescens*	Plant growth promoting bacterium, native to the rhizosphere of many plant species (Hol et al., 2013a).
P. syringae	*Pseudomonas syringae*	Plant pathogenic bacterium with a T3SS (Thomas et al., 2009a).
E. coli	*Escherichia coli*	Gram-negative gut bacterium. Commonly used for genetic work.
etHAn	Effector-to-Host-Analyzer	*P. fluorescens* strain with a functional T3SS (Thomas et al., 2009a).
SBW25	Sugar Beet Wytham, isolate 25	*P. fluorescens* strain that is commonly used in scientific publications.
T3SS	Type-3-Secretion System	Bacterial nano-syringe, structurally related to bacterial flagella but with the function of protein-secretion (Blocker et al., 2008).
GFP	Green Fluorescent Protein	Protein from *Aequorea victoria* that exhibits fluorescence when excited at λ=488 nm (Johnson & Shimomura, 1978).
RFP	Red Fluorescent Protein	Firstly introduced as "DsRed", RFP is a Protein from *Discosoma* that exhibits fluorescence when excited at λ= 558 nm (Gross et al., 2000).
FRUIT	Fluorescence Root-exudate Unbiased In-situ Test	A new fluorescence microscopy-based procedure for the measurement of inducible expression systems on solid surfaces.
SCC	Single Cell Colony	
hrp	hypersensitive response and pathogenicity	*hrp* genes encode for the outer membrane component of the T3SS
HDM	*hrp*-derepressing medium	Activates the gene expression of T3SS related genes (e.g. *hrp)*
LSF	Late Spring Frost	Frost events occurring after germination. Definition from:Lamichhane, (2021)
SAM	Shoot Apical Meristem	Upper part of the shoot in plants.
iGEM	international Genetically Engineered Machine	Global synthetic biology competition.

1 Introduction

This master thesis is an integral component of a broader project, the iGEM competition. iGEM is a global synthetic biology competition in which students from all over the world can contribute to solving a major problem using the tools of synthetic biology. Therefore, a brief introduction to this year's Wageningen iGEM project is given before placing it in the context of the master thesis.

1.1 PseuPomona: iGEM Project 2023, Wageningen

Climate change is one of the most serious environmental threats the world currently faces, manifesting itself in shifts in temperature, precipitation, wind patterns and extreme weather events. Among others, climate change has also consequences for the flowering time of many fruit trees. A prime example of how anthropogenic influences change the flowering time of cherry trees has been observed in Kyoto, Japan (Christidis et al., 2022). Since the beginning of industrialisation, blossom continuously started earlier, which is projected to get even more severe in the future (see Figure 1). This trend of early flowering can be explained by warmer temperatures in spring which again triggers early blooming in cherry trees (Pfleiderer et al., 2019). Nevertheless, late-spring frost still occurs in times of global warming, resulting in an overall increased susceptibility to frost damage on early-blooming fruit trees (Pfleiderer et al., 2019). (Pfleiderer et al., 2019).

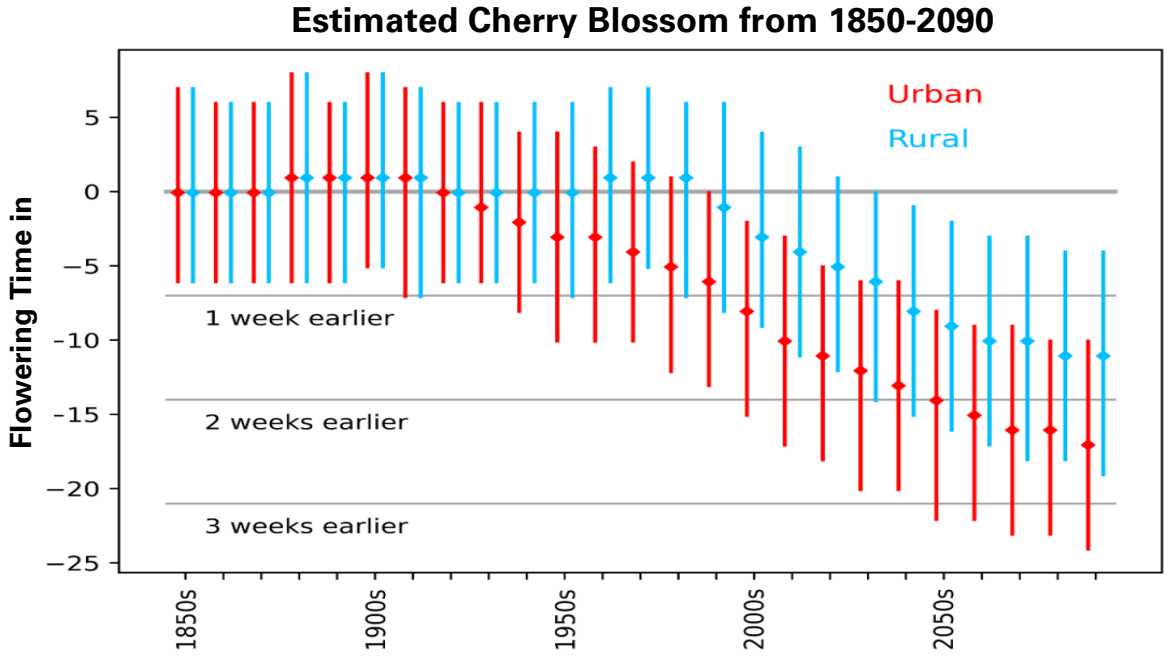

Figure 1: Observed and predicted change in flowering time in cherry trees from 1850 until 2090. *Anthropogenic influences change the blooming time of cherry trees in Kyoto, Japan. Figure adapted from: Christidis et al. (2022)*

A Nature article from 2021 stated that late spring frost (LSF) causes more economic losses to the North American and European agriculture than any other climate-related hazards (Lamichhane, 2021). A single LSF event in Europe in 2017 caused economic losses of approximately 3.3 billion euros (Faust and Herbold, 2018). This is not only devastating for the farmers but, if it occurs more often, could destabilize the agricultural sector and is therefore of everyone's concern. Current solutions include spraying trees with water, covering orchards with foil, or heating the air around the trees with heat cannons or fire pits. From interviews with diverse farmers in Wageningen, the iGEM team concluded that these options have many drawbacks. They are very expensive, not environmentally friendly, and labour-intensive, making farmers seek other solutions.

The aim of PseuPomona, the Wageningen iGEM project 2023, is to fight the negative impact climate change has on the agricultural sector. The project wants to collaborate with farmers to create a sustainable solution using the tools of synthetic biology. To this end, a bacterium was genetically engineered to inhibit flowering up to a point where no late-spring frost is to be expected anymore.

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*"This year, **3-4 days** of flowering delay would have **saved 30 %** of the total harvest."*

Farmer Johan

May 2023, from an interview with PseuPomona.

1.2 Overview

The aim of the iGEM project 2023 is to restore the flowering time to what it was before global warming. To this end, a peptide-based phytohormone (further referred to as antiflorigen) is produced in *Pseudomonas fluorescens*, secreted into the plant tissue and delivered from the roots to the shoot apical meristem (SAM).

Most aspects of antiflorigen production, secretion, and transport in *P. fluorescens*, as well as mechanisms of biosafety were explored by the PseuPomona team.

This thesis explores the bacterial colonisation on plant roots and the protein delivery from the bacterium to and within the plant. This work is crucial in the context of the PseuPomona project to investigate whether the genetically engineered bacterium can live and colonise the roots of fruit trees and deliver antiflorigens into the trees. An overview of the PseuPomona project is given in Figure 2, the subproject that is investigated in this master thesis is highlighted in the red box.

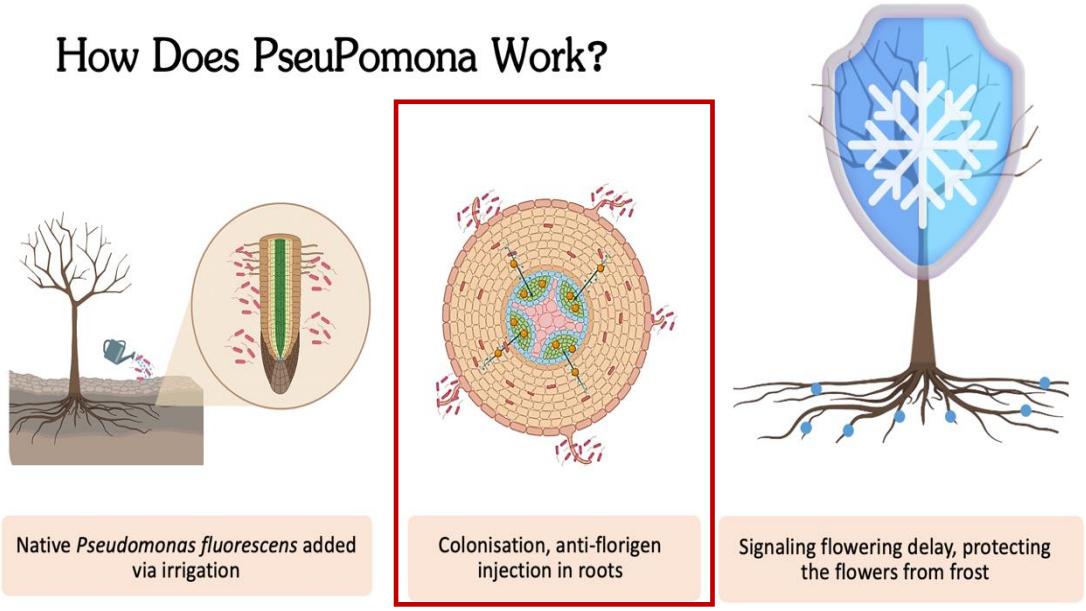

Figure 2: Schematic overview of the Wageningen iGEM project 2023, PseuPomona. *The three main points of the PseuPomona project are (1) Application of the* P. fluorescens, (2) *colonisation of* P. fluorescens *and protein-injection into the plant and (3) phenotypical effect on the plant. The work of this master thesis is highlighted in the red box. General remark: unless otherwise specified, all figures are self-made/ self drawn.*

1.3 Phytohormones & Intercellular Transport of Phytohormones

Since the discovery of photoperiodism in plants and its role in flowering in the early 1920 by Garner and Allard, much effort has been made to understand the principles of flowering and its control mechanisms (Allard & Garner, 1920). Like animals, plants use hormones to regulate the development of their sex organs (Khryanin, 2002). Traditionally, these phytohormones fall mainly under the category of small chemical molecules (Ethylene, Auxin etc.). However, peptide-based phytohormones gained increasing attention (Zhao et al., 2021). Major regulators of the flower-development are the *FLOWERING LOCUS T* (FT) and the floral repressor *FLOWERING LOCUS C* (FLC) (Luo et al., 2019). FT is not only produced in the flower itself but also in the companion cells of the leaves (McGarry & Kragler, 2013). To induce flowering, FT is transported from the leaves through the phloem to the shoot apical meristem (SAM) (McGarry & Kragler, 2013).

In consequence, it is demonstrated that peptide-based phytohormones can travel as mobile elements through the phloem to distinct areas of the plant.

In animals, the transport of substances is facilitated via transport tissue like veins, arteries, or the lymph system. In higher plants, bulk transport is mediated by vascular tissues, namely the xylem and the phloem. The transport of nutrients, genetic material and peptides occurs in the phloem (Aoki et al., 2005). Specialised companion cells are in direct contact with the phloem and can load and unload molecules from it (Imlau et al., 1999). Intercellular transport of nutrients and other molecules from one to the other cell is mediated by the cell walls (apoplastic transport) or occurs symplastic, thus intracellular by plasmodesmata (PD, see Figure 3) PD are cytoplasmic channels that connect the endoplasmic reticulum and cytoplasm of adjacent cells (Cilia & Jackson, 2004).

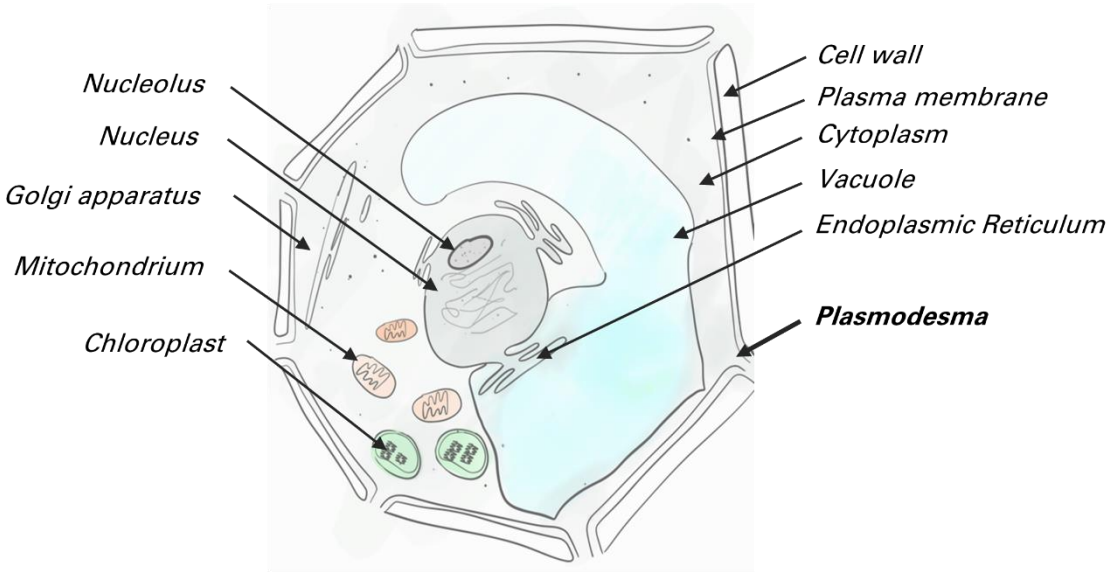

Figure 3: Simplified drawing of plant cell. *Main organelles are depicted. Plasmodesmata (sing. Plasmodesma) connect adjacent cells, Nucleolus: Subnuclear organelle where rRNA synthesis takes place. Nucleus: Stores DNA. Golgi Apparatus: Storage and Modification of Proteins. Mitochondria: ATP-synthesis. Cell wall: Rigid barrier of the cell. Plasma membrane: Flexible lipid barrier of the cell. Vacuole: Storage and Turgor. Endoplasmic Reticulum: Protein and lipid storage. Chloroplast: Site of Photosynthesis.*

Experiments with small fluorescent dyes revealed that free diffusion of molecules between cells is only possible with molecules smaller than 1 kDa (Jackson, 2000). However, in some cases, the size exclusion limit (SEL) for PD can be much larger (Cilia & Jackson, 2004). Plant pathogenic viruses have so-called movement proteins that modify the SEL to allow the transport of entire viral genomes from one to the other cell (Cilia & Jackson, 2004). Further, the green fluorescent protein (GFP, 28kDA) can traffic freely from one to the other cell in specific tissues like petals and root tips (Cilia & Jackson, 2004). Finally, the SEL of companion cells allows the unloading of proteins as big as 67 kDA into the sieve tissue (Stadler et al., 2005).

1.4 *Pseudomonas fluorescens* – Chances and Limitations

Unseen by most, bacteria and other microbes live in close symbiotic relationships with many plants to exchange nutrients, toxins, and hormones. Whereas some bacteria such as *P. syringae* have pathogenic interactions, other microbes live in cooperative communities with plants. The gram-negative bacterium *P. fluorescens* is a cooperative, plant-growth-promoting rhizobacterium (Lugtenberg & Kamilova, 2009). Though the exact mechanisms of how *P. fluorescens* promote plant health are still not fully known, research was carried out to understand the physiology of *P. fluorescens* and its microbe-plant interaction (Silby et al., 2009). Dr Gera Hol from the University of Wageningen states that not only direct interaction between bacteria and the plant but specifically interactions between *P. fluorescens* and other rhizospheric bacteria lead to the plant-growth-promoting effects of *P. fluorescens* (Hol et al., 2013b). She reviews that collaborative relationships between *P. fluorescens* and other plant mutualists are frequently documented while the secretion of secondary metabolites from *P.*

fluorescens was shown to suppress the growth of plant-pathogenic bacteria (Hol et al., 2013b).

A special, and highly interesting feature of *P. fluorescens* is its type-3-secretion system (T3SS) with which it can inject proteins into the plant cells (Notti & Stebbins, 2016). This characteristic makes it an ideal vector for the delivery of heterologous proteins into plant tissue. *P. fluorescens* has garnered significant attention due to its dual attributes of promoting plant growth and possessing the T3SS. Despite its importance, common protocols for genetic modification such as heat shock, electroporation and conjugation protocols are not yet optimised for *P. fluorescens* (García, 2022). Dedicated efforts have been made by researchers like Dr. Enrique García from the University of Wageningen to advance the development of genetic tools, to the end of enabling the presented work (García, 2022).

Among all *P. fluorescens* strains, the strain SBW 25 belongs to the best-studied one (García, 2022). However, to activate the T3SS in *P. fluorescens* SBW25, genetic modifications involving the overexpression of the *hrpL*-gene, an alternative sigma factor, and other genetic regulators are necessary (Preston et al., 2001).

A continuously active T3SS can be established by introducing the "pathogenicity island," a 26-kilobase DNA fragment containing over 20 T3SS-related genes from *Pseudomonas syringae*, into the genome of *Pseudomonas fluorescens* (Thomas et al., 2009b). Using recombineering, Thomas et al. (2009) performed this work and created the "Effector to Host Analyzer" strain, denoted as *P. fluorescens* etHAn. This strain allows secretion studies in *P. fluorescens* by adding a T3SS-specific secretion signal to the gene of interest. It is an ideal organism to investigate the effect of toxic “effector proteins” on plants, as the strain itself neither elicits a hypersensitive response in plants nor interferes with the secreted proteins (Thomas et al., 2009b). Many researchers were able to study the effect of secreted effector proteins on plants using *P. fluorescens* etHAn (Jensen et al., 2022a; Thomas et al., 2009b; Upadhyaya et al., 2014).

Taking these findings together, *P. fluorescens* etHAn is a promising candidate for the secretion of GFP into plants and was therefore used for the here described work.

1.5 The Type-3-secretion System

The Type-3-Secretion System (T3SS) is a nano-scale machinery embedded in the membranes of various Gram-negative bacteria, including species of *Pseudomonas* and *Salmonella*. Once these bacteria come into contact with host cells, the syringe-shaped T3SS serves as a conduit for delivering specific proteins from the bacterial cytoplasm to the cytoplasm of a target cell (Puhar & Sansonetti, 2014).

The T3SS is present in organisms that engage in both commensal and pathogenic interactions with their host, as noted by Preston in 2007. The duality of interactions is well exemplified by the human gut microbiome, where both pathogenic Salmonella strains and mutualistic *E. coli* strains have been observed to express T3SS genes (Preston, 2007).

The expression of T3SS-related genes is considered energy-consuming and therefore tightly regulated (He et al., 2004). For soilborne *Pseudomonas* species, a nutrient-rich medium with high pH and complex carbon and nitrogen sources represses, whereas a minimal medium of lower pH and simple carbon sources like fructose or sucrose induces the expression of T3SS genes (Tang et al., 2006; Upadhyaya et al., 2014). Further, Kim et al. (2009) showed that the addition of iron oxalate can additionally enhance the expression of genes in the pathogenicity island. Additionally, there are indications that root exudates, in particular fatty acids, are implicated in the regulation of T3SS genes, as reported by Tang et al. (2006).

Once expressed, around 25 structural and ancillary proteins of the T3SS are relocated to the plasma membrane and assemble themselves in a hierarchical order to the functional nano-syringe. Proteins are secreted through the 25-Å channel – enough space for proteins in their unfolded, but not tertiary, globular conformation (Blocker et al., 2008). Therefore, specific chaperons can bind to proteins with the T3SS secretion signal, facilitating the unfolding process and allowing passage through the T3SS (Puhar & Sansonetti, 2014). The T3SS secretion signal plays a pivotal role in the recognition of the protein by chaperons and, in contrast to other bacterial secretion signals, is not cleaved during the secretion process (He et al., 2004).

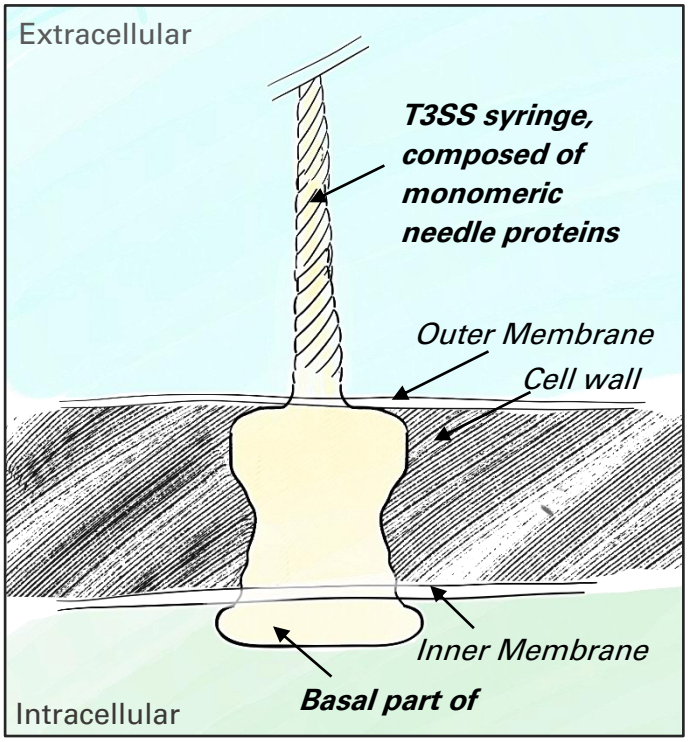

Figure 4: Schematic overview of the Type-3-secretion system. *Scheme inspired by electron microscopy pictures from Blocker et al. (2008).*

In the context of this thesis, it is presumed that *P. fluorescens* etHAn will express T3SS-related genes in a minimal medium and the presence of root exudates of *A. thaliana*. Subsequently, GFP with a T3SS secretion signal will be recognised by T3SS chaperons, unfolded, and secreted through the T3SS into the epidermic cells of *A. thaliana*.

1.6 Aim

The aim of this master thesis is to carry out a comprehensive study of the colonisation behaviour of *Pseudomonas fluorescens* etHAn on living root cells and its ability to secrete GFP with a T3SS secretion signal into *A. thaliana* Col-0. It combines techniques from plant experiments with microbial protocols and experiments. Interdisciplinary plant-microbe protocols were extrapolated to explore how living *A. thaliana* roots can induce responses in bacteria that are sensitive to root signals.

To this end, *P. fluorescens* etHAn was transformed with the vector pSEVA64 to express superfolder green fluorescent protein (further referred to as GFP) linked to a T3SS secretion signal from the AvrRPM1 protein of *P. syringae* under the control of the constitutive promoter J23100 and the Ribosome binding site RBS0034. Both, the used promoter and ribosome binding site were proven strong in *Pseudomonas putida* as described in Damalas et al. (2020). In former experiments, proteins coupled to the AvrRPM1-secretion signal were secreted into plants via the T3SS in *P. fluorescens* etHAn (Upadhyaya et al., 2014). In consequence, it was expected that secretion of GFP into the cells of plant roots is possible.

It was presumed that *P. fluorescens* etHAn expresses T3SS-related genes in a minimal medium and the presence of root exudates of A. thaliana (Tang et al., 2006; Upadhyaya et al., 2014). Subsequently, GFP with a T3SS secretion signal is recognised by T3SS chaperons, unfolded, and secreted through the T3SS into the epidermic cells of *A. thaliana* (Puhar & Sansonetti, 2014).

Once secreted into the epidermis, plasmodesmata and the phloem facilitate the systemic transport of proteins (Cilia & Jackson, 2004; Stadler et al., 2005). Therefore, it was expected that GFP is transported through the PD from the root epidermis to the companion cells. Once in the companion cells, GFP can be loaded into the phloem and distributed within the plant.

Additionally, in collaboration with iGEM teammate Nico Maarten van Donk, protocols for microscopic studies of bacteria on living plants were applied to investigate the interaction of root exudate-sensitive transformants to living roots of *A. thaliana*. This additional experiment will be further referred to as "FRUIT", an acronym for fluorescence root exudate unbiased *in-situ* test.

An overview of the thesis work is illustrated in Figure 5 on the next page.

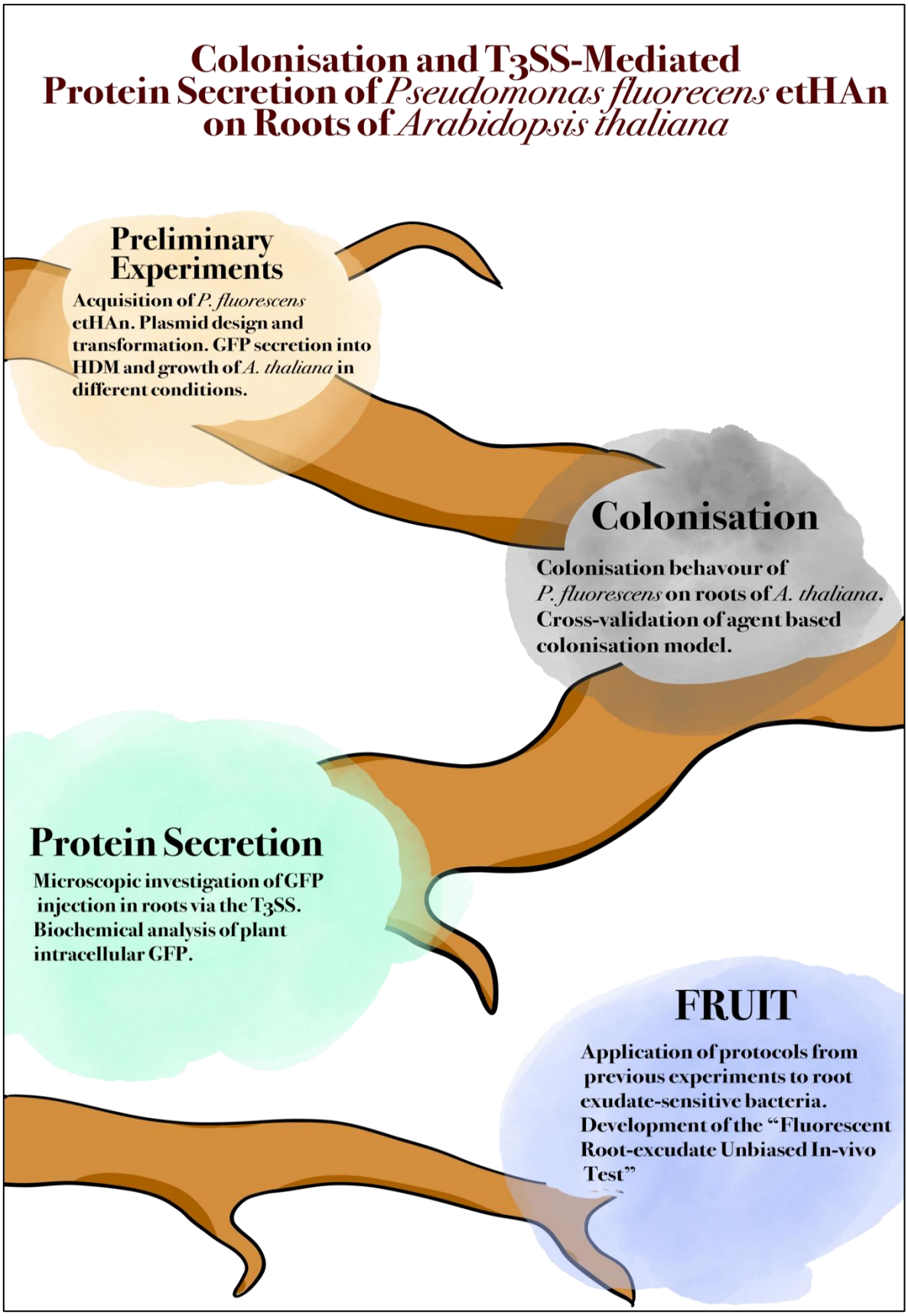

Figure 5: Overview of thesis-work: "Colonisation and T3SS-Mediated Protein Secretion of* Pseudomonas fluorescens *etHAn on Roots of* Arabidopsis thaliana *Columbia 0".

2 Material & Methods

2.1 Bacteria and Plants

The Effector-to-Host-Analyzer (etHAn) strain of *Pseudomonas fluorescens* (Thomas et al., 2009a) was kindly provided by the Department of Plant and Environmental Sciences from the University of Copenhagen, Denmark. The transformation strain *E. coli* DH10β (Grant et al., 1990) was supplied by the Department of Microbiology, Wageningen University and Research (WUR), Netherlands.

E. coli and *P. fluorescens* were stored with 20 % glycerol at -70 °C in 50 µl PCR tubes (ThermoFisher Scientific, Waltham, USA) and 1 ml cryovials (Sigma-Aldrich, St. Louis, USA), respectively.

For plant experiments, non-sterile seeds of *Arabidopsis thaliana* strain Columbia-0 (Lee et al., 1993) were provided by the Department of Plant Physiology and Plant Biosciences, WUR.

2.2 Media and Growth Conditions

Growth media and -conditions were adjusted for the respective experiments as shown in Table 1 and Table 2.

2.2.1 Plant growth

A. thaliana seeds were sterilised for one hour in a covered glass box using a solution of 50 ml household bleach ("Bleekwater," HANOS, Apeldoorn, the Netherlands) and 1 ml 37 % fuming hypochlorous acid (Sigma-Aldrich, St. Louis, USA).

Plants were seeded in one of the following conditions:

Table 1: Overview of media composition and growth for plant growth in different experimental setups. *The media for ½ MS10 Agar, ½ MS and soil are provided. Growth conditions apply to A. thaliana Col-0.*

Experimental setup	Media	Growth condition
Vertical Petri dishes	½ MS10 Agar Medium: • 2,3 g/L Murashige-Skoog Powder (MS Medium, Phygenera, Germany) • 10 g/L sucrose (Sigma Aldrich, St. Louis, USA) • 7 g/L Plant-Agar (Agar (Type A), Phygenera, Germany) Adjust with KOH to pH: 5,7 and autoclave.	Artificial long days with a 16-hour photoperiod at 20 °C in plant growth chambers.
Fåhraeus slides	½ MS Medium: • 2,3 g/L Murashige-Skoog Powder (MS Medium, Phygenera, Germany) Adjust with KOH to pH: 5,7 and autoclave.	Next to a window with approximately 14 hours of light and 9 hours of darkness (long summer days from June to August).
Soil:	Nonsterile, commercial garden soil.	Artificial long days with a 16-hour photoperiod at 20°C in plant growth chambers.

2.2.2 Bacterial Growth

P. fluorescens etHAn was grown on media as described in Table 2 and supplemented with Ampicillin 100 µg/ml, Chloramphenicol 30 µg/ml and Tetracyclin 25 µg/ml. Further, transformants of the pSEVA64 plasmid were selected on 10-20 µg/ml Gentamycin.

For all experiments, bacteria were freshly taken with a sterile inoculation loop (FisherScientific, Hampton, USA) from cryostocks stored at –70 °C and inoculated on media described in Table 2.

Table 2: Overview of media composition and growth for bacterial growth in different experimental setups. *The media for LB, SOC and HDM are given. Growth conditions are specified for* P. fluorescens *(30 °C) and* E. coli *(37 °C). If not indicated differently, all chemicals are supplied by: Sigma Aldrich, St. Louis, USA).*

Experimental setup	Media	Growth condition
Recovery after transformation	Super Optimal broth with Catabolite repression (SOC-Medium): • 2 g/L NaCl (FisherScientific, Hampton, USA) • 5 g/L Yeast Extract • 20 g/L Tryptone • 2,46 g/L $MgSO_4$ • 2,26 g/L $MgCL_2$ • 0,12 g/L KCl Adjust with NaOH to pH 6,8 – 7.	Innova 42 Stackable Incubator Shaker (Eppendorf AG, Hamburg, Germany): • 180 RPM • 30 °C for the first 24 hours & 33 °C until day 3 after transformation (*P. fluorescens*) • 37 °C (*E. coli*)
Overnight cultures	Lysogeny Broth Medium (LB-Medium): • 10 g/L Tryptone • 5 g/L Yeast extract • 10 g/L NaCl (FisherScientific, Hampton, USA) Set to pH 7 by adding one or two drops of NaOH	Innova 42 Stackable Incubator Shaker (Eppendorf AG, Hamburg, Germany): • 180 RPM • 30 °C (*P. fluorescens*) • 37 °C (*E. coli*)
Secretion into Medium	*hrp*-derepressing medium (HDM) as described in Stauber et al. (2012): • 2 g/L Fructose • 45 g/L potassium phosphate monobasic • 25 g/L potassium phosphate dibasic • 0,1 g/L NH_4SO_4 • 0,26 g/L $MgCl_2$ • 0,1 g/L NaCl For HDM + Iron citrate as described in Kim et al. (2009) additionally: • 0,012 g/L Iron citrate (≙ 12 mg in 1 L HDM) Adjust with NaOH and HCl to pH 5,5 – 6.	Innova 42 Stackable Incubator Shaker (Eppendorf AG, Hamburg, Germany): • 180 RPM • 30 °C (*P. fluorescens*)

2.3 Plasmid Design

Plasmids were designed with the cloning software SnapGene (San Diego, USA). Three plasmids were designed:

1. The experimental plasmid with AvrRPM1-secretion signal coupled to His-tagged GFP under the control of the strong constitutive promoter J23100 and the strong Ribosome binding site RBS0034 as described in (Damalas et al., 2020)
2. Like (1), but under the control of the native AvrRPM1 promoter as described in Jensen et al. (2022).
3. The control plasmid with His-tagged GFP under the control of J23100 and RBS0034 but without T3SS secretion signal. This plasmid serves as the control for the functionality of the secretion Signal in the upper groups.

Red fluorescent protein (RFP) was inserted in an operon-like structure behind GFP to counterstain extracellular, thus secreted, GFP to intracellular GFP/RFP positive bacterial cells (see Figure 6).

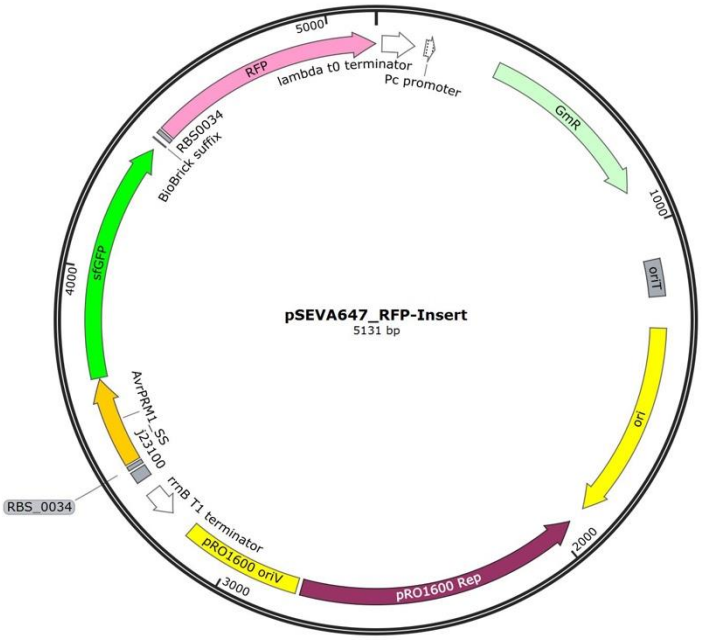

Figure 6: Schematic overview of sfGFP_RFPpSEVA647-plasmid. *The T3SS secretion signal-coupled sfGFP and RFP are under the control of the strong constitutive promoter J23100. Both genes have the strong RBS0034 six base pairs prior the ATG-start codon. Overview made with SnapGene (San Diego, USA).*

2.4 Genetic Material & Plasmid Construction

Apart from GFP and RFP, all gene constructs and primers were synthesised by Integrated DNA Technologies (IDT, Coralville, USA). The DNA sequence of synthesised gene fragments and all plasmids can be found in Table 8 and Table 9 in the appendix.

GFP was PCR amplified with "sfGFP_fwd" and "sfGFP_rev" from pSEVA2311-msfGFP-pBBR1.

2.4.1 Introduction of Promoter Systems and bsa1 Recognition Site

Plasmid 1 (test sample 1):

The bsa1-recognition site *(5'-GGTCTC(N1)/(N5)-3')* was introduced to GFP via overlap extension PCR using the forward primer "sfGFP_promoter_fwd" and the reverse primer "sfGFP_rev".

RFP was PCR amplified with "RFP_RBS_fwd" and "RFP_rev" while introducing the bsa1-recognition site to both ends of the gene and ribosome binding site RBS0034 to the *5'*-end.

The bsa1-recognition site, the promoter J23100 and the ribosome binding site RBS0034 system were introduced to the T3SS secretion signal using the primer pair "J23100:Avr_fwd" and "J23100:Avr_rev".

GFP, RFP and the T3SS secretion signal were inserted into the "Pv" (forward primer) and "Ev" (reverse primer) linearised vector pSEVA64 via golden gate cloning.

Plasmid 2 (test sample 2):

GFP and RFP constructs from Plasmid 1 were used. The T3SS secretion signal insert was synthesised with the AvrRPM1 promoter. The bsa1 recognition site was introduced using the primer pair "Avr_fwd" and "J23100:Avr_rev".

GFP, RFP and the T3SS secretion signal were inserted into the linearised vector pSEVA64 via golden gate cloning.

Plasmid 3 (control sample):

The Promoter J23100 and RBS0034 were introduced to GFP using the forward primer "sfGFP_promoter_fwd" and the reverse primer "sfGFP_rev" while adding the bsa1-recognition site to GFP. The RBS0034 carrying RFP construct with bsa1 recognition site from the previous primers could be used.

GFP and RFP were introduced into the linearised pSEVA64 vector via golden gate cloning.

PCR products were checked on a 1,5% agarose gel in the Biorad Gel Doc XR Imaging System (Bio-Rad Laboratories, Hercules, USA) and analysed on Image Lab (Image Lab Software, Bio-Rad, Hercules, USA). Nucleic acids were stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA) and separated for 30 minutes in an electrophoresis chamber (100 Volt).

2.4.2 Golden Gate Cloning

Golden Gate cloning with bsa1 was performed with the settings shown in Table 3 and Table 4. The "Pv", "Ev"-linearised pSEVA64 served as vector for the above-presented inserts for both test plasmids and the control plasmid. The T4 DNA-Ligase and Bsa1 enzyme was supplied by New England Biolabs Inc. (Ipswich, USA).

Table 3: Reaction mix for bsa1-golden gate cloning.

Component	**Volume in µl**
Vector (10fmol)	1
Insert (10 fmol)	3
T4 DNA-Ligase (2000 Units/µl)	0,5
BsaI-HF®v2 (20 Units/µl)	0,5
ddH20	5

Table 4: Thermocycler-protocol for bsa1 golden gate cloning (LabCycler, BIOKÉ, Leiden, the Netherlands).

Step	**Temperature in °C**	**Time in min**	**Iteration**
Primary Digestion	37	20	1
Ligation	16	4	30
Digestion	37	3	30
Final Ligation	16	10	1
Heat inactivation 1	50	10	1
Heat inactivation 2	80	10	1

2.5 Transformation

The GFP/RFP-containing plasmids were transformed into competent *E. coli* DH10ß, according to the heat shock transformation protocol of New England Biolabs (NEB Inc., USA, Ipswich). The transformants were grown on a selective LB Agar plate containing 10 µg/ml gentamycin at 37 °C. On the subsequent day, colonies were picked from the selective plates and transferred to a 5 mL liquid LB medium. The overnight culture was incubated at 37 °C at 180 RPM in the Innova 42 Stackable Incubator Shaker (Eppendorf AG, Hamburg, Germany). On the next day, plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen; Venlo, Netherlands).

Pure plasmid DNA was eluted with Milli-Q Water (Arium Pro, Sartorius AG, Göttingen, Germany) and the isolated plasmid concentration was determined on the DNA-quantification spectrometer from DeNovix (Wilmington, USA). *P. fluorescens* etHAn.

First, *P. fluorescens* etHAn was made competent via inoculation in 10 % glycerol. Then, competent *P. fluorescens* etHAn were transformed via electroporation (247 V, 200 Ω) using a protocol suggested by the Department of Plant and Environmental Sciences at the University of Copenhagen from where the etHAn strain was delivered. The detailed protocol for the preparation and transformation of electrocompetent cells of *P. fluorescens* etHAn can be found in the appendix (Protocol 1 in the appendix). Transformants were selected on selective LB-Agar Plates containing ampicillin (Amp: 100 µg/ml), chloramphenicol (Cl: 30 µg/ml), tetracycline (Tet: 25 µg/ml) and gentamycin (Gen: 15 µg/ml). Concentrations for Amp, Cl and Tet are suggested by the provider of the bacterial strain. Concentrations for gentamycin were picked according to recommendations for *Pseudomonas* species by the official websites of SEVA-plasmids (https://seva-plasmids.com).

Colonies were detected 2 days after plating. Coinciding with the procedure for *E. coli* DH10ß transformants, colonies were picked, overnight incubated in liquid LB medium (30 °C) and plasmids were miniprepped. After checking the transformation results via sequencing according to the sanger sequencing sample-preparation-guide (Macrogen, Seoul, South Korea), cryostocks of the transformants in 20 % Glycerol were made and stored at -70 °C (Macrogen sequence alignment in Figure 53, appendix). Only fresh cryostock cultures were used in the experiments.

2.6 Plant Experiments

Plants were grown under three different growth conditions, namely in (1) Soil, (2) ½ MS10 Agar and (3) ½ MS medium.

2.6.1 Growth in Unsterile Soil

Two to three seeds of *A. thaliana* were seeded in approximately 20 grams of commercial garden soil in a plastic garden pottery. Plants were watered twice a week (Monday and Thursday) with approximately 20 ml of tap water.

2.6.2 Growth on Agar Plates

Arabidopsis was (1) grown on top of approximately 40 ml ½ MS10 Agar, with roots growing into the growth medium, (2) sideways to allow the roots to grow in contact with the medium but without entering it or (3) on autoclaved, semipermeable household cellophane between agar and roots to prevent entry of the roots into the agar while allowing free diffusion of water and nutrients. As for the growth conditions, in which roots did not enter the Agar-medium (see Figure 7, C-E), square Petri dishes (Greiner Bio-One B.V., Alphen aan den Rijn, the Netherlands) were sealed with Micropore tape (3M, Saint Paul, USA).

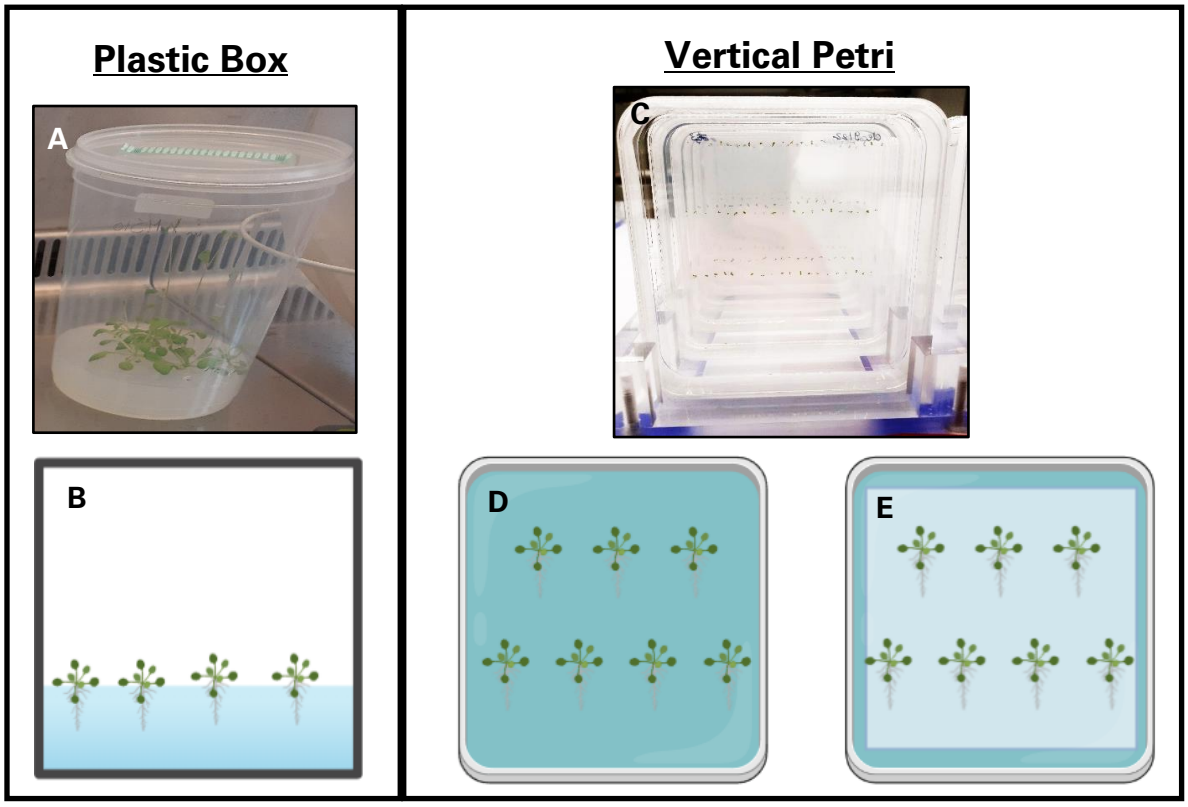

Figure 7: Growth conditions for sterile* A. thaliana *in ½ MS10 Agar medium. *A+B: Growth of* A. thaliana *on top of Agar in plastic box. C-E: Growth of* A. thaliana *at the side of vertical Petri dish filled with Agar. E: With cellophane in between agar and plant.*

2.6.2.1 Transfer from Growth Medium to Microscope

For microscopic work, plants were removed with a forceps or a 10 µl pipet tip from the ½ MS10 Agar and gently placed in 500 µl ½ MS medium on a microscope slide (Thermo Fisher Scientific Inc., USA, Waltham) Depending on the length of the root, a 1,5 cm or 4 cm long cover slip (Knittel Glasbearbeitungs GmbH, Bielefeld, Germany) was placed on top of the root system (see Figure 8)

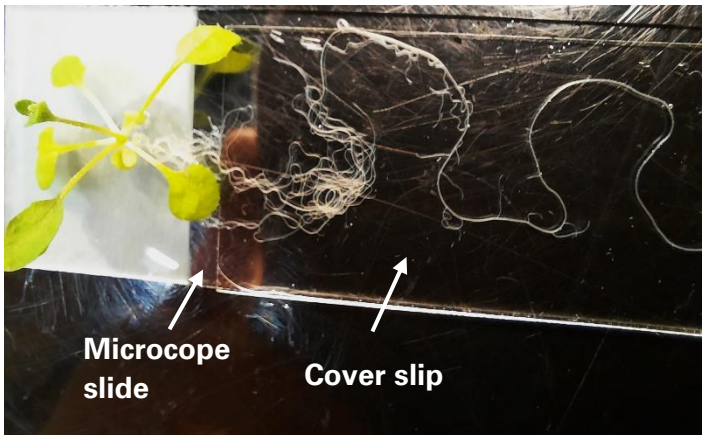

Figure 8: Root system of* A. thaliana *in between Microscope slide and Coverslip. *The pant was grown for 15 days in ½ MS10 Agar in a sterile plastic box.*

2.6.3 Fåhraeus Slides

Fåhraeus slides for microscopic *in vivo* analysis of *A. thaliana* seedlings were prepared and sterilised according to a protocol of the Department of Cell and Developmental Biology, WUR (Protocol 2, in appendix). In short, Fåhraeus slides are made by mounting a coverslip (40 x 24 mm; Knittel Glasbearbeitungs GmbH, Bielefeld, Germany) with household silicone grease to a glass slide (Thermo Fisher Scientific Inc., Waltham, USA) and thereby creating a microchamber of about 0,4 mm that gets filled with ½ MS medium in which roots of *A. thaliana* can grow.

Plants were grown in ½ MS medium at room temperature at a place near the window (14 - 16 hours of light). Plants were analysed seven to ten days after sowing. By this time, the main morphology of the roots was developed such as the primary root and several side roots.

2.7 Studies on the Secretion of GFP into HDM

Bacteria were grown in HDM as described above in Table 2. For the analysis of GFP in the growth medium, cells were spun down at 17 000 g. in a tabletop centrifuge ("Centrifuge 5425 R", Eppendorf AG, Hamburg, Germany). The supernatant was transferred into sterile 96 well-plates (ThermoFisherScientific, Waltham, USA) and the fluorescence intensity was measured in the BioTek Synergy Neo2 plate reader (Agilent Technologies Inc., Santa Clara, USA).

For the data processing, samples of untransformed *P. fluorescens* served as a negative control to measure the fluorescence intensities in the samples with the *gfp*-expressing cells. The negative control served as blank and fluorescence values of the negative control were subtracted from the measured fluorescence in the test samples.

2.8 Studies on Cross-Sectional Root Slices

2.8.1 Sample Preparation

Information about the location of *P. fluorescens* and secreted GFP inside the roots of *Arabidopsis thaliana* was collected on thin root slices of *Arabidopsis* plants (1 - 15 days upon germination).

First, Agar plate-grown plants were inoculated with a bacterial overnight culture for 2 hours. Then, plants were washed twice in a sterile bath of liquid MS-medium. Finally, plants were replaced on the agar plate until fixation.

After 2 days, plants were gently removed with a sterile forceps from the agar plate and fixated in 4 % paraformaldehyde in 0,1 M PBS for 1 hour at room temperature. Subsequently, the fixated root material was washed in distilled water and approximately 5mm of the basal root region was vertically embedded into 6 % Agarose gel in a 1,5 ml Eppendorf tube. Embedded plants were either immediately sectionized on the vibratome or stored at 4 °C.

2.8.2 Sectionizing

Embedded root material was glued on the vibratome tissue holder with "Superglue" (Turbolijm, Dr. Turbo, Maarn, the Netherlands) and subsequently sectionized in a water bath with a Leica VT1000 S Vibrating Blade Microtome (Leica Biosystems, Wetzlar, Germany, further referred to as "Vibratome") at a Frequency of 80 Hz and a feed of 50-75 µm.

A detailed protocol for vibratome sectioning can be found in the appendix (protocol 3).

2.9 Microscopy

2.9.1 Fluorescence Microscopy

Plant tissue was studied under two microscopes. Most microscopic studies were carried out on the ECLIPSE Ts2R microscope (Nikon Instruments, Tokyo, Japan). Vibratome sectioned samples were analysed with the Nikon Eclipse 80i (Nikon Instruments, Tokyo, Japan). For pictures with a 1000 x magnification, Nikon F2 Immersion Oil was used (Nikon instruments, Japan, Tokyo). As for the different objectives, instruments from the CFI Plan Apochromat Lambda D Series were used (Nikon instruments, Tokyo, Japan).

Table 5 displays the microscopy settings.

Table 5: Applied settings for fluorescence microscopy images. *Settings for images were adjusted to the analysed fluorophores (GFP, RFP) and tissue conditions (sectioned and not sectioned roots).*

Application	**Description**	**Setting GFP**	**Setting RFP**
Unsectioned roots	Excitation wavelength	*λ=485 nm*	*λ= 532 nm*
	Emission wavelength	*λ= 510 nm*	*λ= 555 nm*
	Exposure Time	*1 sec*	*4 sec*
	Magnification strength	*As indicated in caption 100x (pH 1 annular ring), 1000x (pH3 annular ring)*	
	Image Application software	*Nis Elements (Nikon instruments, Japan, Tokio)*	
Sectioned roots	Excitation wavelength	*λ=485 nm*	*λ=532 nm*
	Emission wavelength	*Short-pass filter: λ= 510 nm Long-pass filter: λ>510 nm*	*Short-pass filter: λ=588 nm Long-pass filter: λ>588 nm*
	Exposure Time	*1 sec*	*3 sec*
	Manual gain	*11,4 x*	*11,4 x*
	Magnification strength	*As indicated in caption 200x, 400x*	
	Image Application software	*Nis Elements (Nikon instruments, Japan, Tokio)*	

2.9.2 Light Microscopy

For light microscopic studies, differential interference contrast (DIC, further referred to as phase contrast) were employed to facilitate the detection of the unstained, living bacteria under the microscope. Microscopic settings align with the ones used for fluorescence microscopy images in Table 5. In contrast to the fluorescence pictures, an exposure time of 2-5 milliseconds was sufficient for phase contrast pictures.

2.10 Silver Staining

A. thaliana was grown in an ½ MS10 Agar medium in vertical Petri dishes. After 7 days, bacteria from an overnight culture in LB medium were inoculated to the plants for one hour. Non-attached bacteria were washed away with ½ MS medium. After two days, bacteria were removed from the plant surface and the medium following a protocol inspired by the root cleaning protocol from Siqueira et al. (2007). First, bacteria-attached plants were washed in distilled H_2O. Then, plants were inoculated in 5 % sodium hypochlorite (NaOCl) for two minutes. Subsequently, plants were rinsed in 70 % isopropanol and washed twice in distilled H_2O.

For protein extraction, a protocol from the Department of Plant Physiology, Wageningen University & Research, was applied. Bacteria-free plant tissue was lysed in SDS-Buffer and cooked at 99°C for 5 minutes in an Eppendorf ThermoMixer C (Eppendorf SE, Hamburg, Germany). A detailed protocol for protein extraction from plant tissue can be found in the appendix (Protocol 4). As a positive control, the pellet of a 5 ml *P. fluorescens* overnight culture was lysed and examined as well.

100 µl of plant lysate was His-tag purified according to the protocol of the His-tag purification kit (Qiagen N.V, Germany, Hilden). The remaining plant lysate (30 µl) was directly loaded on a Novex 4-20 % SDS-PAGE gel (ThermoFisher Scientific, Waltham, USA). Samples were stained with 4x SDS-sample buffer (containing 1mM bromophenol blue) and separated in the electrophoresis chamber at 220 Volt for 30 minutes.

Proteins were visualised via silver staining, following the instructions of the Pierce Silver Stain Kit (Thermo Fisher Scientific Inc., USA, Waltham). Photos of the respective gels were captured on the Biorad Gel Doc XR Imaging System (Bio-Rad Laboratories Inc., Hercules, USA)

2.11 Additional Experiments – Protein Expression in the Presence of Root Exudates (FRUIT)

2.11.1 Experimental Setup

In a collaborative experiment with the iGEM colleague Nico van Donk, it was tested whether the inducible expression systems pSAL/nahR, pCym/CymR, pTtg/TtgR, and pBAD/AraC are activated in the presence of root exudates from *Arabidopsis thaliana* Col-0. Therefore, root exudate sensitive *P. fluorescens* SBW25 were grown on a selective agar plate and three colonies per transformant were picked for liquid overnight cultures (biological triplicates).

The following day, one-week-old *A. thaliana* seedlings were incubated with cells (OD: 0,1), analogously to the previous microscopic experiments. The plants were grown in ½ MS medium in vertical Petri dishes.

In the negative control, the secretion of root exudates was prevented by plant fixation in 4 % paraformaldehyde in 1 mM PBS and 1 mM DTT. To further dilute the concentration of root exudates in the negative control, fixed samples were stored in tap water for one day.

The controls were alike except for synthetically adding 1 mM of inducer to the positive control. The Inducer were salicylic acid (pSAL/nahR), cuminic acid (pCym/CymR), naringenin (pTtg/TtgR) and arabinose (pBAD/AraC).

Induced *gfp*-expression of plant-attached bacteria was analysed on the ECLIPSE Ts2R microscope (Nikon Instruments, Tokyo, Japan), one day after incubation as described before in the chapter for microscopy.

2.11.2 Image Processing

Images were processed and analysed using FIJI (Schindelin et al., 2012). The following steps were applied for the processing of FRUIT-related data:

1. **Subtraction of the background/noise fluorescence:** Background fluorescence was determined as the mean fluorescence of a root picture with *P. flourescens* harbouring an empty, *gfp*-free vector (subtracted value: -450). Everything that showed higher fluorescence values was accepted as "truly fluorescent".

2. **Removal of autofluorescence in stele:** The autofluorescence emitted by the stele of the roots was removed. The stele is the inner region of the root, containing main vascular tissues like the phloem and xylem and was found to show high autofluorescence after fixation with 4% paraformaldehyde. Therefore, the stele of the roots was manually selected in the phase contrast pictures of the roots. The selection was transferred to the fluorescence pictures and subtracted the autofluorescence for fixated and non-fixated samples, respectively (see Figure 9).

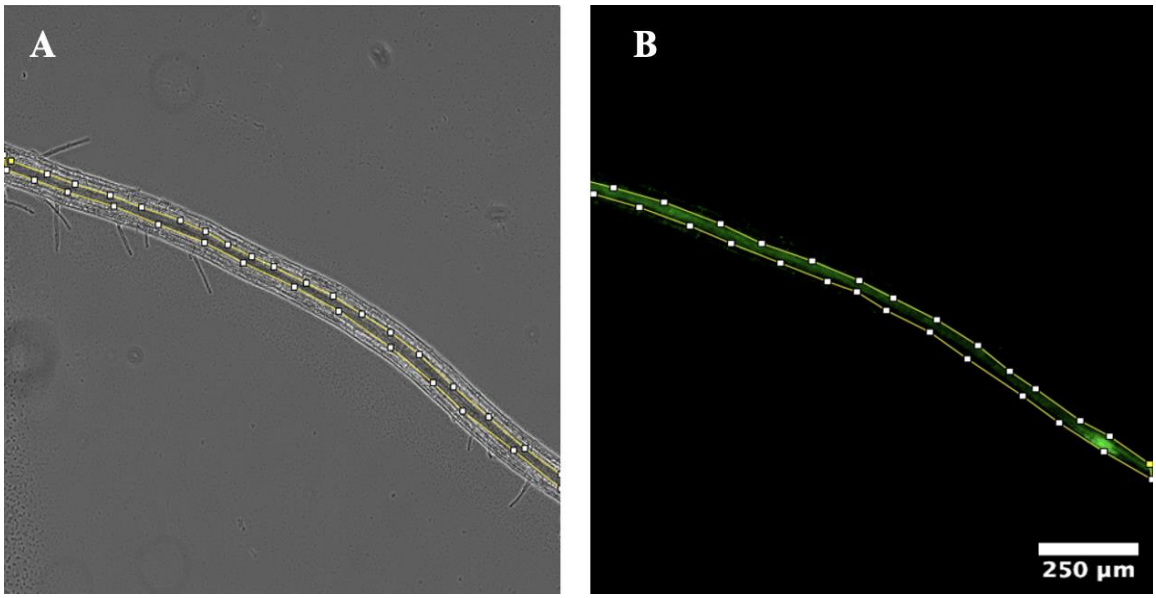

Figure 9: Microscopy pictures of* P. fluorescens *attaching to the roots of* A. thaliana *(negative contro* *1, 100x magnification, 1 day after inoculation). *A: Phase contrast picture with stele in selection, the stele is easily detectable as a darker structure going across the length of the root. B: The selection from the phase contrast picture is transferred to the fluorescence microscopic picture and the autofluorescence is subsequently subtracted. The scale bar for B applies also to A.*

3. **Bacterial fluorescence measurements:** First, a mask was created to include fluorescence signals from bacteria but exclude remaining autofluorescence from the roots (fluorescence value of 125-200 after subtraction of background). Then, particles above a minimal size of 3 µm were selected. Smaller particles are not likely to be bacteria as the mean size of *P. fluorescens* is 3 µm (Turnbull et al., 2001). Finally, the mean fluorescence of particles larger than 3 µm with more than 125 fluorescence was measured to represent bacterial presence. A representative imageJ macro from pSAL/nahR samples is presented in Figure 48, in the appendix.

2.12 Safety Considerations

Biosafety serves as not merely a component but the very foundation of sustainable synthetic biology.

During this master thesis, GMOs were produced, and GFP was potentially secreted into plants. The delivered GFP did not change the genetic material of the plant itself but could very well change its physiology.

The organisms used in this study include *Pseudomonas fluorescens*, a non-pathogenic bacterium that promotes plant growth, and *Arabidopsis thaliana*, a widely distributed plant in Europe with no identified safety concerns. Nevertheless, plant material was discarded as a biohazard. Further, to ensure the safe management of both plants and bacteria, all experiments will be carried out in a biosafety level 1 laboratory. Experiments with GMOs are authorized in these facilities. All parts used in this project are considered safe by the iGEM safety committee. The adherence to high standards of Good Laboratory Practice (GLP) was a priority to ensure the absence of harm. Specific attention was given to:

1. **Quality Assurance:** All experiments were designed, presented, and discussed with researchers from Wageningen University and Research to address safety concerns from the outset. In cases of uncertainty, consultations were conducted with lab safety manager Steven Aalvink.
2. **Standard Operating Procedures:** Established protocols for the transformations of bacteria and handling of plants and microorganisms were used. Any deviations from standard procedures were documented and self-developed protocols are comprehensively described in the material and method section.
3. **Documentation:** Experiments and procedures are recorded from Mai 10th, 2023 (start date of lab work) until September 16th, 2023 (end date of lab work) both in a handwritten lab-journal and the online E-labJournal (eLabNext, Groningen, Netherlands).

Following these safety considerations, no social, environmental, or medical risks are expected to be associated with the outcomes of this master's thesis.

3 Results

3.1 Construction of *P. fluorescens* Strains Expressing *gfp* With and Without a T3SS-Secretion Signal

This study aimed to genetically modify *P. fluorescens* etHAn to develop transformants to study the colonisation of *P. fluorescens* on *A. thaliana* roots and the secretion of GFP by bacteria into the epidermis of *A. thaliana* roots through the T3SS.

To this end, plasmids were designed for the constitutive expression of *gfp*. Control Plasmid 1 expresses *gfp* under the control of the promoter system J23100/RBS0034. Plasmid 2 carries *gfp* under the same promoter system but includes a T3SS-secretion signal at its N-terminal end (further referred to as GFP-SS). The third plasmid contains GFP-SS under the control of the AvrRMP1 promoter from *Pseudomonas syringae*.

3.1.1 An *In Silico* Assessment Predicts the Folding of GFP-SS

As a preliminary step for the plasmid design, the structure of GFP-SS was predicted using AlphaFold2 (Jumper et al., 2021). The predicted protein conformation indicated that the incorporation of the T3SS secretion signal and the N-terminal HIS-tag does not interfere with the native structure of GFP, as illustrated in Figure 10. Subsequent alignment of the heterologous GFP with native GFP (UniProt accession number: P42212) using the PyMol software from Schrödinger (2022) substantiated this observation, revealing a low root-mean-square deviation (RMSD) of 0.262 Å between native GFP and GFP-SS.

In summary, the *in silico* assessment confirmed that GFP-SS has similar properties to native GFP and can be used for subsequent fluorescence microscopic studies.

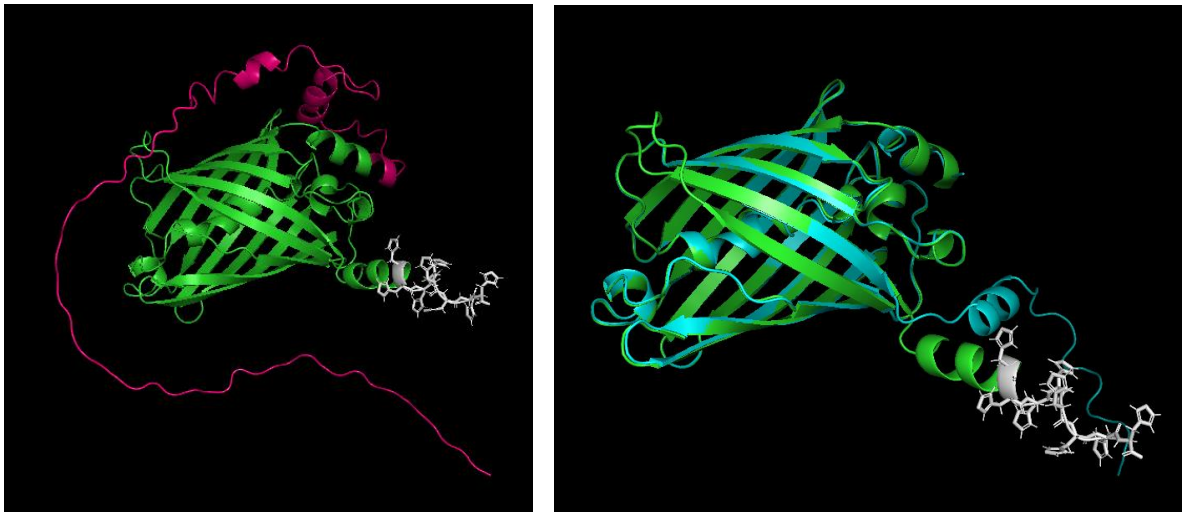

Figure 10: AlphaFold2 predicted 3D-structure of a HIS-tagged GFP construct with a T3SS secretion signal. *Left picture: Green: GFP, Grey: HIS-tag, Magenta: T3SS secretion signal (first 89 AA of AvrRPM1). Right picture: Alignment of GFP (P42212, indicated in blue) to GFP as used in this thesis. No major difference in structure is visible. Structure varies at the N-terminal end (HIS-tag end). Protein visualisation made in PyMol (Schrödinger, 2022).*

3.1.2 Agarose Gel Pictures Verifies the Plasmid Constructs

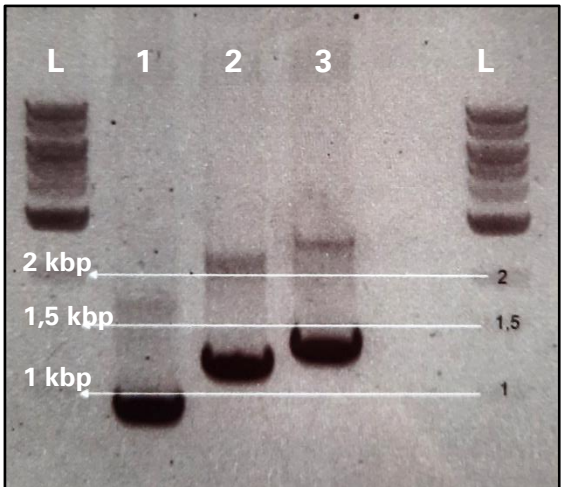

Figure 11: Qualitative inspection of* gfp*-inserts in three different* P. fluorescens *in 1 % agarose gel. L: 1kb ladder (NEB, USA, Ipswich). *1:* gfp *under control of J23100+RBS0034 (787 bp). 2:* gfp*+T3SS secretion signal under control of J23100-RBS0034 (1102 bp). 3:* gfp + *T3SS secretion signal under control of avrRPM1 promoter (1250bp).*

Gfp and *gfp-ss* under the control of different promoter systems were introduced into the vector pSEVA64. The genetic work was regularly checked via DNA-electrophoresis.

Figure 11 shows a representative agarose gel as it was used for the confirmation of PCR results. Plasmid 1 for which the *gfp*-gene without a secretion signal was PCR-amplificated resulted in bands below the larger plasmid 2 and 3 for which *gfp-ss* under their respective promoters was amplified. The expected length of the DNA fragments are 787np, 1102 and 1250 bp for plasmids 1, 2 and 3, respectively. The bands confirmed the expected size, indicating that the plasmids carry the intended GFP inserts (see Figure 11).

3.1.3 Plasmid Constructs were Successfully Transformed into *E. coli* and *P. fluorescens*

Plasmids were constructed through golden gate cloning and introduced into *E. coli* DH10ß and *P. fluorescens* etHAn. Sequencing of the relevant gene regions confirmed the success of the assembly. No mutations were identified in the promoter and coding regions. The transformation process comprised two stages: initial heat-shock transformation into *E. coli* for plasmid accumulation, followed by plasmid extraction and electroporation into *P. fluorescens*. Unlike *E. coli*, which exhibited single-cell colonies (SCC) on selective plates one day after heat-shock transformation, *P. fluorescens* naturally grows slower and therefore required three days for SCC appearance (cf. protocol 1, Appendix). Using untransformed cells as a negative control, the colonies were analysed for green fluorescence under blue light (λ=470 nm). No difference in fluorescence between transformants and negative control was detectable (data not shown).

3.1.4 A Spacer Between RBS and Initial Start Codon is Required for Protein Expression in *P. fluorescens*

A comparison with the plasmid that was used for gene expression in *P. fluorescens* as presented in Jensen et al. (2022) led to the hypothesis that the absence of fluorescence was the result of the lack of a spacer between RBS and initial start codon. Therefore, the plasmid design was optimised by introducing a spacer of 6 bp between RBS and the start codon of gfp (see Figure 12).

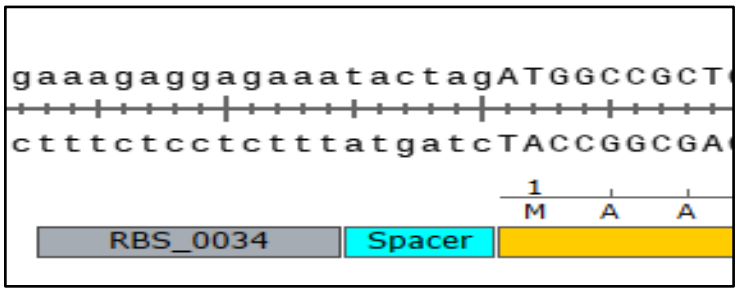

Figure 12: Section of 19 nucleotides upstream the initial start codon of T3SS - secretion signal coupled* gfp *gene**. A 6 bp long spacer is introduced in between the Ribosome Binding site and the initial start codon. Screenshot from SnapGene (San Diego, USA).

Transformants carrying the new plasmids were illuminated at λ=485 nm. Direct visual inspection revealed that the GFP-positive control carrying plasmid 1, as well as the test sample with GFP-SS (Plasmid 2) were fluorescent. Transformants for which GFP-SS was under the control of the AvrRPM1 promoter (Plasmid 3) showed minor fluorescence (see **Fehler! Verweisquelle konnte nicht gefunden werden.**, sample 3).

The visual inspection shows that the J23100 promoter is stronger in *P. fluorescens* than the AvrRPM1 promoter. J23100 leads to a higher GFP-production than AvrRPM1 which leads to more fluorescence in J23100-carrying SCC. The differences in fluorescence were observed both on a macroscopic basis as well as under the fluorescence microscope (see **Fehler! Verweisquelle konnte nicht gefunden werden.**, lower panels).

The positive control for which native GFP was produced, showed similar fluorescence levels to the test sample in which GFP was tagged to a T3SS secretion signal (see **Fehler! Verweisquelle konnte nicht gefunden werden.**, samples 1 and 2). This alignment coincides with the *in silico* prediction, confirming that the T3SS secretion signal likely does not significantly alter the structural characteristics of GFP.

In summary, three constructs for constitutive GFP expression were successfully introduced into *P. fluorescens* etHAn. Plasmids were designed and optimized *in silico*, including a comparison of the 3D structure of the GFP construct to native GFP. The engineered plasmids were then introduced into the cells, and GFP expression was confirmed.

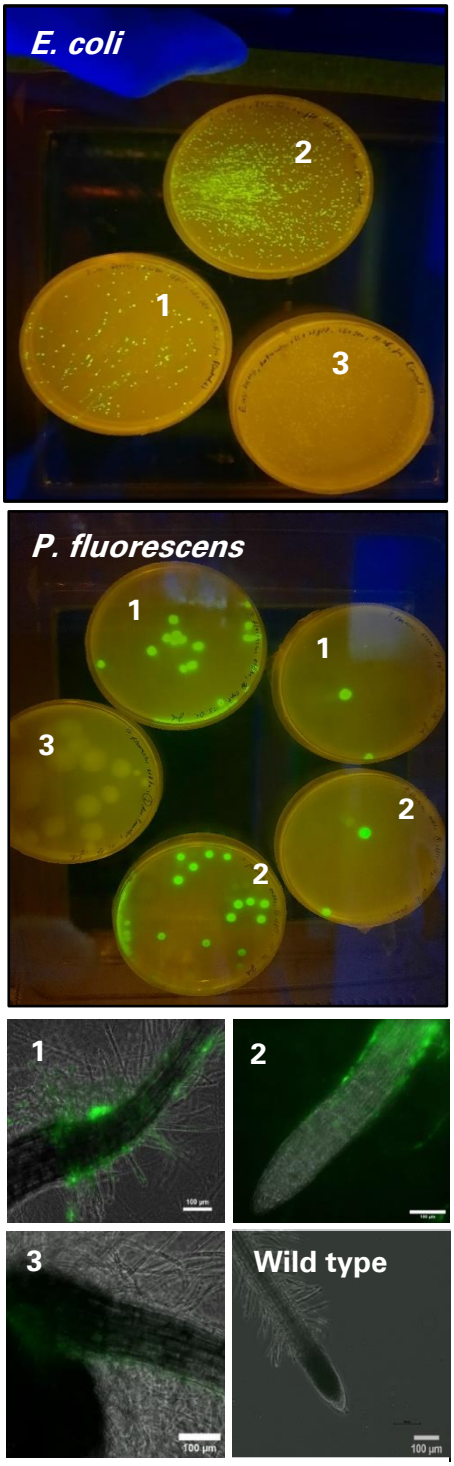

***Figure 13: Single cell colonies and microscopic pictures of* gfp-*expressing* E. coli *and* P. fluorescens.** *Upper and middle panel: Single cell colonies under blue light (λ=470 nm). Lower panel: Merged channel microscopic picture. 1: Positive control,* gfp *under control of J23100+RBS0034. 2: Test sample 1,* gfp*-ss under the control of J23100-RBS0034. 3: Test sample 2,* gfp*-ss under the control of avrRPM1 promoter.*

3.2 GFP Secretion into T3SS-Inducing Media

Before exploring GFP secretion into plant tissue, an assessment was conducted to determine if *P. fluorescens* can secrete GFP into its growth medium. This preliminary experiment was performed because the growth medium presents fewer confounding factors than experiments involving live plant roots.

To this end, *P. fluorescens* etHAn was grown in two different T3SS-inducing media, namely *hrp*-derepressing medium (HDM), and HDM with 50 µM iron citrate and LB medium as negative control. T3SS-related gene expression in *P. fluorescens* etHAn is suppressed in a nutrient-rich LB medium and activated in a minimal *hrp*-derepressing medium (Thomas et al., 2009). Additionally, Kim et al.(2009) explored that 50 µM iron citrate enhances T3SS-related gene expression in *P. syringae*. The assumption was made that elevated gene expression leads to increased protein secretion into the medium.

3.2.1 HDM Does Not Promote Growth of *P. fluorescens*

As shown in Figure 14, *P. fluorescens* grew in LB-medium to stationary phase in about 3 days. A marginal increase in the OD600-value is visible in the HDM medium within 12 days.

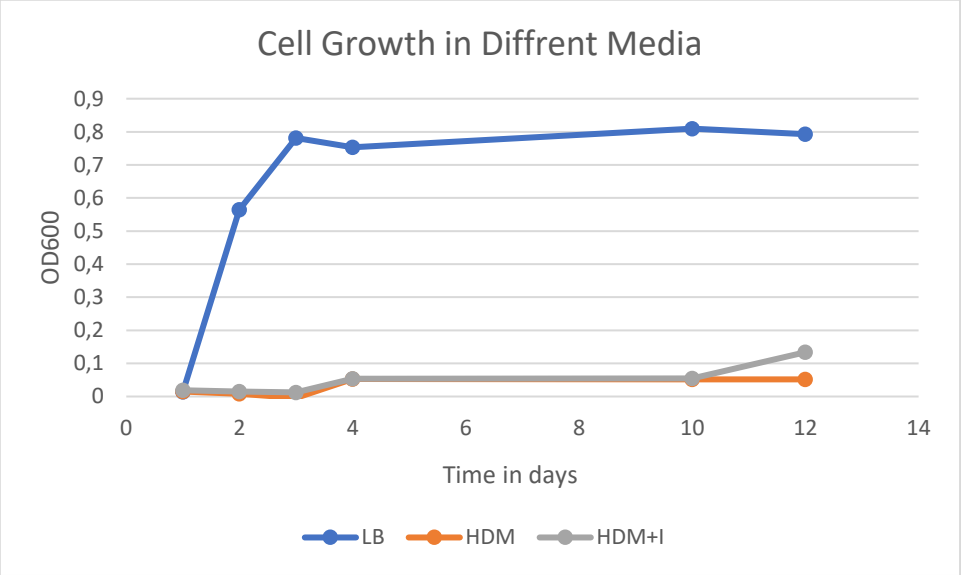

Figure 14: Average growth of P. fluorescens etHAn in LB and HDM medium. *After incubation at OD600=0,01 cells in LB medium grow within three days to its maximum density. Cells growing in HDM medium remain below OD600>0,2 within a time span of 12 days.*

3.2.2 GFP-Secretion into HDM is Not Measurable

The nutrient-rich LB medium showed increasing fluorescence intensities until day 10 with a decrease in fluorescence on day 12 (see Figure 15, upper panel). The negative control (sample 1) without GFP-SS, showed higher fluorescence levels than the test samples, implying that the shown fluorescence did not originate from GFP secretion but from other sources (e.g. cell lysate).

In the minimal HDM fluorescence levels in the cell-free medium fluctuated at low levels. Neither the simple HDM nor the one with iron-citrate supplementation showed differences between negative control and test samples.

In summary, the results showed that *P. fluorescens* could not secrete detectable quantities of GFP-SS in the HDM.

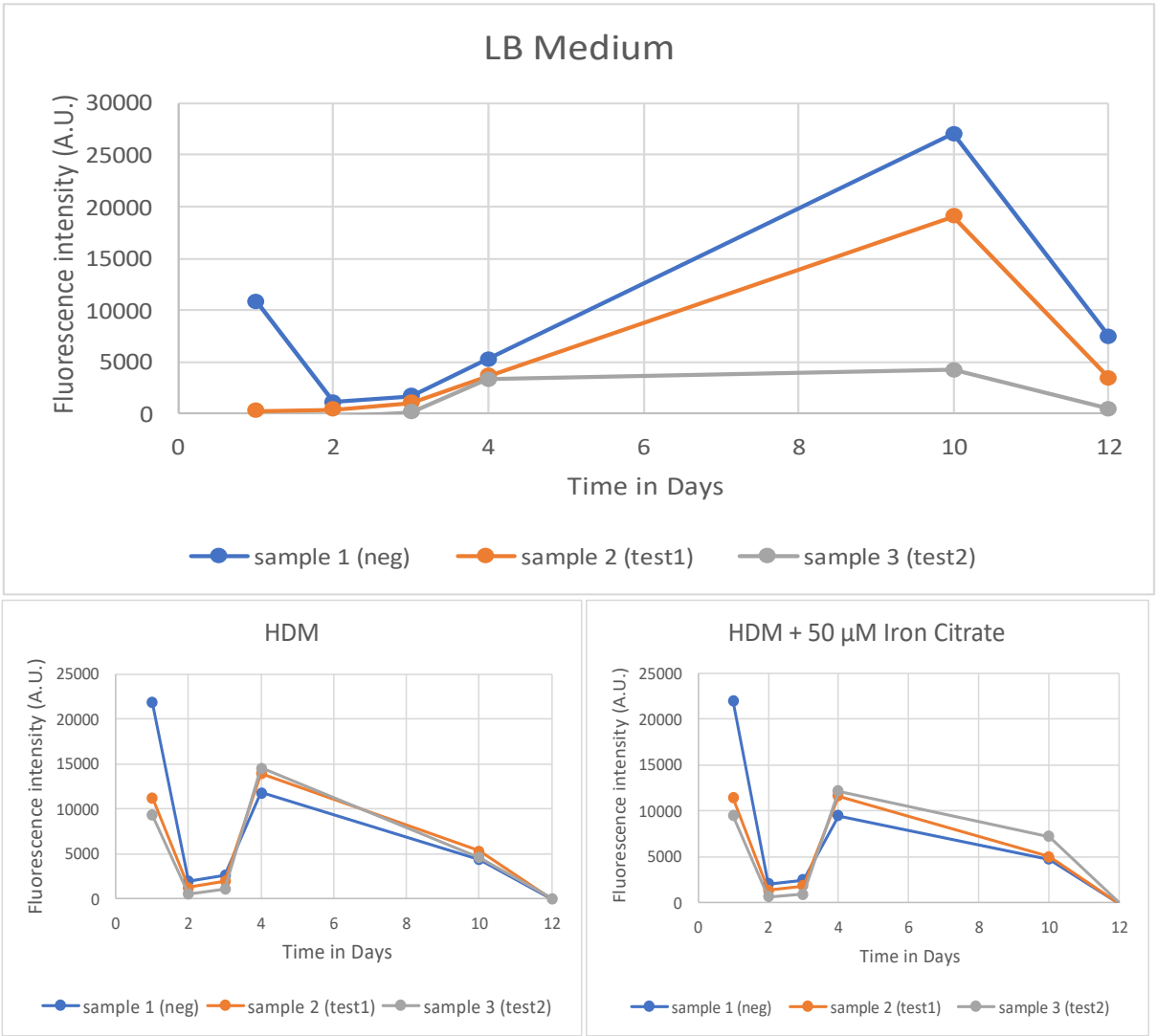

Figure 15: Fluorescence intensities in HDM and LB growth medium of* P. fluorescens *etHAn (Day 1 – Day 12 after incubation). *Cells were removed and the remaining medium was analysed. 1: negative control,* gfp *under control of J23100+RBS0034. 2: Test sample 1, GFP-SS under control of J23100-RBS0034. 3: Test sample 2, GFP-SS under control of avrRPM1 promoter.*

3.3 Growth of *A. thaliana* in Different Conditions

The inability to detect GFP-SS in HDM led to investigations of an alternative environment. For certain T3SS, attachment to an eucaryotic surface is a prerequisite for the secretion of protein (Gosh; 2004). Therefore, root attachment of *P. fluorescens* and GFP-SS secretion into *A. thaliana* should be investigated.

The effectiveness of four different conditions on the growth and root development of *A. thaliana* was evaluated on their suitability for colonisation and secretion experiments as outlined in the following chapters.

The growth methods were analysed on:

1. **Root morphology:** The root should be fully developed, including the presence of root hairs, lateral roots, and well-defined regions for differentiation and elongation.
2. **Feasibility for microscopic studies:** An essential criterion for microscopic analysis was to identify growth conditions that do not harm the root system of A. thaliana during transfer and analysis on a microscopic slide.
3. **Easiness of cultivation:** For the thesis work, approximately 500 plants were sown. It was an objective to find growth conditions that allow the efficient cultivation of many plants.

First, *A. thaliana* was grown in nonsterile soil, simulating a natural, non-laboratory environment. Second, plants were grown in a sterile environment in a plastic box containing 1/2 MS10 Agar. Third, seedlings were placed on a vertical Petri dish filled with ½ MS10 Agar, allowing the roots to grow on top of the agar, but not entering the agar. Finally, seeds were placed in a Fåhraeus slide containing ½ MS liquid medium, allowing the observation of roots at different time points under the microscope without touching the plant.

3.3.1 Soil Adheres to the Root-System and Complicates Microscopic Studies

As a preliminary experiment, *A. thaliana* was seeded on household garden soil, imitating the *in natura* growth condition. One week old plants were gently removed from the soil for microscopic studies.

The assessment of the root morphology was hindered by soil particles that adhered to the root system (see Figure 16). Removal and washing of the plants from the soil turned out to be difficult without damaging the delicate roots. In consequence, the feasibility of the growth method was considered unsuitable for the study of bacteria-root interactions under the fluorescence microscope.

Finally, in contrast to other growth conditions, only 4-6 plants could be cultivated in one growth box.

***Figure 16: Soil-grown* A. thaliana *in Eppendorf tube**. Soil is tightly attached to the root system of the plant.*

3.3.2 ½ MS10 Agar Allows Root Development

The Murashige-Skoog medium (MS-medium) is a plant growth medium that supports the optimal growth of many plant species (Murashige & Skoog, 1962).

Growth on ½ MS10 Agar medium was performed in three ways: (1) Growth in a Plastic box, filled with ½ MS10 Agar medium, (2) growth on a vertical Petri dish, and (3) growth on a cellophane sheet that was placed on a vertical Petri dish. The used cellophane was permeable to nutrients and water but avoided the growth of roots into the ½ MS10 Agar.

The ½ MS10 medium supported a normal root development in all three conditions. One week after seeding, roots were on average 11 mm long.

The agar-grown roots in the plastic box were fully developed and easy to remove from the growth surface, allowing the study of the roots under the fluorescence microscope (see Figure 17). No lesion of the roots was noticed. However, in comparison to the setup with vertical Petri dishes, only a few plants had space in the plastic box (c.f. Figure 18, A). With more than five plants in the plastic box, neighbouring roots became entangled, complicating plant removal from the growth surface. Furthermore, the roots in plastic boxes grew inside the medium. Removal from the medium potentially stressed or damaged the roots.

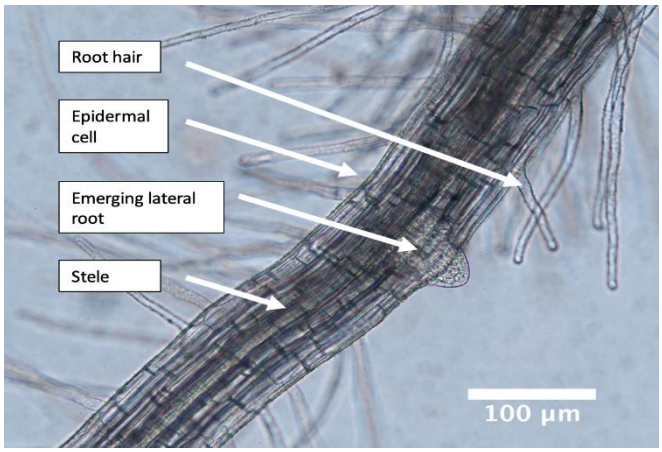

Figure 17: Phase contrast picture of root from* A. thaliana *Col-0, grown in ½ MS10 Agar medium (400 x magnification, one week after germination). *Root structures like the emerging lateral root and root hairs are developed.*

In comparison, roots on vertical Petri dishes remained on top of the medium, minimising the potential of harming the roots while removing them from the growth surface (see Figure 18, B & C). This applied for both, the cellophane carrying and setup without cellophane.

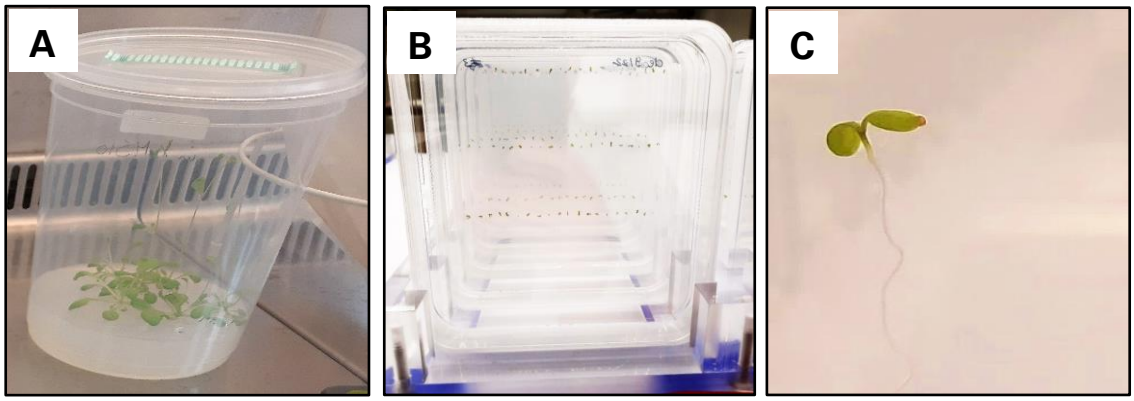

Figure 18:* A. thaliana *grown in ½ MS10 Agar. *A: Growth of* A. thaliana *on top of Agar in a plastic box. B: Growth of* A. thaliana *on top of Agar in a vertical Petri dish. Up to 50 Plants can be seeded on a single vertical Petri dish. C: Close-up picture of "B". Roots do not enter the medium but grow on top of it, allowing easy removal from the surface.*

3.3.3 Fåhraeus Slides are Optimal for Microscopic Studies of Root-Systems

Developed in 1967, G. Fåhraeus introduced a device in which plants grow in between a glass slide and coverslip to allow the easy study of roots under the microscope (Fåhraeus, 1957).

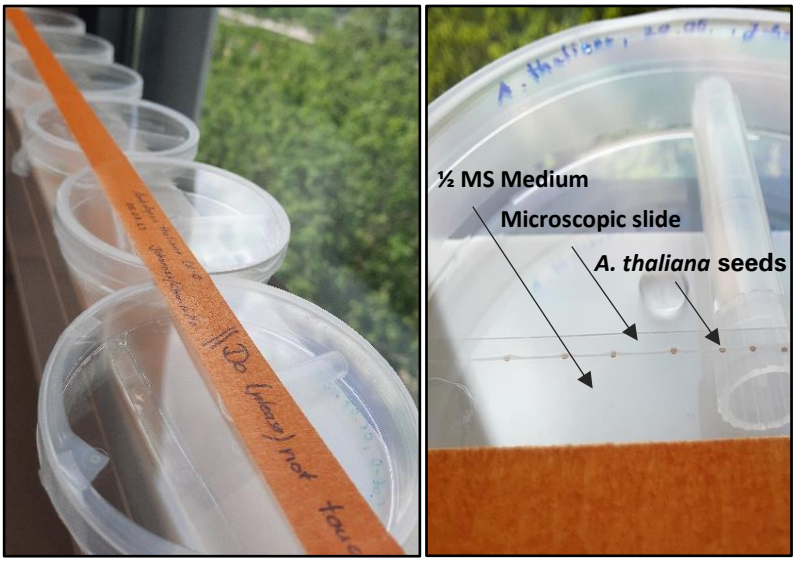

Figure 19: Fåhraeus slides with* A. thaliana *seedlings on window desk in MIB Lab, Helix (day of seeding). *Seeds are placed in between microscopic slide and cover slip. After one day, roots start growing into the ½ MS medium.*

Plant growth and root development were successful in Fåhraeus slides (see Figure 19). Concerning the root morphology, plants grew slower than in the ½ MS10 Agar, resulting in roots with a length of approximately 5 mm after one week. Fåhraeus slides-grown plants showed a promoted development of root hairs as compared in panel A and B in Figure 20. Moreover, fungal infection was detected in Fåhraeus slides (see Figure 20, C). Several iterative cycles of disinfection of both plant seeds and equipment were needed to avoid fungal contamination. Disinfection of seeds with hypochlorous acid as well as sterilisation of Fåhraeus slides in 70 % ethanol for 1 hour was proven effective in avoiding fungal infections.

Regarding the feasibility of microscopic studies, Fåhraeus slides are considered a non-invasive way to study the plant root systems under the microscope as roots already grow in between glass slide and coverslip. In consequence, stressful transfer of the plants from growth media to microscopic slides is not needed.

Furthermore, a Fåhraeus slide can carry 12-15 plants and therefore ease the cultivation of many plants at once.

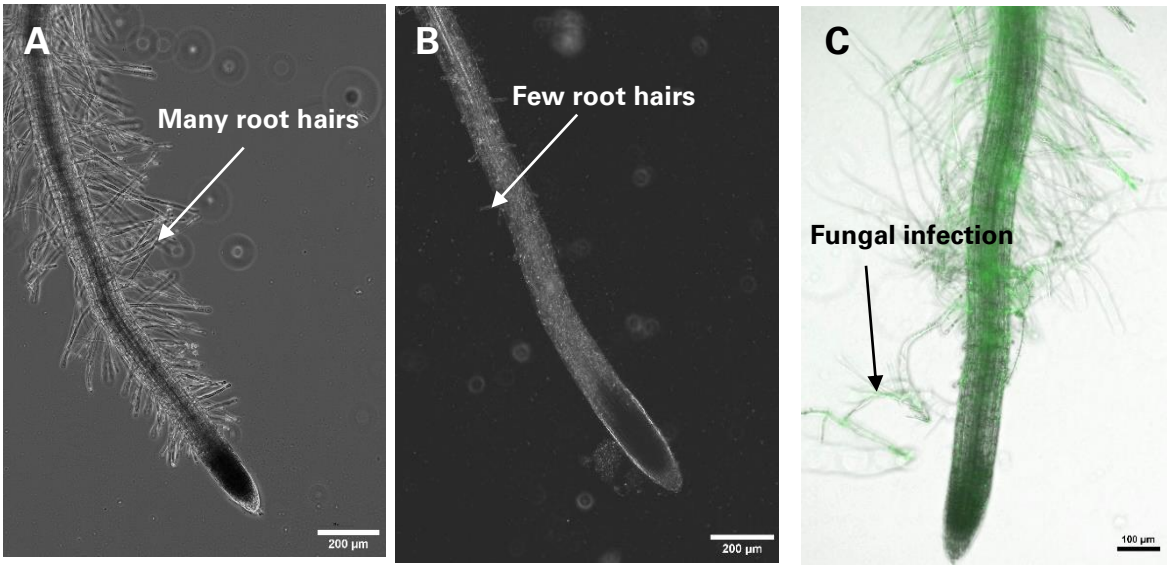

Figure 20: Microscopic pictures of A. **thaliana** ***roots grown in different conditions (100x magnification).*** *A: Phase contrast picture,* A. thaliana *root grown in Fåhraeus slides (6 days from seeding, phase contrast, pH1 objective-lens). B:* A. thaliana *root grown on ½ MS10 Agar on a vertical Petri dish (8 days from seeding, Phase contrast, pH3 objective-lens). C: Merged Channel picture,* A. thaliana *grown in Fåhraeus slides.*

3.3.4 Summary

All three tested growth conditions allowed root growth of *A. thaliana*. Table 6 summarises the strengths and limitations of the applied growth conditions in terms of (1) root morphology, (2) feasibility for subsequent experiments and (3) efficiency of plant cultivation.

Table 6: Overview of strengths and weaknesses of tested growth conditions for colonisation and secretion experiments. *"+" indicates positive attributes whereas "-"denotes areas of improvement. Growth of* A. thaliana *was examined.*

Growth condition	Root morphology	Experimental feasibility	Ease of cultivation	Remarks
Garden Soil	no data	-	-	Simulates natural environment
½ MS10 Agar				
1. Plastic Box	+	+	-	Few plants can be cultivated in one box
2. Vertical Petri dish	+	+	+	
3. Vertical Petri dish with cellophane	+	+	-	Cellophane offers no benefits
Fåhraeus slides (½ MS Medium)	+	+	+	Thorough sterilisation is advised.

In summary, growth in Fåhraeus slides was chosen for the colonisation experiment, as colonisation was investigated over several days in which the same plant was repeatedly inspected under the fluorescence microscope. The inconvenience of showing an abnormal number of root hairs is made up by its optimal design to investigate roots under the microscope.

The results showed that *A. thaliana* develops larger roots when grown in ½ MS10 Agar. Larger roots release in general more root exudates than small roots (Inderjit & Weston, 2003). A high concentration of root exudates is a beneficial property to see the inductive capacity of *A. thaliana* on root exudate sensitive promoters. Therefore, growth in vertical Petri dishes was selected for the FRUIT-induction experiment.

3.4 Colonisation of *P. fluorescens* on the Root of *A. thaliana*

A microscopic approach was employed to investigate the colonisation behaviour of the bacterium *in vivo*. The results were crucial to obtain a comprehensive understanding of how the bacterium interacts with plant roots and the rhizosphere. Further, it gave valuable insights for the subsequent secretion experiment.

The outcomes of the microscopic investigations were compared to the results of an agent-based colonisation model made by the iGEM teammate Zhongyuan Lu. *Arabidopsis thaliana* was utilized as the plant model to explore the behaviour of *P. fluorescens* within the root system for its characteristics of being well-characterized, fast-growing, and easy to handle.

First, it was investigated whether *P. fluorescens* attaches to the roots or remains in the rhizosphere of *A. thaliana.* Second, specific root tissues like root hairs or emerging lateral roots were studied to see if the bacteria have preferred attachment sites. Finally, experimental results were compared to the agent-based colonisation model.

For the colonisation experiment, plants were grown in Fåhraeus slides in ½ MS medium for 5-7 days. At this point, the roots were developed, and the main structures as shown in Figure 21 were visible.

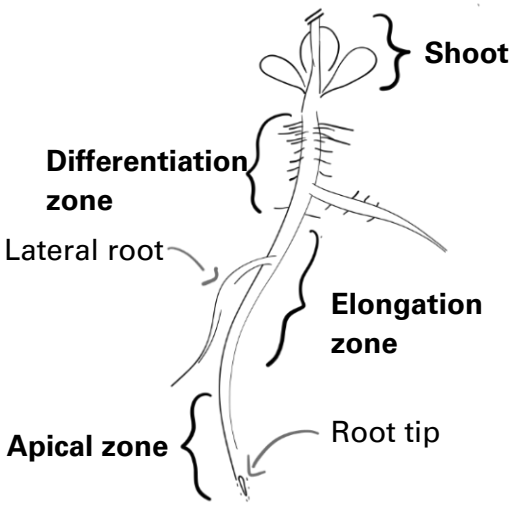

***Figure 21: Main structures and tissues of the root system from* A. thaliana.**

3.4.1 *P. fluorescens* Colonises Roots of *A. thaliana*

It was investigated whether *P. fluorescens* colonises the roots of *A. thaliana* and to which extent the bacteria remain in the rhizosphere. The findings were pivotal for subsequent experiments, as root colonisation is a prerequisite for protein secretion into the epidermis.

Root attachment of *P. fluorescens* occurred from day 0 onwards (Figure 49, Appendix). Two days after exposure to *P. fluorescens*, the roots of *A. thaliana* were uniformly colonised with bacteria. Examinations on a 100x magnification showed that fluorescent bacteria had affixed themselves to the root, with only a few bacteria existing in the rhizosphere (see Figure 22, A). When employing a 1000 x objective with a higher magnification strength, it could be discerned that a minority of bacteria (ca. 10 %) were not bound to the plant's surface but actively swimming within the surrounding medium, as shown in Figure 22, B. Bacterial quantity further decreased in plant remote areas.

In conclusion, two days upon incubation the majority of *P. fluorescens* colonised the root epidermis with a fraction of bacteria remaining in the rhizosphere.

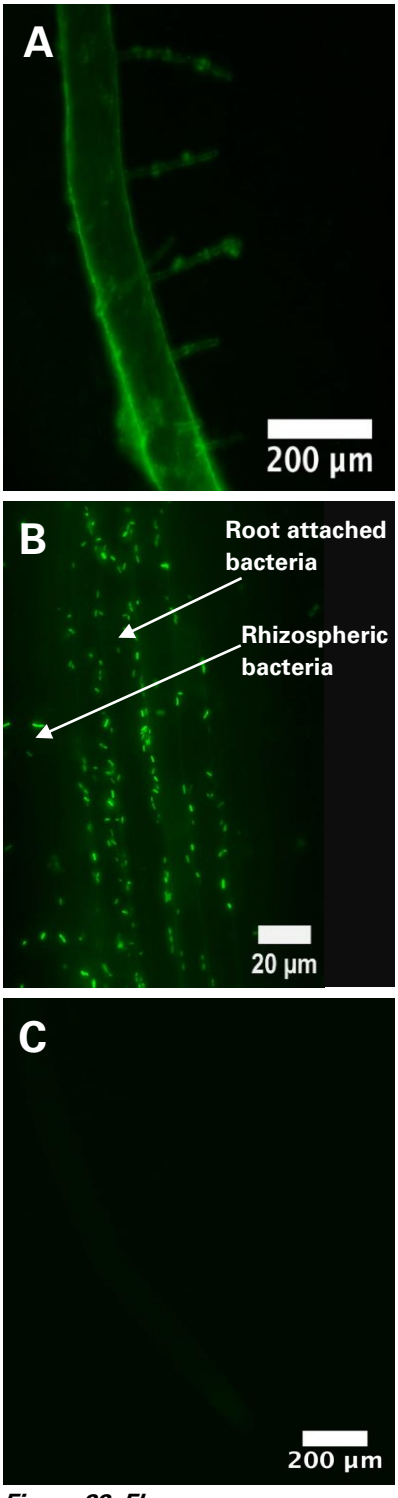

Figure 22: Fluorescence microscopy picture with* gfp-*expressing* P. fluorescens *and* A. thaliana *root (28 hours upon incubation). *A. 100 x magnification, bacteria are evenly attached to the root epidermis and root hairs. B:1000 x magnification, single cells are attached to the root or move freely in the rhizosphere. C: negative control with wild type P. fluorescens.*

3.4.2 *P. fluorescens* Does Not Exhibit Preferred Structures for Attachment or Microcolony Formation

Plant physiologist Prof. Wouter Kohlen from Wageningen University emphasised that rhizospheric bacteria often exhibit a preference for specific regions of the roots to form colonies. For example, plant pathogenic *Bacillus megaterium* infect and colonise roots at the junction of primary and lateral root of maize species (Liu et al., 2006). Therefore, attachment sites of *P. fluorescens* to the root surface were studied.

The microscopic studies showed that bacteria evenly distribute across the root epidermis and root hairs, without displaying a specific preference for particular root structures. In the 1000 x magnification, it was observed that bacteria do not attach on top, but mainly in the groove in between the epidermal cells (Figure 23). Three days after the first attachment, the formation of microcolonies was visible on different parts of the roots, indicating the start of a biofilm formation (see Figure 23).

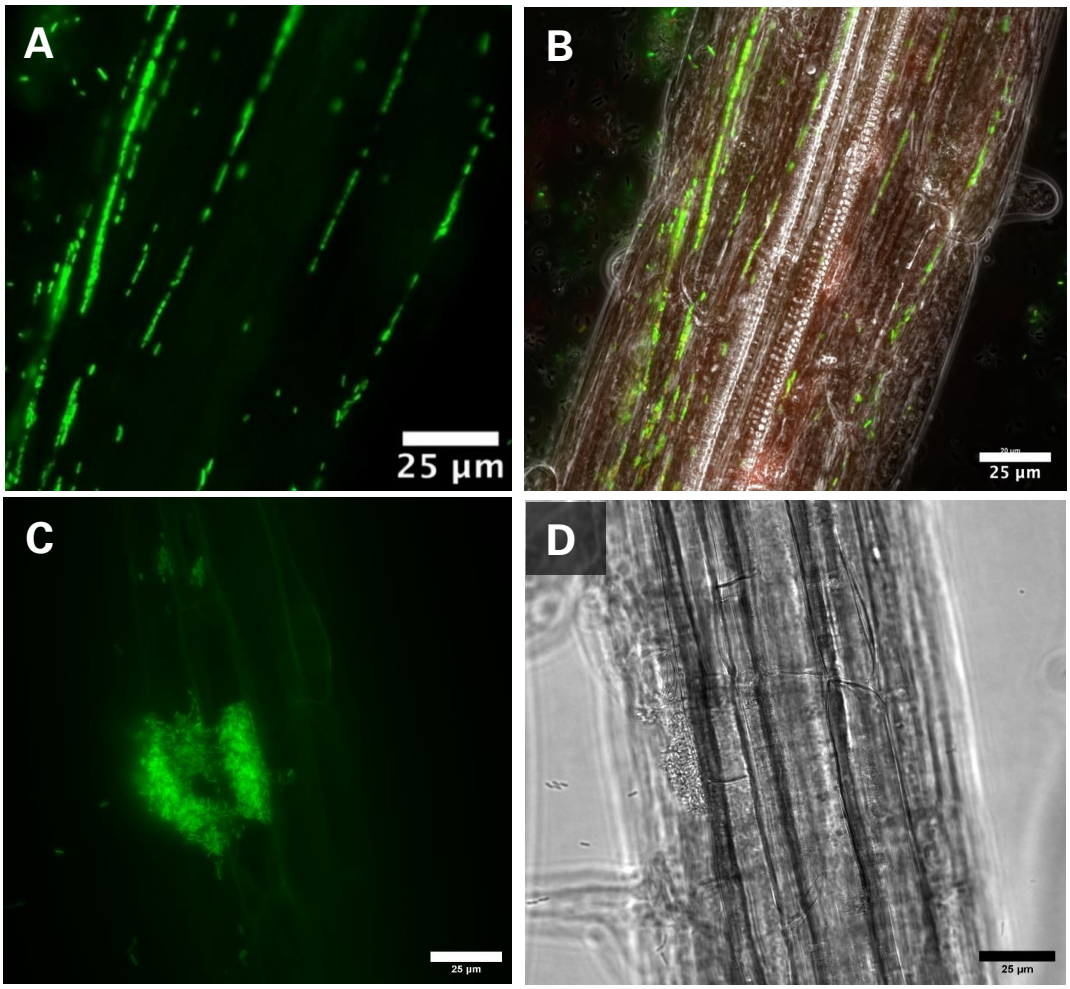

Figure 23: Microscopy image showing attachment of* P. fluorescens *on epidermic cells of* A. thaliana *roots (1000 x magnification) *A. fluorescence microscopy picture of* P. fluorescens *attached in grove of epidermic cells, 1 day after attachment; B: Merged Channel picture of A. C: Fluorescence microscopy image, microcolony formation (3 days after first attachment). D: Phase contrast picture of C.*

Overall, there are no discernible patterns observed for attachment or microcolony formation. Specific root tissues, such as root hairs or emerging lateral roots did not show increased quantities of bacteria.

3.4.3 Experimental Results Coincide with Agent-Based Colonisation Model

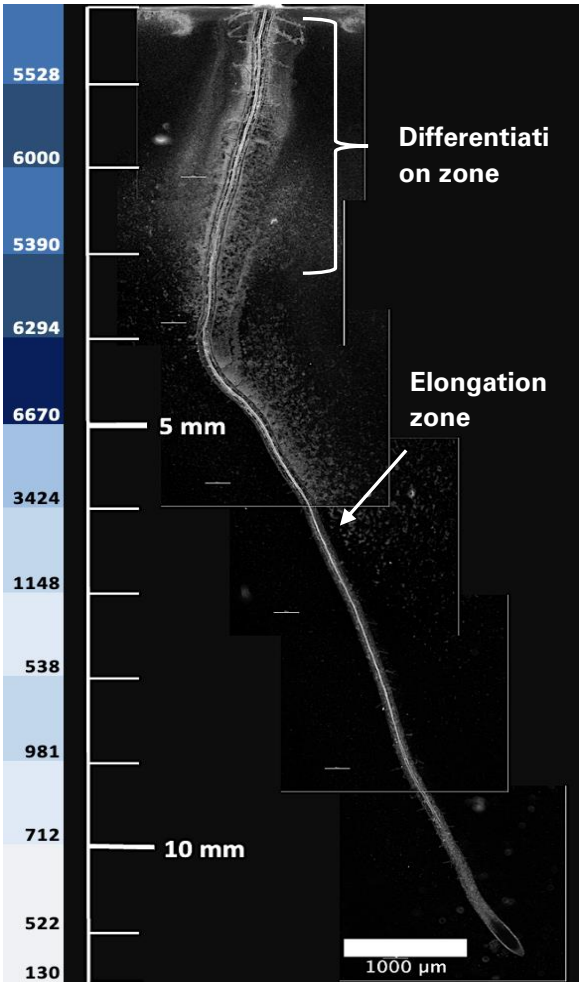

Figure 24: Overview of* A. thaliana *root, bacterial colonisation over length of whole root. *Pictures is merged from 6 phase contrast, pH3, 100× magnification images, 1 day after first attachment. Bacteria are white structures around the root. A heat map on the right site shows the measured number of bacteria per millimetre.*

An agent-based model was programmed by iGEM teammate Zhonguan Lu (2023) to predict the colonisation of *P. fluorescens* on plant roots. The model predicted that colonisation mainly occurs in the basal part of the root with decreasing quantities of bacteria towards the apical root region. To cross-validate the agent-based model to experimental measurements of colonisation of *P. fluorescens* on roots, bacterial quantities of *P. fluorescens* on roots of *A. thaliana* were analysed two days after attachment.

The microscopical pictures show that approximately ¾ of all bacteria attach to the basal region of the root, with diminishing numbers of bacteria towards the root tip. In Figure 24 and Figure 25 two representative roots are displayed. One can see the change in bacterial quantities when going down the root.

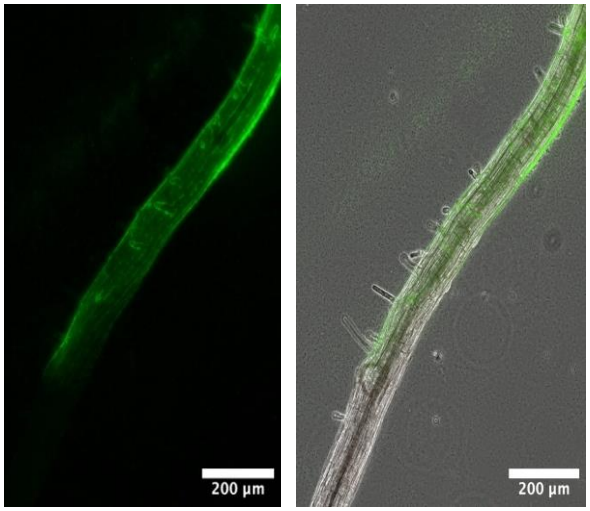

Figure 25: Fluorescence microscopic (left) and merged channel (right) picture of* P. fluorescens *attached to the elongation zone of A. thaliana (2 days after incubation, 100× magnification). *A decrease in green fluorescent bacteria towards the apical site is detected.*

The decrease of bacteria could best be seen when looking at the whole root system from the basal to the apical site. Under the phase contrast microscope equipped with a pH3 objective that creates an optimal visibility of bacteria. In phase-contrast microscopy, bacteria appear as discernible white spots, while the roots themselves are mostly imperceptible, except for a white outline in the stele - the core tissue of the root containing the root vascular tissue. Employing these settings (phase contrast, pH3), six individual images were captured and combined to create an overarching representation of the entire root (see Figure 24). The

overview picture was analysed with imageJ in two ways. First, the brightness of the microscopic pictures of every millimetre from basal to apical root part was determined (see Figure 50, Appendix). The degree of brightness in the image correlates with the abundance of bacteria in the rhizosphere. Second, the quantity of single bright particles was analysed, corresponding to the number of bacteria. The results are shown in a heat map with the according values on the left side of Figure 24. Highest bacterial quantities are observed in the differentiation zone with diminishing numbers of bacteria at the elongation zone of the roots. In the basal half of the root (<5 mm), 29 882 bacteria were counted, whereas in the apical region (> 5 mm) 7 455 bacteria remained (Figure 24).

Overall, experimental results on the spatial distribution of *P. fluorescens* coincide with patterns that were predicted by the agent-based colonisation model (see Figure 26).

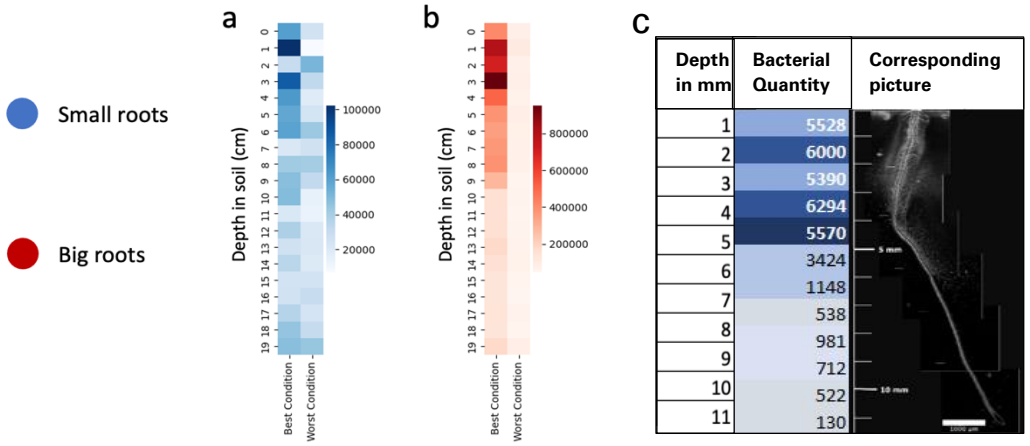

Figure 26: Spatial distribution of* P. fluorescens *on roots*. a & b: results from agent-based model, high quantities of bacteria are expected in the first few centimetres below the surface, with decreasing numbers of bacteria in deeper soil layers, both in small (a) and big (b) roots. c: Experimental results. Bacterial quantities in different parts of the microscopic picture of a one-week-old long* A. thaliana *root, 1 day after inoculation with* P. fluorescens.

3.5 Secretion of GFP into *Arabidopsis thaliana* via Type-3-secretion system

P. fluorescens etHAn is capable of injecting toxic effector proteins into leaves of crop species like wheat (Upadhyaya et al., 2014). Until present, no investigations have explored the secretion of GFP into the roots of *A. thaliana*. Therefore, microscopic examinations were conducted on both live roots and fixed root sections to detect plant-intracellular, thus secreted, GFP.

To this end, GFP with a T3SS-specific secretion signal (GFP-SS) was introduced to the pSEVA64 vector and transformed into *P. fluorescens* etHAn. *Rfp* is expressed as a second protein under the same promoter in an operon-like manner. RFP does not carry a secretion signal and was used as a control to localize the bacteria and distinguish secreted GFP secretion from RFP/GFP-producing bacteria (see Figure 27).

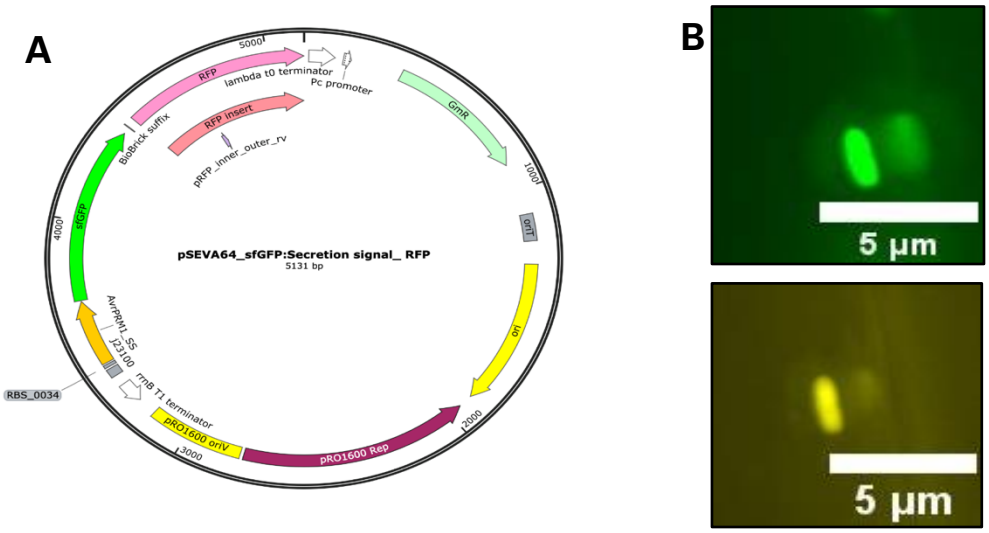

Figure 27: Plasmid map of pSEVA64 for integration into* P. fluorescens *and microscopic image of corresponding bacteria. *A: Plasmid map,* gfp-*SS and* rfp *under the control of the strong constitutive promoter J23100. B: Fluorescence microscopy pictures of* P. fluorescens *etHAn on root surface, 1000x magnification. Upper panel: GFP excitation at λ=485 nm. Lower panel: RFP excitation at λ=555 nm.*

3.5.1 Preliminary Considerations

The results of the colonisation experiment were implemented in the design of the following secretion experiment. First, since colonisation initiates from day one, microscopic examination of secreted GFP within plant tissue commenced on day two to allow sufficient time for the secretion process after attachment (B. J. Kim et al., 2009). Second, *P. fluorescens* colonises mainly in epidermal grooves but was also visible on other root structures, including the root hairs. Therefore, particular attention was directed towards root hairs, given their unicellular and transparent nature where intracellular GFP secretion was anticipated to be most visible. Lastly, recognizing that the basal root region harbours the majority of bacteria, plant material from this region is used for cross-sectioning in the vibratome experiment.

3.5.2 Living Roots Expose no Bacterial GFP

Plant-microscopy expert Norbert de Ruijter, the manager of the Wageningen Light Microscopy Centrum, advised to first study the injection of GFP from attached bacteria to the epidermis on whole, living roots, particularly at root tissues like developing lateral roots and the transparent root hairs. First microscopic studies showed that the roots of *A. thaliana* remain transparent when grown in ½ MS medium (Figure 28). This allowed the detection of intracellular GFP without damaging the roots. Even inner root structures like the stele where xylem and phloem transport take place can be monitored.

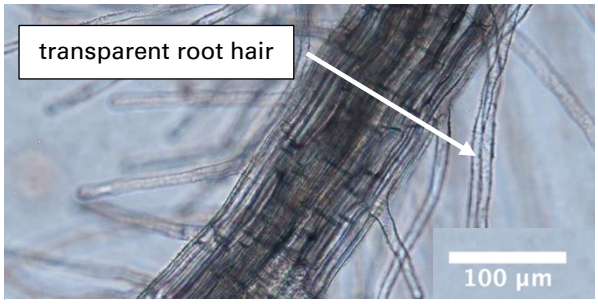

Figure 28: Phase contrast picture of* A. thaliana *Col-0 root, one week after germination (400x magnification). *The transparent cells allow the easy study of epidermal cells with root hairs as well as an emerging lateral root and the internal stele.*

As shown in Figure 29, bacteria expressing *P. fluorescens* attached to root hairs of *A. thaliana*. However, no visible accumulation of GFP inside the root hairs was detectable. The primary root showed elevated fluorescence, the same region with settings adjusted to the RFP, displays similar patterns of fluorescence (see Figure 29, B). These findings led to the hypothesis that the roots of *A. thaliana* produce autofluorescent substances that are nonrelated to the potentially secreted GFP from *P. fluorescens*. The fluorescence microscopic approach was unable to differentiate between root autofluorescence and GFP.

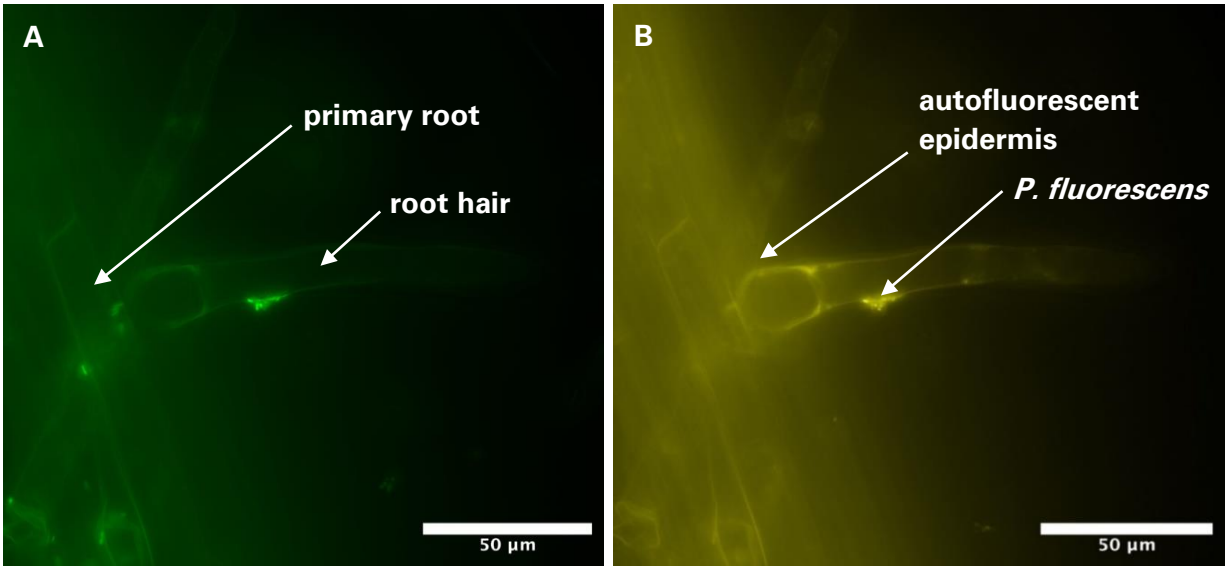

Figure 29: Fluorescence microscopic picture of* P. fluorescens *attaching to root hair of* A. thaliana *(1 day after first attachment, 1000x magnification). *A: GFP-SS producing, and because of the secretion signal also potentially secreting colony of* P. fluorescens *attached to root hair (exposure time 1sec, ext: λ=485, ems: λ=510) B: Same picture showing fluorescence settings for RFP (exposure time: 3 sec, ext: λ=555, ems: λ=583).*

To obtain more informative results regarding the injection of GFP from bacteria into plant cells, vibratome sections with a thickness of 25-50 µm were made. In the previous colonisation experiments, it was determined that bacteria mainly attach to the upper

part of the root. Assuming that all bacteria secrete similar amounts of GFP into the plant, it has been anticipated that in the densely colonised basal parts of the root, more GFP is secreted into the roots compared to rarely colonised parts such as the root tip. Therefore, plant material of the basal root regions was used for vibratome sectioning.

Using cross-sectional and longitudinal sections, the internal structures of the plant were microscopically examined, with a special focus on fluorescent structures that might be caused by GFP secreted by bacteria.

3.5.3.1 Longitudinal Root Section

Both the fluorescence and the multichannel images in Figure 30 depict green *P. fluorescens* colonies adhering to the root epidermis of *A. thaliana*. However, it can be noted that there is neither observable secretion of GFP from the bacteria into the epidermis, nor any evident accumulation of a green fluorescent signal within the phloem of the root.

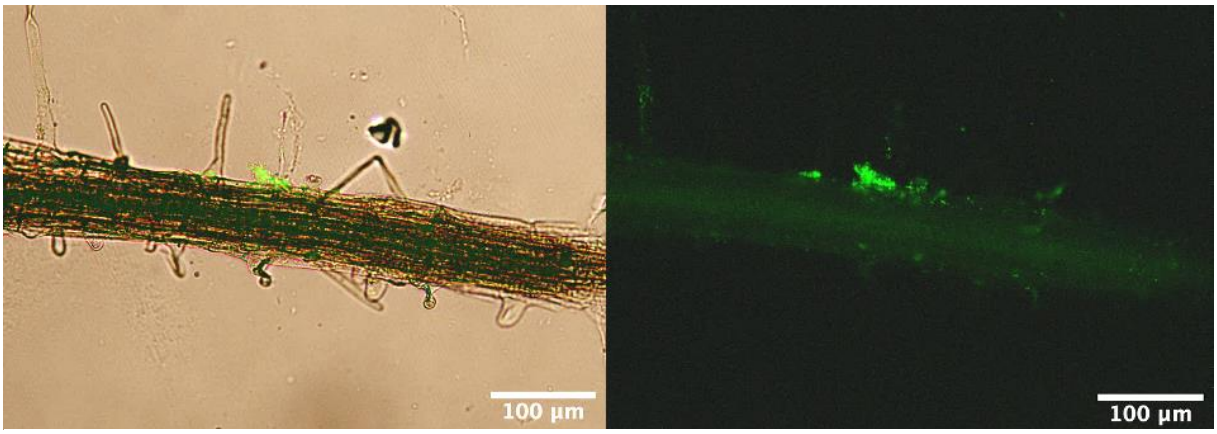

Figure 30: Longitudinal vibratome section of* A. thaliana *root with GFP producing* P. fluorescens *(thickness: 50 µm). *Left: Multi-channel picture with green bacteria attached to root-epidermis. Right: Fluorescence microscopy picture, same section shown as on the left image.*

3.5.3.2 Cross section

Cross-sections of the roots showed high quantities of bacteria coated to the epidermis, but epidermal GFP remains below the detection limit. The cell wall of the epidermis showed faint green fluorescence. However, the intensity was similar to what was seen in the negative control. Green fluorescent structures were visible on the right side of the outermost layer of the plant's vascular tissue (see Figure 31, B). However, cross-sections of the negative control showed similar patterns of fluorescence in the stele (see Figure 31, D).

Therefore, no conclusions could be made of whether the green fluorescent structures observed at the stele are a result of GFP being secreted into this region or if they originate from natural plant compounds.

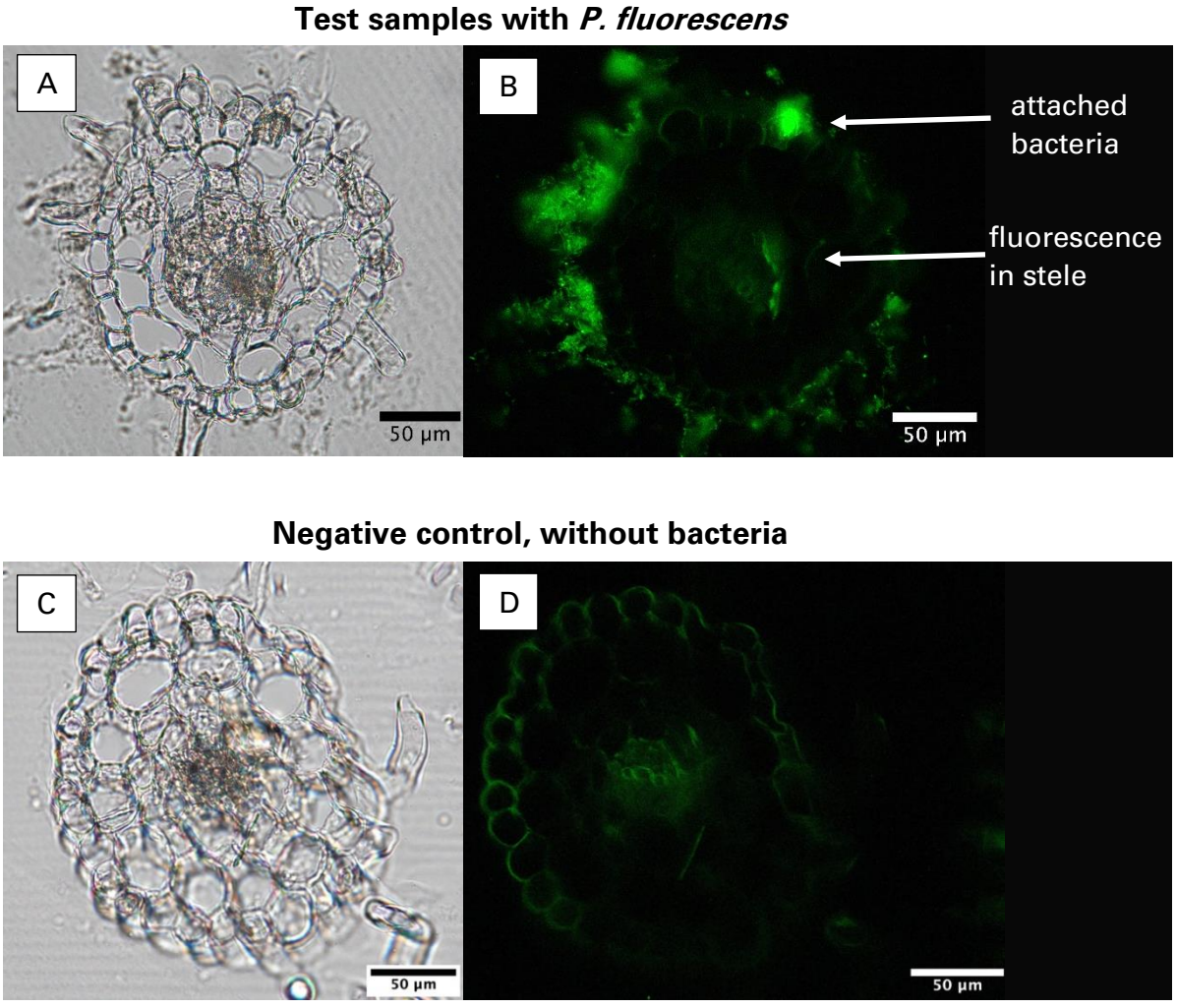

Figure 31: Phase contrast-(left) and fluorescence microscopy (right) picture of A. thaliana root cross sections (thickness: 25µm, 200x magnification). *A+C: Phase contrast pictures. The main structures of the root are visible such as root hairs, epidermis, cortex, and the slightly brown stele. B: Fluorescence microscopy picture. Bacteria are visible as green dots surrounding the epidermis, no major accumulation of GFP is visible inside the epidermis. Green fluorescent structures are visible in the stele. D: Fluorescence microscopy picture. Autofluorescent structures in epidermis and stele.*

3.5.4 Silver Staining Reveals no Bacterial GFP

The inability of fluorescent microscopic techniques to detect GFP inside plant tissue led to the design of an alternative, biochemical detection method.

3.5.4.1 Root Inspection

To avoid false positive results, roots were washed using sodium hypochlorite and isopropanol and subsequently inspected under the microscope before and after the wash procedure to ensure that all bacteria were removed. The wash procedure was effective in removing bacteria from the roots and their environment (Figure 32, C & D). Further, the removal of bacteria did not visibly damage the root itself. Even the delicate root hairs remained intact (see Figure 32, C).

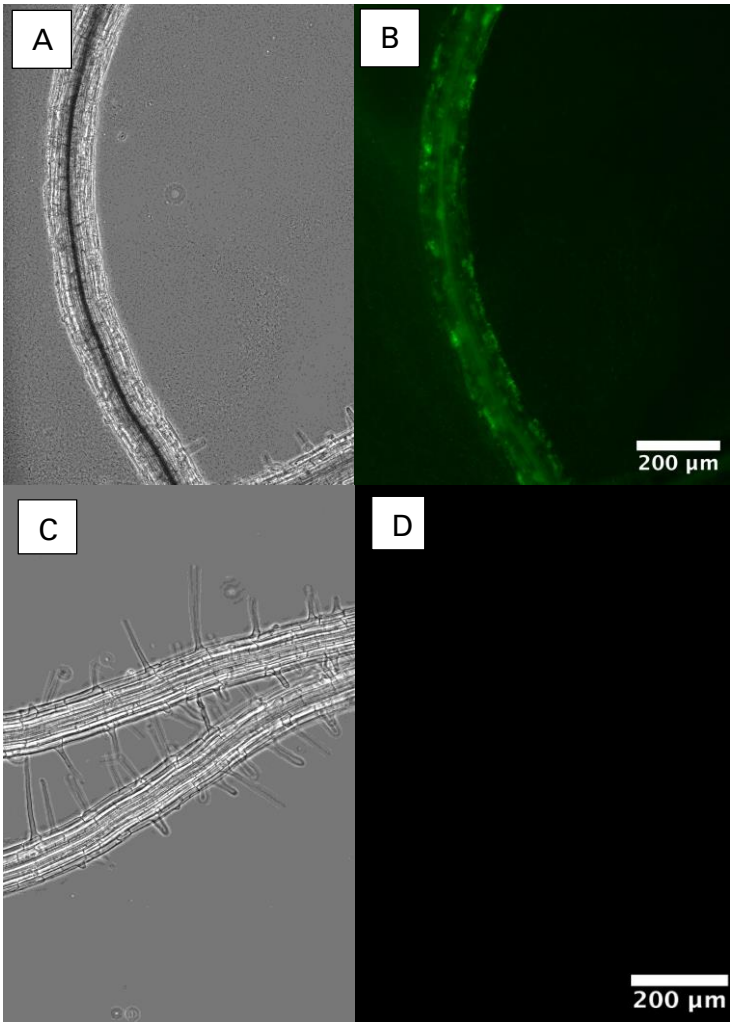

Figure 32: Microscopic pictures of roots before and after removal of bacteria. *A: Phase contrast picture, bacteria are visible as black dots. B: fluorescence microscope picture, bacteria are green fluorescent. C: Phase contrast picture after washing, no bacteria are discernible. D: Fluorescence microscope picture after washing, no fluorescence is visible.*

3.5.4.2 Gel Inspection

Silver stained proteins were studied on two SDS gels. First, the isolated proteins of bacteria-inoculated plants and the bacterial proteins were inspected. No bands for the plant lysate were detected (see Figure 33, left image). Protein bands at the length of the ladder were not considered plant-derived. The bacterial lysate showed many bands with typical patterns of a cell-derived proteome. Column 1, respective for cells expressing but not secreting GFP showed a band at 27 kDa, the molecular weight of GFP (see Figure 33, left picture, bacterial lysate, 1). As for the gel with His-purified proteins, no bands of His-tagged GFP at the molecular weight of 27 kDa were discovered (see Figure 33, right image).

In conclusion, insufficient amounts of proteins from plant lysate were released to be analysed using a silver staining method. A band at the molecular weight of GFP was only visible for the 1 sample of bacterial cell lysates. Bands for GFP were neither discernible in the columns of plant lysate nor for the His-purified samples. Therefore, the secretion of GFP from *P. fluorescens* etHAn into the roots of *A. thaliana* could not be confirmed.

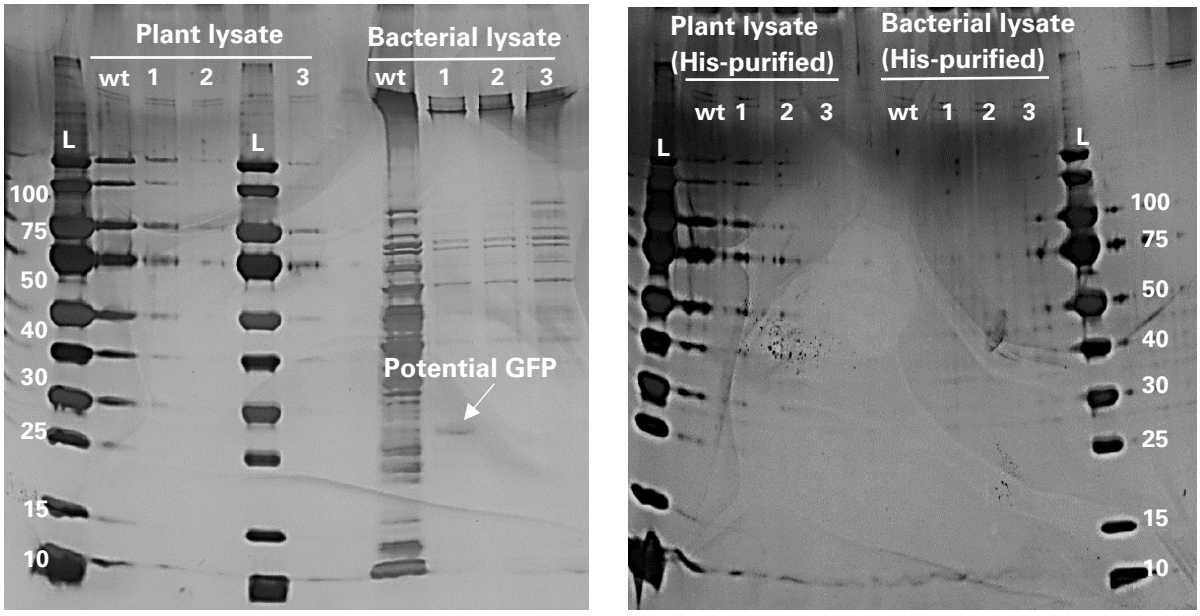

Figure 33: Silver stained SDS-gels with different protein samples. *Left image: proteins of plant lysate, 1 day after incubation with* P. fluorescens *etHAn. Left image: His-purified GFP from plant- and cell lysate. No clear band is discernible apart from the ladder. wt:* P. fluorescens *wild type, GFP is neither expressed nor secreted. 1: GFP is expressed, but not secreted (lack of T3SS secretion signal). 2&3: GFP is expressed and potentially secreted. Right image: Respective samples after His-purification. Numbers next to the ladder (L) indicate proteins in their respective size in kDA.*

3.6 FRUIT: Fluorescence Root-exudate Unbiased *In-vivo* Test

While plate reader assays are commonly employed in studying inducible systems in synthetic biology (Chavez et al., 2017), they often require bacteria to be in a transparent suspension. However, these conditions may not accurately reflect the behaviour of bacteria in their natural environment.

The objective was to address limitations associated with plate reader experiments when measuring inducible expression systems on solid surfaces. Fluorescence microscopic techniques from prior experiments were implemented to measure the inductive capacity of root exudates from *A. thaliana* on four different inducible expression systems in *P. fluorescens*. The applied procedure was combined into the self-developed fluorescence root exudate unbiased *in-vivo* test, short: FRUIT. FRUIT is an optimised data-analysis protocol to measure the inductive capacity of solid surfaces on promoter systems.

The FRUIT protocol consists of two parts, (1) a qualitative study under the fluorescence microscope and (2) the transformation of qualitative into quantitative data, including statistical analysis.

In the presented experiment, the inducible expression systems pCym/CymR, pTtg/TtgR pBAD/AraC and pSal/nahR were tested upon their activation by root exudates of *A. thaliana*. Inducible expression systems are transcription factor/promoter arrangements that allow the gene regulation upon a certain inducer. In the presented case, the inducer is root exudates (e.g. cuminic acid, arabinose & salicylic acid). Salicylic acid is commonly found in the rhizosphere of *A. thaliana* (Strehmel et al., 2014a). The respective inducible expression system that is activated in the presence of salicylic acid is pSal/nahR. The palL/nahR sample is presented in the subsequent chapter.

3.6.1 Qualitative Studies Reveal Autofluorescence in Root and Local Patterns of Induction

3.6.1.1 Autofluorescence

Table 7: Mean autofluorescence in the stele of living and fixated roots. *Fixation in 4% paraformaldehyde resulted in a high autofluorescence in the stele of* A. thaliana *roots.*

Living Root:	Autofluorescence in stele (A.U)	Mean
Sample 1	18,7	**10,13**
Sample 2	1,5	
Fixated Root:	Autofluorescence in stele	Mean
Sample 1	552	**503,95**
Sample 2	455,9	

An initial qualitative assessment with fluorescence microscopic pictures was performed. It was observed that the stele of fixed roots emits higher fluorescence than live roots (see Figure 34). An ImageJ analysis of the fluorescence values in the stele region shows that after removal of the background fluorescence, the fixed roots were 50x more autofluorescent than living roots (see Table 7). To prevent inherent differences in autofluorescence between experimental and control samples from affecting the measurements, respective autofluorescence values were systematically removed from the stele region in the corresponding samples, as outlined in "Material & Methods".

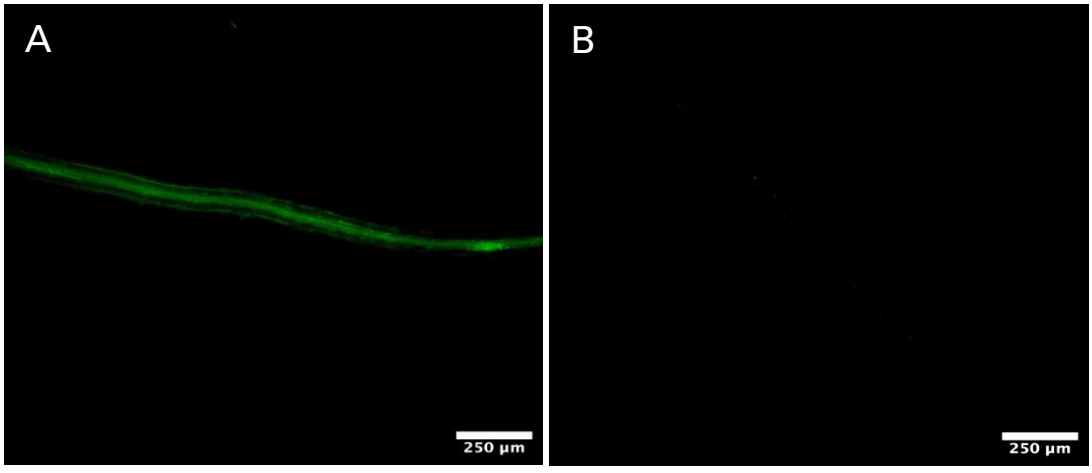

Figure 34: Fluorescence microscopic picture of* P. fluorescens *(non* gfp*-expressing) attached to roots of* A. thaliana *(1 day after first attachment). *A: Root that was fixated in 4 % paraformaldehyde for 1 hour with wild type* P. fluorescens*. B: Non-fixated, living root of* A. thaliana *with wild type* P. fluorescens.

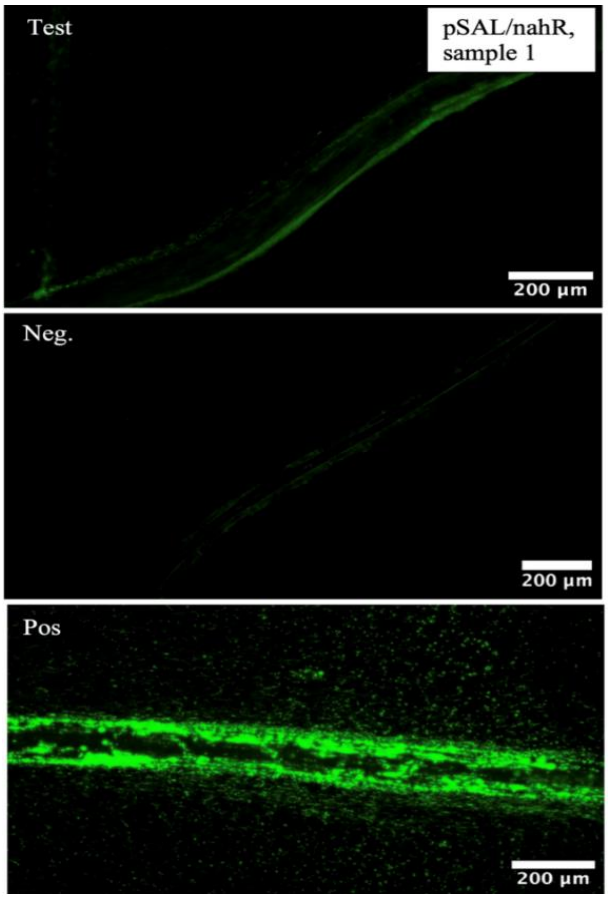

Figure 35: Fluorescence microscopy picture of* P. fluorescens *(pSAL/nahR) root-sensitive bacteria with roots of* A. thaliana *(100x magnification, 1 day after inoculation). *Test: Bacteria with living, exudate-secreting roots. Neg.: Bacteria with fixated, non-secreting, roots. Pos: Bacteria with fixated roots, in presence of 1 mM salicylic acid.*

3.6.1.2 Qualitative Assessment of Induction

The fluorescence microscopy pictures revealed that the strain harbouring the inducible expression system pSal/nahR was induced in response to live *A. thaliana* roots as shown in the upper panel of Figure 35.

For the negative and positive control samples (Figure 35, middle and lower panel) the concentration of root derived exudates was reduced as far as possible by fixating roots in 4 % paraformaldehyde. Paraformaldehyde stops metabolic processes and cross-links subcellular structures (Kim et al., 2017). Therefore, it is assumed that the fixated control groups do not produce root exudates anymore. In the middle panel of Figure 35, it is shown that the reduction of root exudates results in the decrease of bacterial fluorescence. The remaining fluorescence appeared to originate from autofluorescence in the root itself rather than from induced bacteria.

In the positive control group, for which 1 mM salicylic acid was added, induction occurred not only in bacteria directly attached to the root but also in the surrounding medium. This aligns with the fact that the synthetic inducer was added to the 1/2 MS medium and is not directly secreted by the root itself. Moreover, the fluorescence intensity, correlating to the induced expression of GFP, appeared to be stronger in bacteria of the positive control compared to the test sample, and negative control.

3.6.1.3 Local Induction Patterns

The fluorescence microscopy pictures not only give first insights into whether bacteria are induced by root exudates or not but also about local patterns of induction. Coinciding with observations from the pSal/nahR inducible expression system, bacteria carrying pBAD/AraC showed GFP expression when being attached to the root surface but not in the distant surroundings of the roots (see Figure 36). This indicates that a certain threshold concentration of root exudates is needed to induce the tested inducible expression systems and initiate *gfp*-expression. Presumably, this threshold concentration is only found at the direct proximity of the roots.

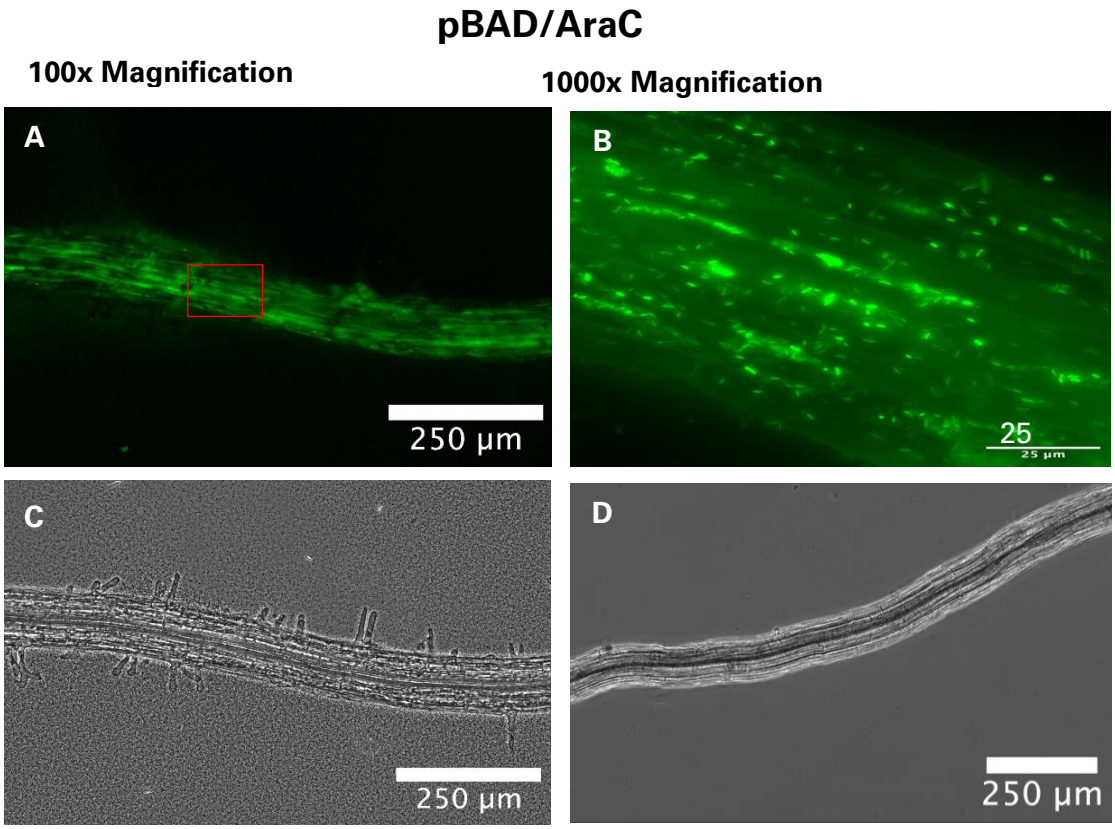

Figure 36: Microscopic images of* P. fluorescens *carrying the pBAD/AraC inducible expression system attached to living roots of* A. thaliana *(6 hours after bacterial inoculation). *A: Fluorescence microscopy overview picture (100x); bacteria are mainly fluorescent when attached to the roots. B: 1000x magnification of region indicated by red box in A. Induced bacteria are attached to the root. C: Phase contrast picture of sample shown in A (100x). Bacteria are visible as dark spots everywhere in the root surrounding medium. D: Phase contrast picture (100x) of* A. thaliana *root without bacteria. An image of the uninduced negative control can be found in the appendix (Figure 52).*

In general, this qualitative study provided first impressions of whether bacteria are induced by root exudates of living roots. Bacteria were mainly induced when being attached to the root with less induction in plant remote areas.

3.6.2 ImageJ Analysis of Microscopic Pictures Demonstrate that pSal/nahR Samples are Significantly Induced by *A. thaliana* Root Exudates

Fluorescence pictures of *P. fluorescens* with root exudate inducible expression systems on living roots were analysed in FIJI/ imageJ as described in the Materials & Methods. The respective imageJ macro can be found in Figure 48, in the appendix.

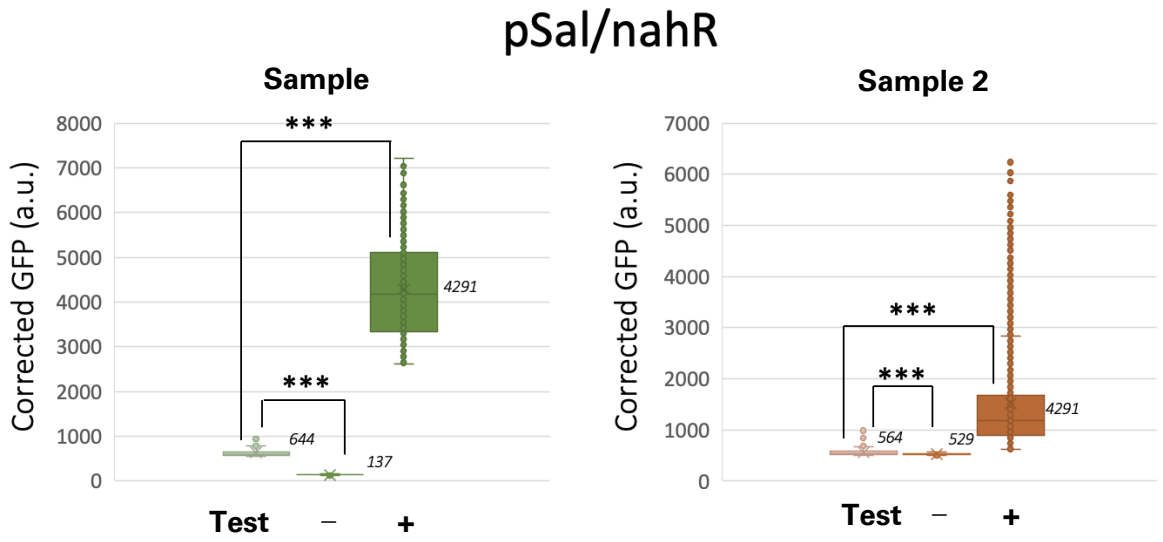

Figure 37: Fluorescence response of FRUIT-measured image analysis after 24 hours of* P. fluorescens *with pSal/nahR inducible expression system cultivated on* A. thaliana *roots (two representative samples). *Test: bacteria with living, exudate secreting roots."-": Negative control, bacteria with fixed, non-secreting roots. "+": Positive control, bacteria with fixated roots, in presence of 1mM of salicylic acid. *** indicates a student t test p-value < 0.001.*

FRUIT quantitively showed that the pSal/nahR inducible expression system can be activated by live roots. Higher fluorescence in the sample containing live roots was detected compared to the negative control (see Figure 37). Both, test samples and negative controls show fluorescence values below the positive control, matching the qualitative observations from Figure 35.

Students' t-tests were applied to the data matrices resulting in significant differences in fluorescence between all three samples (p-value < 0,001). However, it remains to be determined if these significance levels persist when the t-test is corrected for the large sample size. Moreover, one out of the three biological triplicates (sample 3) showed no expression of GFP, irrespective of the condition (see Figure 54, in the appendix).

No significant induction was observed in the other inducible expression systems including pCym/CymR, pTtg/TtgR and pBAD/AraC, (Figure 55-63, Appendix).

In summary, the inducible expression systems pSal/nahR, pCym/CymR, pTtg/TtgR, and pBAD/AraC were examined for their responsiveness to induction by root exudates from living *A. thaliana* roots. Two out of the three biological triplicates of the pSAL/nahR

inducible expression system exhibited elevated GFP expression in close proximity to *A. thaliana* roots.

3.6.2.1 Limitations of FRUIT

The shortcomings of the FRUIT processing protocol are demonstrated by the pTtg/TtgR samples. Living roots induce strong GFP production in bacteria with the pTtg/TtgR inducible expression system attached to the root surface (Figure 38, upper panel). Further, it is visible that few bacteria express *gfp* in the negative control. Medium levels of fluorescence are visible in the 1 mM naringenin-induced positive control (Figure 38, lower panel).

When analysed with FRUIT, no major difference in fluorescence between the three conditions is measured (see Figure 40). Ideally, FRUIT is supposed to measure the fluorescence intensity of every single bacterium. In practice, the FRUIT protocol identifies a group of bacteria that are attached to the root surface in the test sample as a single fluorescent particle (see Figure 39).

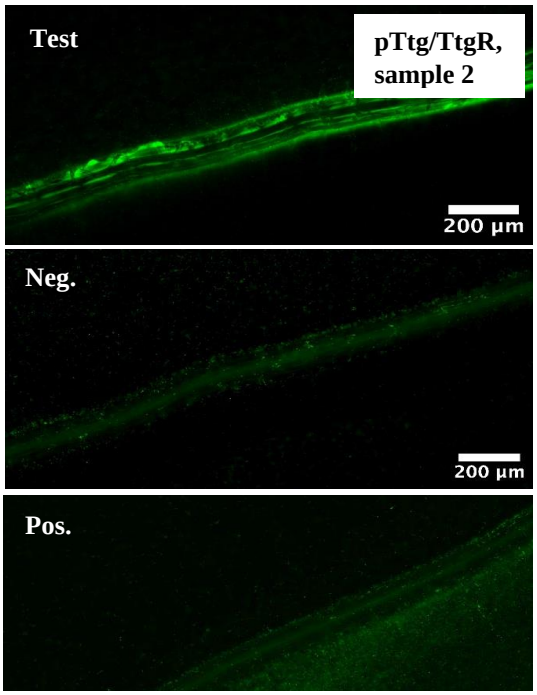

Figure 38: Fluorescence microscopy picture of P. fluorescens (pTtg/TtgR) root-sensitive bacteria with roots of A. thaliana (100x magnification, 1 day after inoculation). *Test: Bacteria with living, exudate-secreting roots. Neg.: Bacteria with fixated, non-secreting, roots. Pos: Bacteria with fixated roots, in presence of 1 mM Naringenin.*

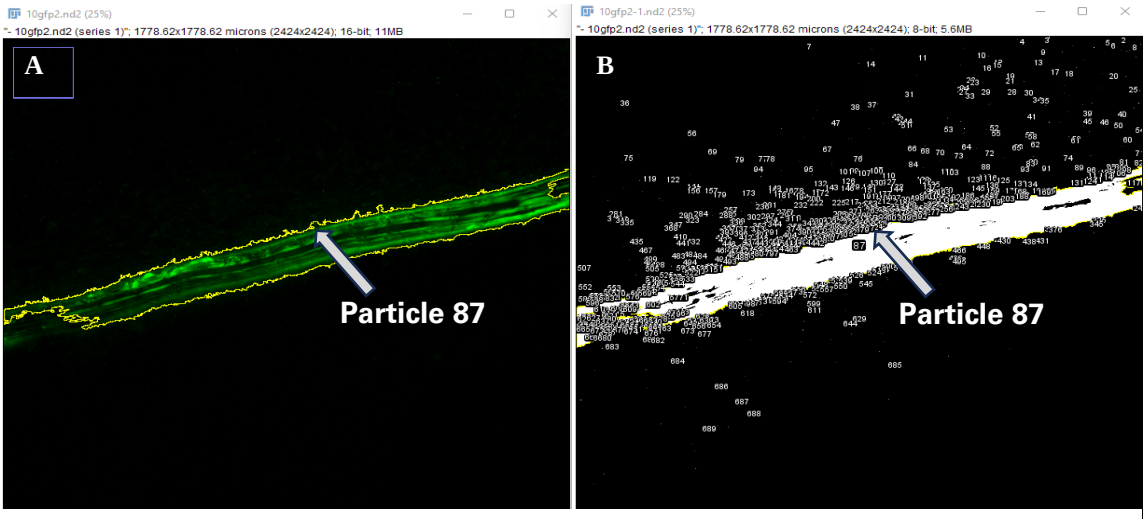

Figure 39: ImageJ screenshot from with pictures for pTtg/TtgR inducible expression system (sample 2, fluorescence microscopy, 100x magnification). *A: Fluorescence microscopy image with "particle 87" in selection. It is evident, that the particle does not represent a single bacterium but rather a major part of all root-attached bacteria. B: Mask of pTtg/TtgR sample with fluorescence threshold as described in the "Material and Methods". White points represent fluorescent particles.*

3.6.2.2 Alternative Image Processing Protocol

The limitations of FRUIT can be addressed and counteracted by measuring the mean fluorescence of the entire captured fluorescence picture instead of the mean fluorescence of a single bacteria.

In the previous section, samples of the pTtg/TtgR inducible expression system were presented. These samples are now processed to exemplify to which extent the image processing can support different statements.

A visual inspection of the fluorescence microscopic pictures with the pTtg/TtgR samples showed that bacteria are induced in the presence of root exudates from *A. thaliana* (see Figure 38). The mean fluorescence of the entire fluorescence microscopic picture for the pTtg/TtgR test sample supports this observation and shows fluorescence values that are twice as high when compared to the control samples, which are not induced by root exudates (Figure 40, left graph). However, when the fluorescence microscopic pictures are analysed according to FRUIT differences in fluorescence intensity vanishes (Figure 40, right graph). In FRUIT, the mean fluorescence of a single fluorescent particle is measured whereas in the alternative image processing protocol the mean fluorescence of the entire picture is measured. In other words, the 77 and 98 fluorescent particles from the positive and negative controls had similar mean fluorescence values as the 338 fluorescent particles in the test sample.

Overall, a measurement of the mean fluorescence of the whole picture is considered more appropriate to investigate whether root exudates of *A. thaliana* are able to induce *gfp*-expression in root exudate-sensitive *P. fluorescens.*

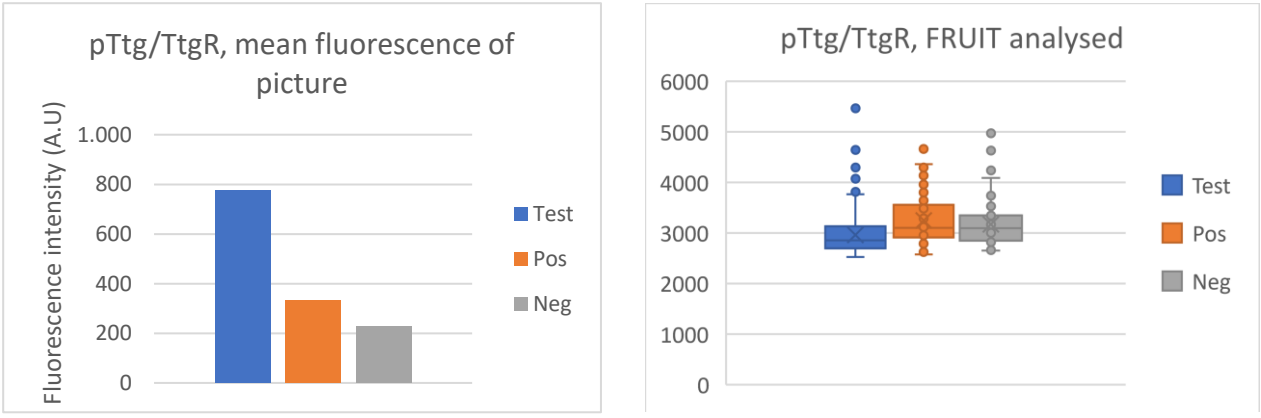

Figure 40: Fluorescence values of fluorescence microscopic picture with* P. fluorescencs *carrying the pTtg/TtgR inducible expression system on* A. thaliana *roots. *Left: Bar chart of mean fluorescence of the entire image. Right: Box Plot diagram of mean fluorescence of single fluorescent particles. Sample 2 of the biological triplicates is presented.*

4 Discussion

4.1 Construction of *P. fluorescens* Strains Expressing GFP With and Without a T3SS-Secretion Signal

After the transformation of *E. coli*, it became evident that *gfp* was insufficiently expressed. Berwal and co-workers elaborate in a publication from 2010 that the distance between RBS and the start codon is crucial for the expression of genes in *E. coli*. Although some genes require spacers up to 70 bp and longer for optimal expression, most spacers are found to be adequate in the range of 6-9 bp (Berwal et al., 2010). Therefore, the plasmid design was optimised by introducing a 6 bp long spacer after the RBS.

4.1.1 A Spacer Between RBS and Initial Start Codon is Prerequisite for Protein Expression in P. fluorescens

Transformants with the new plasmids expressed *gfp* and therefore showed fluorescence when illuminated at the wavelength of λ=485 nm. Bacteria that expressed native *gfp* under the control of the promoter J23100 exhibited the highest fluorescence intensities with similar levels of fluorescence intensity for GFP-SS and minor fluorescence in bacteria carrying the AvrRPM1 promoter. The differences in fluorescence were observed both on a macroscopic basis as well as under the fluorescence microscope (Figure 13).

In a study by Upadhyaya et al. (2014), the AvrRPM1 promoter was identified as a suitable promoter for expressing effector proteins in *P. flourescens* etHAn. However, the promoter region consisting of the J23100 promoter and RBS0034 ribosome binding sites was optimised for high expression in *Pseudomonas putida* (Damalas et al., 2020). The findings presented here suggest that observations of GFP-strong expression from experiments with *P. putida* can be extrapolated to *P. fluorescens*.

4.2 GFP Secretion into T3SS-inducing Media

In this initial secretion experiment, the ability of *P. fluorescens* etHAn to secrete GFP with a T3SS-secretion signal into the HDM medium was investigated. The expression of T3SS-related genes is suppressed in a nutrient-rich medium and activated in a minimal medium (Thomas et al., 2009b). Therefore, *P. fluorescens* etHAn was grown in minimal, *hrp*-derepressing-medium (HDM) and compared to cells grown in a nutrient-rich LB (negative control). HDM was chosen over other minimal media like the M9 minimal medium because of its capacity to activate gene expression of T3SS related genes like the hypersensitive response and pathogenicity gene (*hrp*; Xie et al., 2023). Kim et al. (2009) describe that the addition of 50 µM iron citrate enhances the expression of T3SS-related genes in *P. syringae*. In the assumption that an elevated expression of T3SS related genes results in more secretion through the T3SS, one HDM medium was enriched with 50 µM iron citrate.

Following the procedure of previous studies, cells were inoculated at an OD600 = 0,01 into the two different HDM media (Huynh et al., 1989; Thomas et al., 2009b). In a study by Stauber et al. (2012), cells of *P. syringae* could double their OD600 values in 30 hours when grown in HDM. Own measurements on the growth of *P. fluorescens* etHAn showed slower growth rates, resulting in an OD600 of 0,13 for the HDM and iron citrate cells and 0,053 for HDM-grown cells after 12 days (doubling time of two to four days).

As for the secretion into HDM medium, the measured fluorescence intensities in the supernatant after removal of all cells remained at low levels over the examined period (see Figure 15).

From previous research, it is known that elevated expression of T3SS-related genes starts 2 hours after incubation in minimal media (Stauber et al., 2012). Further, it was proven that the secretion of proteins starts after six hours when cells are inoculated on the leaves of wheat plants (Jensen et al., 2022a). Taking these findings together, it can be concluded that enough time has passed for the cells to secrete GFP into the medium and that fluctuations in measured GFP levels were not the result of GFP-secretion but rather of cells that released GFP into the medium due to cell lysis.

The lack of GFP detection in the HDM may be attributed to both technical and biological factors. As for the technical issues, the cell growth remained at low levels for the examined period. On the biological site, *P. fluorescens* could be inherently incapable of secreting GFP into the HDM medium. A more thorough discussion of both explanations follows in subsequent sections.

4.2.1 GFP-Secretion Remained Under the Detection Limit

P. fluorescens remained the whole time at very low cell densities (OD600 value < 0,2). In consequence, low amounts of GFP were produced and thus small quantities could be secreted into the medium. In this case, the correction of fluorescence intensity to OD600 values did not result in meaningful data (see OD-corrected fluorescence in Figure 53, Appendix).

4.2.2 Incapacity of *P. fluorescens* to Secrete GFP into Extracellular Medium

Functions of the T3SS are highly diverse, ranging from the secretion of proteins into the extracellular medium as shown for Yersinia effector protein YopE in the plant bacterium *Erwinia chrysanthemi* and interbacterial injection to the secretion of proteins into eucaryotic cells (Anderson & Schneewind, 1999, Ghosh, 2004). Gosh reviewed in 2004 that for certain T3SS, attachment to an eukaryotic surface is a prerequisite for the secretion of proteins. In the case of *Pseudomonas fluorescens* etHAn, previous research supports the statement that proteins can be secreted into plant cells (Upadhyaya et al., 2014). A potential restriction of the T3SS in *P. fluorescens* to the secretion into plant cells but not into the extracellular medium is possible. Finally, proteins that are secreted by the T3SS often own a specific structure which makes them suitable for T3SS secretion (Bergeron et al., 2016). It is possible, that GFP is inherently ineligibly to be secreted by the T3SS. Limitations of GFP secretion via the T3SS will be discussed in more detail in chapter 4.5: "Secretion of GFP into *Arabidopsis thaliana* via Type-3-Secretion System".

In summary, fluorescence intensities of the LB medium and HDM did not support the hypothesis that *P. fluorescens* can secrete GFP through the T3SS into the extracellular growth medium, independent of the media composition. It is likely that insufficient growth of *P. fluorescens* in HDM and the related lack of protein production as well as the absence of a plant attachment site resulted in the presented outcomes.

4.3 Growth of *A. thaliana* in Different Conditions

The effect of four different conditions on the growth and root development of *A. thaliana* was evaluated on their suitability for colonisation and secretion experiments. Although growth and root development were observed in every condition, growth in Fåhraeus slides and sterile Petri dishes was chosen for experiments.

4.3.1 Soil Adheres to the Differentiation Zone on Roots of *A. thaliana*

With the motivation to simulate non-laboratory conditions for the experiments, *A. thaliana* was grown on non-sterile garden soil.
Soil particles adhered in large quantities to the soil-grown roots of *A. thaliana*. Removal of soil particles from the roots led to the damage of roots. In 2007, Moreno-Espíndola and coworkers studied the mechanisms of adhesion of soil particles to small roots and emphasised that soil particles are tightly enmeshed to root hairs. Both, experimental findings and the publication from Moreno-Espíndola et al. (2007) determined that soil particles mainly attach to the differentiation zone, where most root hairs are found (cf. Figure 21).

To avoid the labour-intensive and potentially root-harming wash procedure of soil-grown plants, this growth condition was not selected for subsequent experiments.

4.3.2 ½ MS Agar in Vertical Petri dishes are Optimal for FRUIT

Three growth methods were evaluated for ½ MS10 Agar medium: Plastic boxes, a vertical Petri dish, and a cellophane sheet on a vertical Petri dish. Growth in vertical Petri dishes was considered optimal for the FRUIT experiment.

For FRUIT, a large sample size is required with bigger roots having an advantage for the procedure. ½ MS-grown plants combine these attributes. The roots are approximately twice as big as those of the Fåhraeus slide-grown plants and vertical Petri dishes allow the uncomplicated seeding of fifty or more plants on a single, vertical Petri dish.

4.3.3 Fåhraeus Slides Presumably Promote Elevated Ethylene Concentration

In 1957, G. Fåhraeus developed a growth technique for studying bacterial-root interactions on microscopic slides (Fåhraeus, 1957). This growth technique was adopted and evaluated to study the colonisation behaviour of P. fluorescens on A. thaliana roots. Initial observations confirmed that Fåhraeus slides are a suitable method for the growth and development of *A. thaliana* roots. However, fungal contamination and high amounts of root hairs were observed compared to plants grown in ½ MS medium.

It is assumed that the co-inoculation of up to 12 seedlings on one Fåhraeus slide led to elevated levels of the phytohormones auxin and ethylene in the ½ MS medium which resulted in a short root physiology and many root hairs. The phytohormones auxins and ethylene are pivotal for the quantity of root hairs in *A. thaliana* (Li et al., 2022; Lu et al., 2018). Ethylene was also found to further inhibit root elongation, resulting in smaller

roots (Li et al., 2022). This coincides with the observation that roots grown in Fåhraeus slides showed impaired root growth compared to plants grown in ½ MS10 Agar, enforcing the hypothesis that elevated levels of ethylene were present in the medium.

A possible way to counteract the elevated root-hair development would be to lower the number of plants that are seeded per Fåhraeus slide. A lower number of plants would lead to lower ethylene production and presumably to normal root development. However, the elevated number of root hairs did not disturb the colonisation experiment. Therefore, the growth procedure was not adapted to a lower number of plants per Fåhraeus slide.

In a future experiment, one could test whether ethylene is the crucial factor for the observed root morphology or if other factors, such as the auxin concentration play a role as well. If an ethylene-insensitive *A. thaliana* strain as presented in Bleecker et al. (1988) would show no excess of root hairs in the Fåhraeus slide, one could conclude that ethylene is a decisive factor.

4.3.4 Summary

In conclusion, all growth conditions allow the growth of *A. thaliana* and show their strengths and limitations. In the proof-of-concept stage, the MS-grown plants are favoured over the soil-grown Plants because of their ease of cultivation. In forthcoming experiments, methods should be developed to investigate the root system of soil-grown *A. thaliana* without damaging the roots to allow experiments in a nonsterile soil condition. In the tested setup, garden soil was used. However, *A. thaliana* can also grow in many other soils, including clay and sandy soil (Truyens et al., 2016). Future experiments can test whether one of these growth media is leading to less soil adhesion.

4.4 Colonisation of *P. fluorescens* on the Root of *A. thaliana*

Quantitative and qualitative insights into the colonisation behaviour of *P. fluorescens* on plant roots were obtained.

It has been demonstrated that *P. fluorescens* etHAn does indeed colonise the root and barely remains in the rhizosphere (see Figure 24). Root attachment was anticipated as previous research from Utrecht University showed that *P. fluorescens* WCS417r can attach to the roots of *A. thaliana* to perform plant health-promoting plant responses (Hase et al., 2003).

Further, it has been shown that not all bacteria are attached to the plant surface. Approximately 10 % of bacteria remained motile in the ½ MS medium with decreasing numbers of bacteria in plant-remote areas. First, the results indicate that direct root contact is preferred but not prerequisite to sustain growth in *P. fluorescens*. Second, in the context of this investigation it is plausible to assume that the quantity of bacteria is correlated to the availability of nutrients. Therefore, it can be inferred that the availability of root exudates as the sole carbon source in the employed settings decreases with increasing distance to the root. Third, even one week after first colonisation some bacteria remain unattached. This supports the hypothesis, that a share of bacteria always remains motile to colonise new parts of the root system if available. The observed colonisation behaviour corresponds with the model of the "biofilm life cycle" in which a certain share of bacteria remain motile and disperse from an attached surface to colonise new areas (Figure 41) (Sauer et al., 2022). In correlation with the model of the "biofilm life cycle", microcolonies were observed on the roots of *A. thaliana* in which *P. fluorescens* remained mostly immotile. Still, there were always some bacteria that remained motile.

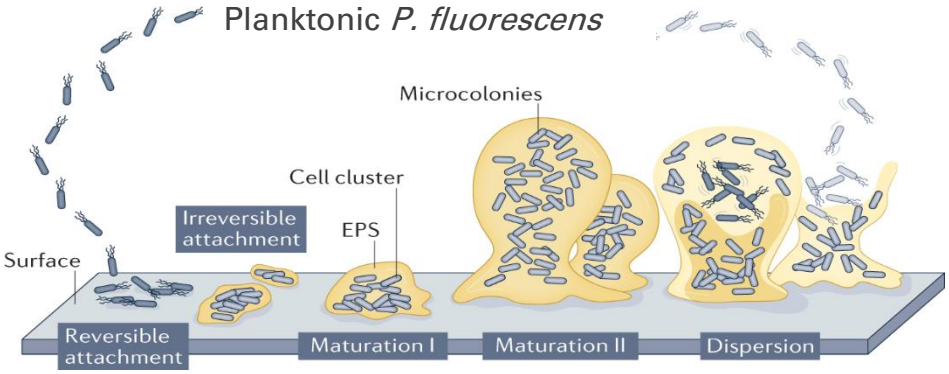

Figure 41: Microcolonies of* P. fluorescens *on plant root surface and schematic overview of the "Biofilm Life Cycle". *The "Biofilm Life Cylce" Plantonic cells, e.g.* P. fluorescens *attaches to root-surface to form cell clusters and microcolonies. However, not all bacteria remain on the plant surface. Instead, some disperse again to find new attachment sites. Scheme adopted from: Sauer et al. (2022).*

4.4.1 Cross-validation of Experimental Results to Agent-based Model

Within Wageningen's iGEM project 2023, an agent-based model was programmed to predict the colonisation patterns of a genetically engineered *P. fluorescens* strain on live roots in orchards. Experimental data from this thesis cross-validates colonisation patterns. It was confirmed that bacteria mainly attach to the upper part of the root system near the soil surface. Decreasing quantities of bacteria were observed in the apical root region with a minimum of bacteria at the root tip (See Figure 26).

Previous work on the colonisation patterns of *P. fluorescens* supported these findings (Turnbull et al., 2001). It was determined that *P. fluorescens* SBW25 mainly colonises the first 15 mm of the root of young wheat plants, 9 days after inoculation with the bacteria (Figure 42, Turnbull et al., 2001). Nutritional advantages and oxygen availability could explain the observed colonisation patterns.

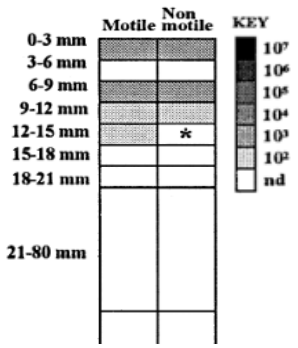

***Figure 42: Part of experimental results from previous research on root colonisation of motile and non-motile* P. fluorescens *SBW25 on the first 14 mm of a wheat root**. Main colonisation is detected in the first 15 mm below ground. Scheme adopted from: Turnbull et al. (2001)*

4.4.1.1 Nutritional advantages

The composition of root exudates varies at different zones of the wheat roots (Rovira, 1973). Root exudates are the main carbon source in the rhizosphere and can be used by *Pseudomonas* species (Jacoby et al., 2018). Further, they are the main contributors to colonisation and chemotaxis of *P. fluorescens* towards the roots of plant cells (Oku et al., 2012). The unique composition of root exudates from the mature, basal region of *A. thaliana* might have enabled the growth and colonisation of *P. fluorescens* etAHn.

4.4.1.2 Oxygen limitation

Pseudomonas fluorescens is an aerobic bacterium. Both, in the soil and under the glass cover, the oxygen content diminishes in the direction of the root tip. The microbial soil community differs depending on the availability of oxygen (Hoorman; 2011). In an oxygen-deficient environment, the growth of *P. fluorescens* bacteria might be impeded.

4.4.2 Summary

The observed colonisation behaviour of *P. fluorescens* was contextualised using the "biofilm life cycle" model, and the impact of root exudates and oxygen availability was discussed. However, it is acknowledged that the presented colonisation results were raised under laboratory conditions and might substantially differ in a nonsterile soil environment.

The predicted colonisation behaviour of *P. fluorescens* on plant roots from an agent-based model could be cross-validated by experimental results from own experiments and experiments described in the literature. Nevertheless, one should acknowledge the limitations of the cross-validation: For the model, the first 20 cm of a root system from

A. thaliana was predicted, whereas the experimental data used small roots that did not exceed the depth of 11 mm. Finally, the study by Turnbull et al from 2001 used a different *P. fluorescens* strain and studied its colonisation behaviour on wheat instead of *A. thaliana*.

Further research in more comparable conditions is needed to make general statements about how *P. fluorescens* exactly colonises on roots of plants. In a forthcoming experiment, it is recommended to investigate the colonisation behaviour of *P. fluorescens* on wheat roots. Wheat roots are bigger which would make results more comparable to both the agent-based colonisation model and the colonisation assay from Turnbull et al. (2001).

4.5 Secretion of GFP into *Arabidopsis thaliana* via Type-3-Secretion System

4.5.1 Autofluorescence in Living Root Hairs Could be Stress-Related

P. fluorescens etHAn can inject toxic effector proteins into leaves of crop species like wheat to elicit a hypersensitive plant response, i.e. the apoptosis of plant cells around the injection site (Upadhyaya et al., 2014). Until present, no studies on the secretion of GFP into the roots of *A. thaliana* have been conducted. Therefore, roots of *A. thaliana* were microscopically examined to detect plant intracellular, thus bacteria secreted GFP. Studies were performed on both live roots and fixated root sections. Further, plant roots were lysed, His-tag purified and examined for the presence of GFP via silver staining.

For the living roots, a focus was laid on root hairs, as they are unicellular structures in which any secreted GFP should be most discernible. It was expected that GFP accumulates outside of the vacuole as it has no vacuole-targeted tag (Tamura et al., 2003). Knowing that the vacuole takes up approximately 90 % of the plant cell, it was assumed that GFP, if secreted, accumulates as a green lining next to the cell wall of the root hairs. Intriguingly, a green fluorescent lining was observed on root hairs (see Figure 29, A). However, similar fluorescent patterns could be seen when the RFP was illuminated. RFP was lacking a T3SS secretion signal and therefore remained in the bacteria and could not be secreted. In consequence, it was concluded that roots of *A. thaliana* show general autofluorescence independent of the secretion of GFP.

An explanation for the observed autofluorescence in plants is the stress-related production of secondary, autofluorescent metabolites. The experimental setup was designed to create minimal stress for the plants. However, the sterile environment, the abundance of *P. fluorescens* and the continuing transport of the Fåhraeus slides from the microscope and back to the growth chamber can be considered stress factors. Stress factors can lead to the production of defensive secondary metabolites in plants and many secondary metabolites are known to be autofluorescent (García-Plazaola et al., 2015; Aguirre-Becerra et al., 2021). In a forthcoming experiment, one might use a shorter exposure time for the fluorescence microscopic pictures. A shorter exposure time would

lower the threshold of what is detected as “fluorescent”. This could facilitate the differentiation of GFP to autofluorescent secondary metabolites.

4.5.2 Fixated Vibratome Sections Show Fluorescence at Casparian Strip

The basal region of bacteria-colonised roots was fixated in 4 % paraformaldehyde, embedded in agarose and sections via the vibratome. Interesting insights into intracellular root structures showed a new perspective on how bacteria attach to the root surface. However, no conclusive evidence could be found for the secretion of GFP into the epidermis of *A. thaliana* roots and an accumulation of GFP in the phloem.

Interestingly, enhanced fluorescence is observed in the endodermis, the outmost layer of the stele. The endodermis carries the Casparian strip, a universal semi-permeable barrier in angiosperms (Geldner, 2013). The Casparian strip-bearing endodermis is of crucial importance for the determination of what can enter the phloem of the plant and thus be systematically distributed in the plant and what remains in the cortex of the roots (Geldner, 2013). Having this in mind, it is possible that GFP was secreted into the plant and transported through the cortex but remained in the endodermis because of the incapability of traversing the Casparian strip-carrying endodermis.

Nevertheless, it is questionable how GFP might have reached the stele without accumulating in the epidermis of the cell. Additionally, it remains questionable why GFP has only accumulated at one side of the stele and not also on the other sides where bacteria are attached in high quantities (see Figure 31, B).

In a future experiment, one could perform immunostaining on the respective cross-section with GFP-targeted antibodies to verify whether the observed fluorescence is plant or GFP derived.

4.5.3 Silver Staining was Ineffective Due to Lack of Proteins

From the previous fluorescence microscopic experiments, it became clear that plant root tissue from *A. thaliana* shows autofluorescence. Autofluorescence complicates the interpretation of whether fluorescent structures originate from plant material or GFP. These limitations led to the design of a detection method that is not biased by autofluorescent particles. Firstly introduced by Switzer and coworkers in 1979, protein detection via silver staining is highly sensitive with a detection limit below 1 ng (Switzer et al., 1979). After the removal of bacteria from the plant root surface, plants were cooked in SDS buffer and the lysate was purified for His-tagged GFP.

4.5.3.1 Root Inspection

The efficacy of bacterial removal was checked under the microscope. Bacteria were neither visible on the roots nor in the root environment (Figure 32, C & D). However, a microscopic evaluation serves as an indication for the removal of all bacteria. For example, a bacterium attached to the root surface is less visible and easily overlooked. In a forthcoming experiment, one should consider performing a colony PCR on the washed roots. This molecular approach could detect the presence of bacterial DNA, even if the bacterium itself might not be visible under the microscope. In detail, one could use 1-3 mm of the washed root as template material and perform a PCR on the insert (in this case GFP-SS) or a 16S rRNA-gene region that is specific for *P. fluorescens*. Either way, the transformant would be detected.

4.5.3.2 Gel Inspection

The plant and the bacterial proteome were investigated on a silver-stained SDS gel. Only the non-purified cell lysate showed sample-derived protein bands with a band for GFP being detected in sample 1 (see Figure 33, left image, bacterial lysate). The results can be explained by the lack of proteins in plant and bacterial lysate.

4.5.3.2.1 Lack of Proteins in the Plant Lysate

No, or too little amounts of the plant-proteome were gathered. This can have many reasons. First, the roots of *A. thaliana* at the point of the experiment were very small (approximately 10 mm in length, 100 µm in width). The small root size limits the availability of proteins. Second, the wash procedure for the removal of bacteria also washed intracellular proteins away. From the microscopic inspection (see Figure 32) one can see that the wash procedure did not disrupt the morphology of the plant. However, Isopropanol (70 %) was used as one wash detergent to remove bacteria from the plant surface. Isopropanol is known to disrupt plant cells which can in turn lead to the release of proteins (Linke et al., 2010). In consequence, part of the root proteome may be removed in the wash steps. Finally, the plant lysation procedure was designed for the extraction of proteins from cauline leaves. An extrapolation of this protocol for roots of *A. thaliana* was supposably not successful and led to small or no protein extraction from the here-described plant samples. Roots and cauline leaves differ in their function and morphology. In contrast to leaves, roots need to penetrate the soil and are therefore likely to be more rigid in their structure. The robust structure of roots might have lowered the efficiency of the plant lysation. In a future experiment, one should consider using a plant lysation protocol that is specialised for root lysation. Further, a preliminary step for cell-wall disruption via bead beating could increase the protein extraction efficiency.

4.5.3.2.2 Lack of Proteins in the Bacterial Cell Lysate

From previous fluorescence experiments and sequencing results, it is known that the bacterial cells expressed *gfp*. However, only sample 1 shows a vague band at the respective molecular weight of GFP. Further, this band is not visible anymore after the His-purification (see Figure 33, right image). The proteins of the wild-type cell lysate show more protein than the ones of the transformants. Knowing that the same protein extraction protocol was used for both GFP-producing as well as wild-type cells, one can conclude that wild-type cells grew to a higher density than GFP-producing cells after 1 day of incubation, resulting in more cells and a higher protein concentration. In a forthcoming experiment, one should consider either extracting proteins from a higher quantity of cells or using a more efficient protein extraction protocol for GFP-producing cells. French pressing, sonication and many other described mechanical, chemical and thermal lysis methods are known to be efficient in the lysis of bacterial cells and could be used in a future procedure (Shehadul Islam et al., 2017).

In conclusion, the here-developed silver-staining procedure for the detection of GFP derived from *P. fluorescens* in roots of *A. thaliana* was not efficient for detecting plant-intracellular GFP. Recommendations for optimisation of the procedure were provided.

4.5.4 Summary

Neither microscopic nor biochemical experiments confirmed the secretion of GFP from *P. fluorescens* via the T3SS into the roots of *A. thaliana*. Experimental results were discussed and recommendations for upcoming experiments were given. However, one might also consider that GFP is inherently not able to be secreted in the given circumstances.

GFP was designed with a T3SS-secretion signal to allow its secretion via the T3SS. Previous research showed, that this approach was suitable to deliver effector proteins into leaves of wheat cells (Shehadul Islam et al., 2017).

However, as previously described in the introduction, the secretion of proteins through the T3SS is not only dependent on the presence of a T3SS secretion signal. The T3SS consists of a 25 Å nanochannel (Blocker et al., 2008). 25 Å is enough space for proteins in their unfolded, but not tertiary, globular conformation (Blocker et al., 2008). Therefore, specific chaperones must bind to proteins with the T3SS secretion signal, facilitating the unfolding process and allowing passage through the T3SS (Puhar & Sansonetti, 2014). Refolding in the host cell occurs unchaperoned (Puhar & Sansonetti, 2014). In the here described experiments superfolder GFP was used for its high fluorescence ability and good stability when produced as a heterologous protein (Pédelacq et al., 2006). However, the high stability of sfGFP might have hindered the crucial unfolding process of the protein that is needed for transportation through the T3SS.

In upcoming experiments, one might consider using a less stable fluorescent protein for the investigation of protein secretion through the T3SS. The decrease in protein stability possibly will result in less fluorescent bacteria but might enhance the quantity of secreted protein into the host cell. A first proof of concept could even be made using the effector protein AvrBs2 (UniProtKB accession number: Q9Z3F4) as previously used in Upadhyaya et al. (2014). In the context of this year's iGEM project, this approach was not favoured as the delivery of a non-toxic protein was regarded as crucial. Nevertheless, a toxic effector protein would be an efficient way to show that secretion of this AvrBs2 is not only possible in the leaves of wheat plants but also into the roots of *A. thaliana*.

4.6 FRUIT: Fluorescence Root-exudate Unbiased *In-Vivo* Test

Techniques and protocols established in this master thesis were applied to root exudate sensitive *P. fluorescens* SBW25, engineered by the iGEM teammate Nicolaas Maarten van Donk, to create a "Fluorescence Root-exudate Unbiased *In-Vivo* Test", short FRUIT. The goal was to overcome constraints associated with plate reader experiments and investigate the inductive capacity of root exudates from living *A. thaliana* roots on the root-exudate inducible system of four different transformants.

4.6.1 Oxidative Stress Induces Autofluorescence in Stele

The first observations showed that the autofluorescence of the fixed roots (used for positive and negative controls) exceeds the autofluorescence of test samples by a factor of 50.

A review of the experimental results with plant microscopy expert Norbert van Ruijter led to the hypothesis that the fixation process with 4 % paraformaldehyde led to oxidative stress in the plant tissue. Oxidative stress is known to stimulate the creation of secondary metabolites in plants which in return are known to be autofluorescent (García-Plazaola et al., 2015; Hong et al., 2013). For FRUIT, the differences in autofluorescence were eliminated by subtracting the respective autofluorescence value from the stele of living and fixated roots (compare Table 7).

When repeated, one might consider fixating plant tissue with a higher concentration of the reducing agent DTT to avoid oxidative stress in plant tissues.

4.6.2 Fluorescence Origins from Autofluorescent Plant Tissue and Induced Bacteria

In a first qualitative inspection of fluorescence images with the strain carrying the pSAL/nahR inducible system, one could already determine a strong difference in induced fluorescence between negative and positive control and slightly higher fluorescence levels for the test samples when compared to the negative control. The control samples consisted of fixated thus metabolically inactive roots, with 1 mM inducer (positive control) or without inducer (negative control). As for the test sample bacteria were induced when being attached to the root, but not in plant remote areas (< 50 50 µm). Green fluorescence was also visible in the image representing the negative control. However, when inspected at a higher magnification, it became evident that fluorescent structures in the negative control mainly originate from the autofluorescent stele and its surrounding tissue whereas fluorescence in the test sample comes from fluorescent bacteria that are attached to the epidermis of the root. Induced and therefore green fluorescent bacteria attach to the surface of the root epidermis and are recognizable as green dots (see Figure 43, A). The green fluorescent stele is a continuous green lining in the centre of the roots (Figure 43, B).

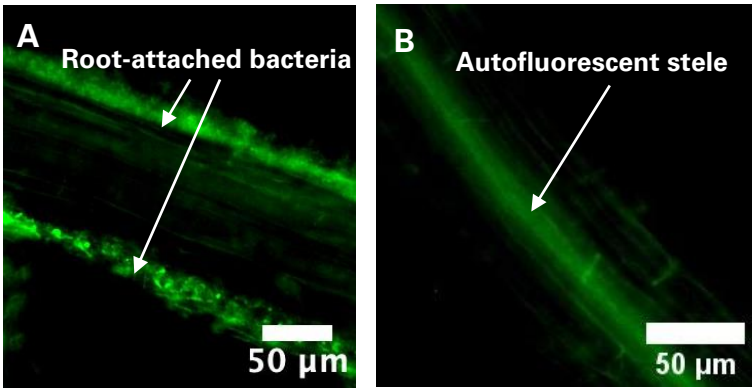

***Figure 43: Close up picture of* P. flourescens *carrying the pSAL/nahR inducible expression system attached to roots of* A. thaliana (10x objective)**. *A: Test sample: Bacteria are attached to a live root. Attached bacteria express GFP and therefore show green fluorescence. B: Negative control: Bacteria are attached to a fixated root. Fluorescence is limited to the autofluorescence of the root.*

4.6.3 FRUIT Allows Investigation of Local Induction

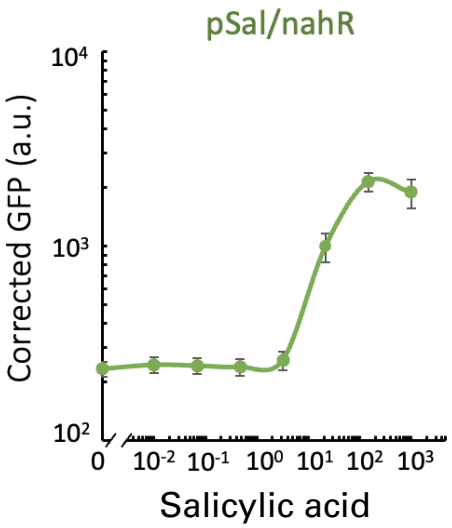

Figure 44: Corrected fluorescence (GFP/OD600) of the pSAL/nahR inducible systems in* P. fluorescens *after 24 hours of exposure to a range of its inducer salicylic acid. *The system is induced at salicylic acid concentration above 5 µM. Graph kindly provided by N.M. van Donk.*

The qualitative studies of fluorescence microscopy images further allowed the investigation of local induction. The pSal/nahR carrying strain was for example only induced in the direct environment of the root, but not in remote areas. From a plate reader experiment of fellow iGEM teammate Nico Maarten van Donk, it is known that bacteria carrying pSAL/nahR show no response to the inducer salicylic acid up to a concentration of 5 µM (see Figure 44). From the literature, it is known that salicylic acid is a root exudate of *A. thaliana* (Strehmel et al., 2014b). Taking these findings together, it is probable that live roots of *A. thaliana* secrete salicylic acid and create a microenvironment where the salicylic acid concentration exceeds 5 µM, thus inducing the *gfp*-expression in pSAL/nahR carrying *P. fluorescens*. Beyond a distance of approximately 50 µm from the root (c.f. Figure 43), the concentration falls below the critical threshold, resulting in the absence of induced *gfp*-expression.

In conclusion, the fluorescence microscopic inspection of bacteria with inducible expression systems can be used to predict the inducer concentration in the microenvironment of *A. thaliana* roots.

4.6.4 Transformation of Qualitative into Quantitative Data with ImageJ

Images were processed with FIJI/imageJ. A mask for fluorescent particles was selected and the fluorescence intensity of all particles above a threshold-fluorescence was measured. As a result, the mean fluorescence of every fluorescent particle, ideally corresponding to a single bacteria, was measured and visualized in a box-plot diagram (see Figure 37).

For two out of three samples from the pSAL/nahR inducible expression system, FRUIT was successful in determining significant differences between fluorescence intensity of particles in the test samples and negative control using a student's t-test.

However, for samples with the bacteria carrying pCym/CymR, pTtg/TtgR and pBAD/AraC no significant differences in the mean fluorescence of particles were detected. The lack of differences in fluorescence intensity can be explained by the absence of the inducive root exudate and the limitations of the FRUIT image processing protocol.

4.6.4.1 Noninductive Root Exudate

One reason for the absence of significant differences could be that *A. thaliana* did not exudate the inducer for the respective inducible expression system. Therefore, no difference in fluorescence was measured between the negative control and test samples.

4.6.4.2 Disproportional Influence of Outliers

The box plot graphs of FRUIT show the mean fluorescence intensity of fluorescent particles above a certain threshold, unbiased by the number of bacteria. On the one hand, this can be regarded as advantageous as stochastic differences in bacterial quantities cannot disturb the output. On the other hand, a single, highly fluorescent bacterium in the negative control can lead to high values in the box plot diagram whereas many, moderately fluorescent bacteria lead to lower values.

4.6.5 Limitations of FRUIT and Alternative Image Processing

The shortcomings of FRUIT were presented on the pTtg/TtgR samples. Some bacteria are induced in the negative control of pTtg/TtgR. Reasons for activated GFP production in the negative control range from the potential leakiness of the promoter to point mutations in the promoter region that led to constitutive expression of GFP in the respective cells. Further, it was demonstrated that at a 100x magnification, FRUIT was not able to discern between single bacteria and merged many fluorescent bacteria in the test sample to one big fluorescent particle. In conclusion, few fluorescent bacteria in the control samples show similar levels of mean fluorescence as the many induced bacteria of the test sample.

In a forthcoming experiment, one might consider examining the roots with a higher magnification (40x or 100x objective) in which bacteria appear bigger and are easier to discern. Further, it is advised to create an imageJ protocol that can better distinguish between single bacteria, even if they are closely attached to each other. Finally, one might expand the protocol with a step to correct for “particle size”. In consequence,

bigger particles that are likely to represent a group of bacteria have a higher impact on the box-plot results than smaller particles that probably embody a single bacterium.

For now, an alternative image processing was presented that analyses the mean fluorescence intensity of an entire microscopic picture. In the context of this experiment, this image processing is considered as more appropriate, because it is less prone to the discussed limitations of FRUIT. However, it's important to note that this approach consolidates the information from an entire image into a single data point which in turn cannot be used for the statistical analysis of the results. To facilitate statistical tests multiple images should be analysed. For example, a sample size of thirty pictures would be appropriate for a student's t-test.

4.6.6 Summary

A new procedure for the measurement of inducible expression systems on solid surfaces was developed. The procedure includes co-inoculating bacteria with inducible expression systems to an inducive, living surface - in the presented case, the living roots of *A. thaliana*. After a first qualitative inspection, in which local patterns of induction can be identified, microscopic pictures are analysed with ImageJ.

An advantage of the procedure is that one can analyse many data points within a single picture which in turn facilitates the application of statistical tests for comparing test and control samples. However, it was discussed that the image processing protocol of FRUIT is prone to the overrepresentation of outliers. Furthermore, the particles do not always correspond to single bacteria. Therefore, an alternative image processing was presented in which the mean fluorescence of the entire picture was measured rather than the fluorescence of single particles.

FRUIT combines disciplines from plant sciences, microbiology and microscopy and can easily be adapted to other experiments in which cell-cell interactions are measured via fluorescence induction. It can expand and complement plate reader experiments by looking at local patterns of induction and analysing fluorescence intensities on a single cell level.

5 Conclusion and Future Perspectives

5.1 Conclusion

A comprehensive study on the colonisation behaviour of *P. fluorescens* etHAn on roots of *A. thaliana* was performed. Further, investigations concerning the secretion capacity of GPF into plant cells via the type-3-secretion system were conducted.

5.1.1 Preliminary experiments

Growth in Fåhraeus slides (½ MS medium) and on top of ½ MS10 Agar medium in vertical Petri dishes was considered optimal for the experiments. Both conditions enable the seeding of many (15+) plants in a small space and reduce damage to plant material in microscopic studies to a minimum.

Plasmids for transformation were designed and transformed, first into *E. coli* DH10ß and subsequently into *P. fluorescens* etHAn. It was confirmed that the genetic construct as shown in Figure 45 enables the expression of the genes *gfp* and *rfp.*

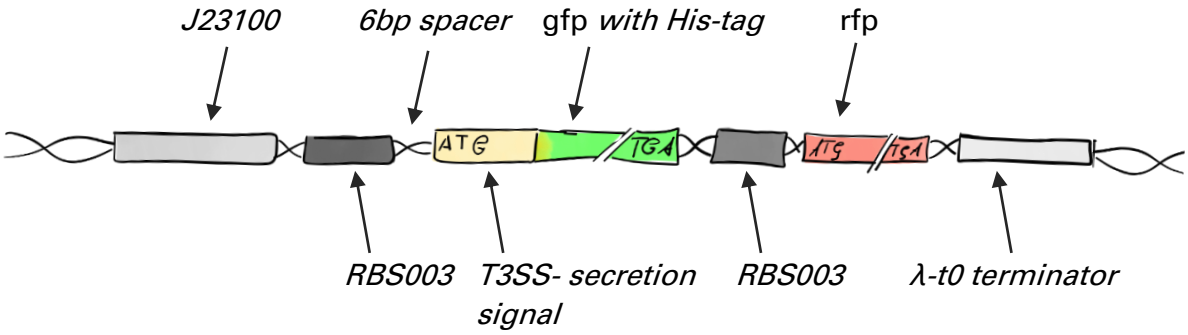

Figure 45: Schematic overview of the pSEVA64 vector insert as found in the GFP-secreting, RFP-producing* P. fluorescens *etHAn strain used in this thesis (coinciding with experimental group 2). *The* gfp *-gene is coupled to a T3SS secretion signal whereas* rfp *is only produced but cannot be secreted. Start and stop codons are indicated with "ATG" and "TGA", respectively.*

Previous work noted that genes related to the T3SS are active in a minimal medium (Stauber et al., 2012). Therefore, *P. fluorescens* with the above-mentioned gene construct was inoculated in HDM and the fluorescence intensity in the HDM was measured over 12 days. The data did not allow a statement on whether GFP was secreted into the HDM. It was discussed that the absence of GFP in the medium was presumably related to low bacterial quantities in the HDM and the lack of a plant attachment site.

5.1.2 Colonisation experiments

A microscopic approach was applied to study the colonisation behaviour of *P. fluorescens* on the roots of *A. thaliana*.

In conclusion, the investigation showed that *P. fluorescens* mainly attaches in the grooves, in between epidermic cells with no preference for specific root structures such as root hairs or emerging lateral roots. Three days after the first attachment, microcolonies started to form in various regions of the epidermis. Further, phase contrast pictures were processed with FIJI and compared with the results of an agent-based colonisation model. Both, the model and experimental data demonstrated that bacteria mainly attach to the basal region of the root with decreasing bacterial quantities towards the root tip.

Colonisation patterns were discussed, and it was acknowledged that lab results might deviate in non-sterile conditions.

5.1.3 Secretion experiments

Microscopic and biochemical methods were employed to investigate whether *P. fluorescens* etHAn can inject GFP-SS into the roots of *A. thaliana*. In summary, one could not affirm the thesis that proteins can be delivered from bacteria to plants. The results were thoroughly discussed and suggestions for improvement were given to reduce disturbing factors for both microscopic analysis of GFP-SS in plant cells and biochemical analysis of proteins in plant lysates.

5.1.4 Fluorescence Root-exudate Unbiased *In-Vivo* Test (FRUIT)

A novel method was developed to measure inducible expression systems on solid surfaces. This approach involves co-inoculating bacteria with inducible expression systems onto a living surface with inducible properties, exemplified by the living roots of *A. thaliana* in this instance. It was discussed that FRUIT excels in the analysis of local patterns of induction and facilitates the statistical analysis of samples. However, FRUIT also shows weaknesses in the recognition of single bacteria and is highly influenced by outliers. Therefore, an alternative image process protocol was presented that counteracts some of the shortcomings of FRUIT.

5.2 Future Perspectives

Procedures and protocols from the described master thesis can be used to further explore bacteria-plant interactions. As summarised above, genetic expression systems and plant growth procedures were developed. Namely, the J23100 promoter and RBS0034 Ribosome binding site were proven successful for the constitutive expression of a target gene in *P. fluorescens* etHAn. The plant growth protocols for Fåhraeus slides and vertical Petri dishes can be extrapolated for future experiments in this field.

P. fluorescens etAHn is stored as cryocultures and can be used for future investigations on the secretion of proteins via the T3SS.

As discussed in chapter 4.5.3, "Silver Staining", it is advised to use a different protein than superfolder GFP for protein-secretion experiments. In this context, the protein AvrBs2 (UniProtKB accession number: Q9Z3F4) as previously used in Upadhyaya et al. (2014) might be a suitable candidate.

As for the choice of plants, it is endorsed to use a plant with bigger roots for microscopic work. *A. thaliana* is an excellent model organism for genetic studies and shows some beneficial properties for microscopic studies, namely the transparent nature of the roots. However, they were found to be easily harmed when removed from the growth surface. Further, more sturdy roots are recommended for the creation of vibratome sections. Therefore, *Zea mays,* a plant-model organism is suggested for upcoming experiments using vibratome sections. As to be seen in Figure 46, the bigger root of *Zea mays* allows an in-detail analysis of plant intracellular structures and is presumed to give more informative results than the ones obtained with *A. thaliana*.

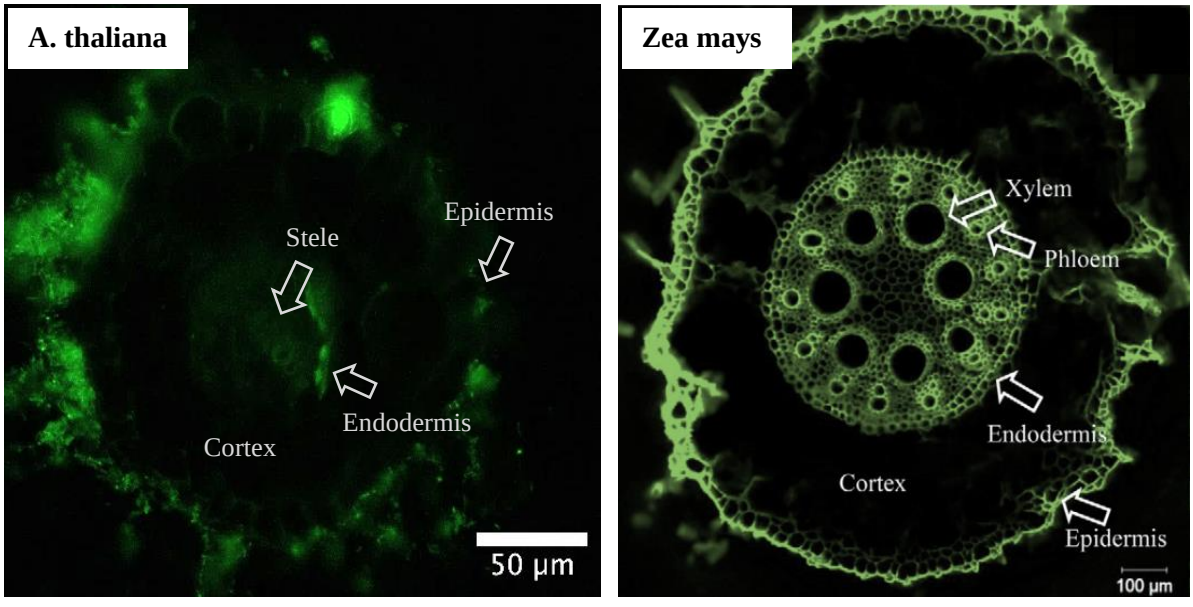

Figure 46: Fluorescence microscopy picture of cross-section from the model organisms* A. thaliana *(A) and* Zea mays *(B). *Main plant structures are indicated with arrows. The roots of* Zea mays *are substantially bigger than the one from* A. thaliana *(compare scale bars) which facilitates the creation of cross sections. The microscopic picture of* Zea mays *is adopted from: Zhao et al. (2012)*

As for the detection of intracellular proteins, fluorescence microscopy was proven to be not ideal. Autofluorescent structures of the plant complicated the microscopic analysis of intracellular GFP. Therefore, an unambiguous detection method is recommended for future experiments. Western blotting can be applied to determine whether the protein can be detected in a plant lysate. Further, fluorescent antibodies can be used (immunostaining) to investigate the location of the bacteria-derived protein in cross-sectioned plant tissue.

Future experiments should simulate natural, non-laboratory conditions. A laboratory, thus often sterile, condition does not represent the behaviour of the bacteria in their natural environment. To investigate what is happening in the field, one should grow the plant in non-sterile soil. The removal of soil particles from the fragile root system of *A. thaliana* was challenging in this thesis. However, the roots of *Zea mays* are bigger and therefore expected to be more resilient against physical damage. The presence of other rhizospheric bacteria might change the colonisation behaviour and protein secretion of *P. fluorescens.*

Finally, future experiments can apply the FRUIT procedure to give quantifiable and significant evidence to fluorescence pictures. Nonetheless, one should be aware of the limitations of FRUIT and evaluate to which extent the protocol needs to be deviated or refined for the respective experiment.

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*"Properly speaking, such work is **never finished**; one must declare it so when, according to time and circumstances, **one has done one's best.**".*

Johann Wolfgang von Goethe

March 16, from "Italian Journey"

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8 Appendix

8.1 Protocols

8.1.1 Protocol 1: Preparation and Transformation of Electrocompetent Cells of *P. fluorescens* etHAn

Preparation of Electrocompetent P. flourescens *etHAn*

DAY 1:

- Grow up EtHAn in 5ml LB + Amp (100 µg/ml)+Chlo(30 µg/ml)+Tet(50 µg/ml) at 33 ℃ with shaking, O/N.

DAY 2:

- Inoculate 100ml LB + Amp(100 µg/ml)+Chlo(30 µg/ml)+Tet(50 µg/ml) with 2.5 ml pre-culture of EtHAn, incubate at 30 ℃ with shaking for ~18h until OD600 reaches 0.8-1.2.

DAY 3:

- Cool down the EtHAn culture on ice for ~20 min, also pre-cool 10 % glycerol and pre-cool the centrifuge to 4 ℃.
- Centrifuge at 4 ℃, 3000 g for 10 min.
- Discard supernatant, resuspend the pellet in 20 ml cold 10 % glycerol.
- Centrifuge at 4 ℃, 3000 g for 6 min.
- Discard supernatant, resuspend cells in 4 ml cold 10 % glycerol. Repeat this step 3 times.
- Make 50 µl aliquots and immerse in liquid N2, save the cells at -80 ℃.

Transformation of Electrocompetent Cells

1. Use one vial of competent cells with 500 ng-1 ug plasmid.
2. Electroporate cells at 247 V, 200 ohms, 25 uF.
3. After shocking cells add 1 ml SOC and shake for 2-3 hours at 28 °C.
4. Plate cells on LBA plus appropriate selection and incubate at 33 °C for 1 day followed by incubation at 30 °C for 2 days.

Protocol from From the Department of Plant and Environmental Sciences at the University of Copenhagen

8.1.2 Protocol 2: Slides for Live Observation of Root Hairs

Fåhraeus slides are microchambers for growth of seedlings in sterile conditions allowing microscopic observations of the roots. A microchamber is made by mounting a coverslip (40 x 24 mm) to a glass-slide. The space in between slide and cover is plantspecies specific, related to width of primary root. For *Arabidopsis* roots the spacer should be 0.4 mm, for *Medicago* 1.2 mm. As spacers we use 2 cover slips (*Arabidopsis*) or one glass slide (*Medicago*). The spacers will be removed when the silicone-grease has solidified (24h). A Fåhraeus slide with 40 x 24 mm coverslip for *Arabidopsis* holds approx. (see Figure below). 300 µl, for *Medicago* 1 ml medium.

Before use, the slides need to be boiled several times (2-3x) in demi-H2O with a spoon tip of EDTA to remove excess of calcium and solvents and acids from silicon. Slides can be cleaned and reused.

Prior to the insertion of the seedlings (or imbibed seeds), 'sterilization' is carried out either with 70 % EtOH (several hours) or with boiling the slides in demi-H2O for at least 15 min. The slides are air dried in the Laminar Air Flow hood by tilting them a little until the EtOH is evaporated.

Fill the microchamber with 1 ml PGM medium. The 1 cm long primary root of the seedling is carefully placed into the medium. When seedlings are placed into the slides they are transferred to round Petri dishes, where they rest at the thick side of a sterile 200 µl pipette tip, so that they are tilted. Add 1ml additional PGM medium to the slides to prevent that roots can fall dry.

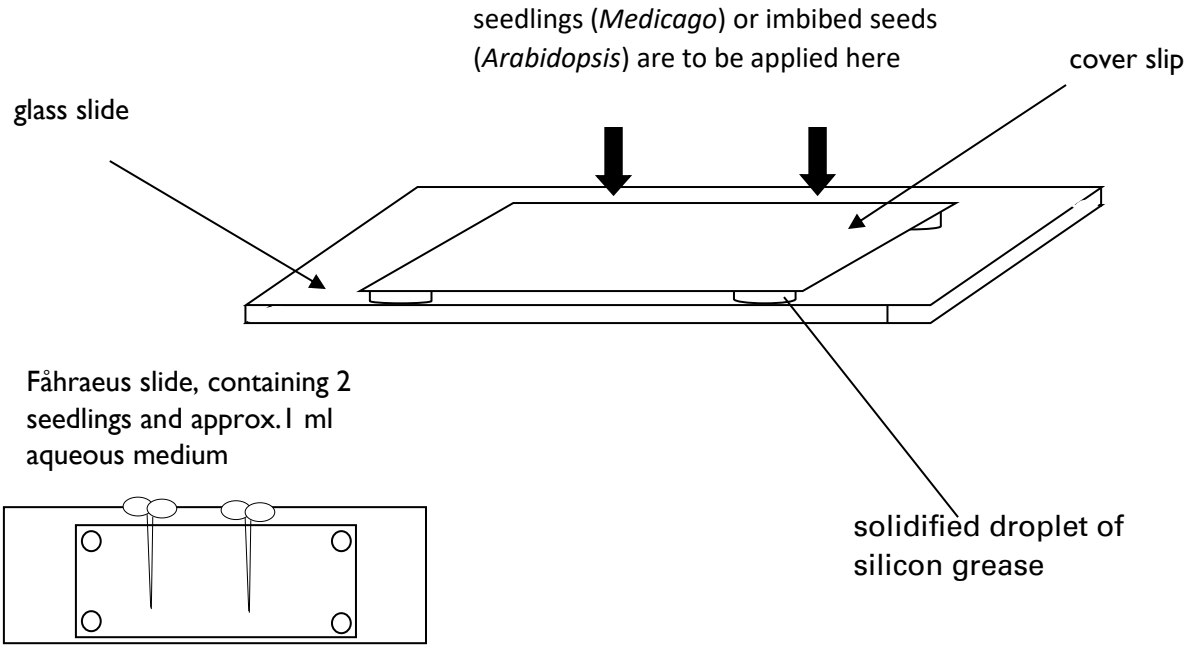

Figure 47: Schematic overview of components from a Fåhraeus slide.

Protocol from the Department Cell and Developmental Biology, WUR; Norbert de Ruijter

8.1.3 Protocol 3: Protocol for Vibratome Sectioning

1. Prepare sample (optional)

- Fix in 4 % paraformaldehyde in 0.1 M PBS pH 7.2-7.4.
- Vacuum for 15 minutes or longer if needed.
- Store sample in fridge overnight.
- remove excess fixative by washing tissues in 1 x PBS.

2. Prepare a 6 % low-melt agarose solution.

- Add 3 g of low-melt agarose to 50 mL of sterile PBS in a in an Erlenmeyer flask.
- Heat in the microwave by heating the solution under medium power in short 10- to 15-sec bursts, stirring after each pulse. Transfer the flask with the dissolved agarose to 65 ˚C oven and allow all of the water bubbles to escape the viscous 6 % agarose-PBS.
- Keep the dissolved agarose on 48 ˚C hot plate before use. (use long hot bar)

3. Embed sample

- Trim sample to make sure if it can fit in the eppendorf tube (1.5 ml or 2 ml) lid or other preferred molds.
- Briefly wipe your sample with tissue paper to remove excessive liquid.
- Place the sample in the tube lid, slowly add melted agarose in the lid. Try to avoid introducing bubbles.
- Orientate your sample in the agarose within 1-2 minutes (Root samples can be easily placed vertically). Make sure there are no bubbles around the sample. Let the agarose sit in the room temperature for 10 min or longer.
- Demold the lid loaded sample by clicking the flat side of the lid with finger nail. Slide a piece of agarose with (root) samples out of the tube with gentle push from cut-open bottom side. Trim the agarose if needed.
- Place a piece of plastic adhesive tape on the black plate.
- Place super gel on the adhesive tape, then place the sample on the gel. Sit in the room temperature for 5 min.

4. Read the manual before using the vibratome. The followings are some tips.

- Set vibrating frequency at 80 Hz as default.
- Start sectioning at the speed around 5. If sectioning is going well, you can increase the speed, but at a risk of having the blade push your sample out of agarose. If sample easily falls out of agarose while sectioning, set the speed lower than 5 on your next sample.
- Having multiple samples in one mold is not advisable, since the thin (50-75 um) agarose slice can be easily folded.
- Keeping agarose during florescent signal imaging is possible.
- Make sure the blade submerged in the 1 x PBS about 1 cm. This is to help the cut agarose slice flow.
- If you prefer working with sample in water, then replace all 1 X PBS with water.

Protocol from the Department of Plant Biosciences, WUR; Defeng Shen

8.1.4 Protocol 4: Protocol Protein Extraction from Plant Tissue

Plant material:

Cauline leaf: 11753 (GFP positive) & Col-0 (GFP negative)

In this thesis: Roots from A. thaliana (1/2 MS grown, 7 days old plants)

Procedure:

1-2 young cauline leaves were harvested and put in 200ul SDS sample buffer.

Material was roughly grinded with small blue pestle.

Put at 99 °C 600rpm in Temp block for 5 min.

Cooled on ice

Spinned down and supernatant moved to fresh epp.

- ready for SDS-PAGE

SDS-Lysis Buffer

2X SDS-sample buffer, w/o DTT, 40 ml

0.01 g bromophenol blue (0.02 % final)

2.0 g SDS – electrophoresis grade (4 % final)

10 ml glycerol (20 % final)

5 ml 1 M Tris-HCl pH 6.8 (100 mM final)

Add H2O to 40 ml. Store at RT.

Before use add 1 M DTT to reach 200 mM DTT final (800 µl + 200 µl 1M DTT).

Protocol from the Department of Plant Physiology, Wageningen University & Research; Froukje van der Wal

8.2 DNA Sequences

Table 8: Plasmid sequence of pSEVA2311-msfGFP-pBBR1 and pSEVA647. *Sequences from official pSEVA website: https://seva-plasmids.com/.*

Plasmid name	Sequence	Remarks
pSEVA2311-msfGFP-pBBR1	0001 TTAATTAATC AAAAAACAAT AGAGGAGACT GAATTTTCAG ACACGAGATT 0051 GGTGTGTTCC TGTCTGATTT CGATGGGTGT TTTCCCAAAA AATTTTTGAA 0101 ATACTCGGGT CATATGGCTC TGATCGCAAA AACCGGCTTC ACTGGCAATC 0151 ACTTTCAGTG GAATTTCTTT TTTTGAAGTG AGCAAACGTT TAGCAAATTG 0201 CACCCTGAGA TTCATGATGT AGGCAGCAGG GGAATTACCA AAATCGACTT 0251 TAAATTTTCT CATTAAATGA GGCACGCTCA TCTTGACTAA TCCCGCTAAA 0301 TCTTCGAGGG TAATTTTAAT GCTCATGTTT TCATTGATAA ATTCTAATAA 0351 CAGTCTGCGT TGTAGGGGAG TAAAACCAGA ACGGCACTGT CCTTCCTTAA 0401 AATCTAATTT GGCATACTGA CGGAGTAAAT GCAGGGCGAT CTGGTTTTTA 0451 ATGCTCTCTA AATACAGGTT TCCACCTAAA CCACCTTGCT TTAATTCTAG 0501 TTCTAAAAGT GAAGTCAGAG CAGGTAAAAC ATGATCTTCC ACACCGACTT 0551 CGTCTCTGAT TCGGATCGAA GCGATATCAT AATCAAACAC CTTATTCGCC 0601 ATTGAGGTGA TGGAGTCATG CGAAATGTAG ACATGGGTGA CGGCAATATG 0651 GTCATTCCAC GCCCAGCGGG AATCTTCACC ACAGGTCAAC AAGGAGACAT 0701 AACCCGGCTT AACCACTTGG GTATCCCAAG AGGCATCGCC TTTTCTACGC 0751 ATTTCCGCCG CACTTTTTTT ATAGTTGACG ATCATGTAGT CACGCATGGC 0801 AGGAATATGC ACATCGAGGT TGGTATATTC GTACCCTCTG ATTGCAATGT 0851 TTTCCCAACC GGCATTCATG CTGTCAATTG TGACATTACC CGGAATCCAT 0901 AACACGACAT CTTCGGGTTT GATCAGCGTA TTTGCTTTGT CTGTGCTCAT 0951 GCTAGCTTTT TCCTCCTTAT AAAGTTAATC AACACCCCTT GTATTACTGT 1001 TTATGTAAGC AGACAGTTTT ATTGTTCATG ATGATATATT TTTATCTTGT 1051 GCAATGTAAC ATCAGAGATT TTGAGACACA AGACGTGGCA AAAAACATTA 1101 TCCAGAACGG GAGTGCGCCT TGAGCGACAC GAATTATGCA GTGATTTACG 1151 ACCTGCACAG CCATACCACA GCTTCCGATG GCTGCCTGAC GCCAGAAGCA 1201 TTGGTGCACC GTGCAGTCGA TGATAAGCTG TCAAACAGCG GATAACAATT 1251 TCACACAGGA GCAACTAAAA GAGATTGTTT GGATCAGTTA CCCAAAATCG 1301 TTGAAAAGAT TTTAACTCTT CGATTTTTAT TTTTTAGGTA ATCCTAGCCC 1351 TCTCGGGGGC TAGGATTAAA AATTTTAAGT TATTCCAACA CGAATGACAA 1401 ATTGTTCAAT GCAAAATAAA AACATACAAT ATATAAATAT ATTTTTTAAA 1451 TAAAACATAA GATTACAATA AAATAAGAAT TTTTATTTGG AGTTTGTTTT 1501 TTTTCTACAA TGATCATTAT GTACAATTTT TAGGTTCACC CCATCCAAGC 1551 CTTGTGATTG CATTCCTGCG ATTCTTTATT CAATGAATAA GCAATGCTAT 1601 TAATCAGCAA TGAATAACCA GCAGTGCAGA TTTTGAATAA ATTCACATGT 1651 CGTAATCCTA GGCCGCGGCC GCGCGAATTC GAGCTCGGTA CCCGGGGATC 1701 CTCTAGAGTC GACCTGCAGG CATGCAAGCT TAGGAGGAAA AACATATGCG 1751 TAAAGGTGAA GAACTGTTCA CCGGTGTTGT TCCGATCCTG GTTGAACTGG 1801 ATGGTGATGT TAACGGCCAC AAATTCTCTG TTCGTGGTGA AGGTGAAGGT 1851 GATGCAACCA ACGGTAAACT GACCCTGAAA TTCATCTGCA CTACCGGTAA 1901 ACTGCCGGTT CCATGGCCGA CTCTGGTGAC TACCCTGACC TATGGTGTTC 1951 AGTGTTTTTC TCGTTACCCG GATCACATGA AGCAGCATGA TTTCTTCAAA 2001 TCTGCAATGC CGGAAGGTTA TGTACAGGAG CGCACCATTT CTTTCAAAGA 2051 CGATGGCACC TACAAAACCC GTGCAGAGGT TAAATTTGAA GGTGATACTC 2101 TGGTGAACCG TATTGAACTG AAAGGCATTG ATTTCAAAGA GGACGGCAAC 2151 ATCCTGGGCC ACAAACTGGA ATATAACTTC AACTCCCATA ACGTTTACAT 2201 CACCGCAGAC AAACAGAAGA ACGGTATCAA AGCTAACTTC AAAATTCGCC 2251 ATAACGTTGA AGACGGTAGC GTACAGCTGG CGGACCACTA CCAGCAGAAC 2301 ACTCCGATCG GTGATGGTCC GGTTCTGCTG CCGGATAACC ACTACCTGTC 2351 CACCCAGTCT AAACTGTCCA AAGACCCGAA CGAAAAGCGC GACCACATGG 2401 TGCTGCTGGA GTTCGTTACT GCCGCAGGTA TCACGCACGG CATGGATGAA 2451 CTCTACAAAT AAACTAGTCT TGGACTCCTG TTGATAGATC CAGTAATGAC 2501 CTCAGAACTC CATCTGGATT TGTTCAGAAC GCTCGGTTGC CGCCGGGCGT 2551 TTTTTATTGG TGAGAATCCA GGGGTCCCCA ATAATTACGA TTTAAATTTG 2601 TGTCTCAAAA TCTCTGATGT TACATTGCAC AAGATAAAAA TATATCATCA 2651 TGAACAATAA AACTGTCTGC TTACATAAAC AGTAATACAA GGGGTGTTAT 2701 GAGCCATATT CAGCGTGAAA CGAGCTGTAG CCGTCCGCGT CTGAACAGCA 2751 ACATGGATGC GGATCTGTAT GGCTATAAAT GGGCGCGTGA TAACGTGGGT 2801 CAGAGCGGCG CGACCATTTA TCGTCTGTAT GGCAAACCGG ATGCGCCGGA 2851 ACTGTTTCTG AAACATGGCA AAGGCAGCGT GGCGAACGAT GTGACCGATG 2901 AAATGGTGCG TCTGAACTGG CTGACCGAAT TTATGCCGCT GCCGACCATT 2951 AAACATTTTA TTCGCACCCC GGATGATGCG TGGCTGCTGA CCACCGCGAT 3001 TCCGGGCAAA ACCGCGTTTC AGGTGCTGGA AGAATATCCG GATAGCGGCG 3051 AAAACATTGT GGATGCGCTG GCCGTGTTTC TGCGTCGTCT GCATAGCATT 3101 CCGGTGTGCA ACTGCCCGTT TAACAGCGAT CGTGTGTTTC GTCTGGCCCA 3151 GGCGCAGAGC CGTATGAACA ACGGCCTGGT GGATGCGAGC GATTTTGATG 3201 ATGAACGTAA CGGCTGGCCG GTGGAACAGG TGTGGAAAGA AATGCATAAA 3251 CTGCTGCCGT TTAGCCCGGA TAGCGTGGTG ACCCACGGCG ATTTTAGCCT	*superfolder gfp* highlighted in green.

	3301 GGATAACCTG ATTTTCGATG AAGGCAAACT GATTGGCTGC ATTGATGTGG 3351 GCCGTGTGGG CATTGCGGAT CGTTATCAGG ATCTGGCCAT TCTGTGGAAC 3401 TGCCTGGGCG AATTTAGCCC GAGCCTGCAA AAACGTCTGT TTCAGAAATA 3451 TGGCATTGAT AATCCGGATA TGAACAAACT GCAATTTCAT CTGATGCTGG 3501 ATGAATTTTT CTAATAATTA ATTGGACCGC GGTCCGCGCG TTGTCCTTTT 3551 CCGCTGCATA ACCCTGCTTC GGGGTCATTA TAGCGATTTT TTCGGTATAT 3601 CCATCCTTTT TCGCACGATA TACAGGATTT TGCCAAAGGG TTCGTGTAGA 3651 CTTTCCTTGG TGTATCCAAC GGCGTCAGCC GGGCAGGATA GGTGAAGTAG 3701 GCCCACCCGC GAGCGGGTGT TCCTTCTTCA CTGTCCCTTA TTCGCACCTG 3751 GCGGTGCTCA ACGGGAATCC TGCTCTGCGA GGCTGGCCGT AGGCCGGCCC 3801 TACCGGCGCG GCAGCGTTAC CCGTGTCGGC GGCTCCAACG GCTCGCCATC 3851 GTCCAGAAAA CACGGCTCAT CGGGCATCGG CAGGCGCTGC TGCCCGCGCC 3901 GTTCCCATTC CTCCGTTTCG GTCAAGGCTG GCAGGTCTGG TTCCATGCCC 3951 GGAATGCCGG GCTGGCTGGG CGGCTCCTCG CCGGGGCCGG TCGGTAGTTG 4001 CTGCTCGCCC GGATACAGGG TCGGGATGCG GCGCAGGTCG CCATGCCCCA 4051 ACAGCGATTC GTCCTGGTCG TCGTGATCAA CCACCACGGC GGCACTGAAC 4101 ACCGACAGGC GCAACTGGTC GCGGGGCTGG CCCCACGCCA CGCGGTCATT 4151 GACCACGTAG GCCGACACGG TGCCGGGGCC GTTGAGCTTC ACGACGGAGA 4201 TCCAGCGCTC GGCCACCAAG TCCTTGACTG CGTATTGGAC CGTCCGCAAA 4251 GAACGTCCGA TGAGCTTGGA AAGTGTCTTC TGGCTGACCA CCACGGCGTT 4301 CTGGTGGCCC ATCTGCGCCA CGAGGTGATG CAGCAGCATT GCCGCCGTGG 4351 GTTTCCTCGC AATAAGCCCG GCCCACGCCT CATGCGCTTT GCGTTCCGTT 4401 TGCACCCAGT GACCGGGCTT GTTCTTGGCT TGAATGCCGA TTTCTCTGGA 4451 CTGCGTGGCC ATGCTTATCT CCATGCGGTA GGGGTGCCGC ACGGTTGCGG 4501 CACCATGCGC AATCAGCTGC AACTTTTCGG CAGCGCGACA ACAATTATGC 4551 GTTGCGTAAA AGTGGCAGTC AATTACAGAT TTTCTTTAAC CTACGCAATG 4601 AGCTATTGCG GGGGGTGCCG CAATGAGCTG TTGCGTACCC CCCTTTTTTA 4651 AGTTGTTGAT TTTTAAGTCT TTCGCATTTC GCCCTATATC TAGTTCTTTG 4701 GTGCCCAAAG AAGGGCACCC CTGCGGGGTT CCCCCACGCC TTCGGCGCGG 4751 CTCCCCCTCC GGCAAAAAGT GGCCCCTCCG GGGCTTGTTG ATCGACTGCG 4801 CGGCCTTCGG CCTTGCCCAA GGTGGCGCTG CCCCCTTGGA ACCCCCGCAC 4851 TCGCCGCCGT GAGGCTCGGG GGGCAGGCGG GCGGGCTTCG CCCTTCGACT 4901 GCCCCCACTC GCATAGGCTT GGGTCGTTCC AGGCGCGTCA AGGCCAAGCC 4951 GCTGCGCGGT CGCTGCGCGA GCCTTGACCC GCCTTCCACT TGGTGTCCAA 5001 CCGGCAAGCG AAGCGCGCAG GCCGCAGGCC GGAGGCTTTT CCCCAGAGAA 5051 AATTAAAAAA ATTGATGGGG CAAGGCCGCA GGCCGCGCAG TTGGAGCCGG 5101 TGGGTATGTG GTCGAAGGCT GGGTAGCCGG TGGGCAATCC CTGTGGTCAA 5151 GCTCGTGGGC AGGCGCAGCC TGTCCATCAG CTTGTCCAGC AGGGTTGTCC 5201 ACGGGCCGAG CGAAGCGAGC CAGCCGGTGG CCGCTCGCGG CCATCGTCCA 5251 CATATCCACG GGCTGGCAAG GGAGCGCAGC GACCGCGCAG GGCGAAGCCC 5301 GGAGAGCAAG CCCGTAGGGG GGGCGCGCCC AGCTGTCTAG GGCGGCGGAT 5351 TTGTCCTACT CAGGAGAGCG TTCACCGACA AACAACAGAT AAAACGAAAG 5401 GCCCAGTCTT TCGACTGAGC CTTTCGTTTT ATTTGATGCC T	
pSEVA 647	0001 TTAATTAAAG CGGATAACAA TTTCACACAG GAGGCCGCCT AGGCCGCGGC 0051 CGCGCGAATT CGAGCTCGGT ACCCGGGGAT CCTCTAGAGT CGACCTGCAG 0101 GCATGCAAGC TTAGGAGGAA AAACATATGC GTAAAGGTGA AGAACTGTTC 0151 ACCGGTGTTG TTCCGATCCT GGTTGAACTG GATGGTGATG TTAACGGCCA 0201 CAAATTCTCT GTTCGTGGTG AAGGTGAAGG TGATGCAACC AACGGTAAAC 0251 TGACCCTGAA ATTCATCTGC ACTACCGGTA AACTGCCGGT TCCATGGCCG 0301 ACTCTGGTGA CTACCCTGAC CTATGGTGTT CAGTGTTTTT CTCGTTACCC 0351 GGATCACATG AAGCAGCATG ATTTCTTCAA ATCTGCAATG CCGGAAGGTT 0401 ATGTACAGGA GCGCACCATT TCTTTCAAAG ACGATGGCAC CTACAAAACC 0451 CGTGCAGAGG TTAAATTTGA AGGTGATACT CTGGTGAACC GTATTGAACT 0501 GAAAGGCATT GATTTCAAAG AGGACGGCAA CATCCTGGGC CACAAACTGG 0551 AATATAACTT CAACTCCCAT AACGTTTACA TCACCGCAGA CAAACAGAAG 0601 AACGGTATCA AAGCTAACTT CAAAATTCGC CATAACGTTG AAGACGGTAG 0651 CGTACAGCTG GCGGACCACT ACCAGCAGAA CACTCCGATC GGTGATGGTC 0701 CGGTTCTGCT GCCGGATAAC CACTACCTGT CCACCCAGTC TAAACTGTCC 0751 AAAGACCCGA ACGAAAAGCG CGACCACATG GTGCTGCTGG AGTTCGTTAC 0801 TGCcGCAGGT ATCACGCACG GCATGGATGA ACTCTACAAA TAAACTAGTC 0851 TTGGACTCCT GTTGATAGAT CCAGTAATGA CCTCAGAACT CCATCTGGAT 0901 TTGTTCAGAA CGCTCGGTTG CCGCCGGGCG TTTTTTATTG GTGAGAATCC 0951 AGGGGTCCCC AATAATTACG ATTTAAATTT GACATAAGCC TGTTCGGTTC 1001 GTAAACTGTA ATGCAAGTAG CGTATGCGCT CACGCAACTG GTCCAGAACC 1051 TTGACCGAAC GCAGCGGTGG TAACGGCGCA GTGGCGGTTT TCATGGCTTG 1101 TTATGACTGT TTTTTTGTAC AGCCTATGCC TCGGGCATCC AAGCAGCAAG 1151 CGCGTTACGC CGTGGGTCGA TGTTTGATGT TATGGAGCAG CAACGATGTT 1201 ACGCAGCAGC AACGATGTTA CGCAGCAGGG CAGTCGCCCT AAAACAAAGT 1251 TAGGTGGCTC AAGTATGGGC ATCATTCGCA CATGTAGGCT CGGCCCTGAC 1301 CAAGTCAAAT CCATGCGGGC TGCTCTTGAT CTTTTCGGTC GTGAGTTCGG 1351 AGACGTAGCC ACCTACTCCC AACATCAGCC GGACTCCGAT TACCTCGGGA 1401 ACTTGCTCCG TAGTAAGACA TTCATCGCGC TTGCTGCCTT CGACCAAGAA 1451 GCGGTTGTTG GCGCTCTCGC GGCTTACGTT CTGCCCAAGT TTGAGCAGCC 1501 GCGTAGTGAG ATCTATATCT ATGATCTCGC AGTCTCCGGA GAGCACCGGA 1551 GGCAGGGCAT TGCCACCGCG CTCATCAATC TCCTCAAGCA TGAGGCCAAC 1601 GCGCTTGGTG CTTATGTGAT CTACGTGCAA GCAGATTACG GTGACGATCC 1651 CGCAGTGGCT CTCTATACAA AGTTGGGCAT ACGGGAAGAA GTGATGCACT 1701 TTGATATCGA CCCAAGTACC GCCACCTAAC AATTCGTTCA AGCCGAGATC 1751 GGCTTCCCGG CCGCGGAGTT GTTCGGTAAA TTGGACAACG GTCCGCGCGT 1801 TGTCCTTTTC CGCTGCATAA CCCTGCTTCG GGGTCATTAT AGCGATTTTT	Vector backbone for transformations.

	1851 TCGGTATATC CATCCTTTTT CGCACGATAT ACAGGATTTT GCCAAAGGGT 1901 TCGTGTAGAC TTTCCTTGGT GTATCCAACG GCGTCAGCCG GGCAGGATAG 1951 GTGAAGTAGG CCCACCCGCG AGCGGGTGTT CCTTCTTCAC TGTCCCTTAT 2001 TCGCACCTGG CGGTGCTCAA CGGGAATCCT GCTCTGCGAG GCTGGCCGTA 2051 GGCCGGCCGA TAATCTCATG ACCAAAATCC CTTAACGTGA GTTTTCGTTC 2101 CACTGAGCGT CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC 2151 TTTTTTTCTG CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC 2201 CAGCGGTGGT TTGTTTGCCG GATCAAGAGC TACCAACTCT TTTTCCGAAG 2251 GTAACTGGCT TCAGCAGAGC GCAGATACCA AATACTGTTC TTCTAGTGTA 2301 GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC 2351 TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG 2401 TGTCTTACCG GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG 2451 GTCGGGCTGA ACGGGGGGTT CGTGCACACA GCCCAGCTTG GAGCGAACGA 2501 CCTACACCGA ACTGAGATAC CTACAGCGTG AGCTATGAGA AAGCGCCACG 2551 CTTCCCGAAG GGAGAAAGGC GGACAGGCAT CCGGTAAGCG GCAGGGTCGG 2601 AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC TGGTATCTTT 2651 ATAGTCCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTTGTGA 2701 TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCGT 2751 GAAAGGCAGG CCGGTCCGTG GTGGCCACGG CCTCTAGGCC AGATCCAGCG 2801 GCATCTGGGT TAGTCGAGCG CGGGCCGCTT CCCATGTCTC ACCAGGGCGA 2851 GCCTGTTTCG CGATCTCAGC ATCTGAAATC TTCCCGGCCT TGCGCTTCGC 2901 TGGGGCCTTA CCCACCGCCT TGGCGGGCTT CTTCGGTCCA AAACTGAACA 2951 ACAGATGTGT GACCTTGCGC CCGGTCTTTC GCTGCGCCCA CTCCACCTGT 3001 AGCGGGCTGT GCTCGTTGAT CTGCGTCACG GCTGGATCAA GCACTCGCAA 3051 CTTGAAGTCC TTGATCGAGG GATACCGGCC TTCCAGTTGA AACCACTTTC 3101 GCAGCTGGTC AATTTCTATT TCGCGCTGGC CGATGCTGTC CCATTGCATG 3151 AGCAGCTCGT AAAGCCTGAT CGCGTGGGTG CTGTCCATCT TGGCCACGTC 3201 AGCCAAGGCG TATTTGGTGA ACTGTTTGGT GAGTTCCGTC AGGTACGGCA 3251 GCATGTCTTT GGTGAACCTG AGTTCTACAC GGCCCTCACC CTCCCGGTAG 3301 ATGATTGTTT GCACCCAGCC GGTAATCATC ACACTCGGTC TTTTCCCCTT 3351 GCCATTGGGC TCTTGGGTTA ACCGGACTTC CCGCCGTTTC AGGCGCAGGG 3401 CCGCTTCTTT GAGCTGGTTG TAGGAAGATT CGATAGGGAC ACCCGCCATC 3451 GTCGCTATGT CCTCCGCCGT CACTGAATAC ATCACTTCAT CGGTGACAGG 3501 CTCGCTCCTC TTCACCTGGC TAATACAGGC CAGAACGATC CGCTGTTCCT 3551 GAACACTGAG GCGATACGCG GCCTCGACCA GGGCATTGCT TTTGTAAACC 3601 ATTGGGGGTG AGGCCACGTT CGACATTCCT TGTGTATAAG GGGACACTGT 3651 ATCTGCGTCC CACAATACAA CAAATCCGTC CCTTTACAAC AACAAATCCG 3701 TCCCTTCTTA ACAACAAATC CGTCCCTTAA TGGCAACAAA TCCGTCCCTT 3751 TTTAAACTCT ACAGGCCACG GATTACGTGG CCTGTAGACG TCCTAAAAGG 3801 TTTAAAAGGG AAAAGGAAGA AAAGGGTGGA AACGCAAAAA ACGCACCACT 3851 ACGTGGCCCC GTTGGGGCCG CATTTGTGCC CCTGAAGGGG CGGGGGAGGC 3901 GTCTGGGCAA TCCCCGTTTT ACCAGTCCCC TATCGCCGCC TGAGAGGGCG 3951 CAGGAAGCGA GTAATCAGGG TATCGAGGCG GATTCACCCT TGGCGTCCAA 4001 CCAGCGGCAC CAGCGGCGCC TGAGAGGGGC GCGCCCAGCT GTCTAGGGCG 4051 GCGGATTTGT CCTACTCAGG AGAGCGTTCA CCGACAAACA ACAGATAAAA 4101 CGAAAGGCCC AGTCTTTCGA CTGAGCCTTT CGTTTTATTT GATGCCT	

Table 9: DNA-sequence g-blocks and oligonucleotides (Primers) as synthesised by IDT (Coralville, USA).

G-block and oligonucleotide	**Sequence in 5′- 3′ direction**	**Remarks**
Type-3-secretion system, secretion signal from AvrRPM1 in *P. syringae.*	ATGGCCGCTGTATCGAGCACTTCAAGAAGTACTGGA TATTATTCCGGTTACGAAAATCATGAGGAGCCAAGGGT GGCAAGTAGCCCGACGAACAGGCATGATCGCGGCTACG AAACCGATTCCGAGCATTCCAATGTTAACTTAATACCCA ATGCGAGGAGAGTGTATAAAGAGTCTGTTTTGTGGCATG GTACATCTATGCAATCAAAAATAGATTTGAGAAGGCAAG GGTTTGATGTGAATAGAAAAACTGATGGTGCAAC	DNA sequence retrieved from supplementary data of Jensen et al. (2022).
T3SS secretion signal with AvrRPM1 promoter	taattcggcaaaaatcgtacgcaggctttttcggcctgagcctgttcagatctgtagatttgtgatgga cgcaggcgagtatgcgcagtctaaaaaatcgttttccgccaatggagtctgttccagcagggaactc attttctttttaaaaccacacatgtactccatactttcaatcaaaaggtttttttATGGCCGCTGTATC GAGCACTTCAAGAAGTACTGGATATTATTCCGGTTACGAAAATCATGA GGAGCCAAGGGTGGCAAGTAGCCCGACGAACAGGCATGATCGCGGCT ACGAAACCGATTCCGAGCATTCCAATGTTAACTTAATACCCAATGCGAG GAGAGTGTATAAAGAGTCTGTTTTGTGGCATGGTACATCTATGCAATCA AAAATAGATTTGAGAAGGCAAGGGTTTGATGTGAATAGAAAAACTGAT GGTGCAAC	DNA sequence in capitals (AvrRPM1 secretion signal), Sequence in lower case (AvrRPM1 promoter) as described in

		(Jensen et al., 2022b).
sfGFP_fwd	TATGGTCTCAcgagatgcgtaaaggcgaagaactgtt	Bsa1 recognition site in capital letters
sfGFP_rev	TATGGTCTCAactgtcaatgatgatgatgatgatgatgcttatacagctcgtccataccgtgg	Bsa1 recognition site in capital letters
sfGFP_promoter_fwd	TATGGTCTCAactgtcaatgatgatgatgatgatgatgcttatacagctcgtccataccgtgg	Bsa1 recognition site in capital letters
RFP_RBS_fwd	TATGGTCTCAagggaaagaggagaaatactagATGGCTTCCTCCGAAGATGTTATCA	RBS0034 in small letters
RFP_rev	TATGGTCTCAGCCCTAAGCACCGGTGGAGTGACG	
J23100:Avr_fwd	TATGGTCTCAgaatttgacggctagctcagtcctaggtacagtgctagctactagtgaaagaggagaaatactagATGGCCGCTGTATCGAGCAC	Bsa1 recognition site and start of gene In in capital letters
J23100:Avr_rev	TATGGTCTCACTCGGTTGCACCATC	
Avr_fwd	TATGGTCTCAgaattaattcggcaaaaatcgtac	Bsa1 recognition site in capital letters
Pv	aggtctcacagtccggcaaaaaaggg	Linearisation of pSEVA647
Ev	ccgggtctcaattccagaaatcatccttagcgaaagc	Linearisation of pSEVA647

8.3 Figures

Fiji/imageJ Macro for the samples carrying the pSal/nahR inducible expression system (ref. "Material & Methods", FRUIT experiments).

Image J Macro (pSAl/nahR):

```
run("Subtract...", "value=450");
run("Duplicate...", " ");
setMinAndMax(125, 2189);
setAutoThreshold("Default dark no-reset");
//run("Threshold...");
setOption("BlackBackground", true);
run("Convert to Mask");
run("Close");
run("Analyze Particles...", "size=3-Infinity clear add");
close();
roiManager("Measure");
String.copyResults();
```

Figure 48: Image J Macro for the processing of fluroescence microscopic pictures from the pSAL/nahR inducibile expression system according to the FRUIT protocol. *Input: Fluorescence microscopy pictures after uploading to FIJI (image J). Output: Fluorescence intensities of single fluorescent particles in the*

Attachment of *P. fluorescens* on roots of *A. thaliana* occurred from day 0 after addition of bacteria to plants (ref. "Results", *P. fluorescens* Colonises on Roots of *A. thaliana*).

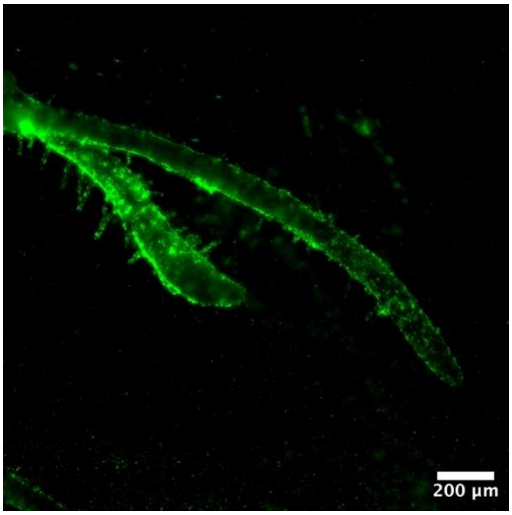

Figure 49: Fluorescence microscopy picture showing attachment of* P. fluorescens *on roots of* A. thaliana *(one hour after incubation). *On the day of incubation, attachment occurs on the basal and apical region of the roots.*

Colonisation of *P. fluorescens* etHAn on a root of *A. thaliana.* The bacterial quantity is indicated on the left side in arbitrary units. The bacterial quantity correlates with the brightness of the image at the respective millimetre (Ref. "Results", Colonisation experiment).

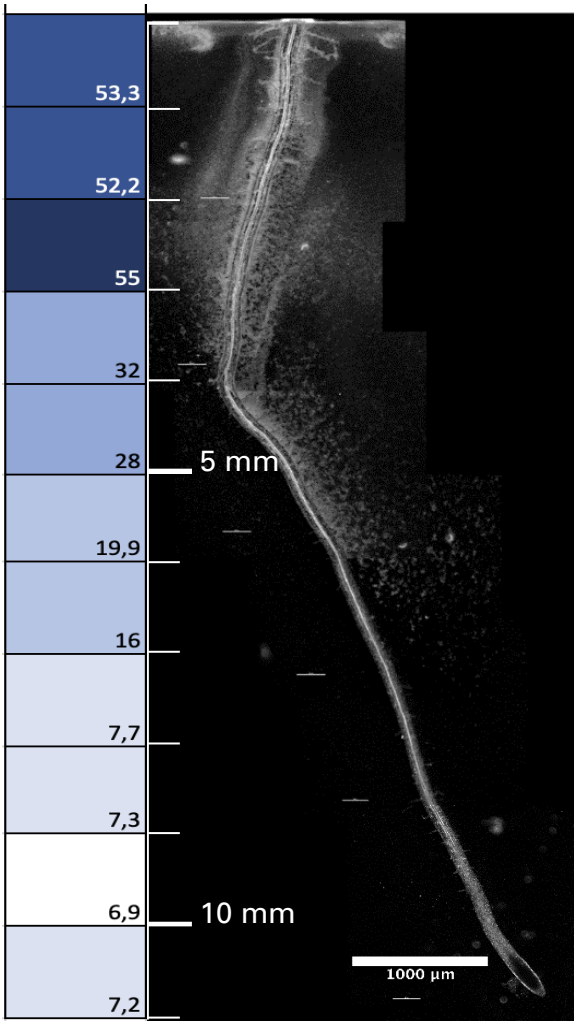

Figure 50: Overview of* A. thaliana *root, bacterial colonisation over length of whole root. *Pictures is merged from 6 phase contrast, pH3, 100x magnification images, 1 day after first attachment. Bacteria are white structures around the root. A heat map on the right site shows the measured brightfield values correlating with bacterial densities.*

Macrogen (Macrogen, Seoul, South Korea) sequencing results. The sequence alignment is representative for all samples.

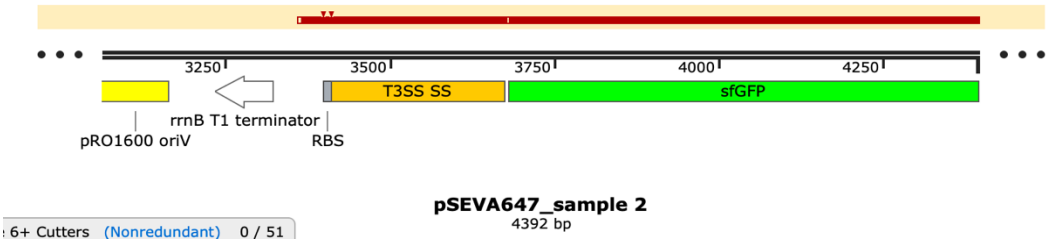

Figure 51: SnapGene (San Diego, USA) screenshot showing the gfp-ss gene in sample 2 (test plasmid 2). Red line above the plasmid map indicates matching base pairs.

Negative control of pBAD/AraC inducible expression system. Bacteria are numerous (black spots in phase contrast picture), but few bacteria are induced (*gfp*-expression).

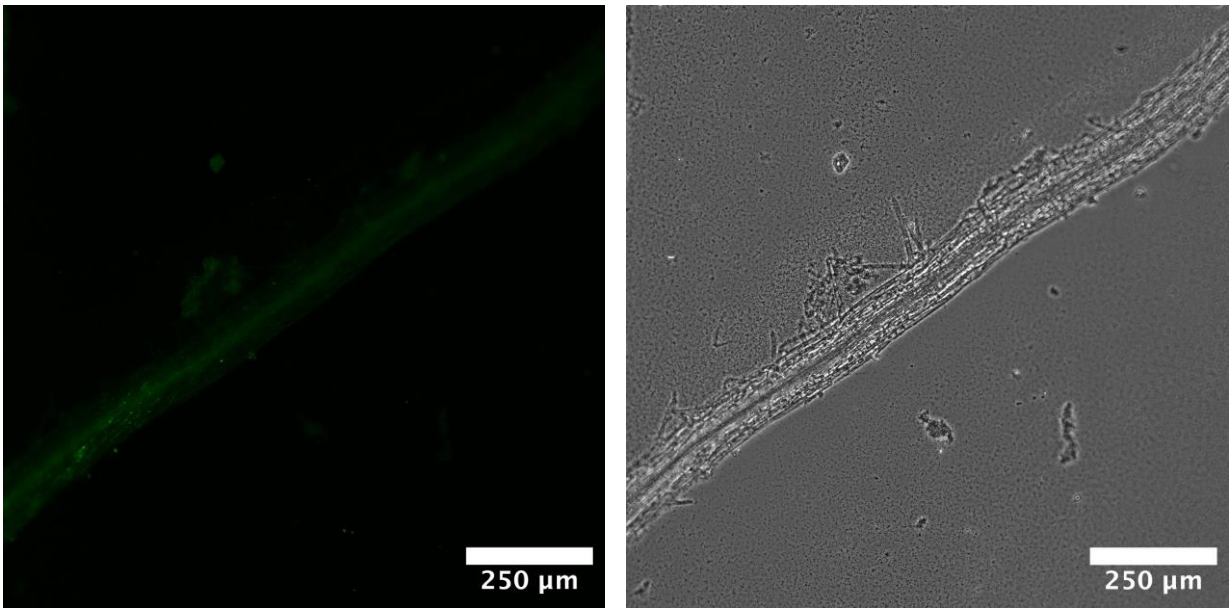

Figure 52: Microscopic images of* A. thaliana *root with root exudate sensitive* P. fluorescens *SBW25 (pBAD/AraC inducible expression system, 100x magnification, 24 hours after first attachment, negative control). *Left: Fluorescence microscopic image. Right: Phase contrast image.*

Growth of *P. fluorescens* remained on a low level when grown in HDM. A correction for OD (fluorescence intensity divided by OD-value) does not produce meaningful data (ref. Chapter 3.2, "Secretion into T3SS-Inducing Media")

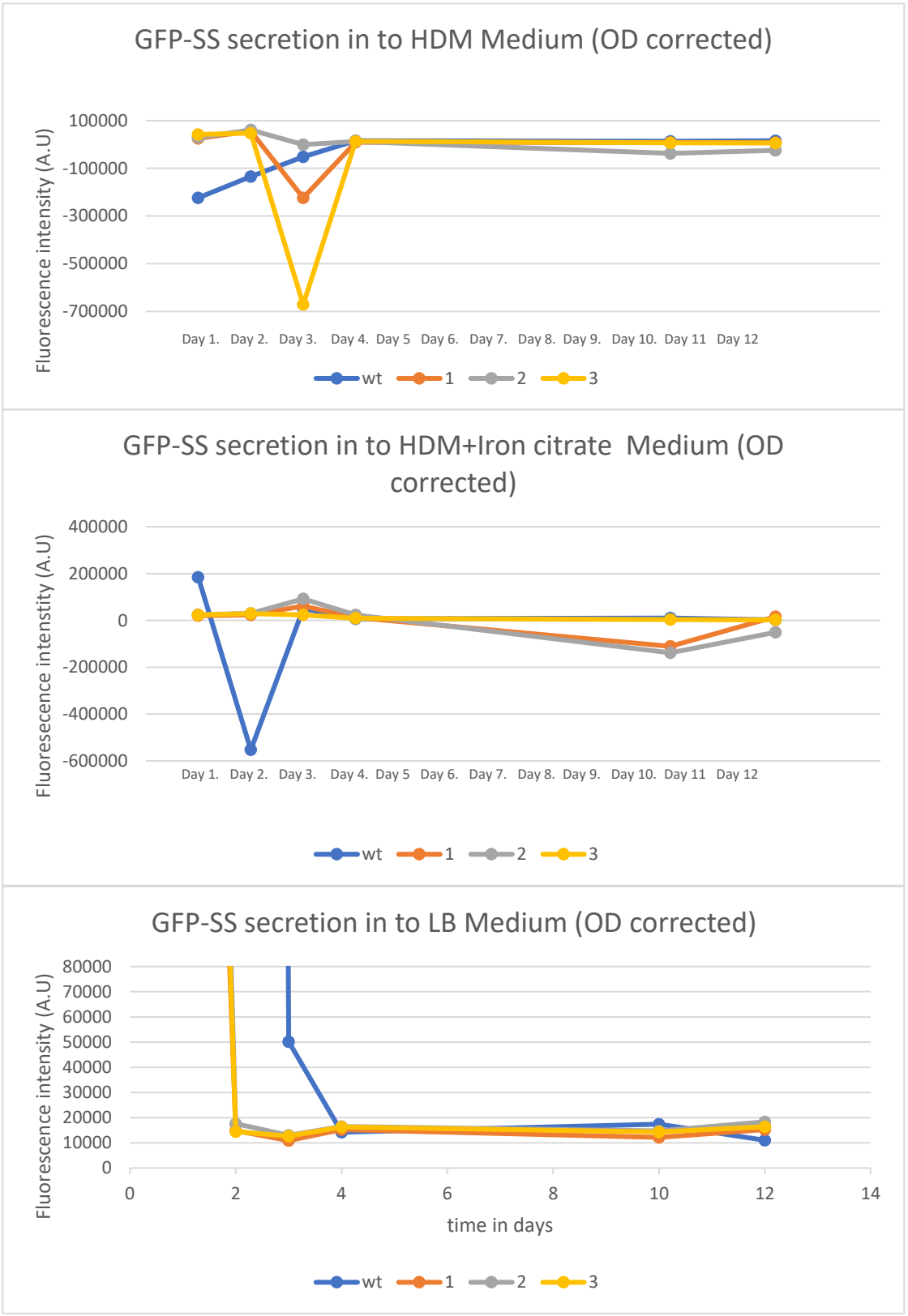

Figure 53: OD-corrected values for measured GFP-SS inside the growth medium of* P. fluorescens *etHAn.

Box-Plot diagrams showing the mean fluorescence intensity of the fluorescent particles in microscopic pictures with root exudate sensitive *P. fluorescens* SBW25 on roots of *A. thaliana* (ref. "Results", FRUIT experiments). Sample 3 of the pSAL/nahR inducible expression system is shown (**Figure 54**Further all samples for the inducible expression system for pCym/CymR, pTtg/TtgR, and pBAD/AraC (Figure 55, Figure 56 and Figure 57)

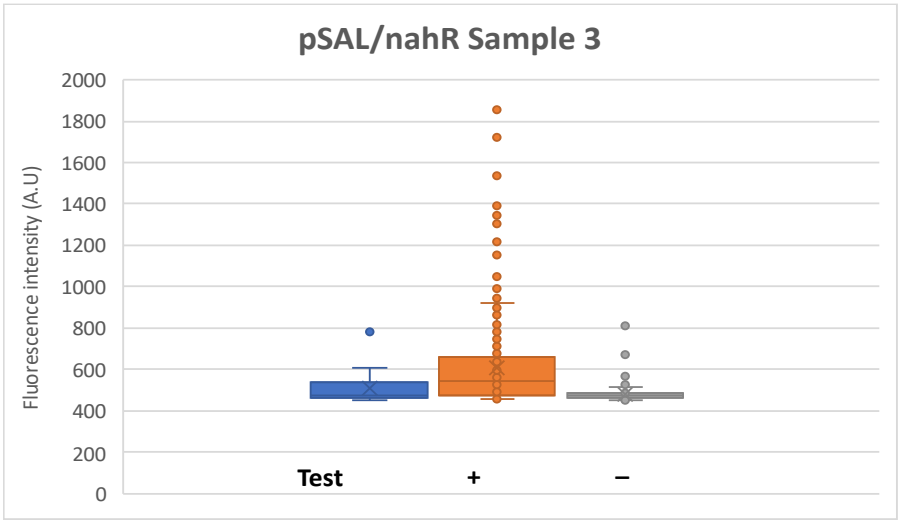

Figure 54: Fluorescence response of FRUIT-measured image analysis after 24 hours of* P. fluorescens *with pSal/nahR inducible expression system cultivated on* A. thaliana *roots (Sample 3). *Test: bacteria with living, exudate secreting roots."-": Negative control, bacteria with fixed, non-secreting roots. "+": Positive control, bacteria with fixated roots, in presence of 1mM of salicylic acid.*

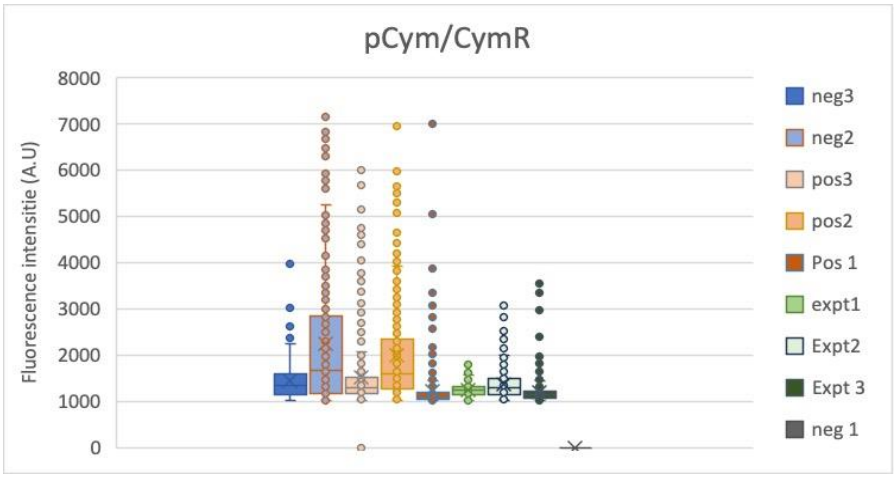

Figure 55: Fluorescence response of FRUIT-measured image analysis after 24 hours of* P. fluorescens *with pCym/CymR inducible expression system cultivated on* A. thaliana *roots. *"expt 1-3": bacteria with living, exudate secreting roots. "neg 1-3": bacteria with fixed, non-secreting roots. "neg 1" did not show any fluorescent particles. "pos 1-3": Positive control, bacteria with fixated roots, in presence of 1mM of cumic acid.*

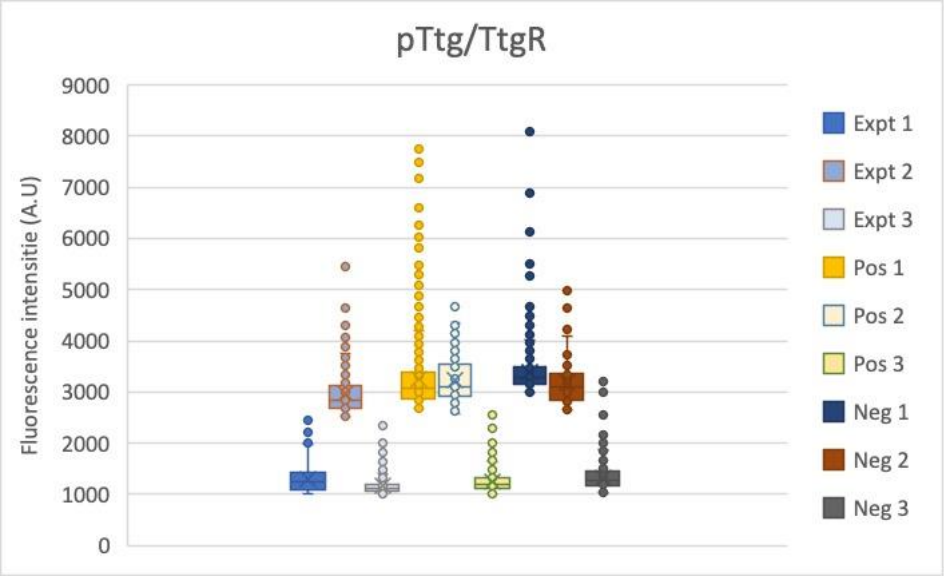

Figure 56: Fluorescence response of FRUIT-measured image analysis after 24 hours of* P. fluorescens *with pTtg/TtgR inducible expression system cultivated on* A. thaliana *roots. *"Expt 1-3": bacteria with living, exudate secreting roots. "Neg 1-3": bacteria with fixed, non-secreting roots. "Neg 1" did not show any fluorescent particles. "Pos 1-3": Positive control, bacteria with fixated roots, in presence of 1mM of naringenin. Sample 3 shows no induction, irrespective of the condition.*

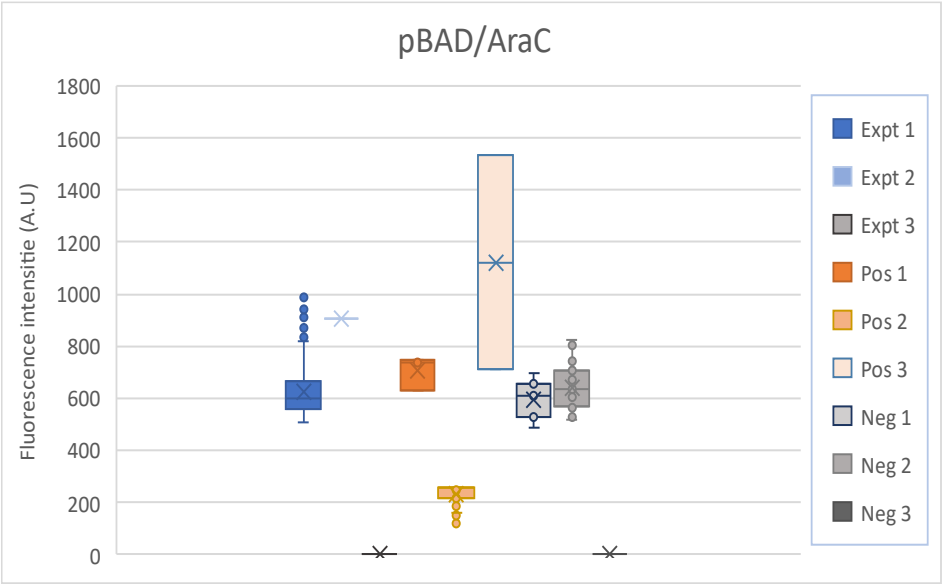

Figure 57: Fluorescence response of FRUIT-measured image analysis after 24 hours of* P. fluorescens *with pBAD/AraC inducible expression system cultivated on* A. thaliana *roots. *"Expt 1-3": bacteria with living, exudate secreting roots. "Neg 1-3": bacteria with fixed, non-secreting roots. "Neg 1" did not show any fluorescent particles. "Pos 1-3": Positive control, bacteria with fixated roots, in presence of 1mM of arabinose. Expterimental and negative control of Sample 3 shows no induction.*

9 Declaration of Authenticity

I hereby declare that I completed the master thesis with the topic "Colonisation and T3SS-Mediated Protein Secretion of *Pseudomonas fluorescens* etHAn on Roots of *Arabidopsis thaliana* Columbia 0" independently and used only the materials that are listed. All materials used, from published as well as unpublished sources, whether directly quoted or paraphrased, are duly reported.

Furthermore, I declare that the master thesis, or any abridgement of it, was not used for any other degree-seeking purpose.

06.12.2023, Wageningen

Time, Place

Johannes Heisterberg

Signature