Octavian MURGU Bakk.rer.nat

Interactions of beneficial bacteria with fungi and their host plants

Master thesis

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Science (M.Sc.)

in “Plant science”

Institute of Environmental Biotechnology

Graz University of Technology

Supervisor: Univ.-Prof.Dipl.-Biol.Dr.rer.nat Gabriele Berg

April 2014

EIDESSTAATLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am …… ……
(Unterschrift)

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…….. ……
date (signature)

Acknowledgement

The research presented in this master thesis was carried out at the Laboratory of Environmental Biotechnology, Graz University of Technology between March 2013 and April 2014.

First, I would like to express my gratitude to my supervisor Prof. Gabriele Berg for giving me an insight into to fascinating area of microbial diversity and for giving me the opportunity to be a part of her team.

I am also very thankful to my co-supervisor Dr. Henry Müller for supporting me during my whole researches and his results-orientated thinking which helps me reaching my scientific aims. Additionally I want to thank him for proofreading this thesis.

I am grateful for the scientific support from Dr. Christin Zachow, Dr. Anastasia Bragina, M.Sc. Tomislav Cernava, Alexander Mahnert and to all co-workers from the work group of Prof. Berg.

The whole groups of researches, teachers and students have earned my compliments for the atmosphere they have created to the laboratories in Graz. Finally I would like to thank my parents for giving me the opportunity to study and supporting me in my choices and dreams and to my sister and my friends for their endless patience with me.

Abstract

The aim of this work was to design different approaches allowing an easy but significant analysis of interactions of biological control agents (BCAs) with themselves but also with plants and fungi. Six BCAs were used in total, including the gram negative bacteria *Stenotrophomonas rhizophila* ep-17 and P69, *Serratia plymuthica* 3Re4-18, Rp5 and Rp8, and the gram positive bacterium *Bacillus subtilis* B2g. The interactions were studied by applying molecular, microbiological and microscopic methods. To simultaneously detect several strains using confocal laser scanning microscopy (CLSM), the BCAs should be transformed with the same vector construct bearing different fluorescent genes. The rhizosphere stable plasmids pJH, pBAH8 and pIN 29 were used for the cloning approaches. Only four fluorescent dyes (DsRed2, GFP, eBFP2 and mNeptune) could be used in this study due to the fixed laser sources of the CLSM. Whereas the two plasmids pJH and pBAH8 were unsuitable for the cloning approaches, the DsRed2 gene of the original pIN 29 was successfully restricted by using *Xba*I and *Bgl*II and replaced by the other three fluorescent genes. All BCAs except B2g were transformable using the pIN 29 vector. The stable expression of the fluorescent genes allowed detection on tomato and maize roots two weeks after inoculation. The simultaneous detection of all four fluorescent dyes was also shown for four ep-17 transformants each labeled with a different fluorochrome. Analyses of *in vitro* interactions between the BCAs showed no inhibitory effects of ep-17, Rp8 and 3Re4-18 when mixing them together in dual culture assays suggesting potential compatibility for multi-strain inoculations. The *ad planta* interaction studies of the BCAs were done with pIN 29-DsRed2-transformed ep-17, P69 on tomato and maize plants. Culture collection derived fungi but also newly isolated fungi were tested for their BCA transport abilities. Only B2g cells could be transported by the fungi BR4-1-11, BR1-1-5 und BE1-1-3. The identification of the *Bacillus* strain was done by comparison of BOX fingerprints and the viability check by using a Live/Dead staining kit.

Kurzfassung

Im Rahmen der Masterarbeit wurden die Interaktionen verschiedener bakterieller Biokontrollstämme (BCAs) untereinander sowie mit Pflanzen und Pilzen untersucht. Bei den BCAs handelte es sich um die fünf gram negativen Bakterien *Stenotrophomonas rhizophila* ep-17 und P69, *Serratia plymuthica* 3Re4-18, Rp5 und Rp8 sowie um das gram positive Bakterium *Bacillus subtilis* B2g. Die Interaktionen wurden unter Verwendung von molekularbiologischen, mikrobiologischen und mikroskopischen Methoden analysiert. Um die Stämme zeitgleich mittels eines konfokalen Laser-Scanning-Mikroskops (CLSM) an der Pflanze visualisieren zu können, war es wichtig, sie mit unterschiedlichen fluoreszierenden Genen im gleichen Vektorkonstrukt zu transformieren. Als Ausgangsvektoren für die Klonierungsarbeiten wurden die Plasmide pJH, pBAH8 und pIN 29 herangezogen. Bedingt durch die fixen Laserquellen des CLSM konnten vier verschiedene Fluoreszenzfarbstoffe selektiert werden. Die Nutzung von DsRed2, GFP, mNeptune und eBFP2 ermöglichte eine gleichzeitige Visualisierung von allen vier BCAs. Während die Klonierungsarbeiten mit den Vektoren pJH und pBAH8 nicht zielführend waren, konnte das DsRed2-Gen des pIN 29 Vektors erfolgreich mit den Restriktionsenzymen für *Xba*I und *Bgl*II herausgeschnitten und durch die Gene der alternativen fluoreszierenden Proteine ersetzt werden. Die Transformation von allen BCAs mit Ausnahme von B2g erfolgte mit Hilfe des modifizierten pIN 29-Plasmids. pIN 29 zeigte eine stabile Expression des fluoreszierenden Farbstoffes ohne dabei die Vitalität der Zellen zu beeinflussen. Kolonisierungsmuster konnten auch an 14 Tage alten Mais- oder Tomatenwurzeln noch dargestellt werden. Mit der CLSM-Analyse von Transformanten des Isolats *S. rhizophila* ep-17, die jeweils mit einem der vier modifizierten Vektoren ausgestattet wurden, konnte die gleichzeitige Darstellung aller vier Fluoreszenzfarbstoffe in einem Ansatz gezeigt werden. Die Analyse der Interaktionen zwischen den einzelnen BCAs zeigte, dass vor allem die gram negativen Bakterien ep-17, Rp8 und 3Re4-18 für eine kombinierte Anwendung in Frage kommen könnten, da diese keine inhibierenden Effekte untereinander aufwiesen. Die Interaktionen zwischen den pIN 29-DsRed2 *Stenotrophomonas* Stämmen ep-17 und pBAH8-markierten P69 an der Pflanzenwurzel, wurden anhand von Mais und Tomaten getestet. Bei der Suche nach einen potentiellen Transporter für die BCAs wurden sowohl Pilze aus einer Stammsammlung als auch aus landwirtschaftlich genutztem Boden für den Maisanbau isoliert und getestet. Von allen sechs Stämmen konnten lediglich Zellen von *Bacillus subtilis* B2g durch die aus der Stammsammlung stammenden Pilze BR4-1-11, BR1-1-5 und BE1-1-3 transportiert werden. Die Identität von B2g wurde in einem BOX PCR Ansatz geprüft. Des Weiteren konnte durch ein Live/Dead Färbenachweis die Vitalität der *Bacillus* Zellen bestätigt werden.

List of contents

1 Introduction

1.1 Agriculture/crop plants

Biocontrol in sustainable agriculture

It is expected that the earth`s population will increase up to 8.3 billion people by 2025. To ensure adequate food nutrition a simultaneous increase in crop production is necessary. Humans use plants up to 80% as nutriment source. Therefore, it will be an important goal of the twenty-first-century to ensure this supplemental challenge (Pimentel & Pimentel, 2006). For decades, synthetic pesticides and chemical fertilizers were used for industrial agriculture. Out of that, a range of problems results, like chemical-resistant pathogens and high pollution of arable land. Besides environmental damages, the chemical pesticides are very expensive and could harm human health. A trend-setting method for solving these troubles could provide biological control agents (Sayeed et al., 2010). Microorganism can be used as natural pesticides and protect the plants against different pathogens but also increase their tolerance towards abiotic stress and act as plant growth promoting agents. A high abundance of these beneficial microorganisms were already described for the rhizosphere of plants. Different strains colonize roots and affecting them positively. These bacteria were designated as plant growth promoting rhizobacteria (PGPR) (Lugtenberg & Kamilova, 2009). Together all these properties of PGPR could be used in the commercialized agriculture and offer new ecofriendly methods in biocontrol. Nowadays there are many commercial used microbial inoculants of PGPR summarized as biological control agents (BCA) which lead to a higher crop production and reduced predisposition against plant diseases. The use of bacterial mixtures as biocontrol product could amplify the positive effects. Therefore many strains have already been tested.

Crop plants

For studying the bacteria-plant interactions two different plant species were chosen for this work. In both cases the plants are worldwide intensively in use as crop plants.

- The usage of maize (*Zea mays* L.) as a crop plant becomes very important nowadays. The plant is not only used as a source for food production. A high amount of maize goes directly into fabrication of biofuel. To guarantee a high yield a lot of fertilizer such as nitrogen is applied. To obtain 1 t maize ha^{-1} it requires the application of 9 to 11 kg of chemical nitrogen. This leads one site to the pollution of the soil and on the other to high upcoming costs for the farmers. A more profitable solution would deliver the usage of BCAs as biofertilizers. PGPRs could increase yields without negative effects of agrochemicals. Beside their function of nitrogen fixation, they also provide a brought range of positive effects on plants like induction of plant resistance against pathogens (Montañez et al., 2012).

- The main cultivation areas for tomatoes are located in the USA, China and Italy. For an ideal fruit yield the nitrogen (N) uptake plays an important role. For optimal growth conditions an uptake up to 450 kg of N ha^{-1} must occur. This data depends on cultivar and machining method of crop land (Elia & Conversa, 2012). Further, the usage of high amounts of fertilizers lead to an environmental problem because most of the chemical nutrients couldn`t be metabolized by plants and were washed out and accumulating in the ground water. PGPR could play an important role in the nutrient management. They could optimize the nutrient content in fertilized soil to improve the plant uptake and simultaneous prevent the soil against overfertilisation (Yang et al., 2009). Soils have to deal with higher temperature due to the climate change, leading to an increase of soil salinity worldwide. Using PGPRs like *Achromobacter piechaudii* for tomato, could led to a decreased level of ethylene by production of 1-aminocyclopropane-1-carboxylate deaminase (ACC) under this conditions. Tomato seedlings treated with PGPR showed 66% higher growth under high salt conditions in comparison to the untreated approaches (Maya et al., 2004).

1.2 Rhizosphere/Plant (Fungi)-microbe-Interactions

Unlike bulk soil the rhizosphere is a very nutrient-rich environment. Plants exsudate up to 50 % of their photosynthetic products through their roots. Beside sugars, vitamins, organic and amino acids different purines, pyrimidines and enzymes could be found in plant root exudates (Barriuso et al., 2008; Farrah et al., 2008). Hence, a lot of microorganisms colonize the soil habitat. The diversity of the rhizosphere microbiome is very high. PGPRs constitute a special part within this community. A lot of plant exudates were taken up by PGPRs and used for their own metabolism (Vacheron et al., 2013). On the other site, PGPRs produce plant growth promoting substances like auxines, gibberellins and cytokines and have therefore a direct influence on plant growth. Indirect interactions between microorganisms and plants take also place. Plant pathogens are inhibited through synthesis and contribution of antibiotics or cell wall lysing enzymes by bacteria (Farah et al., 2008).

Interactions of bacteria with their host plants play important roles in the rhizosphere. The so called quorum sensing (QS) describes the communication between different microorganism by producing molecules which could be recognized and used as a kind of language (Witzany, 2011). The term is generally used to describe the change in the genome expression pattern of bacteria in response of different stimuli. These cues could derive from environmental changes or density of bacterial population. For their communication the bacteria possess so called auto-inducers (*N*-Acyl-homoserine lactone (AHL)). The binding of this substance on specific receptors led to an expression of different genes including these for the production of AHL. If a high density of the same kind of bacteria relasae AHLs into the surrounding medium it led to increasing production of AHL (= positive feedback) of the microorganisms when the concentration reaches a threshold.

A variety of other communication signals exists. Especially gram negative bacteria use AHL for communication. Different mechanisms are regulating by QS like the symbiotic, pathogenic or commensally relationship of bacteria with their eukaryotic host. Biofilm formation or production of different compounds like toxins or exopolysaccharide is regulated by QS. This knowledge is used to generate adorable effects on the plant-bacteria interaction level. AHL analogs could be produce synthetically and implement into field approaches. Also transgenic plants which produce AHL could therefore influence their bacterial colonization pattern (Iqbal et al., 2010).

Beside plant-bacteria interactions also interactions between fungi and bacteria exist in nature. The pathogenic fungi *Didymella bryoniae* which causes black rot and gummy stem blight on Styrian oil pumpkin for example was identified as "bacterial transporting organism". *D. bryoniae* interacts with different pathogenic bacteria like *Pseudomonas viridiflava*. Through the co-infection of pumpkin with both organisms a high number of the yield lost occurred in the past years. An assay was set up which demonstrated that the fungi is able to transport the bacteria by mycelium growth (Grube et al., 2011).

1.3 Plant beneficial bacteria

In this work, the interactions and effects of three BCAs among themselves, plants and fungi were analyzed. In total, investigations of six strains including *Bacillus*, *Serratia* and *Stenotrophomonas* were analyzed on different models.

- ***Bacillus*** is a gram positive bacterium with a high abundance in soil. It is resistant to extreme conditions due to production of endospores. The high potential of *Bacillus* as PGPR and BCA is already known and in comparison to other natural occurring bacteria it produces different antibiotics including bacitracin and gramicidin. Many studies have shown the ability of *Bacillus* strains as biocontrol agents against many fungi. *Bacillus brevis* produces unknown antibiotics and inhibited the growth of *Fusarium oxysporum f.* sp. *udum.* Studies of *Bacillus subtilis* AF1 and *F. udum* showed that the strain is able to produce extracellular proteins which reduce the growth of the fungus (Dardanelli et al., 2010;Pandey et al., 2010). *Bacillus subtilis* 3A25, a strain isolated from Indian soil, mediate an increased ozon stress tolerance of *Brassica juncea* (Holzinger et al., 2011). In the industrialized agriculture *Bacillus*-based products offer the advantage of easy formulation and storage due to production of endospores. There are many commercial products already available on the market which were used as biofertilizer, -pesticides and -fungicides. Kodiak for example is a *Bacillus subtilis* derived product used as biofungicid. Additionally, it protects cotton, legumes, soybean and vegetable crops against *Rhizoctonia*, *Fusarium*, *Pythium* and *Aspergillus*. Some *Bacillus* strains produce different molecules like lipopetides which induce the resistance response (induced systemic resistance, ISR) in plants and make

them more resistant against different diseases. The lipopetid surfactin for example, showed an important effect of the swarming ability of *Bacillus*. By releasing this substance a higher root colonization of *Bacillus* occurred (Pérez-García et al., 2011). Another example of BCAs is *Bacillus subtilis* B2g. It was first isolated in 1993 out of oilseed rape. The strain is known for its antifungal activities. The bacterium is able to suppress soil-borne fungi like *Rhizoctonia solani* by secreting lytic exoenzymes like β-1,3-glucanase. The production of the plant growth hormone indole-3-acetic acid (IAA) as well as chitinases couldn't be identified for this strain (Marten et al., 2000).

- ***Serratia plymuthica*** occurs in high abundance in the rhizosphere of different plant species. It has a high potential as BCA against soil borne pathogens. One of these strains is *S. plymuthica* HRO-C48. Different work groups have tested its antagonistic activities and plant growth promoting effects. *S. plymuthica* HRO-C48 was isolated from the *Brassica napus* rhizosphere. The strain produces volatile compounds which work against fungi like *V. dahlia* or *R. solani* (Vacheron et al., 2013). The commercial product based on this strain called RhizoStar® is already in use. A further advantage of this bacterium is the long colonization ability of roots which provides a long time effect as biocontrol agent. The reason of this effect is because the gram negative bacteria communicate by the production of N-acylhomoserine lactones (AHLs) which regulates gene expression. This regulatory network is very important because it affects the symbiosis-, virulence and colonization- behavior of the bacterium. Also, the production of antagonistic and plant growth promoting factors is AHL-dependent. *S. plymuthica* is classified within the risk group 1 by the DSMZ (German Collection of Microorganisms and Cell Cultures) therefore the use as BCA is correlated without risks on human health (Müller et al., 2009; Liu et al., 2011). One *Serratia* strain with high antagonistic effects against fungi is 3Re4-18. The strain was isolated of the endorhiza of potato. Due its strong antagonistic effects against soil born fungi like *R. solani* or *Botrytis cinerea* it was characterized as high potential BCA (Berg et al., 2005).

- ***Stenotrophomonas*** belongs to the gram negative bacteria and has a high abundance in different environments all over the world. *S. maltophila* and *S. rhizophila* were determined as PGPR`s. In comparison to *S. rhizophila* the *maltophila* strains have become important as nosocomial human pathogen. It is known that *S. rhizophila* DSM14405^T (= e-p10^T = P69^T) produce trehalose and glucosylglycerol under salt stress conditions (Hagemann et al., 2008). It mediates osmoprotective features to plants. These results were getting from plant assays in Uzbekistan which were done under high salinity conditions. Different genes were expressed when the bacterium was exposed to salt stress. One of them, the *ggpS* gene leads to expression of

glucosylglycerole-phosphatesynthase which regulates the production of glucosylglycerol (GG). The osmoprotective substances are stabilizing enzymes under higher salt conditions. Synthesis of osmoprotectives and their extracellular transport could be disprove by using genomic sequencing methods (Alavi et al., 2013). *S. rhizophila* P69 showed also high plant growth promoting effects on tomato plants when applying the BCA under salt stress conditions. Seed treatment of tomato resulted in higher germination rate, shoot and root growth of the plants in comparison to the untreated samples. The *S. rhizophila* strain has also antifungal effects against the pathogens *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae*. Different substances are involved in the inhibition process. Experiments were done revealing that the volatile organic compound (VOC) α-phenylethanol produced by *S. rhizophila* is able to inhibit the mycelia growth of *R. solani* up to 90%. It was also found that *S. rhizophila* produces lantibiotics (peptide antiobiotic) by expression of the *LanC* gene. Several other genes were identified which led to an expression of different substances which are involved in mechanisms like root colonization, plant host cell adhesion or adaption to environmental changes (Berg et al., 2013).

The effect of *Serratia plymuthica* 3Re4-18, *Stenotrophomonas rhizophila* P69 and *Bacillus subtilis* B2g as BCAs and PGPRs were already approved (Zachow et al., 2010; Alavi et al., 2013).

1.4 Objectives of the study

The overall aim of this work was to analyze the root colonization pattern in maize and tomato plants for defined bacterial mixtures with the aforementioned strains additionally including two strains of *S. plymuthica* (= Rp5 and Rp8) and one strain of *S. rhizophila* (= ep17). The study was segmented in 3 sub studies.

1. The aim of the first study was to envelope a reliable method for implementing different fluorescent genes (4 in total) in a rhizosphere-stable plasmid. The six environmental strains should be transformed with these plasmids. By transforming the strains with different fluorescenting genes an easy differentiation of the bacteria would be possible. Therefore the rhizosphere stable plasmid pIN 29 was taken and equipped with different fluorescent proteins (Vergunst et al., 2010). After successful implementation of the fluorescent gene into the vector the bacterial strains were transformed. Additional to the pIN 29 a second, pBAH8 called plasmid (which is also determined as rhizosphere stable plasmid and carries the green fluorescent protein (GFP)) was also taken to transform the bacteria. Except for *Bacillus subtilis* B2g (= gram positive bacteria) it was possible to transform each strain with a different color including pIN 29 vector.

2. The aim of the second study was to test bacterial interactions (pIN 29 transformed bacteria) of the six strains and analyze the colonization behavior in rhizosphere experiments on maize and tomato plants. The strains were tested for their antagonistic features against each other to avoid an inhibiting effect when mixing them together. Therefore specific plate tests were performed. The differentiation of the bacterial strains could be done by using time intensive visualization methods like fluorescent *in situ* hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM). The transformed bacteria were analyzed by a less time cosuming but equally method to FISH. This allows a quick and simplified study of colonization patterns of potential BCA *ad planta* (on roots). The root colonization studies of tomato and maize were done for the *Stenotrophomonas* strains by using CLSM. In mixtures of transformed strains it was possible to differentiate between all four fluorescent proteins by using specific excitation and emission filters during microscopy. Studies of different fluorescent *Pseudomonas* and *Bacillus* mixtures already exists (Pandey et al., 2010).

3. The aim of the third and last study was to find possible transport vectors for the bacterial strains. This could offer a quicker colonization of plants by bacteria and therefore a more efficient usage as BCAs when applying them in field approaches. Motility of bacteria plays an important part in the root colonization (Czaban et al, 2007). For the six strains no far distance motility in soil was known. These suppositions could be confirmed by simple approaches. Possible “bacterial vectors” could derive from the field of fungi. Different fungi were taken out of a stock collection and also isolated out of natural occurring crop land where maize was cultured for more than ten years. The fungi were chosen by different features like growth rate, formation of hyphae and of course their synergistic interaction with the different strains. By designing a transport approach based on already existing system (Grube et al., 2011) it was able to show that only *Bacillus subtilis* B2g could be transported by different fungi. Additionally further assays were done which come to the point that B2g was not transported as spores but as living cells. These tests were done by using a Live/Dead staining kit and optical analyses with epi- fluorescence microscopy.

2 Material and Methods

2.1 Materials

2.1.1 Growth media

All used media and solutions were autoclaved at 121°C for 15 min to ensure sterility.

LB Agar (Roth; Karlsruhe; Germany)

Tryptone	10 g
Yeast	5 g
NaCl	10 g

Filled up with 1 l ddH_2O and mixed with 18 g of Agar (Agar Kobe 1)

Potato Dextrose Agar (PDA) (Roth)

Potato infusion	6.5 g
Glucose	20.0 g
pH 5.6 ± 0.2	
Agar	15 g

Synthetic Nutrient Agar (SNA, minimal medium)

Glucose	0.16 g
Sucrose	0.16 g
KH_2PO_4	0.8 g
KNO3	0.8 g
KCl	0.4 g
$MgSO_4$ 7*H_2O	0.4 g
Agar-agar	17.6 g

Filled up with 800 ml of ddH_2O and autoclaved.

Sabouraud 2 % Glucose-Bouillon (SAB, full medium)

Meat- and caseinpeptone	8 g
Glucose	16 g
pH	5.6 ±0.2
Agar-Agar	17.6 g

Filled up with 800 ml of ddH_2O and autoclaved.
Sterile filtered antibiotics (0.2 µm pore size filters) were added to the autoclaved SNA and SAB medium:

Tetracycline (10 mg ml^{-1})	800 µl
Streptomycine (50 mg ml^{-1})	800 µl
Penicillin (100 mg ml^{-1})	800 µl

2x TY Medium

Peptone	16 g
Yeast extract	10 g
NaCl	5 g

LB Medium (Roth;Karlsruhe;Germany)

Tryptone	10 g
Yeast	5 g
NaCl	10 g

Filled up with 1 l ddH_2O

Nutrient Broth II Medium (Sifin)

Peptone from casein	3.5 g
Peptone from meat	2.5 g
Peptone from gelatin	2.5 g
Yeast extract	1.5 g
NaCl	5.0 g

pH 7.0 ± 0.2

Filled up with 1 l ddH_2O

SOC Medium

Tryptone	20 g
Yeast Extract	5 g
$MgSO_4$	4.8 g
dextrose	3.603 g
NaCl	0.5 g
KCl	0.186 g

Filled up with 1 l ddH_2O

Fungi conservation medium

Glycerol [99 %]	600 ml/l
Glucose [50 %]	200 ml/l
Pepton [20 %]	200 ml/l
Yeast extract [10 %]	100 ml/l

The components were autoclaved separately and mixed together after cooling.

2.1.2 Solutions

TAE buffer [50 x] (Tris-Acetate-EDTA)

Tris (99.9%)	242 g
Glacial acetic acid	57 ml
0.5 M EDTA pH 8	100 ml
Filled up with 1 l ddH_2O	

TBE buffer [5 x] (Tris-Borat-EDTA)

Tris (99.9%)	54 g
Boric acid (99.8 %)	27.5 g
0.5 M EDTA pH 8	20 ml
Filled up with 1 l ddH_2O	

10 % Glycerine solution

100 ml glycerine was added to 900 ml of ddH_2O and autoclaved.

Antibiotics

All antibiotics used for the preparation of selective media were listed in table 1.

Table 1: Antibiotics used for preparing selective media in this study

Antibiotics	Description	Stock concentration (mg ml^{-1})	Work concentration (µg ml^{-1})	Source
Ampicillin	Ampicilin sodium salt; M 371.39 g mol^{-1}; 99 %; β-Lactam antibiotic; broad-spectrum antibiotic	50	50	ROTH
Gentamycin	Aminoglycoside antibiotic	50	20	ROTH
Tetracycline	M 480.9 g mol^{-1}; >95 %; Tetracycline hydrochloride, bacterostatic	50	50	ROTH
Trimethoprim	M 290.32 g mol^{-1}; > 99% crystallized; dihydrofolate reductase inhibitor (prokaryotes)	50	50	Sigma Aldrich

Loading Dye [6 x]

Bromphenol blue	0.25 %
Xylencyanol	0.25 %
Glycerol	30.0 %
EDTA Na_2 x $2H_2O$ [0.5 M]	50 mM

Standard DNA Ladder

The Fermentas Life Sciences GeneRuler 1 kb DNA ladder (Figure 1) with a [0.1 µg/µl] concentration was used for all gel electrophoresis assays in this study. Depended on thickness of the agarose gel 3 µl up to 6 µl of the ladder was used as reference.

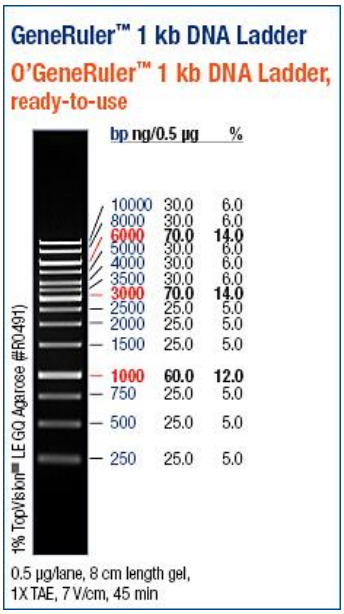


Figure 1: GeneRuler 1kb DNA ladder

2.1.3 Strains

Different BCAs and vector providing strains were used in this study (table 2).

Table 2: Properties and description of the used strains in this study

Name/Strains	Properties/Description	Reference
***Pseudomonas fluorescens* L13-6-12**	Source for isolation of plasmid pJH_dsred	Zachow et al. 2010
***Burkholderia terricola* ZR2-12**	Source for isolation of plasmid pIN 29 and pBAH8	Gasser et al. 2011
***E. coli* 5-alpha**	(Low and high efficiency) competent cells for transformation approaches	NEB Biolabs
List of BCAs	*Bacillus subtilis* B2g	Marten et al., 2000
	Serratia plymuthica 3Re4-18	Maurer et al., 2013
	Serratia plymuthica Rp5	
	Serratia plymuthica Rp8	
	Stenotrophomonas rhizophila P69 and e-p 17	Maurer et al., 2013; Alavi et al., 2013

2.1.4 Plasmids

Table 3: Description of vectors used in this study

Name	Description	Antibiotic conc. (µg ml^{-1})	Reference
pJH	PJH is a construct of two different plasmids which was constructed by J. Fatehi. To obtain the pJH vector the DsRed2 gene was integrated into the pBBR1MCS-5 vector and again this vector was merged with the rhizosphere stable plasmid pME6031. The vector contains a Tetracycline resistance cassette (SI Fig. 1 and Tab. 1)	40	Zachow et al., 2010 Kovach et al., 1995 Heeb et al., 2000
pIN 29	PIN 29 is tac promoter region and DsRed gene including vector which was generated out of a non-mobilisable chloramphenicol resistant derivative of pBBR1 MCS (3). In comparison to the original pIN 29 vector the construct used in this study had a trimethoprim resistance gene instead of chloramphenicol. The restriction sites flanking the DsRed gene were the same (SI Fig.2).	50	Vergunst et al., 2010
pBAH8	The pBAH8 plasmid is a pBBR1MCS -5 vector containing a PA1/04/03-gfp mut3-To-T1 cassette and a gentamycin resistance gene.	20	Kovach et al. 1995 Huber et al. 2002
pEX-A-mNeptune_5bglll_3xbal	PEX-A-mNeptune_5bglll_3xbal-vector (see figure 3) is a synthesized plasmid (Eurofins MWG) with coding sequence for the mNeptune gene (ACN FN565569) with restriction sites for *Bgl*II and *Xba*I and an integrated ampicilin resistance gene (SI Fig.3).	50	This study
pEX-A-eBFP2_5bglll_3xbal	pEX-A-eBFP2_5bglll_3xbal is a synthesized plasmid (Eurofins MWG) with coding sequence for the eBFP2 gene (ACN EF517318) with restriction sites for BglII and Xbal and an integrated ampicilin resistance cassette (SI Fig.4).	50	This study
pSM1880	The pSM1880 (pSM1880 with an pUTTc $P_{A1-04/03}$::gfpmut3-cassette) was provided as isolated and purificated solution. It was used for amplifying the implemented GFP gene by using different primers in PCR reaction.		Aspray et al., 2005

2.1.5 Fluorochrome encoding genes

In total, four different fluorescent genes were used in this study. DsRed2 and GFP containing plasmids were provided in the form of cryo stocks (transformed strains, table 2). DsRed2 was implemented in the pIN 29 and GFP in the pBAH 8 and the pSM1880 vector. EBFP2 and mNeptune were synthesized genes and delivered in pEX-A plasmids flanked by specific restriction sites for their further modification. The coding sequences for the different fluorescent dyes together with their characteristic extinction and emission spectra were listed in table 4.

Table 4: Coding sequences, accession numbers, length, maximum extinction and emission spectrum of eBFP2, DsRed2, GFP and mNeptune gene used in this study

Name	ACN*	Base pairs	Ex (nm)	Em (nm)	Sequence*
DsRed2[a]	AJ851284.1	678	563	582	ATGGCCTCCTCCGAGAACGTCATCACCGAGTTCATGCGCTTCA AGGTGCGCATGGAGGGCACCGTGAACGGCCACGAGTTCGAG ATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAA CACCGTGAAGCTGAAGGTGACCAAGGGCGGCCCCCTGCCCTT CGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAG GTGTACGTGAAGCACCCCGCCGACATCCCCGACTACAAGAAG CTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAAC TTCGAGGACGGCGGCGTGGCGACCGTGACCCAGGACTCCTCC CTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGC GTGAACTTCCCCTCCGACGGCCCCGTGATGCAGAAGAAGACC ATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGAC GGCGTGCTGAAGGGCGAGACCCACAAGGCCCTGAAGCTGAA GGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACAT GGCCAAGAAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGA CGCCAAGCTGGACATCACCTCCCACAACGAGGACTACACCATC GTGGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTC CTGTAG
GFP[a]	U50974.1	711	475	505	AAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTG AATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGG AGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAA ATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACA CTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCAAGATA CCCAGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATG CCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGATG ACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTG ATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAA AGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTA TAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAA TGGAATCAAAGTTAACTTAAAAATTAGACACAACATTGAAGAT GGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAA TTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTC CACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGA CCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACA CATGGCATGGATGAACTATACAAATAG
eBFP2[c]	EF517318	720	383	448	ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCC CATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTT CAGCGTGAGGGGCGAGGGCGAGGGCGATGCCACCAACGGC AAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC GTGCCCTGGCCCACCCTCGTGACCACCCTGAGCCACGGCGTG CAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCACGACT TCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCA CCATCTTCTTCAAGGACGACGGCACCTACAAGACCCGCGCCG AGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG CTGAAGGGCGTCGACTTCAAGGAGGACGGCAACATCCTGGG GCACAAGCTGGAGTACAACTTCAACAGCCACAACATCTATATC

					ATGGCCGTCAAGCAGAAGAACGGCATCAAGGTGAACTTCAAG ATCCGCCACAACGTGGAGGACGGCAGCGTGCAGCTCGCCGAC CACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG CTGCCCGACAGCCACTACCTGAGCACCCAGTCCGTGCTGAGC AAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAG TTCCGCACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG TACAAGTAA
mNeptune[b]	FN565569	735	600	650	ATGGTGTCTAAGGGCGAAGAGCTGATTAAGGAGAACATGCA CATGAAGCTGTACATGGAGGGCACCGTGAACAACCACCACTT CAAGTGCACATCCGAGGGCGAAGGCAAGCCCTACGAGGGCA CCCAGACCGGCAGAATCAAGGTGGTCGAGGGCGGCCCTCTCC CCTTCGCCTTCGACATCCTGGCTACCTGCTTCATGTACGGCAG CAAGACCTTCATCAACCACACCCAGGGCATCCCCGATTTCTTT AAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGAGTCACC ACATACGAAGACGGGGGCGTGCTGACCGCTACCCAGGACACC AGCCTCCAGGACGGCTGCCTCATCTACAACGTCAAGATCAGA GGGGTGAACTTCCCATCCAACGGCCCTGTGATGCAGAAGAAA ACACTCGGCTGGGAGGCCAGTACCGAGACGCTGTACCCCGCT GACGGCGGCCTGGAAGGCAGATGCGACATGGCCCTGAAGCT CGTGGGCGGGGGCCACCTGATCTGCAACCTGAAGACCACATA CAGATCCAAGAAACCCGCTAAGAACCTCAAGATGCCCGGCGT CTACTTTGTGGACCGCAGACTGGAAAGAATCAAGGAGGCCGA CAATGAGACCTACGTCGAGCAGCACGAGGTGGCTGTGGCCA GATACTGCGACCTCCCTAGCAAACTGGGGCACAAACTTAATG GCATGGACGAGCTGTACAAGTAA

*Accession numbers and sequences were taken from NCBI (National Center for Biotechnology Information), [a] (clonetech.com) [b] (Lin et al., 2009) [c] (Ai et al., 2007).

2.1.6 Enzymes

Table 5: Description, specifity and source of enzymes used in this study

Enyzme	Description	Specifity	Source
***Ale*I**	Restriction enzyme	5′...CACNNNNGTG...3′ 3′...GTGNNNNCAC...5′	NEB[a]
***Psp*XI**	Restriction enzyme	5′...VCTCGAGB...3′ 3′...BGAGCTCV...5′	NEB
***Sph*I (High fidelity)**	Restriction enzyme	5′...GCATGC...3′ 3′...CGTACG...5′	NEB
***Hind*III**	Restriction enzyme	5′...AAGCTT...3′ 3′...TTCGAA...5′	NEB
***Nde*I**	Restriction enzyme	5′...CATATG...3′ 3′...GTATAC...5′	NEB
***Xba*I**	Restriction enzyme	5′...TCTAGA...3′ 3′...AGATCT...5′	NEB
***Bgl*II**	Restriction enzyme	5′...AGATCT...3′ 3′...TCTAGA...5′	NEB
Terminal Transferase	(TdT)	Catalyzes addition of deoxynucleotides to the 3´ hydroxyl terminus of DNA molecules	NEB
Dream Taq Mastermix [2 x]	enhanced *Taq* DNA polymerase for all standard PCR applications	Catalyzes the incorporation of 10 nmol of deoxyribonucleotides into a polynucleotide in 30 min at 70°C.	TS[b]
FAST AP	Thermosensitive Alkaline Phosphatase	Catalyzes the removal of 5'- and 3'-phosphate groups from DNA, RNA, nucleotides, and proteins	TS
(Quick)T4 DNA Ligase	Phage T4 DNA ligase	Catalyzes the formation of a	NEB

		phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA	
Blunt enzyme mix	T4 phage DNA polymerase + T4 phage Polynucleotide Kinase	T4 DNA polymerase with both 3´→ 5´ exonuclease activity and 5´→ 3´ polymerase activity and T4 Polynucleotide Kinase for phosphorylation of the 5´ends of blunt-ended DNA	NEB
Quick Ligase	T4 phage DNA Ligase	Catalyzes the ligation of cohesive end or blunt end DNA fragments in 5 minutes at room temperature (25°C).	
Taq & Go® Master Mix [5 x]	*Taq* DNA polymerase for all standard PCR applications	For nucleic acid amplification	MP
Instant Sticky-end Ligase Master Mix	ready-to-use 2x solution of T4 DNA ligase	Ligation enhancer in an optimize buffer	NEB

[a] New England Biolabs, [b] Thermo Scientific; Specific information's of the enzymes were taken from the producer- and deliverer internet sites or instruction leaflets.

Table 6: Description of NEB buffers used in this study for restriction

Buffer	NEB 4 buffer	NEB 2 buffer	NEB 3 buffer	NEB 3.1 buffer
Contents	50 mM Potassium-Acetate 20 mM Tris-acetate 10 mM Magnesium-Acetate 1 mM DTT pH 7.9, 25°C	50 mM NaCl 10 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9, 25°C	100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9, 25°C	100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 100 µg/ml BSA pH 7.9, 25°C

2.1.7 Primer

Table 7: Description and sequences of primers used in this study

Primer	Sequence(5´→3´)(underline restriction sites)
GFP_*Ale*I	ATCACNNNNGTGCTATTTGTATAGTTCATCCATGCCATG
GFP_*Psp*XI	ATAAGAATVCTCGAGBATGAGTAAAGGAGAAGAACT
GFP_wild_for	CTATTTGTATAGTTCATCCATGCCATG
GFP_wild_rev	ACGCCAAGCTTGCATGC
pJH_Insert_Check_f	CCAAGCGCGCAATTAACC
pJH_Insert_Check_r	AGCTGATTTAACAAAAATTTAAC
GFP_gene-F	BBBATGAGTAAAGGAGAAGAACT
GFP_gene-R	BBBCTATTTGTATAGTTCATCCATGCCATG
GFP_N	CATCACCATCTAATTCAACAAGA
GFP_C	GGTCCTTCTTGAGTTTGTAACAG
DsRed1_N	GTACTGGAACTGGGGGGACAG
DsRed1_C	AGCTGGACATCACCTCCCACAACG
DsRed-forRC	CTGTCCCCCCAGTTCCAGTAC
DsRed-revRC	CGTTGTGGGAGGTGATGTCCAGCT
RSP	CAGGAAACAGCTATGACC
USP	GTAAAACGACGGCCAGT
GFP1_*Nde*I_Vergunst	*CCC*CATATGAGTAAAGGAGAAGAAC
GFP2_*Xba*I_Vergunst	GCTCTAGACTATTTGTATAGTTCATCC
pIN7_insertcheck_for	CGGCTCGTATAATGTGTGGA
pIN7_insertcheck_rev	TAGTGAGTCGTATTACGCGCG

GFP1_*Bgl*II_Vergunst	CCCAGATCTAGTAAAGGAGAAGA
GFP1_*Bgl*II_rc_Vergunst	CCCTCTAGAAGTAAAGGAGAAGA
GFP1_*Bgl*II_Vergunst_ATG	CCCAGATCTATGAGTAAAGGAGAAGA
A1R	CTACGGCAAGGCGACGCTGAC

2.2 Molecular Methods

2.2.1 Plasmid and insert preparation

Several molecular steps were applied on the untreated plasmids. The base plasmids mostly have to be isolated out of the stock strain, purified and transformed into *E. coli* competent cells. The transformation into *E. coli* was done because of the easier handling of the strain. The end product (purified vector out of *E. coli*) was used for the final cloning approach which consists of a restriction, ligation and transformation step.

Isolation of pJH out of the original strain and transformation into NEB 5-alpha *E. coli* cells

Plasmid bearing strains were cultivated overnight at 30°C in 100 ml LB medium with appropriately concentrated antibiotics (table 3). The isolation of the plasmid was done by using the PureYield™ Plasmid Maxiprep System (Promega) by following the attributive kit protocol.

Transformation of NEB 5 alpha competent *E.coli* with pJH

The isolated pJH vector was transformed into NEB 5 alpha competent *E. coli* cells by following the standard NEB transformation protocol (*E. coli* was easier to handle in comparison to *P. fluorescence*).

Isolation of pJH out of 5-alpha *E. coli*

The pJH containing *E. coli* cells were incubated in 100 ml shaking flask with LB-Tet (80 µl work concentration) at 37°C overnight. 6 ml of this suspension were used for the pJH plasmid isolation. The QIAprep® Spin Miniprep Kit (QIAGEN) protocol was applied including some deviations:

- In total 6 ml of the overnight culture (onc) were taken and separated on three 2 ml reaction tubes. The tubes were centrifuged at 6800 *g* for 3 min at RT.
- The pellets were resuspended in 250 µl P1 buffer and pooled together in one 1.5 ml tube.
- Steps 3 to 10 were done in the same way as described in the instruction manual. Step 7 of the manual wasn't necessary (5-alpha *E. coli* didn't have nuclease activity or high carbohydrates content).
- The elution was done with 50 µl nuclease free H_2O.

Agarose gel electrophoresis

A separation of the restriction approaches (restricted bands) were done by applying an agarose gel electrophoresis. An inactivation step of the restriction reaction wasn't necessary when electrophoresis was done directly after the restriction reaction. Also purification of the restriction reaction by using a commercial kit would inactivate the enzymes.

Introduction agarose gel electrophoresis

With the agarose gel electrophoresis it is possible to separate nucleic acids belonging to their molecular size. The separation took place in a chamber where the DNA is embedded in an agarose gel. By applying a voltage the negatively charged DNA moves through the gel. Smaller DNA fragments are moving faster and wider than bigger ones. The size of the fragments could be estimated by comparison of the running distance with defined standards (Jeppsson et al., 1979).

In this study a 1 kb DNA ladder was used as reference for every gel electrophoresis assay. The DNA which should be separated was mixed with [6 x] Loading Dye and pipette in the gel chambers. The electrophoresis ran for 30 – 45 min depended on the gel size and the wanted accuracy. Labeling of the gel took place in [0.01 %] ethidium bromide solution. The incubation time was between 15 and 25 minutes. Afterwards the gel was washed with some ddH_2O and placed into the Gel Doc 2000 (Biorad) UV photometer. Different images could be done by exposing the gel to UV light.

Production of 0.8 % TAE agarose gel:

20 ml of 50 x TAE buffer were mixed with 980 ml ddH_2O to receive a 1 x TAE buffer solution. When generating 1 l of 0.8 % TAE agarose, 8 g of agarose have to be added to the buffer. Generally 400 ml of 0.8 % TAE agar were prepared. The mixture was micro waved for approximately 5 min until the solution became clear and filled in the electrophoresis chambers. Depended on the size and number of wells different combs could be placed into the liquid gel.

Preparative gels

Preparative gels were generally done for the restriction approaches. The whole amount of the restriction approach was taken and mixed with an adequate amount of [6 x] Loading dye (for example: 50 µl of restriction approach with 8.333 µl of [6 x] Loading dye). The electrophoresis ran approximately for 45 min at 100 V. Higher voltage wasn't recommended because of less accuracy in separation. Preparative gels were much thicker in comparison to the analytical. Therefore it was possible to fill the whole reaction approach in one or two gel chambers. The fragment which represented the restricted fragment (e.g. restricted pJH) was cut out of gel by using a sterile scalpel. The visualization of the bands was done by the utilization of the UV photometer. The scalpel was reused after washing steps with ethanol or

after autoclaving. The gel slices were purified by using different Clean up kits (Promega, QIagen or GE Healthcare).

Quantifying the DNA concentration by NanoDrop

The determination of the DNA concentration was important for generating the cloning approaches. By knowing the DNA concentration of plasmids and inserts it was possible to calculate the required µl for the ligation reactions. The DNA yield of plasmids or inserts was directly measured by using the NanoDrop spectrophotometer. Therefore the "Nucleotide" program had to be chosen on the computer. The device was calibrated with nuclease free H_2O (1.5 µl). The detector was cleaned between every measurement by using delicate task wipers (Kimtech). 1.5 µl of each sample were taken for determination of the DNA concentration.

Ligation

The ligation approach was done by following the NEB protocol including some deviations:

Reaction mix	
50 ng of vector	3.5 µl
3 x molar excess of insert fragment	6.5 µl
H_2O	to 10 µl
Rapid ligation buffer [2 x]	10 µl
Quick Ligase	1.0 µl
Total	21 µl

50 ng of restricted vector (3.5 µl) were mixed with 3-fold molar excess of insert (6.5 µl) and filled with H_2O up to 10 µl. 10 µl [2 x] Quick ligation buffer (or [2 x] Rapid ligation buffer, Promega) was added to the reaction approach and mixed. Afterwards 1 µl Quick T4 DNA Ligase was added to the approach and mixed thoroughly. The reaction was centrifuged quickly (table centrifuge) and incubated for 5 min at 25°C (= room temperature, RT). The approach was chilled on ice and directly used for further transformation reactions. The storage of the ligation approach was also possible for longer time at -20°C.

Calculation of insert amount for the ligation reaction

A common formula was used to calculate the correct insert amounts. The application of this formula starting with cloning approaches of pIN 29 and pBAH8 vector.

$$\left.\frac{ng\ of\ vector\ x\ kb\ size\ of\ insert}{kb\ size\ of\ vector} \times \frac{insert}{vector}\right\} molar\ ratio = ng\ of\ insert$$

Different molar ratios (1:1, 10:1, 1:10) were tested. The most adorable result was gained by using a ratio of 3:1.

Transformation of bacterial strains

Transformation approach: Protocol – NEB 5 alpha competent *E. coli* (Subcloning Efficiency, SE)

The transformation approach according beside some deviations to the NEB instruction manual:

50 µl of competent *E. coli* cells were thawed approximately 30 min on ice until all ice crystals disappeared.

1. 1 – 5 µl (up to 6 µl) plasmid DNA (maximal 1 µg ml^{-1}) were added to 50 µl competent cells and the mixed carefully by tipping the reaction tubes.
2. The approach was incubated for 30 min on ice.
3. Afterwards the reaction tubes were heated for 30 s at 42°C.
4. Again the samples were incubated on ice for 5 min.
5. 950 µl of SOC (or LB medium which reduces the transformation efficiency) was pipette to the approach.
6. The mixture was incubated for 60 min or longer at 37°C in the thermocycler at 250 rotations per minute (rpm).
7. Meanwhile selection plates (normally stored at -4°C) were brought into the clean bench.
8. 100 µl of the transformation reaction were directly plated out on selective LB plates.
9. The remaining mixture was centrifuged at 2500 rpm for 2 min at RT. The supernatant was discarded and the pellet resuspended in 100 µl of SOC (or LB) medium and plated out. Applying this second step a "concentration" of the transformation reaction was done.
10. The plates were incubated at 37°C (for *E. coli*) overnight.

2.2.2 Making electro competent environmental strains

Six different BCA's were used in this study. All six strains were transformed with fluorescent gene including plasmids to study their interactions behaviours. Therefore the strains have to be made competent for their transformation.

Making electro competent cells

The strains (B2g, ep-17, P69, 3Re4–18, Rp5, Rp8) were taken out of cryo stocks plated on LB plates (no selectivity) and incubated at 30°C overnight.

Day 1:

10 ml of [2 x] TY medium were inoculated with a single colony of each strain and incubated overnight at 30°C with ~ 120 rpm.

Day 2:

1. 200ml of [2 x] TY medium were inoculated (using a sterile pimpf) with the onc (Day 1) of each strain.

2. The flasks were incubated at 30°C and the optical density (OD_{600}) was determined (by using the eppendorf biophotometer) each 30 min until it reached a score between 0.5 and 0.7.

3. The 200 ml suspension were separated onto five 40 ml Sarstedt tubes (cooled at 4°C) and incubated for 20 min on ice.

4. The tubes were centrifuged for 8 min with 4000 rpm at 4°C

5. The supernatant (of each tube) was discarded and the pellet resuspended in 40 ml of 10 % glycerine (cooled at 4°C).

6. Again a centrifugation step with the same settings as in point 4 was done.

7. The supernatant (of each tube) was discarded and the pellet resuspended in 20 ml of 10 % glycerine.

8. The tubes were centrifuged with same settings as in point 4.

9. The supernatant (of each tube) was discarded and the pellets resuspended in a total volume of 10 ml of 10 % glycerine. All pellets were pooled in one tube.

10. The tubes were centrifuged for 6 min with 4000 rpm at 4 °C.

11. The supernatant (of each tube) was discarded and the pellet resuspended in 1 ml of 10 % glycerine.

12. 50 µl aliquots (in 1 ml reaction tubes) were prepared out of this stock solution. The aliquots were immediately stored at -70°C.

Transformation protocol of electro competent cells

- Competent cells (50 µl aliquots) were thawed on ice until all ice crystals disappeared.
- The cell suspension was transferred into 2 mm electroporation cuvettes (Biozym, green cap) and variable µl of isolated plasmid suspension (not more than 1 µg of DNA) was added. The reaction was mixed by tipping the cuvette.
- The approach was incubated for 10 min on ice.
- The moisture of each cuvette was whipped of by using a paper towel and the approach placed into to the BIO-RAD Micropulser™ device. A pulse was given to the reaction applying the EC2 program.
- Immediately the reaction was filled up with 950 µl LB or SOC solution (adopted on RT) and incubated for 60 min at 37°C and 250 rpm.

- 100 µl of the approach was directly plated on selective LB plates.
- The remaining solution was centrifuged for 2 min with 2500 *g* at RT. The supernatant was removed and the pellet was resuspended in 100 µl of LB (or SOC) solution.
- The solution was plate on selective LB plates.
- The plates were incubated at 30°C overnight.

Quick analyse by epi-fluorescence microscopy or CLSM

LB plates containing the transformed strains (including pIN 29, pJH and pBAH8) could be analyzed very quickly by using the Motic epi-fluorescence microscope. The Motic device had two implemented settings for GFP and DsRed analysis. Otherwise mNeptune and eBFP2 inserts could only be detected by CLSM.

2.3 Plasmid construction and fluorescent gene cloning

To study the colonization patterns of bacterial strains in the rhizosphere the fluorescent labeling of the bacteria was necessary. Studies were already done with *Pseudozyma flocculosa* which is known as BCA. The strain was transformed with GFP to analyze plant-bacteria interactions (Neveu et al., 2007). Three rhizosphere plasmids were used as base material in our study. Different cloning strategies were tried out to implement the synthetic genes into to the vectors (see table 8). Due the handling and preliminary results of the cloning approach the final work was done by using exclusively the pIN 29 vector. In total four different dyes were implemented into the vector. Further six different BCA strains were transformed with these vectors.

Table 8: Cloning strategies for the different plasmids used in this study

Vector	Methode	Reference
pJH	Double digest with two different restriction enzymes (*Ale*I and *PspX*I) Double digest (*Ale*I and *PspX*I) and blunting of vector and insert Single digest (*Ale*I)	
pBAH8	Double digest (*SpH*I and *Hind*III) and blunting of vector and insert	
pIN 29	Double digest (*Nde*I and *Xba*I) and blunting of vector and insert TA cloning Proofing amplification of AleI/PspXI GFP with Blue/White Screening Double digest (NdeI and XbaI) and	 (Zhou & Gomez-Sanchez, 2000) pGEM®-T Easy Vector manual (Promega)

	blunting of vector and blunted, wild primers amplified GFP Double digest (NdeI and Xbal) pIN 29 and GFP1_NdeI_Vergunst/Xbal amplified GFP Double digest (NdeI and Xbal) pIN 29 and GFP1_Bglll_Vergunst /Xbal amplified GFP Double digest (NdeI and Xbal) pIN 29 and GFP1_Bglll_Vergunst_ATG /Xbal amplified GFP	

2.3.1 Manipulation of pJH

2.3.1.1 Double digest method

The pJH plasmid and the GFP gene were restricted with two different enzymes. For a successful ligation the pJH vector was dephosphorylated directly after the restriction approach. Afterwards a ligation of the fragments was done. The ligation product was transformed into *E. coli* cells.

Preparation of the plasmid

Restriction with AleI and PspXI		
NEB buffer [10 x]	2 µl	5 µl
Ale I [10 U/µl]	1 µl	1 µl
PspX I [10 U/µl]	1 µl	1 µl
pJH	6 µl	30 µl
H2O	10 µl	13 µl
Total	20 µl	50 µl

Two approaches were done in total, each in a 1.5 ml reaction tube. The tubes were incubated for 3 h at 37°C in a thermocycler (HLC) without shaking (related to the producer protocol a minimum of 1 h of incubation is required to digest 1 µg of λ DNA at 37°C in a total reaction volume of 50 µl).

Dephosphorylation

The restricted and purified pJH plasmid was dephosphorylated by using FAST AP. The restriction and dephosphorylation was done simultaneous in the same approach by adding FAST AP and FAST AP buffer directly to the restriction reaction. A heat inactivation of the restriction reaction wasn't necessary when applying FAST AP

directly to the approach. Two different dephosphorylation approaches regarding to the user manual were tried out.

Dephosphorylation: For simultaneous vector linearization and dephosphorylation

FAST AP buffer [10 x]	2 µl
FAST AP	1 µl
Restriction enzyme 1	1 µl
Restriction enzyme 2	1 µl
H_2O	to 20 µl
Template	up to 1 µg of DNA
Total	20 µl

Incubation 10 min at 37°C in thermo cycler without shaking

Inactivation 5 min at 75°C

Dephosphorylation of DNA 5'-termini

FAST AP buffer [10 x]	2.0 µl
FAST AP	1.0 µl
H_2O	to 20 µl
Template	1 µg of DNA
Total	20 µl

Incubation 30 min RT

Inactivation 5 min at 75°C

Preparation of the insert

PCR: GFP gene Amplification

Dream Taq Mastermix [2 x]	25 µl
pSM1880	1 µl
GFP_AleI	1 µl
GFP_PsPXI	1 µl
H_2O	22 µl
Total	50 µl

PCR program : GFP Plas on Cycler 1 (Biometra® Tpersonal)

1. 96°C	5 min	
2. 96°C	30 s	35 x
3. 58°C	30 s	
4. 72°C	40 s	
5. 72°C	5 min	
6. 15°C	∞	

Purification

The PCR product was purified by using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit. All steps according to the GE Healthcare protocol.

Restriction of amplified GFP (PCR-product)

NEBbuffer 4 [10 x]	4 µl
AleI	1 µl
PsPXI	1 µl
PCR-product	25 µl
H_2O	19 µl
Total	50 µl

The restriction reaction was incubated for 3 h at 37°C (additional repetitions were done with different incubation times up to a minimum of 1 h).

Purification

A preparative gel with the restriction reactions was done.

The restricted vectors and GFP bands were cut out of the agarose gel with a sterile scalpel. The gel slices were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega).

Ligation of GFP gene with pJH vector and transformation into NEB 5 alpha *E. coli*

The amplified and restricted GFP gene was ligated to the pJH vector which also was restricted by the same enzymes and additionally dephosphorylated.

Transformation check

Colony PCR of transformants

The outgrowing (transformed) colonies were tested for their insert. Therefore DNA samples of colonies were taken with sterile toothpicks and mixed with 50 µl H_2O in 1.5 ml reaction tubes. The mixtures were incubated for 5 min at 95 - 100°C. The DNA out of these reaction tubes served as further templates for the colony PCR.

Approach

Dream Taq Mastermix [2 x]	5 µl
GFP_gene-F	1 µl
GFP_gene-R	1 µl
Colony DNA	2 µl
H_2O	1 µl
Total	10 µl

PCR program : Colony PCR on Cycler 1

1. 96 °C	5 min	
2. 96 °C	30 s	35 x
3. 55 °C	30s	
4. 72 °C	40 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Analytical gel

A gel electrophoresis of the PCR product was done.

2.3.1.2 Blunting method

The blunting method was done in the same way as the double digest method but additionally with blunting of the vector and insert.

Preparation of the plasmid

Double digest approach with Ale I and PspX I

The restriction approach was done with a total volume of 20 µl (same setting as in 2.3.1.1 pJH- Double digest approach). The reaction was incubated for 1 h 30 min at 37°C followed by an inactivation step of 20 min at 80°C.

Blunting: Quick blunting kit (NEB)

The blunting reaction was directly done in the restriction approach by adding 2 µl of 1 mM deoxynucleotide solution mix (dNTP Mix) and 1 µl of Blunting Enzyme. The reaction was incubated for 30 min at 25°C. Afterwards an inactivation step followed for 10 min at 70°C.

Analytical gel

A gel electrophoresis was done with the restricted/blunted reaction products.

Purification

The remaining solution of the restricted/blunted approaches was purified with the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit. All steps according to the GE Healthcare protocol. The restricted pJH was cut out of the gel using a sterile scalpel.

Dephosphorylation

FAST AP buffer [10 x]	3.5 µl	Incubation 30 min RT	Inactivation 5 min at 75°C
FAST AP	1.0 µl		
pJH (restricted & blunted)	28.5 µl		
H_2O	2.0 µl		
Total	35 µl		

Preparation of the insert

GFP-amplicon

The amplification of the GFP gene (50 µl approach) was done in the same way like it's have been described in the double digest method for pJH (2.3.1.1). Immediately after the PCR a purification of the product was done by using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit. Some µl of the purified solution was analyzed in an analytical gel.

Blunting approach: Quick blunting kit (NEB)

GFP amplicon	19 µl	Incubation 30 min 30°C	Inactivation 10 min 70°C
Blunting buffer [10 x]	2.5 µl		
1 mM dNTP Mix	2.5 µl		
Blunt Enzyme Mix	1.0 µl		
Total	20 µl		

Ligation of blunted GFP and pJH

Quick ligation approach (NEB)

50 ng of vector	2.0 µl	Incubation 5 min RT
3 x molar excess of insert fragment	6.0 µl	
Quick ligation buffer [5 x]	4.0 µl	
Quick Ligase	1.0 µl	
H_2O	to 10 µl	
Total	15 µl	

A purification of the ligation reaction wasn't necessary for chemical transformable cells.

Ligation check

PCR: Identification of blunt ligation
Some material of the blunt ligation reaction and the isolated pJH vector (as positive control) served as template for a control PCR.

Approach

Dream Taq Mastermix [2 x]	5 µl
pJH_Insert_Check_f	1 µl
pJH_Insert_Check_r	1 µl
Ligation approach (1:10)	1 µl
H_2O	2 µl
Total	10 µl

PCR program: LIGCHECK on Cycler 1

1. 94 °C	5 min	
2. 94 °C	30 s	30 x
3. 55 °C	30s	
4. 72 °C	90 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Analytical gel

An analytical gel was done using 5 µl of the ligation product. The electrophoresis ran for approximately 35 min at 100 V.

Transformation

NEB 5-alpha *E.coli* cells were transformed with 5 µl of ligation suspension. The transformation was done by following the instructions of NEB protocol for 5-alpha *E.coli* cells.

2.3.1.3 Simple digest method

The restriction of pJH was done by using only AleI. This was done to check if the implementation of an additional gene into the vector was possible. Simultaneous the function of the dephosphorylation and ligation reaction was checked.

Preparation of the plasmid

Plasmid isolation

An already isolated pJH vector from earlier approaches was taken for the reactions.

Restriction

NEBbuffer 4 [10 x]	2 µl	Incubation	Inactivation
*Ale*I	1 µl	2 h 37°C	20 min 65°C
pJH vector	10 µl		
H_2O	7 µl		
Total	20µl		

Dephosphorylation

The dephosphorylation was done directly in the restriction approach by adding 1 µl FAST AP enzyme to the mix. The reaction was incubated 10 min at 37°C followed by 5 min at 75°C for inactivation.

Preparative Gel
The whole restricted and dephosphorylated reaction was mixed with 3.5 µl Loading Dye [6 x] and filled into a gel chamber.

The DNA fragment which corresponds to the size of the restricted pJH vector was cut out of the gel by using a sterile scalpel. Afterwards a purification of the DNA containing gel piece was done with the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit. Additional a second approach was done using Wizard®SV Gel and PCR Clean-Up System (Promega) for purification. Both purifications were eluted with 35 µl of sterile H_2O.

Ligation of restricted vector to blunted GFP amplicon

Blunting of the pJH after restriction wasn't necessary due to the blunt end generation of AleI. A Quick ligation approach (NEB) was done in a total volume of 15 µl (see 2.3.1.2 Quick ligation approach). The GFP amplicon was taken from an earlier amplification.

Transformation
Two transformation approaches were prepared using the ligation product and the isolated pJH vector. The transformations were done by following the NEB protocol (5-alpha *E.coli*).

Ligation and Transformation check

PCR: Ligation check
Three PCR approaches were done. Each was using 1 µl of ligation product, isolated pJH vector as positive and H_2O as negative control. Every approach was done with a total volume of 10 µl using the LIGCHECK program (see 2.3.1.2 LIGCHECK program with 20 instead of 30 cycles).

Analytical gel:
An analytical gel was prepared with 5 µl of each PCR product.

Colony PCR
The transformation approaches were plated out on selective LB. Some cell material of the outgrowing colonies was taken as template for Colony PCR. The PCR reaction was done with the same settings as for the ligation check before.

2.3.2 Manipulation of pIN 29 and pBAH8 vectors

Both vectors were isolated out of the base strains (*Burkholderia terricola* ZR2-12) and transformed into 5 alpha *E.coli* cells for an easier handling.

Implementing pIN 29 and pBAH8 into competent 5 aplha *E.coli*

Vector isolations out of *Burkholderia terricola* ZR2-12

Burkholderia terricola ZR2-12 with integrated pIN 29 was cultivated overnight at 30°C in 100 ml of LB medium with a 100 µg ml^{-1} trimethoprim work-concentration. The same was done *for Burkholderia terricola* ZR2-12 including pBAH8 but with a 20 µg ml^{-1} gentamycin work concentration. The vectors were isolated by using the QIAprep® Spin Miniprep Kit (QIAGEN).

Analytical gel

The size of both vector sequences was n't known exactly and out of the literature it wasn't possible to get an exact bp length. Therefore an analytical gel was prepared for both isolated vectors following the standard procedure for 0.8 % agarose gel electrophoresis.

Sequencing of pIN 29 und pBAH8 vector

Both vectors were sequenced to get an overview about the DsRed (in case of pIN 29) or GFP (pBAH8) gene flanking restriction sites.

pIN 29 sequencing:	
1 µg of (purified) vector	variable µl
H_2O	up to 10 µl
DsRed1_C or DsRed1_N	4 µl

pBAH8 sequencing:	
1 µg of (purified) vector	variable µl
H_2O	up to 10 µl
GFP_C or GFP_N	4 µl

Transformation

The isolated vectors were transformed into NEB 5 alpha *E. coli* cells. Different amounts of the isolated vectors were taken for the transformation approaches.

2.3.2.1 Double digest (blunting method)

Preparation of plasmids

Isolation of pIN 29 and pBAH8 out of NEB 5 alpha *E.coli*

E. coli transformants (containing pIN 29) were cultivated overnight at 37°C in 100 ml of LB medium with a 50 µg ml^{-1} trimethoprim work-concentration. The same was done for *E. coli* with integrated pBAH8 vector but with a 20 µg ml^{-1} gentamycin work concentration.

The vectors were isolated out of the *E. coli* by using the the QIAprep® Spin Miniprep Kit (QIAGEN). This step was redone using the same kit and settings because of insufficient isolation amount.

Restriction

pBAH8 vector

NEBbuffer 2 [10 x]	2 µl	Incubation 1 h 30 min 37°C	Inactivation 20 min 65°C
*Sph*I	1 µl		
*Hind*III	1 µl		
pBAH8 isolate	10 µl		
H_2O	6 µl		
Total	20 µl		

pIN 29 vector

NEBbuffer 2 [10 x]	2 µl	Incubation 1 h 30 min to 2 h 37°C	Inactivation 20 min 65°C
*Nde*I	1 µl		
*Xba*I	1 µl		
pIN 29 isolate	10 µl		
H_2O	6 µl		
Total	20 µl		

Blunting approach: Quick blunting kit (NEB)

The blunting reaction was done like in 2.3.1.2 with an incubation time of 30 min at 25°C.

Preparative gel

The restricted vectors were cut out of the agarose gel by using a sterile scalpel. The gel slices were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega). The elution amount was between 30 µl (first approach) and 50 µl (second approach) of nuclease free water.

Dephosphorylation approach 1 (30 µ elution amount)
The dephosphorylation of restricted and blunted pIN 29 was done like in 2.3.1.2 in a total volume of 20 µl and an incubation time of 10 min at 37°C. The amount of FAST AP and the inactivation time remained the same.

Dephosphorylation approach 2 (for 50 µl elution)

2 µl of FAST AP and 5.2 µl FAST AP buffer [10 x] was added to the purified suspension and the reaction incubated for 10 min at 37°C. Afterwards an inactivation step followed at 75°C for 5 min.

Speed vac (centrifugal evaporator/concentrator)
The centrifugal evaporator is used to concentrated biochemical specimen by using the centrifugal force coupled with an applied vacuum. The boiling temperature is set down and the solvent evaporates more easy when applying vacuum (Guy Jean L. & Michel, 1993).

The approach was done to concentrate the DNA amount of the restricted, blunted and dephosphorylated reactions.

Therefore half of the isolated pIN 29 vector solution was transferred into new reaction tubes. The speed vac was done until all H_2O evaporated. The specimens were pooled together in a 25 µl nuclease free water containing reaction tube.
The approach was purified by following the manual of the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Preparation of insert

GFP amplicon
The amplification of the GFP gene (50 µl approach) was done in the same way like it's have been described for GFP out of pSM1880. A second approach was also generated by using the double amount of primers (meaning 2 µl) for the PCR reaction. The purification of the PCR products was done by using the Wizard®SV Gel and PCR Clean-Up System (Promega).

Analytical gel
The purified GFP fragments were analyzed in a gel electrophoresis.

Blunting: Quick blunting kit (NEB)

GFP amplicon	40 µl	Incubation 45 min 25°C	Inactivation 10 min 70°C
Blunting buffer [10 x]	6 µl		
1 mM dNTP Mix	6 µl		
Blunt Enzyme Mix	1.0 µl		
Total	60 µl		

Ligation:

Two different ligation approaches were tried out:

1. Quick ligation approach (NEB) of restricted and blunted pIN 29 to blunted GFP fragment

   The ligation approach was done in a total volume of 15 µl using the same setting as in 2.3.1.2. Untreated pIN 29 vector (no speed vac) isolates and 2.2 – 2.4 µl of the Quick ligation buffer [5 x] were used.

   - Four different ligation approaches were prepared by using different amounts of vector, insert, H_2O and ligation buffer.
   - Four additional approaches were done in the same way by using 150 ng of vector and 450 ng of insert material.

   Transformation
   The ligation approaches (each 5 µl) were transformed into NEB 5 alpha *E. coli* cells. Out of the higher concentrated ligation approaches (150 ng of vector) 3 to 5 µl were taken for the transformation approaches.

2. Ligation: restricted/blunted pIN 29 (after speed vac) and blunted GFP amplicon

   The amount of insert was calculated applying the following formula:

$$\frac{50\ ng\ of\ vector\ x\ 0.73\ (\ kb\ size\ of\ insert)}{4.5\ (kb\ size\ of\ vector)} \times \frac{3}{1} \} molar\ ratio = ng\ of\ insert$$

   Additional 1:3, 1:1 and 10:1 molar ratios were applied.

   The standard Quick Ligation protocol (NEB) was used:

50 ng of vector (restricted & blunted)	variable µl	Incubation 5 min 25°C
3 x molar excess (GFP blunted)	variable µl	
Quick ligation buffer [5 x]	5 µl	
Quick Ligase	1.0 µl	
H_2O	variable µl	
Total	maximum of 12 µl	

   Transformation
   5 µl of the ligation approaches were transformed into NEB 5 alpha *E.coli* cells using the standard protocol.

2.3.2.2 TA cloning

Different PCR products could be cloned without any restriction sites into plasmids of choice. A single adenosine base could be added to the 3' end of double stranded DNA molecule by using Taq polymerase for the PCR reaction. For a successful ligation the vector must also be prepared by adding a 3' thymidine overhang on both sites of the molecule. The terminal deoxynucleotidyl transferase catalyzes this reaction (Zhou & Gomez-Sanchez, 2000).

The Terminal Transferase Method was done by following the protocol of Zhou & Gomez-Sanchez (2000) including some deviations:

Material
2',3'-Dideoxythymidine-5'-Triphosphate (ddTTP) (Affymetrix®)
Terminal Transferase (TdT) (NEB)

Generation of T-vector

Restriction (double digest)

NEBbuffer 2 [10 x]	5 µl	Incubation 1 h 37°C	Inactivation 20 min 65°C
*Nde*I	1 µl		
*Xba*I	1 µl		
pIN29 isolate	30 µl		
H_2O	13 µl		
Total	50 µl		

Blunting: Quick blunting kit (NEB)

Blunting was directly done in the restriction approach (50 µl) by adding 5 µl of 1 mM dNTP Mix and 1 µl of Blunting Enzyme to the mixture. The reaction was incubated for 15 min to 20 min at 25°C. Afterwards an inactivation step followed for 10 min at 70°C.

Analytical gel

The restricted and blunted plasmid was used as template for an analytical gel.

Purification

The corresponding gel bands were cut out and purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega).

T-tailed vector:Terminal Transferase Method

TdT buffer [10 x]	7.5 µl	Incubation 1 h 30 min 37°C	Inactivation 20 min 65°C
25 mM $CaCL_2$	7.5 µl		
1 mM ddTTP	1.5 µl		
pIN29 (restricted & blunted)	38 µl		
H_2O	12 µl		
Total	71.5 µl		

Purification

The Terminal Transferase Method reaction was purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega).

Generation of A-tailed PCR Product

GFP amplicon

The amplification of the GFP gene (50 µl approach) was done out of pSM1880 like in 2.3.1.1. The PCR product was purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) and used directly for an analytical gel.

Ligation of A-tailed DNA fragment to the T-vector

60 ng of T-vector	variable µl	Incubation 14°C
72.8 ng A-tailed DNA	variable µl	
T4 DNA Ligase buffer [10 x]	1 µl	
T4 DNA Ligase	1 µl	
H_2O	to 10 µl	
Total	10 µl	

A control ligation approach was done with the same setting but without using an A-tailed DNA fragment.

Five different ligation reactions were generated:

1. x 2 µl β-mercaptoethanol + 2 µl ligation approach
2. x 2 µl ligation approach
3. µl β-mercaptoethanol + 2 µl control ligation approach
4. µl control ligation approach

Procedure:

- The different ligation reactions were added to 50 µl of 5-alpha *E.coli* cells and incubated for 10 min on ice.
- Mixtures were heat shocked for 30 s at 42°C and incubated for further 2 min on ice.
- 450 µl of pre-warmed LB were added to the culture and incubated for 2 h at 37°C with 250 rpm.
- 100 µl of this suspension were plate on selective LB plates.
- The rest of the solution was centrifuged for 2 min with 2500 g at RT.
- The supernatant was discarded and the pellet resuspended in 100 µl of LB.
- Again the suspension was plate out on selective LB plates and incubated overnight at 37°C.

Transformation

NEB 5-alpha *E.coli* (SE) cells were transformed with the ligation approaches following the standard NEB transformation protocol.

Transformation check

Epi - fluorescence microscopy

The fluorescence detection of transformed colonies was done by using the Motic® Type 106 M epi – fluorescence microscope.

Colony PCR: GFPPlas on Cycler 1 (this study)

Dream Taq Mastermix [2 x]	5 µl
GFP_Ale I	1 µl
GFP_PspXI	1 µl
template	1 µl
H_2O	2 µl
Total	10 µl

Different transformed colonies, pSM1880 isolates as positive and pIN 29 isolates as negative control were taken as templates for the Colony PCR. Colony DNA was picked up by a toothpick and dissolved in 50 µl sterile H_2O in a reaction tube. The suspension was heated for 5 min at 95°C. 1 µl of this mixture served as template for the PCR approach.

2.3.2.3 Proofing amplification of AleI/PspXI amplified GFP with Blue/White Screening

The pGEM® - T Easy Vector System contains a linearized vector with a 3'-T overhang. Every (3'-A overhang) generated PCR product could be cloned into to the vector. Important is the use of thermostable DNA polymerase for the PCR reaction. The multiple cloning site of the vector is flanked by T7 and SP6 RNA polymerase promoters. Within the MCS is a region that encodes the β-galactosidase enzyme. A successful insertion of the PCR product leads to an incomplete production of the enzyme. This could be observed by the so called blue white screening. Only the active β-galactosidase is able to convert the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; colorless analog of lactose) by cleavage which leads to a change of color into bright blue. White cells have a interrupted β-galactosidase encoding gene because of the inserted PCR product. Isopropyl β-D-1-thiogalactop (IPTG) is also added to the selective plates. It catalyzes the expression of the β-galactosidase (Promega manual, 2006).

Generating Selective Plates for Blue/White identification

LB agar plates were prepared with a 100 µg ml^{-1} ampicillin concentration. Additionally 50 µl of X-gal (20 mg ml^{-1}) and 50 µl IPTG (24 mg ml^{-1}) were plate together and incubated without cover for 30 min. All procedures were done in the clean bench to avoid contaminations.

Preparation of insert

The amplification of the GFP gene (30 µl approach) was done like in 2.3.1.1. The PCR product was purified using the Wizard®SV Gel and PCR Clean-Up System (Promega).

Ligation of T-tailed pGEM vector to GFP:

The insert/vector molar ratio was calculated with 1:1.

Rapid Ligation buffer [2 x]	5 µl	Incubation overnight 4°C
pGEM® - T Easy Vector	50 ng	
GFP amplicon	11.91 ng	
T4 DNA Ligase	1 µl	
H_2O	2 µl	
Total	10 µl	

Analytical gel

An analytical gel of the GFP product was prepared. Additionally the negative control of GFP amplification and the 0.1 ng uncut pGEM® - T Easy Vector were also analyzed.

Transformation

NEB 5-alpha *E.coli* cells were transformed with the ligation approaches following the standard NEB transformation protocol (this study).

Two transformation approaches were prepared:

- using 2 µl of the ligation approach and
- 2 µl of the 0.1 ng (uncut) pGEM vector.

The ligation approaches were mixed to 50 µl of competent *E.coli* cells and 0.1 ng of pGEM vector to 100 µl. A negative control was done by transforming competent *E.coli* cells only with H_2O. 100 µl of the transformed cells were plate out on agar plates. The rest of the suspension was centrifuged and 100 µl were plate out as concentrated suspension. The plates were incubated overnight at 37°C.

Transformation check

Colony PCR: pGEM specific primers

Dream Taq Mastermix [2 x]	15 µl
USP	1.5 µl
RSP	1.5 µl
template	1 µl
H_2O	12 µl
Total	30 µl

PCR: PGEM-T-E Cycler 1 (Biometra®)

1. 94 °C	5 min	
2. 94 °C	30 s	30 x
3. 55 °C	30s	
4. 72 °C	60 s	
5. 72 °C	10 min	
6. 15 °C	∞	

Analytical Gel:

The PCR products were analyzed in a 0.8 % agarose gel electrophoresis.

Fluorescence check

The fluorescence of the colonies was checked by using the Motic Epi - fluorescence microscope with GFP settings.

Colony PCR: GFP specific primers

Beside the Colony PCR with specific primers for the pGEM vector an additional PCR reaction was done using GFP specific primers (GFP_AleI and GFP_PspXI). The templates for both reactions were the same. Additionally a positive control using the isolated pSM1880 was done. The total volume of the reactions were 20 µl.

2.3.2.4 Double digest (NdeI and XbaI) and blunting of pIN 29 and ligation to (wild primers) amplified GFP

A new GFP_PspXI (= GFP_wild_rev) primer for the GFP amplification out of the pSM1880 vector was designed. The GFP_AleI (= GFP_wild_for) remain the same. The wild primers haven't the same restriction sites as the plasmid. Therefore a blunting approach of the vector was necessary.

Preparation of plasmid

Restriction of pIN 29
The restriction was done with the same settings like in 2.3.2.1. The total reaction volume was 50 µl. The incubation and inactivation time was the same.

Quick Blunting (NEB)
The blunting of pIN 29 was directly done in the restriction approach (50 µl) by adding 5 µl of 1 mM dNTP Mix and 1 µl of Blunting Enzyme to the mixture. The reaction was incubated for 15 min to 20 min at 25°C. Afterwards an inactivation step followed for 10 min at 70°C.

Purification
The restricted and blunted pIN 29 vector was purified directly after blunting using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Dephosphorylation
The dephosphorylation approach was done by using the FAST AP (NEB) kit (see 2.3.2.1 dephosphorylation of pIN 29). A total volume of 50 µl was prepared.

Preparation of insert

The GFP amplification was designed as "hot start" PCR. The product yield should be increased and non-specific amplifications decreased by applying this reaction. Two different primer pairs were used in separate reactions.

Dream Taq Mastermix [2 x]	10 µl
pSM1880	1 µl
GFP_wild_for and (GFP AleI)	1 µl
GFP_wild_rev and (GFP PspXI)	1 µl
H_2O	7 µl
Total	20 µl

PCR program : GFP Plas on Cycler 1

1. 99°C	∞	
2. 96 °C	5 min	
3. 96 °C	30 s	35 x
4. 58 °C	30s	
5. 70 °C	40 s	
6. 70 °C	5 min	
7. 15 °C	∞	

Purification

The GFP amplicon was purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega).

Blunting

The amplicons were blunted in the same way as the vector.

Analytical gel

The purificated sample was further analyzed by running an analytical gel.

Ligation of GFP (wild primers and AleI/PspXI) amplicon with restricted pIN 29

Two ligation approaches were done. The inserts were calculated with a 1:1 molar ratio for both approaches:

GFP amplicon (GFP wild primers)

$$\frac{50\ ng\ of\ vector\ x\ 1.5\ (\ kb\ size\ of\ insert)}{4.5\ (kb\ size\ of\ vector)} \times \frac{1}{1} = 16.6\ ng$$

GFP amplicon (GFP_AleI and GFP_PspXI)

$$\frac{50\ ng\ of\ vector\ x\ 0.75\ (\ kb\ size\ of\ insert)}{4.5\ (kb\ size\ of\ vector)} \times \frac{1}{1} = 8.3\ ng$$

Ligation approach

Rapid Ligation buffer [2 x]	12.5 µl	Incubation over night 4°C
pIN29	50 ng	
GFP (GFP wild primers)	16.6 ng	
T4 DNA Ligase	1 µl	
H2O	to 20 µl	
Total	20 µl	

The same ligation approach was done with GFP_AleI and PspXI in a total volume of 25 µl.

Transformation
2 µl of each ligation approach were added to 50 µl NEB 5-alpha *E.coli* cells. A standard and a "concentrated" transformation were plate out on selective LB plates and incubated at 37°C over night.

Ligation check

The ligation reactions were directly analyzed by preparing an agarose gel electrophoresis.

2.3.2.5 Double digest (NdeI and Xbal) pIN 29 and GFP1_NdeI_Vergunst/Xbal amplified GFP

Vergunst primers according were chosen to amplify the GFP gene out of the pSM1880. The primer sequence for Xbal was identically to the Vergunst work. In case of NdeI a modification of the sequence was done (see table 7). The original primers were used to amplify the GFPmut3 gene out of pBBR1-KGFP vector (Vergunst et al., 2010).

Preparation of plasmid

Restriction
The restriction approaches were done with the same settings like for the pIN 29 reaction in the TA cloning approach (see 2.3.2.2). The total volume was 50 µl. The incubation time was 2 h at 37°C followed by an inactivation step of 20 min at 65°C.

Preparative gel
A gel electrophoresis was done with the restriction approaches. The restricted bands were cut out by using a sterile scalpel. In a further step a purification of the gel slices was done using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Preparation of insert

GFP Amplification
Two different approaches for the GFP amplification were done. One hot start and a standard PCR program were designed. The hot start approach was done only with pSM1880 isolate as template. Otherwise for the standard reaction beside the pSM1880 a pBAH8 isolate served as template. All reaction approaches were done in a total volume of 20 µl.

PCR approach
The reaction was done in the same way like the one for the insert in 2.3.2.4 using the Vergunst instead of the wild primers.

Hot Start PCR program: GFP Plas on Cycler 1

1. 99°C	∞	
2. 94 °C	5 min	
3. 95 °C	30 s	35 x
4. 54 °C	30s	
5. 72 °C	40 s	
6. 72 °C	5 min	
7. 15 °C	∞	

Standard PCR program: GFP Plas on Cycler 1

1. 95 °C	5 min	
2. 95 °C	30 s	35 x
3. 54 °C	30s	
4. 72 °C	40 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Purfication

The PCR products were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit following the standard protocol.

Analytical gel

The band sizes of the purified GFP amplicons were analyzed by doing a gel electrophoresis.

Restriction

The restriction was done by using the same setting like in 2.3.2.2 with a total volume of 40 µl. The incubation and inactivation time was the same like for the pIN 29 restriction in this approach.

Ligation of restricted pIN 29 and Vergunst primers amplified GFP

The GFP amplicons were ligated to the pIN 29 (Ndel & Xbal restricted) vector. Two different ligation approaches were generated:

1. Quick ligation approach (see 2.3.1.2). Two ligation reactions were done using a 3:1 (GFP = 23.9 ng) and a 1:1 (GFP = 7.96 ng) molar ratio. The incubation time of the reaction was 5 min at 25°C.

2. T4 DNA ligation approach (see 2.3.2.2: Ligation of A-tailed DNA fragment to the T-vector). Two reactions using the same molar ratios (3:1 and 1:1) as for the Quick ligation. The incubation time was 16°C over night.

Transformation

The transformation was done correlated to the standard protocol for NEB 5-alpha competent cells. 2 µl of the Quick ligation and 4 µl of each T4 DNA ligation approach were added to 50 µl of *E. coli* cells. Only the concentrated suspensions were plate out on selective plates and incubated at 37°C over night.

Ligation check

The ligation approaches were analyzed by PCR using specific pIN 29 primers. Additional a negative control (only H_2O) and a positive control (isolated pIN 29) were analyzed too.

Dream Taq Mastermix [2 x]	10 µl
Template	1 µl
pIN7_insertcheck_for	1 µl
pIN7_insertcheck_for	1 µl
H_2O	7 µl
Total	20 µl

PCR program: Cycler 1

1. 95 °C	5 min	
2. 95 °C	30 s	30 x
3. 60 °C	30 s	
4. 72 °C	40 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Analytical gel

The PCR products of the ligation check were analyzed by gel electrophoresis.

Gradient PCR

An insufficient PCR product was generated in the Ligation check reaction. Therefore a gradient PCR was generated by using the same primers as before.By varying the annealing temperature of the PCR in a total range of 10°C the ideal temperature for an optimal amplification should be found out.

The Gradient PCR reaction had a total volume of 10 µl and the same templates were used as for the Ligation check reaction. Instead of [2 x] Dream Taq Master Mix the [5 x] Taq & Go Master Mix was used. The PCR program remained the same. The approach was done with Cycler 3 which is able for gradient PCR. Additional a second approach (with the same settings but) with a lower annealing temperature of 55 °C instead of 60 °C was done.

Analytical gel

The PCR products were analyzed in a gel electrophoresis approach.

2.3.2.6 Double digest (NdeI and Xbal) pIN 29 and GFP1_BglII_Vergunst /Xbal amplified GFP

The NdeI Vergunst primer was exchanged by a new primer including a restriction site for BglII. The pIN 29 vector also had a restriction site for BglII at the same site like for NdeI (Vergunst et al., 2010). Additionally a second primer was designed which had the reverse complementary sequence for the BglII restriction site. This was additionally done for testing the recognition specifity of the enzyme.

Preparation of plasmid

Restriction

NEBbuffer 3 [10 x]	5 µl	Incubation 3 h 37°C
BglII	1 µl	
Xbal	1 µl	
Template	30 µl	
H_2O	13 µl	
Total	50 µl	

Preparative gel

The restriction reaction was directly used for gel electrophoresis. An inactivation step wasn't necessary (BglII couldn't be heat inactivated). The restricted pIN 29 bands were cut out of the gel by using a sterile scalpel. The gel slices were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Preparation of insert

GFP amplicon

Two amplification reactions were done using the same settings as for the GFP amplification in 1.3.2.2 (but the Vergunst primers).

PCR program: Cycler 1

1. 95 °C	5 min	
2. 95 °C	30 s	35 x
3. 56 °C	30s	
4. 72 °C	40 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Purification

The PCR products were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Analytical gel

The purified GFP amplicons were analyzed in a gel electrophoresis approach.

Restriction

The GFP amplicons were restricted by using the same reaction settings as for pIN 29 vector in a total volume of 40 µl. The approach was incubated for 2 h at 37°C.

Preparative Gel

The restricted GFP band was cut out of the gel by using a sterile scalpel. The gel slice was purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Ligation of the restricted pIN 29 with the GFP1_Bglll_Vergunst /Xbal amplified GFP

In total 3 ligation approaches were generated by applying different molar ratios. The reactions settings were the same as in 2.3.2.2 (Ligation of A-tailed DNA fragment to the T-vector). Due to the different primers six reactions were done in total.

Calculation of the GFP insert amount (in silico ~ 717bp):

- GFP amount for 3:1 molar ratio = 23.9 ng
- GFP amount for 1:1 molar ratio = 7.66 ng
- GFP amount for 10:1 molar ratio = 76.6 ng

The reactions were incubated at 16 °C overnight.

Transformation

2 µl of the ligation products were added to 50 µl of competent 5-alpha *E.coli* cells. Only the concentrated suspensions were plate out on selective LB plates and incubated at 37°C overnight.

Transformation check

Colony PCR of Transformants:

The preparation of the colony material was done in the same way as for the colony PCR approach in 2.3.1.1 (Colony PCR of transformants).

The GFP_wild, the DsRed_RC and additionally the PspXI and AleI (GFP specific) primers were used for the PCR reaction. Only DNA of one colony was taken as template for all reactions. Additionally negative controls (only H2O) as well as positive controls for the DsRed and GFP_wild and PspXI-AleI primers were used. All reactions had the same settings. The annealing temperature of the PCR reaction with PspXI and AleI was 58°C instead of 60°C.

Reaction settings for all three primer pairs:

Taq & Go Mastermix [5 x]	4 µl
Template	1 µl
GFP_wild or DsRed_RC_rev	1 µl
GFP_wild or DsRed_RC_for	1 µl
H_2O	13 µl
Total	20 µl

PCR program: Cycler 3 CHECK

1. 95 °C	5 min	
2. 95 °C	30 s	35 x
3. 60 °C	30s	
4. 72 °C	40 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Analytical gel

The PCR products were analyzed by gel electrophoresis.

2.3.2.7 Double digest (NdeI and Xbal) pIN 29 and GFP1_Bglll_Vergunst_ATG /Xbal amplified GFP

Preparation of plasmid

The restricted and purified plasmid was taken from 2.3.2.6.

Preparation of insert

GFP amplicon

Two PCR reactions (each 20 µl) were generated in the same way as for the GFP amplicon in 2.3.2.6 (see GFP amplicon using Vergunst primers for BglII and Xbal). The PCR program was made with an annealing temperature of 58°C instead of 60°C.

Purification

One PCR reaction was purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) the other by the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare).

Restriction
The restriction reactions were done in the same way as in 2.3.2.6. Instead of the [10 x] NEB 3 the [10 x] NEB 3.1 buffer was used. Also new ordered restriction enzymes were taken for these reactions. The incubation time of the reaction was 30 min at 37°C because of the time safer properties of the enzymes.

Preparative Gel
A preparative gel was done with the restriction approaches. The gel slices were purfied by using the Wizard®SV Gel and PCR Clean-Up System (Promega) kit.

Ligation of restricted pIN 29 and GFP1_Bglll_Vergunst_ATG /Xbal amplified GFP

The preparation and execution of the ligation reaction was done by following the NEB protocol for Instant Sticky-end ligation. The GFP insert was calculated with a molar ratio of 3:1 (= 23.9 ng).

Ligation approach	
pIN29 (Xbal & Bglll restricted)	50 ng
GFP amplicon	23.9 ng
H_2O	0.43 µl
Sticky-end Ligase Master Mix	5 µl

The reaction was directly used for further transformations. An incubation time of the ligation reaction wasn't necessary because of the instant properties of the used ligase.

Transformation
The transformation was done by following the standard NEB protocol for 5-alpha competent *E. coli* (High Efficiency, HC) cells. 5 µl of the ligation approach was used for the transformation. SOC was used as incubation medium.

2.3.3 Transformation of environmental strains with fluorescent genes

2.3.3.1 Generating pIN 29 vectors including mNeptune and eBFP2 genes

Plasmid preparation 1

Implementation of mNeptune and eBFP2 plasmids into NEB 5-alpha competent *E. coli* (HC) cells

The transformations of the *E.coli* cells were done by following the standard NEB protocol. 5 µl of each plasmid solution (pEX-A-mNeptune_5bglll_3xbal and pEX-A-eBFP2_5bglll_3xbal) was added to 50 µl of *E.coli* cells. The transformed suspension was filled up with 950 µl LB

solution and incubated for 60 min at 37°C. The samples were centrifuged for 2 min at 2500 g at RT. The supernatant was discarded and the cell pellet taken up in 100 µl of SOC. The suspension was plate out on LB ampicillin (50 µg ml^{-1}) plates and incubated at 37°C overnight.

Plasmid isolation

100 ml LB medium with an ampicillin concentration of 50 µg ml^{-1} were inoculated with transformed NEB 5-alpha *E.coli* cells and incubated overnight at 37°C at ~ 120 rpm.
The isolation of the plasmids was done with 6 ml of the overnight culture by using the QIAprep® Spin Miniprep Kit (QIAGEN) applying the same protocol as in 1.2.1 (Isolation of pJH out of *E. coli*).

Restriction of pIN 29 and fluorescent genes

NEBbuffer 3.1 [10 x]	5 µl	Incubation 1 h 37°C
BglII	1 µl	
XbaI	1 µl	
Template	10 µl	
H_2O	33 µl	
Total	50 µl	

Two repetitions of each restriction approach were done. In addition two restriction reactions of pIN 29 were also applied. The total volume of each reaction was 50 µl.

Beside that a further restriction approach was done for each vector. The setting was the same as before (50 µl total). Instead of 10 µl, 20 µl of template were used and an incubation time of 2 h at 37°C.

Preparative gel

A gel electrophoresis was done for each restriction approach. The specific bands were cut out by a sterile scalpel and purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega) and the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) kit.

Ligation of BglII and XbaI restricted pIN 29 to restricted mNeptune and eBFP2

Two ligation reactions were done for each fluorescent gene in the same way as for the instant sticky-end ligation in 2.3.2.7 (Ligation: Instant Sticky-end Ligase). The gene inserts were calculated with a 3:1 molar ratio:

mNeptune – pIN 29:

$$\frac{50\ ng\ of\ vector\ x\ 0.747\ (\ kb\ mNeptune)}{4.5\ (kb\ size\ of\ vector)} \times \frac{3}{1} = 24.9\ ng$$

eBFP2 – pIN 29:

$$\frac{50\ ng\ of\ vector\ x\ 0.732\ (\ kb\ eBFP2)}{4.5\ (kb\ size\ of\ vector)} \times \frac{3}{1} = 24.4\ ng$$

Transformation

The transformation was done by following the standard protocol for NEB 5-alpha competent *E. coli* (HC) cells. 5 µl of the ligation approach was used for each transformation. SOC medium was used as incubation solution. Out of each transformation reaction an undiluted and a concentrated suspension was plate out on selective LB (50 µg ml^{-1} trimethoprim) plates. The petri dishes were incubated overnight at 37°C.

Transformation check of pIN 29 – mNeptune and pIN 29 – eBFP2 *E.coli* cells

CLSM: Fluoresescence check of the transformants

Colony material of the transformants were picked up from the petri dishes and placed on glass slides by using a pimpf. Additionally some µl of 0.85 % NaCl were added to the cell material and the mixture covered with a cover glass. All steps were done beneath the clean bench to avoid contaminations. The colony material was analyzed by using the CLSM with different extinctions and emissions spectra.

Colony PCR of transformants

Additionally to the fluorescence check a colony PCR was done. DsRed_RC primers were used for the PCR reaction. An in silico check of the primers showing no accordance/hybridization to the mNeptune and eBFP2 sequence. The approaches were done identically as for the GFP transformed pIN 29 in 2.3.2.6 (see Colony PCR of transformants). The DNA material of only one colony served as template for the PCR reactions. Additionally DNA from a pIN 29 (DsRed) transformed strain and an isolated pIN 29 vector were taken as positive control. H_2O was used as negative control.

A second PCR check by using the same conditions was done. Beside the mentioned templates (but without the eBFP2 samples) it included also the isolated mNeptune plasmid out of the NEB 5-alpha *E.coli* cells and the restricted and purified mNeptune insert as template.

Colony PCR of random pIN 29-mNeptune transformed *E.coli* cells

Different pIN 29-mNeptune colonies were picked and scratched out on new LB selective plates. The plates were incubated at 37°C overnight. Single colonies were taken from these plates and the DNA material prepared for the colony PCR reaction (see 2.3.1.1).

The PCR reactions and program settings were the same as for the Colony PCR before. Sixteen (8 pIN 29.1-mNeptune and 8 pIN 29.2-mNeptune) colonies were analyzed in total. Additionally the isolated pIN 29-mNeptune plasmid out of NEB 5-alpha cells and the isolated pIN 29 plasmid were used as negative and positive control.

Analytical gel
All PCR reactions were analyzed by gel electrophoresis.

CLSM: Fluoresescence check of pIN 29-mNeptune colonies
Two colonies weren´t amplified by using the DsRed_RC primers. These colonies were tested with the same CLSM settings as for the transformed colonies before.

Several colonies (P69 transformants of pIN 29-mNeptune and pIN 29-DsRed) were mixed together on one glass slide by using a sterile pimpf. Some µl of 0.85 % NaCl were added to the suspension and covered by a glass slide. The slides were analyzed by using the CLSM with the same settings as for mNeptune and DsRed differentiation.

Plasmid preparation 2

After the successful detection of modified pIN 29 vectors the isolation of these vectors out of *E.coli* and transformation into the environmental strains was done.

Isolation of pIN 29-mNeptune and -eBFP2 *E.coli* containing plasmids
Pin29-eBFP2 and -mNeptune 5-alpha *E.coli* cells (which were already tested for their fluorescence) were grown in 100 ml LB trimetohprim (50 µg ml^{-1}) solution at 37°C overnight. The vectors were isolated by applying the QIAprep® Spin Miniprep Kit (QIAGEN) protocol.

Transformation of P69 and ep17
The electro competent P69 and ep-17 cells were transformed with pIN 29-mNeptune and –eBFP2 using the transformation protocol for environmental strains in 2.2.2 (see transformation protocol). 8 transformation approaches were done (4 for each vector) in total. SOC was used as incubation solution. Undiluted and concentrated transformation suspensions were plated out on LB trimethoprim plates and incubated at 30°C overnight.

Transformation check

Colony PCR of ep17- and P69-pIN29-mNeptune transformants
The preparation of the colony DNA was done in the same way like for the colony material in 2.3.1.1 (see Colony PCR of Transformants).

The PCR reaction was done with the DsRed_RC primers in a total volume of 20 µl (see 2.3.2.6: Colony PCR of Transformants). 30 cycles were used for the PCR reaction. Beside the colony DNA, the pIN 29 plasmid (positive control) and the mNeptune plasmid (negative control) were also used as templates for the reaction.

Analytical gel
The PCR products were mixed with [6 x] Loading dye and analyzed by gel electrophoresis.

2.3.3.2 Transformation of B2g

B2g wasn't transformable by using the standard protocol for environmental strains (see 2.2.2). Two B2g specific protocols were applied to generate competent cells:

Transformation protocol (Protocol NO. 4308 915.504, Eppendorf)

Material:

- Growth medium: LB medium containing 0.5 M sorbitol
- Washing solution: 0.5 M sorbitol, 0.5 M mannitol, 10 % glycerol
- Electroporation solution: 0.5 M sorbitol, 0.5 M mannitol, 10 % glycerol
- Outgrowth medium: LB medium containing 0.5 M sorbitol and 0.38 M mannitol
- Cuvettes with 1 mm gap width (Biozym, orange cap)

Making electro competent B2g cells

- B2g was grown in growth medium at 30°C overnight. An OD_{600} of 1.73 was measured after the incubation.
- Out of the overnight culture a 16 x dilution in a total volume of 150 ml of growth medium was done.
- The diluted suspension was incubated at 30°C for 4 h and 15 min until the OD_{600} reached 0.797 (an OD_{600} between 0.85-0.95 was recommended).
- The suspension was separated in four 35 ml aliquots (50 ml Sarstedt tubes) and stored on ice for 20 min.
- The tubes were centrifuged for 8 min with 4000 g at 4°C.
- The supernatant was removed and pellets were washed four times with 10 ml washing solution.
- The remaining solution was filled up with 3.75 ml of electroporation solution.
- Out of the stock suspension several 50 µl aliquots were generated and stored at -70°C.

Transformation (Electroporation)

- Competent B2g aliquots (50 µl) were thawed on ice for 30 min.
- 5 µl of plasmid isolates (pIN 29, pBAH8 and pJH) were added to the suspension and briefly mixed by tipping.
- The suspension was incubated for 20 min on ice and afterwards transferred into 1 mm electroporation cuvettes.
- The moisture of the cuvettes was whipped of by using a paper towel.
- The tubes were electroporated by using the BIO-RAD Micropulser™ device. The standard program with 2.1 kV and a pulse time of 5.9 ms were applied.
- The electroporated cells were filled up with 950 µl of outgrowth medium and transferred into new 1.5 ml reaction tubes.
- The tubes were incubated for 2 h at 37°C and 250 rpm.
- 100 µl were directly plated on selective LB plates.
- The remaining solution was centrifuged for 2 min with 2500 g at RT. The supernatant was discarded and the pellet resuspended in 100 µl of outgrowth medium.
- The concentrated suspension was plate out on selective LB plates. The plates were incubated overnight at 30°C.

Transformation protocol ("Stanford protocol")

Material

10 x Medium A base:

Yeast extracts	1 g
Casamino acids	2 g
Distilled water	to 100 ml

All ingredients of the Medium A base were mixed together and autoclaved. 10 ml of filter sterilized 50 % glucose were added afterwards.

10 x Bacillus salts:

$(NH_4)_2SO_43H_2O$	2 g
$K_2HPO_43H_2O$	18.3 g
K_2HPO_4	6 g
Tri-sodium citrate	1 g
$MgSO_47H_2O$	2 g
Distilled water	to 100 ml

The 10 x Bacillus salts solution was autoclaved after preparing.

Medium A

10 x Medium A base	10 ml
10 x Bacillus salts	9 ml
Sterile L-Tryptophan (11 mg ml^{-1})	0.1 ml
Sterile water	81 ml

Medium B

Medium A	10 ml
50 mM $CaCl_22H_2O$	0.1 ml
250 nM $MgCl_26H_2O$	0.1 ml

The generation of Medium A and B was done under sterile conditions. 10 ml Aliquots of 10 x Medium A base and 9 ml aliquots of 10 x Bacillus salts were stored at 4°C.

Making B2g competent

- B2g (out of cryo stocks) was streaked out on LB plates and incubated for 2 days at RT.
- 10 ml of Medium A was inoculated with several B2g colonies. The OD_{650} was determined by using the photo spectrometer (U – 2001). The start OD_{650} was 0.1.
- The suspension was incubated with vigorous shaking at 37°C.
- The OD_{650} was measured in 20 min intervals.
- The suspension was incubated for further 90 min at 37°C after an exponential growth phase (in this study OD_{650} of 0.64; recommended = between 0.7 and 1.0).

- 50 µl of this culture were transferred into 450 µl pre-warmed Medium B containing reaction tubes and incubated for further 90 min at 37°C (= ready for transformation).

Gylcerol stocks

The competent B2g aliquots (500 µl total) were centrifuged 10 min with 4000 g at RT. The supernatant was discarded and the pellets resuspended in 500 µl of 50 % glycerol.
The tubes were stored at -70°C.

Transformation

- The B2g aliquots were centrifuged at 2000 g for 10 min at RT.
- 400 µl of the medium was discarded and the pellet resuspended in the remaining suspension (approximately 100 µl).
- The suspension was mixed thoroughly.
- Different µl of pIN 29-, pBAH8- and pJH-isolates were added (maximum 600 ng DNA) to the aliquots.
- The mixture was incubated for 30 min at 37°C with 250 rpm.
- 100 µl of the culture was plate on selective LB plates and incubated at 30°C overnight.

Transformation check

Colony PCR

Dream Taq Mastermix [2 x]	5 µl
DsRed-forRC	1 µl
DsRed-revRC	1 µl
template	1 µl
H_2O	2 µl
Total	10 µl

DNA material of different transformed colonies as well as ddH_2O and pIN 29 isolate were used as templates.

PCR program : RED Cycler 2 (Biometra®)

1. 94 °C	5 min	
2. 94 °C	30 s	35 x
3. 62 °C	30s	
4. 72 °C	90 s	
5. 72 °C	5 min	
6. 15 °C	∞	

Analytical gel

A gel electrophoresis of the PCR products were done

2.4 Bacteria-Bacteria Interactions

The interactions of the environmental strains among them were tested in special plate approaches. Overnight cultures of the six environmental strains were mixed with a specific amount of liquid LB agar. After a short cooling time (till the agar became solid) colony material of one of the six environmental strains was streaked out on the surface of the agar-bacteria suspension. The plates were incubated for 1 week at 30°C and the occurred effects noted for each day. Due to the evaluation of the interactions it was possible to make a point about possible BCA mixtures. Further these predicted mixtures could be applied on plant assays to test their effectiveness in comparison to the single application of the BCA.

Day 1: Preparing bacterial cultures

- Overnight cultures were prepared by inoculating 100 ml of LB medium with one of the six strains and incubated at 30°C overnight with ~ 120 rpm.
- Additionally, each strain was recovered out of cryo stocks and plated on LB plates. The plates were also incubated overnight at 30°C (this step could also be done two days before the experimental start to ensure a sufficient amount of bacterial material).

Day 2: Generation of bacterial LB plates

- The OD_{600} of the oncs was measured by generating a 1:10 dilution (0.85 % NaCl). 100 ml of LB were inoculated with a start OD_{600} of 0.05. The samples were incubated at 30°C and 120 rpm and the OD_{600} was measured every half hour until it reached 0.4.
- 200 ml of LB agar was prepared and stored meanwhile at ~65°C in the heat chamber (this step could be done also one day before).
- The flask was cooled down to approximate 50°C using a water bath.
- 12.5 ml of the oncs (OD_{600} = 0.4) were added to 200 ml LB agar.
- The suspension was filled rapidly in approximate 10 petri dishes (this step had to be done quickly to avoid stiffen of the agar).
- Colony material were taken up of the petri dishes by a toothpick and streaked on the agar.
- Each strain was tested for the interaction behavior between the other strains and also against itself. Additionally ddH_2O was also streaked out as negative control.

Monitoring of the interactions

The petri dishes were incubated for one week (first two days at 30°C and at RT for the remaining time).

2.5 Bacteria-fungi interactions

Beside the interactions among the BCAs, the interactions between fungi were also tested.

The aim was to find fungi which were able to transport the different BCAs. In further approaches these fungi could be used as transport vectors of BCAs to enhance their distribution in soil. Fungi of the SCAM collection as well as isolated fungi out of three different soil types were tested.

Due the spore forming features of several fungi it was important to do all works beneath an open flame (air conditioner switched off).

2.5.1.1 Fungi from culture collection SCAM

28 different fungi (see table 9, small PDA plates) were provided from the Strain Collection of Antagonistic Microorganisms (SCAM) maintained at the Institute of Environmental Biotechnology.

A small agar block was cut out of each fungi and placed on 9 cm petri dishes containing PDA. The plates were incubated in the dark (carton box) for one week at RT. During this time the growth rate and the mycelium building capacity were analyzed.

Table 9: 28 fungi out of the stock collection

Taxonomy/Colony morphology	Strain	Origin
***Trichoderma* sp.**	**BE1-1-3**	Berlin, strawberry rhizosphere
	BR3-1-2	Berlin, oilseed rape rhizosphere
	BSE1-1-10	Braunschweig, strawberry rhizosphere
	RE2-1-15	Rostock, strawberry rhizosphere
	RR4-1-11	Rostock, oilseed rape rhizosphere
***Penicillium* sp.**	**BR2-1-2**	Berlin, oilseed rape rhizosphere
	BE2-1-1	Berlin, strawberry rhizosphere
	RB1-1-14	Rostock, soil
	RE2-1-11	Rostock, strawberry rhizosphere
	BSR2-3-4	Braunschweig, oilseed rape rhizosphere
	BSE4-3-1	Braunschweig, strawberry rhizosphere
***Paecilomyces* sp.**	**BE4-1-9**	Berlin, strawberry rhizosphere
	BR4-2-6	Berlin, oilseed rape rhizosphere
	RE2-2-4	Rostock, strawberry rhizosphere
	RR3-1-9	Rostock, oilseed rape rhizosphere
***Plectosporium* sp.**	**RE1-3-6**	Rostock, strawberry rhizosphere
Pink fluffy	**BE3-1-10**	Berlin, strawberry rhizosphere
White flat	**BR4-1-11**	Berlin, oilseed rape rhizosphere
Putative *Trichoderma* sp.	**BE2-1-12**	Berlin, strawberry rhizosphere
	BR1-1-5	Berlin, oilseed rape rhizosphere
Flat fluffy	**BE4-3-6**	Berlin, strawberry rhizosphere
	RE3-2-6	Rostock, strawberry rhizosphere
Air mycellium	**BE4-3-1**	Berlin, strawberry rhizosphere
	RE2-1-9	Rostock, strawberry rhizosphere
Stinky	**RE2-4-3**	Rostock, strawberry rhizosphere
	BR1-6-3	Berlin, strawberry rhizosphere
Till cap	**RR1-5-2**	Rostock, oilseed rape rhizosphere
	BR1-6-8	Berlin, oilseed rape rhizosphere
Epicoccum	**RR3-5-16**	Rostock, oilseed rape rhizosphere

First letter(s): B Berlin, BS Braunschweig, R Rostock

2.5.1.2 Fungi isolated from cropland (maize cultivation)

Fungi out of agricultural used farmland (maize cultivation; google coordinates: 47.053425, 15.418388 47°03'12.3"N 15°25'06.2"E) and two commercial available potting soils were isolated.

Pre-arrangement: Soils used for fungi isolation:

1. DonBosco derived (DonB; see coordinates)
2. Standard potting soil (Rhizo)
3. Profi substrat (PS)

Five small plant pots were filled up with these different soil types. 3 maize seeds (Rhonaldinho) were added to each pot. The pots were incubated for two weeks at approximate 25°C (more than 30°C at direct light exposition) in green house. The maize roots of all 3 soil types were collected and the fungi isolated by applying a specific treatment (see figure 2).

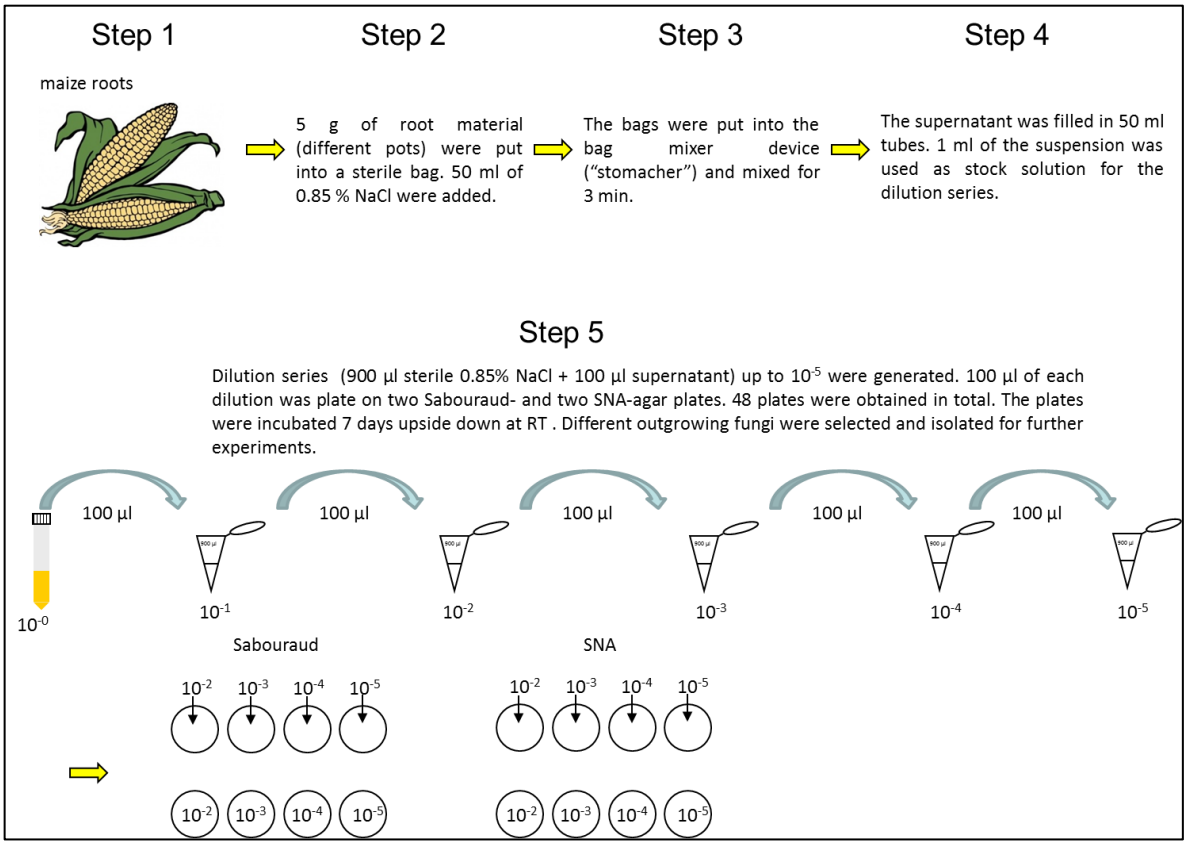


Figure 2: Single steps of fungi isolation from maize roots.

The Sabouraud (SAB) and SNA plates were incubated for a week at RT. The outgrowing fungi were categorized related to their growth rate and the ability of mycelium formation.

Glycerol stocks

Glycerol stocks were done with the selected fungi. Small agar blocks containing the fungi were cut out and put on small PDA plates. These plates were incubated for a week at RT in the dark. Up to 5 small agar blocks were cut out of these plates by using a toothpick and put in 2 ml reaction tubes, containing 1 ml of fungi conservation medium. The reaction tubes were stored at -70°C.

2.5.1.3 Dual culture assay for determining fungi-bacteria interaction

Dual cultures approaches were done to test the interactions between pre-selected fungi and the BCAs.

Pre-arrangement:

- Each of the six BCAs was streaked out on LB plates and incubated for 1 day at 30°C or several days at RT till a moderate cell density was reached.
- Several 1/5 PDA agar plates were generated.

Set up of interaction approach:

Small agar pieces of pre-selected fungi (PDA plates) were cut out using a toothpick and put on 9 cm petri dishes (containing 1/5 PDA). Some colony material of the bacterial strains (pre-arrangement) was picked up with a toothpick and streaked on the PDA plates (see figure 3). Three different bacteria where tested at once in the presence of one of the selected fungi (see table 9). The approaches were incubated for 1 week at RT in a carton box. Several parameters like inhibition of the bacteria by fungi or other interactions (color change of the bacteria) were analyzed during the these time period.

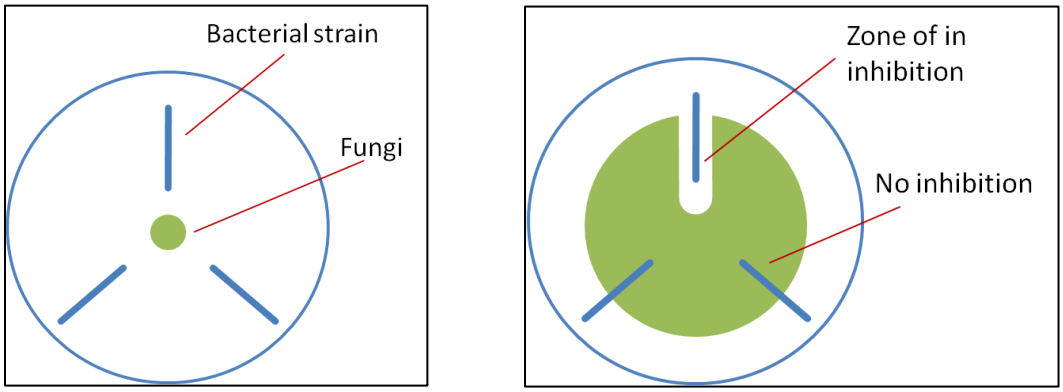


Figure 3: Experimental setup for monitoring the fungi-bacteria interactions (left). Fungi-Bacteria interactions after x days (right).

2.5.1.4 Fungi-Bacteria Transport assay

A list of possible bacterial transports was generated. Based on these predictions two different assays were designed related to the work of (Kohlmeier et al., 2005). The single steps of the assays were shown in figure 4 and 5. In case of the first assay (see figure 4) the bacterial strains were simultaneous put together with the fungi on the PDA agar. In the second assay (see figure 5) the fungi were put first on the agar and incubated until they reached the QPDA media. Only then the bacterial strain was dropped as suspension on the fungi. For the second assay a liquid onc of each (with fluorescent gene transformed) BCA was prepared in selective LB solution.

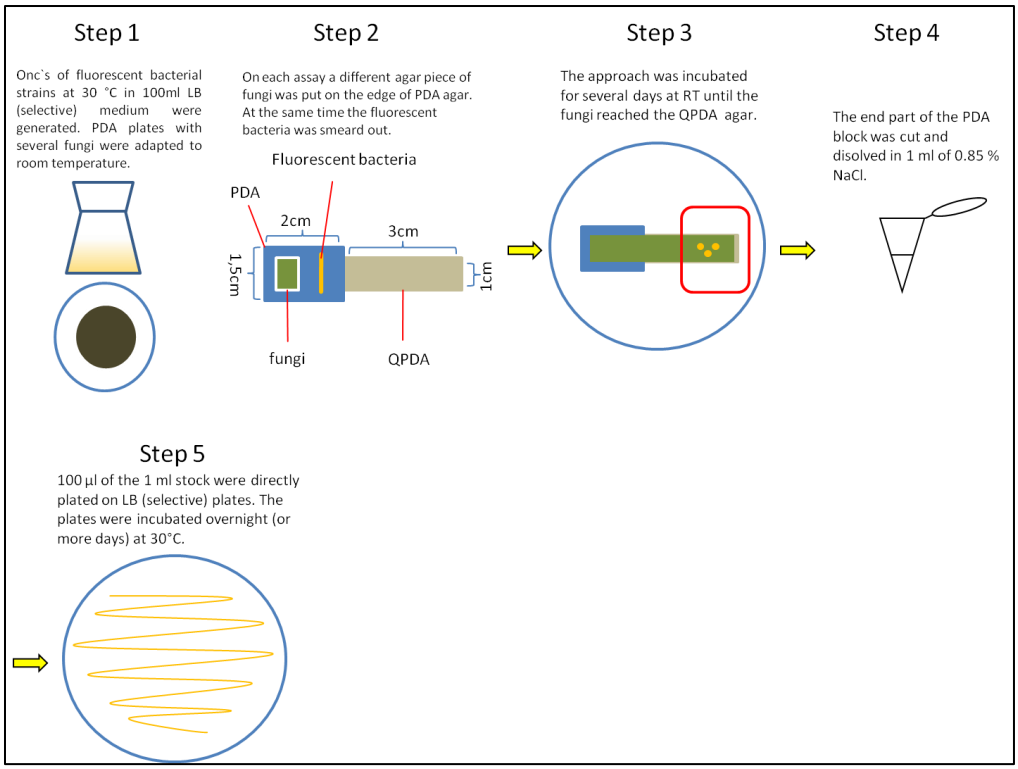


Figure 4: Steps of the first assay for testing the fungi transport abilities.

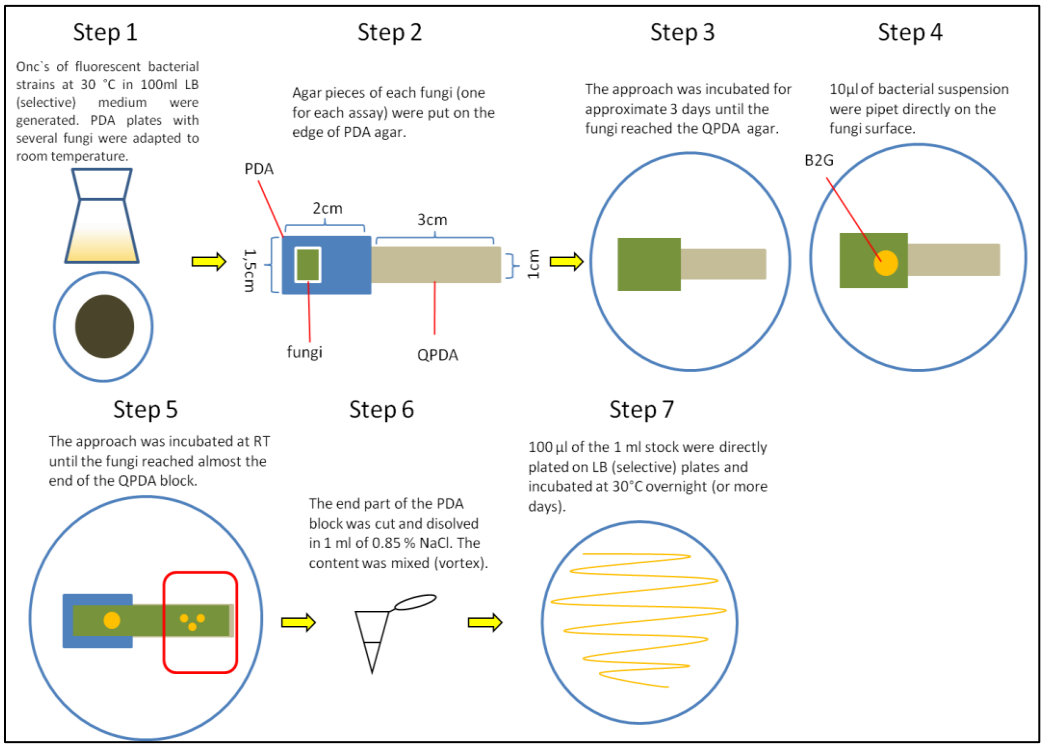


Figure 5: Experimental set up of second assay testing the fungi transport abilities.

2.5.2 – Interactions with maize plants

Two fungi could be identified as possible B2g transporters. An approach was designed to check if the simultaneous application of fungi and B2g together delivered any colonization advantages on maize plants. The Rhonaldinio maize seeds cultivar were used in this approach. The experiment included 6 single steps which were shown in figure 6.

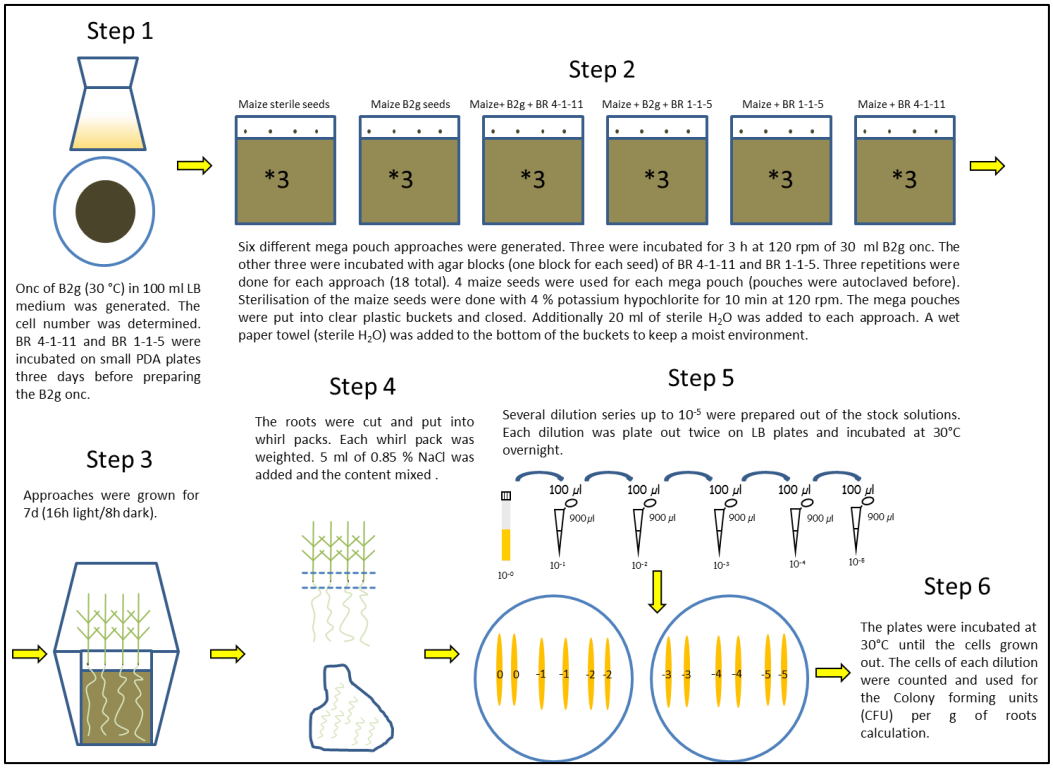


Figure 6: Single steps for calculating the B2g CFU/g of maize root.

Ad. Step 2 (figure 6): Determination of cell number by using a haemocytometer

$$\frac{cell\ number}{ml} = \frac{registered\ number\ of\ cells * 400 * 100000}{\text{registered number of small squares}} * \text{dilution factor}$$

B2g:

$$\frac{78*400*100000}{125} * 10 = \underline{2.496 * 10^8}$$

The B2g onc was used directly for the incubation of maize seeds.

The B2g CFU/g root material was determined by following the single steps in figure 6. Also the "B2g effects" on leave size and weight of root material were investigated.

2.5.3 B2g – BR fungi Live/Dead staining approach

An additional experiment was done by using the B2g transportable fungi. The aim of the experiment was to visualize the ongoing interactions between the B2g strain and the BR fungi. In detail it should be shown if the transported B2g cells were alive or in the form of spores. The single steps of the experiment were shown in figure 7.

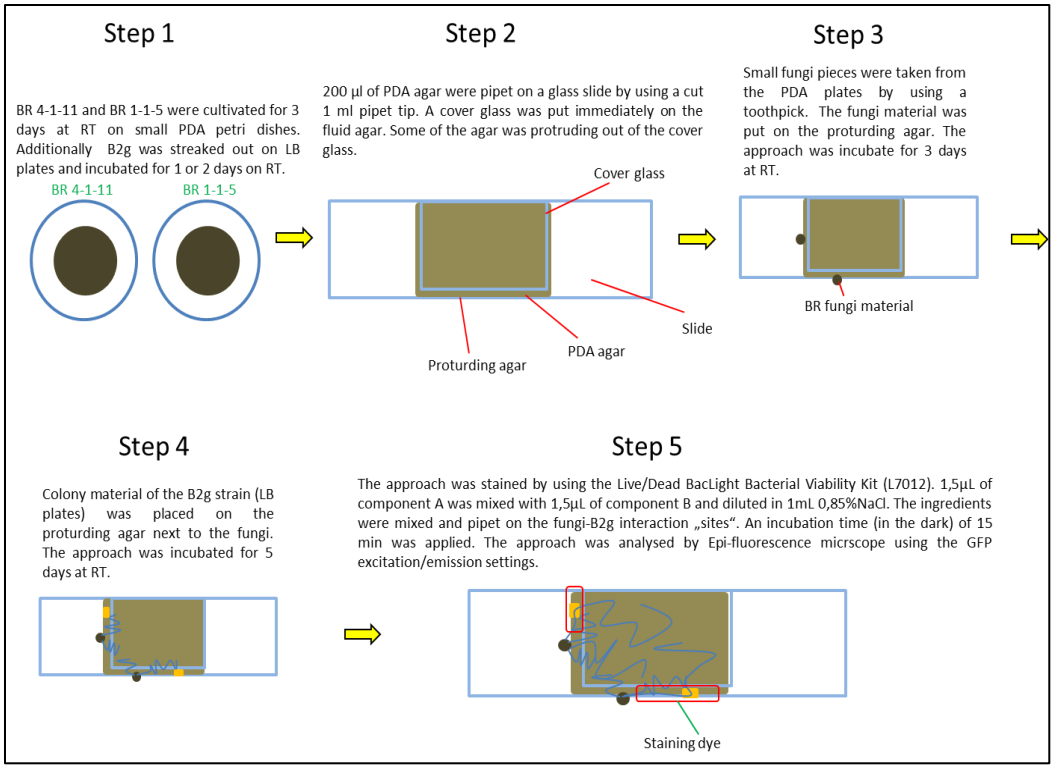


Figure 7: Single steps of the B2g –BR fungi Live/Dead staining approach.

The fluorescence was measured by using the Motic epifluorescence microscope. The fluorescence settings for the live/dead detection were listed in table 10.

Table 10: Extinction and emission parameters for Live/Dead staining approach analysis

Setting	Extinction (nm)	Emisison (nm)	Fluorescence
Live	485	530	Green
Dead	485	630	Red
GFP (settings on Motic)	470	509	green

2.5.4 B2g – BR fungi spores identification

In addition to the Live/Dead staining experiment the viability of B2g colonies were tested in a further approach (see figure 8).

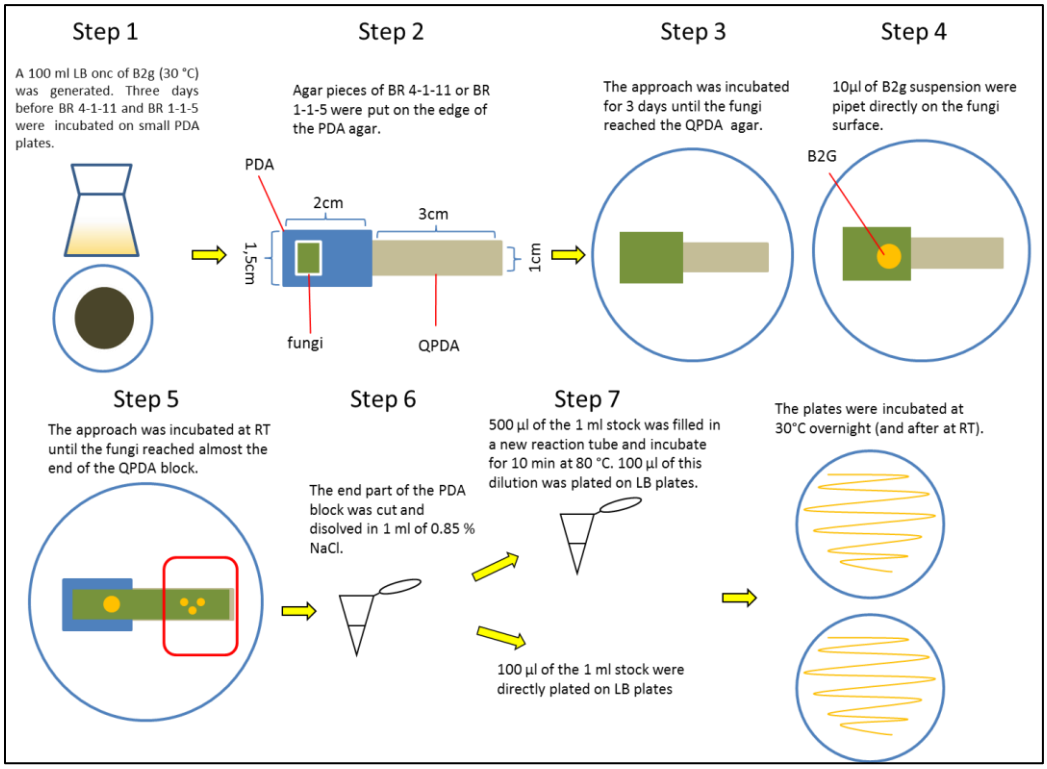


Figure 8: Single steps of B2g – BR fungi interaction approach for spores identification.

It was necessary to maintain a humidity environment in the closed assays during the whole incubation time. Otherwise the agar dried out and the fungi weren't able to growth. This was done by adding a wet (with sterile H_2O) to the approach.

2.5.4.1 BOX PCR of transported B2g colonies

BOX-elements are high repetitive and polymorph sequence elements in genomes. They differ in their length and sequence. These elements could be used for the differentiation of microorganisms by using a BOX-PCR reaction. Only one specific primer is used for the reaction. The primer binds on the repeated elements and initiates the amplification of the sequences. A strain-specific band pattern can be obtained by gel electrophoresis of the PCR products. Each pattern is unique for each microorganism and therefore used as kind of fingerprint.

Only B2g colonies could be transported by applying the fungi-bacteria transport assays. For the identification of the re-isolated strains a BOX PCR was done.

DNA extraction of B2g

1. 2 ml reaction tubes with screwed lid were filled up with autoclaved glass beads (200 mg of 0.15 – 0.21 µm diameter).
2. Some of the colony material was taken from the plates and mixed with 1 ml of PCR water.
3. The mixture was added to the reaction tubes.
4. The tubes were stored for approximate 20 min at -70°C until the content was frozen.
5. The tubes were put into the FastPrep Instrument24 and shaken twice for 30 s at 5.5 m/s (the samples were stored on ice between the runs).
6. The tubes were centrifuged for 5 min at 13.000 rpm and 4°C.
7. The supernatant was used for the PCR reaction.

PCR reaction mix

Taq & Go Mastermix [5 x]	5 µl
A1R	2.5 µl (10 pmol)
Colony DNA	1 µl
H_2O	16.5 µl
Total	25 µl

PCR program : Cycler 1

1. 95 °C	6 min	
2. 94 °C	60 s	35 x
3. 53 °C	60s	
4. 65 °C	8 min	
5. 65 °C	16 min	
6. 15 °C	∞	

Analytical gel

PCR products were separated by gel electrophoresis using a 1.5% agarose gel in 0.5 x TBE buffer. A voltage of 90 V was applied and a run time of 4 h. The gel chambers were loaded with 15 µl of the PCR product.

2.5.5 *Serratia* sp.– *R. solani* – Interactions

Rhizoctonia solani is a soil born disease and causes heavy damages on different agricultural plants like potato, sugar beet, lettuce but also on trees. The fungus is able to infest more than 500 host species. An efficient control agent for the fungus is hard to find. One reason is that there are different mechanisms how the pathogen infects the host plants. There is a high interest to find environmental friendly solutions like bacterial control agents (Grosch et al., 2006). A potential BCA could be derived from the *Serratia* strains.

An experiment was designed where the interactions between *R. solani* and pIN 29 DsRed transformants of *Serratia* Rp5, Rp8 and 3Re4-18 could be analyzed by using the CLSM (see figure 9).

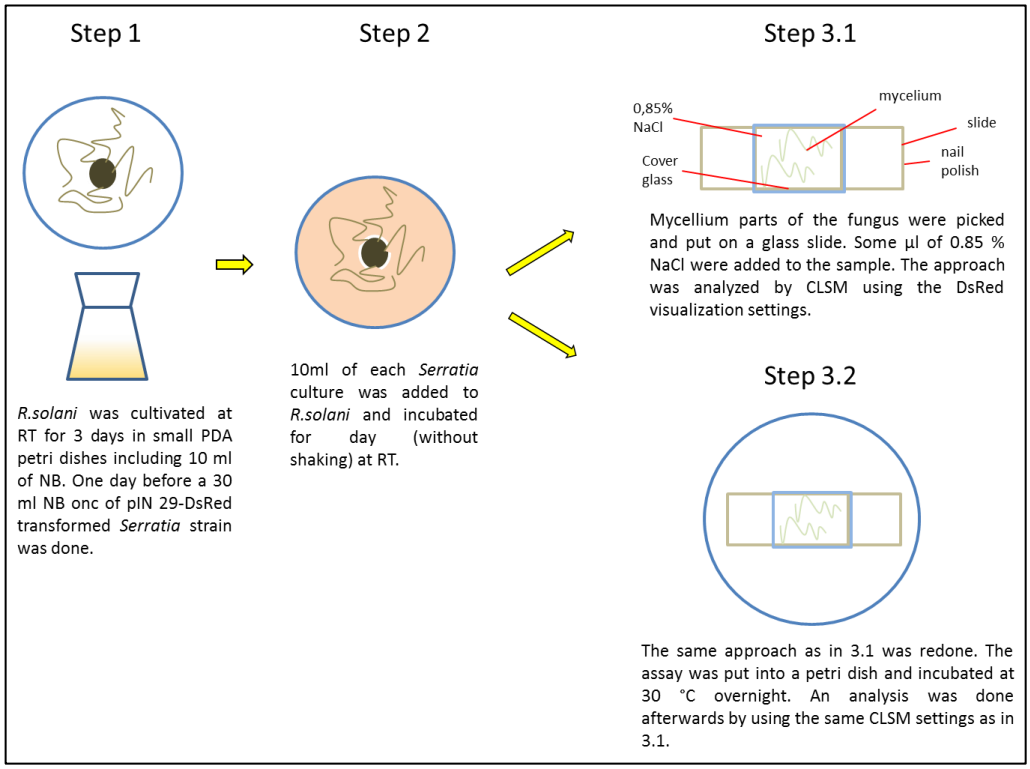


Figure 9: Experimental setup of *R.solani* – *Serratia* Interactions analysis.

2.6 Bacteria – Plant Interactions

2.6.1 Interactions of *Stenotrophomonas rhizophila* P69, ep17 with maize and tomato roots

Only pIN 29-DsRed transformants of ep17 and P69 in combination with Ronaldhino maize seeds and Dirk tomato seeds (Billa) were used. The assay consisted of 7 steps which were shown in figure 10. The whole experiment was done twice.

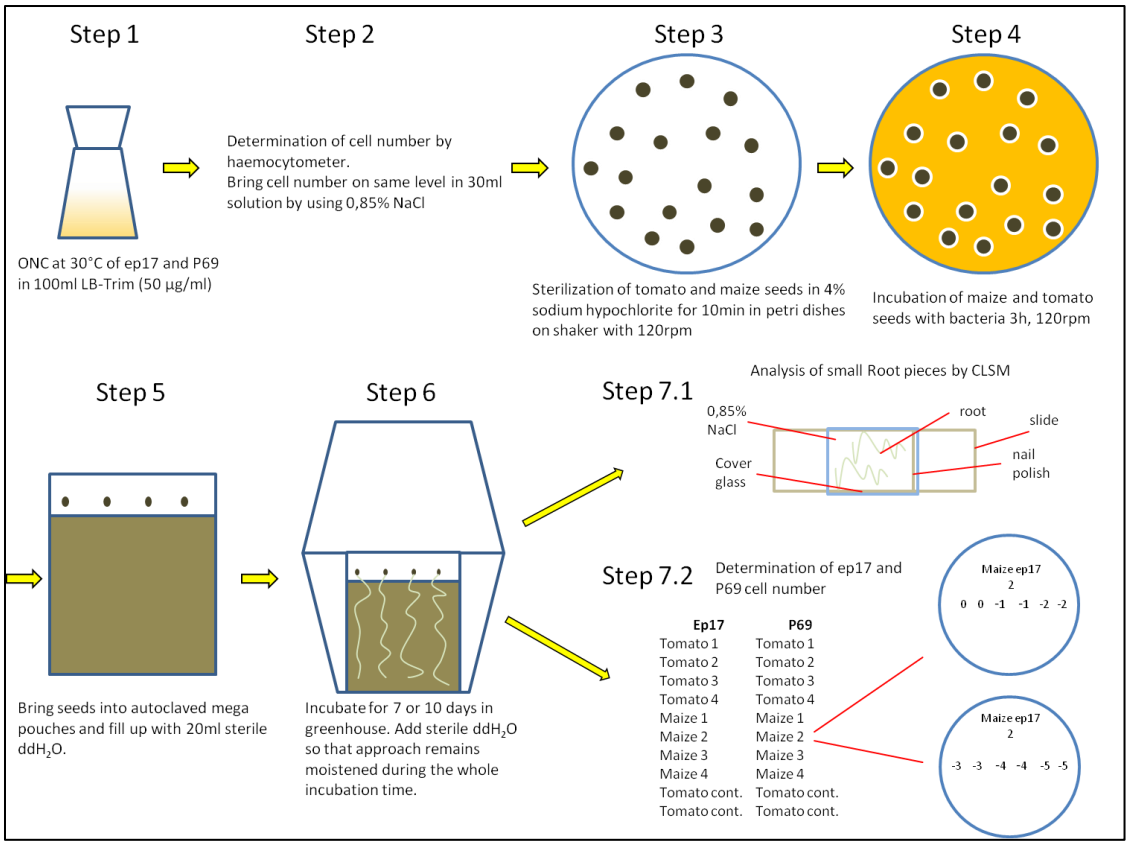


Figure 10: Single steps of *S. rhizophila P69- ep17* - maize roots interactions.

Ad. Step 2 (figure 10): Determination of the cell number by using a haemocytometer
The calculation was done like in 1.5.2:

P69:

$$\frac{50*400*100000}{80} * 10 = \underline{2.5 * 10^8}$$

ep17:

$$\frac{85*400*100000}{80} * 100 = \underline{4.25 * 10^9}$$

The cell density of both strains was brought on the same level of $2.5 * 10^8$ in a 30 ml 0.85 % NaCl solution.

Ad. Step 5 (figure 10): Total amount of germination pouches
In total, four germination pouches (cyg germination pouch, Mega International, USA) were used for each plant. Five seeds were used for the tomato and four seeds for the maize approaches. Additionally to the BCA treated approaches, two control approaches using sterile maize and tomato seeds were also prepared.

Ad. Step 7.2 (figure 10): Determination of ep17 and P69 CFU/g root material
The single steps of the CFU calculation were listed in figure 11.

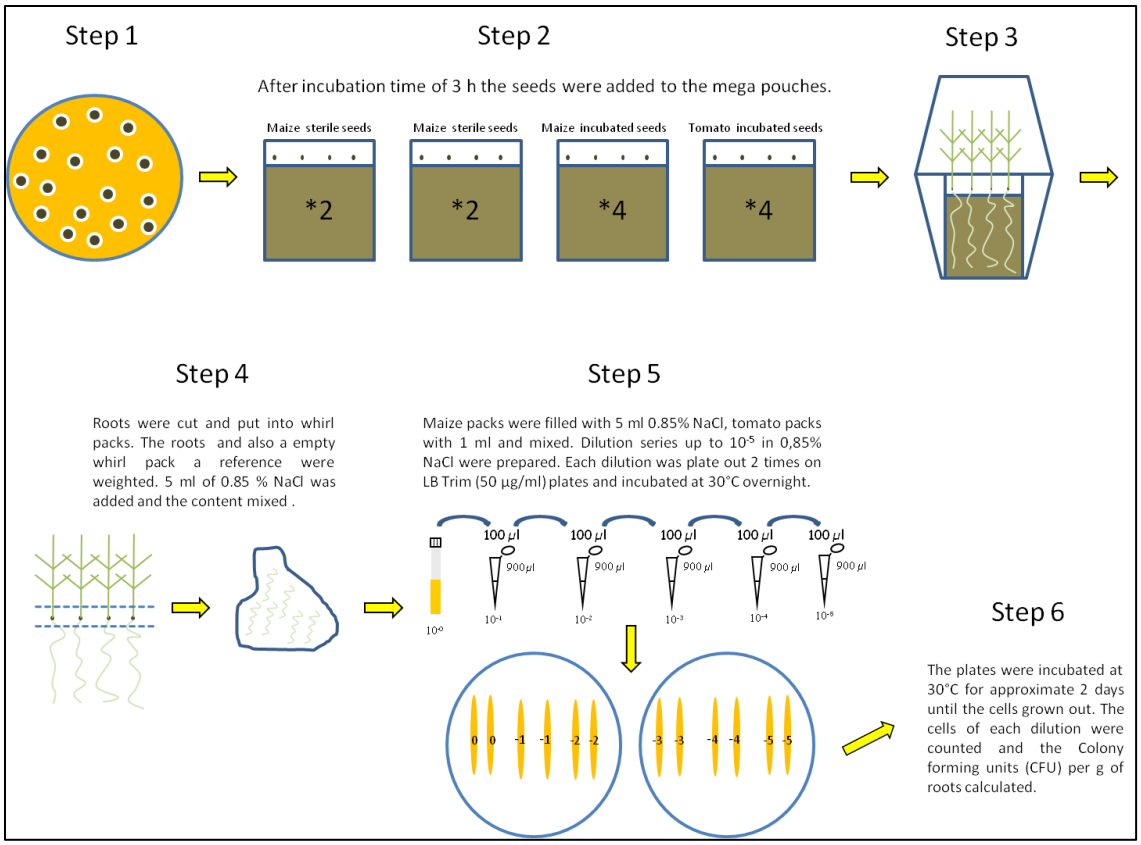


Figure 11: Steps for calculating the P69 and ep17 CFU/g of maize/tomato roots.

Ad. Step 5 (figure 11):

Out of each dilution 10 µl was dropped on LB (containing 50 µg ml^{-1} trimethoprim) plates. The plates were swayed until the suspension moved into the agar. After an incubation time of 1-3 days the outgrowing cells were counted. It was possible to calculate the CFU/g of root material by knowing the root weight and the amount of 0.85 % NaCl which was used for production of the stock solution.

2.6.2 Visualization of ep17 (pIN29 DsRed) and P69 (pBAH8) on maize roots

Additionally to the approach described in 2.6.1, an equal approach was done by using ep17 (pIN29-DsRed) and P69 (pBAH8) transformed strains. Overnight cultures of both strains were diluted to a cell number of 2.5 * 10^8. The sterile maize seeds were incubated in a 30 ml bacterial solution mixture of ep17 (15 ml) and P69 (15 ml) for 3 h at 120 rpm. Two mega pouches were prepared in total. One was done with 5 bacterial threatened maize seeds, the other one with 5 sterile seeds. The approaches were incubated for 7 days. The root material of the plants was prepared like in figure 10 (step 7.1) and analyzed by CLSM.

2.6.3 Mixture of four different fluorescenting ep17 strains on maize roots

One mega pouch was incubated with sterile maize seeds (like in 2.6.1, figure 10, step 6) for 7 days. 20 ml LB oncs of transformed ep17 (pIN29 - DsRed, - mNeptune, - eBFP2 and pBAH8) strains were generated. 5 ml of each culture was mixed together in one petri dish. Several maize roots were cut and put into the suspension for approximate 30 min. Afterwards the roots were prepared like in step 7.1 of figure 10 for CLSM analysis.

3 Results

3.1 Molecular Methods

3.1.1 Making electro competent environmental strains

The environmental strains were made electro competent related to the protocol in the method part. Each strain was transformed with the pIN 29-, pJH- and pBAH8 vector. The transformants were checked by Motic epi-fluorescence microscopy for GFP and DsRed fluorescence (see table 11).

Almost all of the environmental strains were transformable by using the DsRed and GFP carrying vectors. B2g couldn't be transformed neither with pIN 29, pJH nor pBAH8. The transformation of P69 with pJH wasn't also possible.

Table 11: List of transformed environmental strains using the pIN 29-, pJH- and pBAH8 vector

Strain	Vector* used for transformation	Fluorescence of colonies
Rp5	pIN29	✓
	pBAH8	✓
	pJH	✓
Rp8	pIN29	✓
	pBAH8	✓
	pJH	✓
3Re-14	pIN29	✓
	pBAH8	✓
	pJH	✓
ep17	pIN29	✓
	pBAH8	✓
	pJH	✓
P69	pIN29	✓
	pBAH8	✓
	pJH	No
B2g	pIN29	No
	pBAH8	No
	pJH	No

*pIN 29 (141.4 ng μl^{-1}), pBAH8 (128.3 ng μl^{-1}), pJH (115 ng μl^{-1})

3.2 Plasmid construction and fluorescent gene cloning

A standard protocol was generated to clone different fluorescent genes into a rhizosphere stable plasmid. Therefore, several cloning strategies and plasmids were tried (see table 8).

3.2.1 Manipulation of pJH

3.2.1.1 Double digest method

The pJH vector was isolated and purified out of *P. fluorescence* L13-6-12 (154 ng μl^{-1}). This product was restricted with AleI and PspXI resulting in two fragments (figure 12, sample 1, > 10 kb and ~ 6 kb). The amplified (and AleI/PspXI restricted) GFP out of pSM1880 shown a fragment at ~750 bp (see figure 13, sample 1 and 2).

The restricted and dephosphorylated pJH vector was ligated to the restricted GFP amplicon. 5-alpha *E. coli* (NEB) cells were transformed by using this product. Only one colony could be obtained after transformation. Material of this colony was used for colony PCR (see figure 14). The analytical gel was showing a low fragment at ≤ 250 bp (figure 14, sample 1).

Preparation of the plasmid

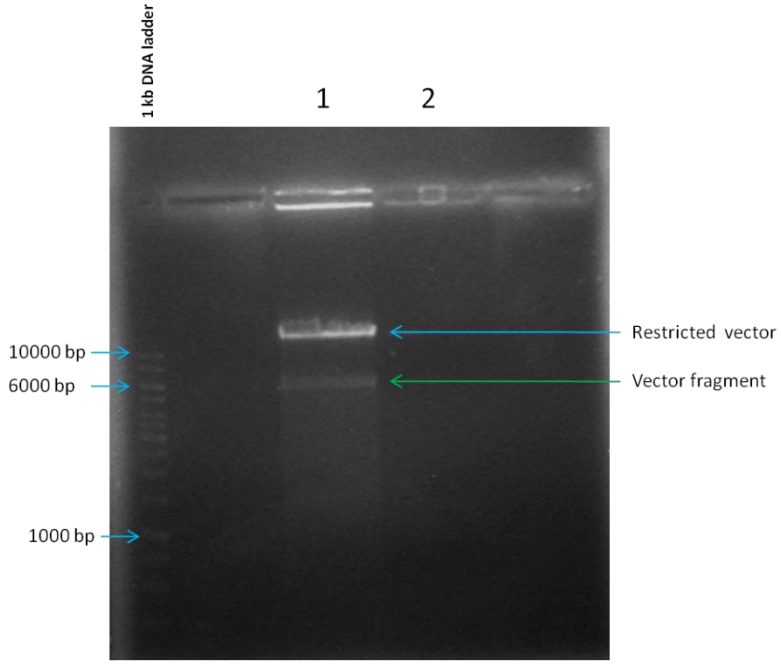


Figure 12: Gel image of restricted and dephosphorylated pJH plasmid. 1 and 2 = each 34 µl of the restriction approach.

Preparation of insert

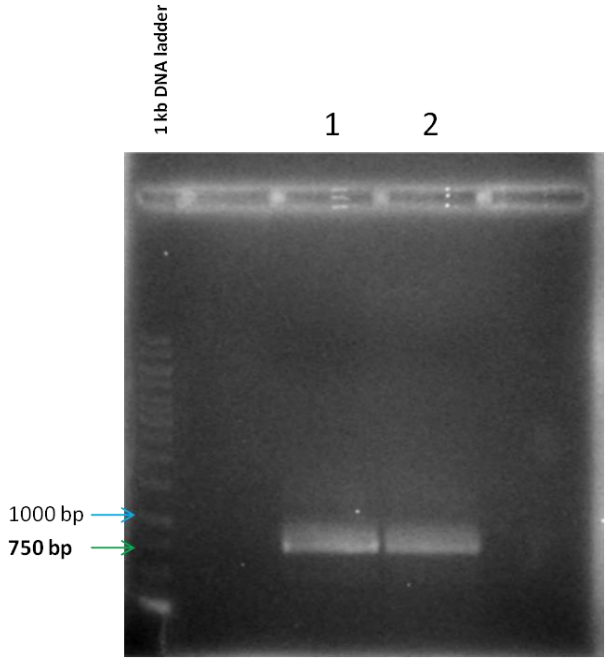


Figure 13: Gel image of amplified and restricted GFP. 1 and 2 = each 25 µl of the restriction product.

Transformation check

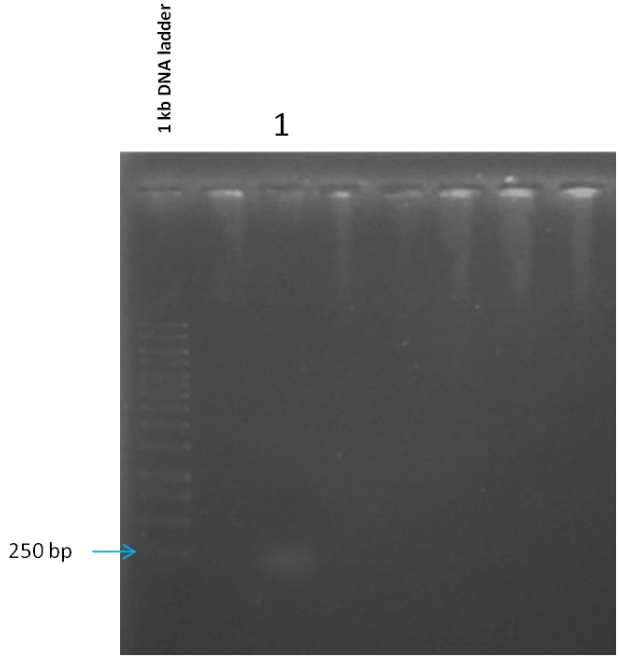


Figure 14: Analytical gel of colony PCR. 1 = 5 µl of the PCR product (= transformed E. coli cells).

3.2.1.2 Blunting method

The pJH isolated out of 5-alpha *E.coli* (157,6 ng μl^{-1}) was restricted with AleI and PspXI. The vector was producing a fragment with a size of ~ 750bp (see figure 15). The amplified GFP out of pSM1880 had a size of < 750 bp (see figure 16).

GFP and pJH were extracted out of the gels and purified with the GE Healthcare kit. The DNA concentrations of the purified samples were listed in table 12.

GFP (54.1 ng μl^{-1}) was blunted and ligated to the purified pJH vector in a Quick Ligation approach. The reaction was analyzed by PCR. The ligation samples were showing the same product as the original pJH out of *E. coli* (figure 17, sample 1-3, ~ 1000 bp). Competent *E. coli* cells were transformed with the ligation product resulting in no transformants.

Preparation of the plasmid

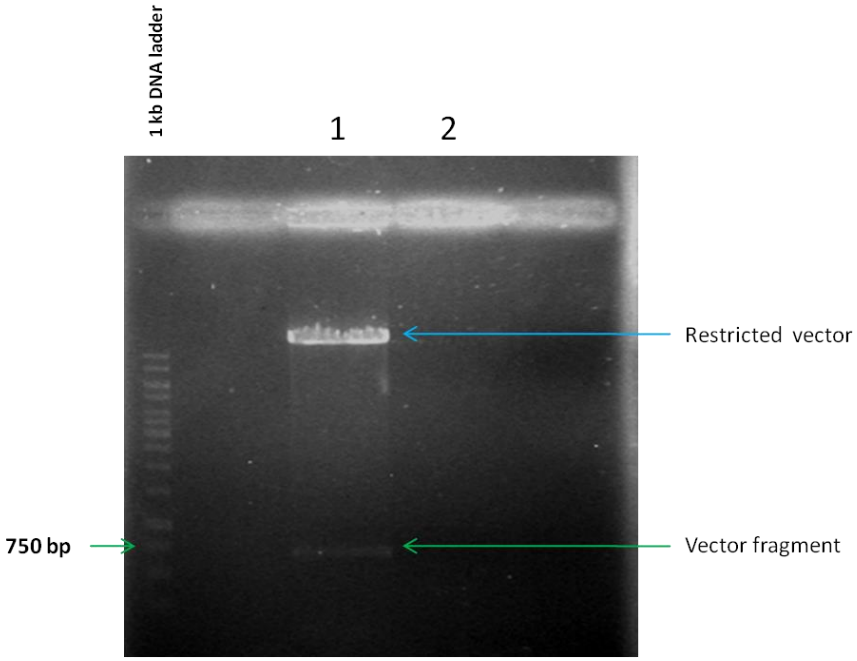


Figure 15: Gel image of blunted and restricted pJH plasmid (157,6 ng μl^{-1}). 1 = 50 µl of pJH vector. 2 = same approach settings with H_2O as negative control.

Preparation of the insert

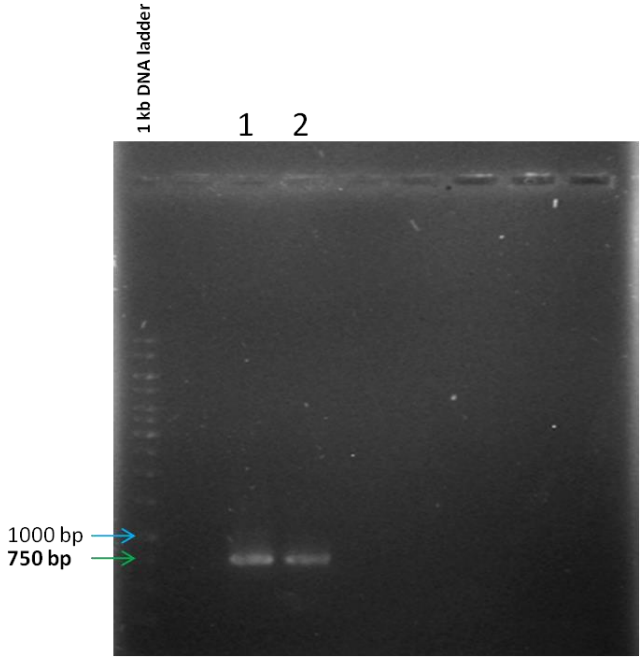


Figure 16: Gel image of amplified GFP out of pSM1880. 1 and 2 = 5 µl of the PCR product.

Table 12: DNA concentration of pJH and GFP

Sample	Purification kit	DNA concentration ng μl^{-1}
Restricted pJH	Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit	51
Amplified GFP	Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit	54.1

Ligation check

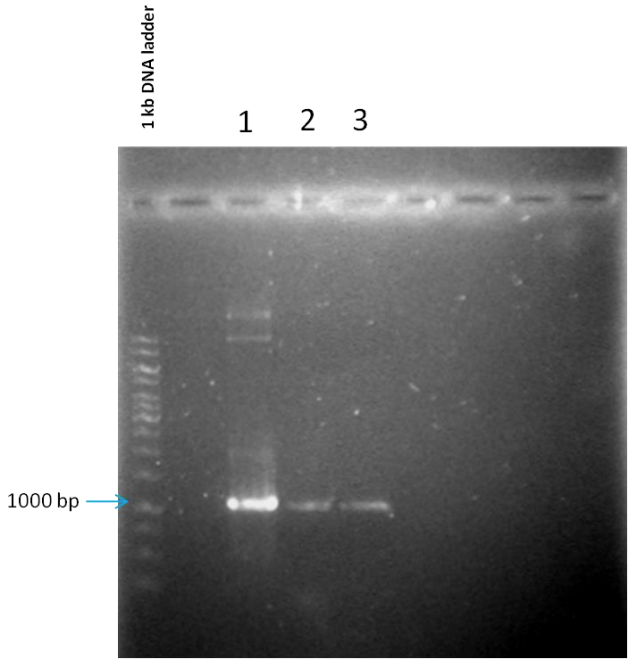


Figure 17: Analytical gel of the ligation check PCR. 1 = 5 µl of pJH out of 5-alpha *E.coli* (157.6 ng μl^{-1}), 2 and 3 = 5 µl of Quick ligation approach.

3.2.1.3 Simple digest method

The isolated pJH vector (out of 5 – alpha *E. coli*) was restricted only by AleI and dephosphorylated. The preparative gel of the reaction (see figure 18, sample 1) was showing no restriction. The purification of the gel slice was done by using the Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit. No sufficient DNA yield was obtained. The same restriction and dephosphorylation approach was done again and the gel slice purified by using the Wizard®SV Gel and PCR Clean-Up System from Promega. The DNA concentration was 32.9 ng μl^{-1} (using an elution amount of 35 µl). The purified vector was ligated to the blunted GFP (54.1 ng μl^{-1}) fragment. The ligation approach was checked by PCR and the product visualized in an analytical gel (see figure 19).

Several *E.coli* cells were transformed by using the ligation product. Only one colony was grown out. Colony material was used for colony PCR. A low amplification at approximate 1 kb (see figure 20, sample 1) was detected.

Preparation of the plasmid

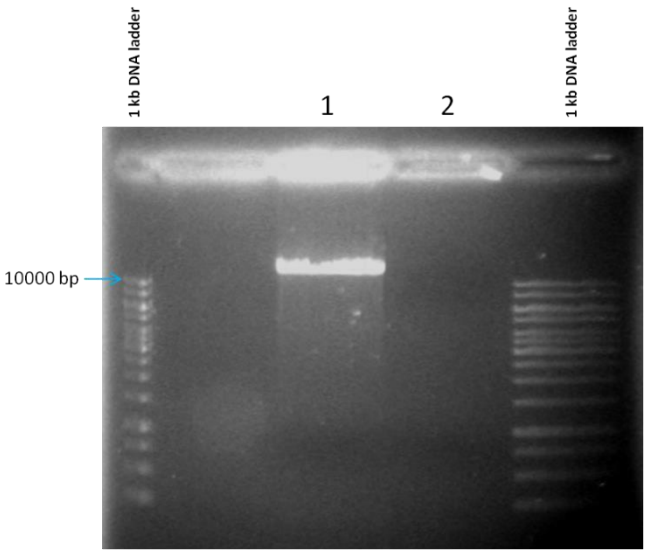


Figure 18: Gel image of AleI restricted pJH (157.6 ng μl^{-1}). 1 = 21 µl restricted and dephosphorylated pJH, 2 = negative control. First DNA ladder (left site) = 3 µl, Second DNA ladder = 5 µl.

Ligation and Transformation check

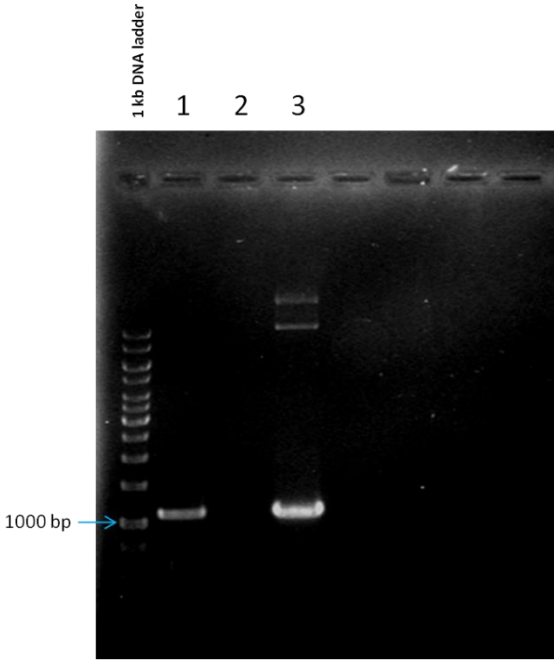


Figure 19: Analytical gel of ligation check by PCR. 1 = 6 µl of ligation reaction, 2 = negative control, 3 = purified pJH vector as positive control.

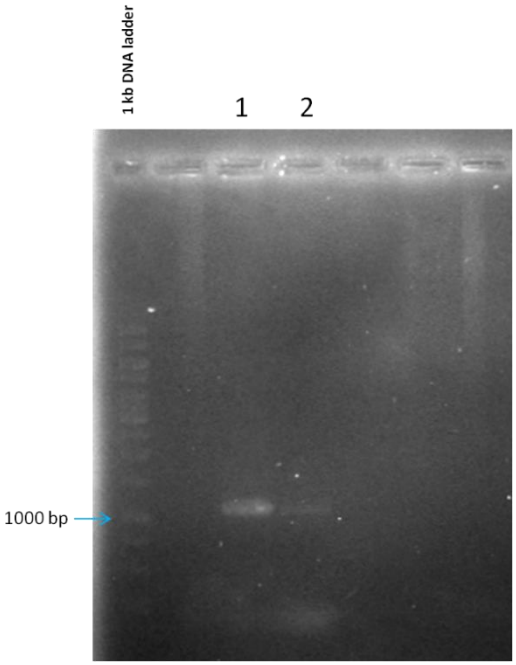


Figure 20: Colony PCR of transformed 5-alpha *E.coli* cells. 1 = 5 µl colony material. 2 = negative control.

3.2.2 Manipulation of pIN 29 and pBAH8 vectors

pIN 29 and pBAH8 were isolated out of *Burkholderia terricola* ZR2-12 strains (see table 13) by using the QIAprep® Spin Miniprep Kit (QIAGEN). Additionally the isolated and purified vectors (pIN 29 and pBAH8 out of *Burkholderia terricola* ZR2-12) were transformed into 5-alpha *E.coli* cells. This was done because of the easier handling of the *E.coli* strain. The vectors were isolated and purified again (see table 14). The pIN 29 vectors were analyzed in a gel electrophoresis (figure 21). PIN 29 was shown four different fragments in a range between 3 and >10 kb (see figure 21, sample 2).The isolated pBAH out of *Burkholderia terricola* ZR2-12 couldn't be transformed into 5-alpha *E.coli* cells.

The restriction sites flanking the fluorescent genes of pIN 29 and pBAH8 were only known by literature research. Both vectors were sequenced to proof the annotation (SI Tab. 2). The sequencing reaction in case of pBAH8 was insufficient and delivered no useful results.

Table 13: DNA concentrations of isolated pIN 29 and pBAH8 vectors out of *Burkholderia terricola* ZR2-12

Isolated vector	OD_{600}	DNA concentration (ng μl^{-1})
pBAH8 (1)	2.29	85.5
PBAH8 (2)	0.92	34.0
pIN29 (1)	3.19	22.6
pIN29 (2)	0.80	12.0
pIN29 (3)	4.44	33.4

Table 14: DNA concentrations of isolated pIN 29 out of 5-alpha *E.coli* strains.

Isolated vector	OD_{600}	DNA concentration (ng μl^{-1})
pIN29	4.44	33.4
pIN29	Not measured	40.2

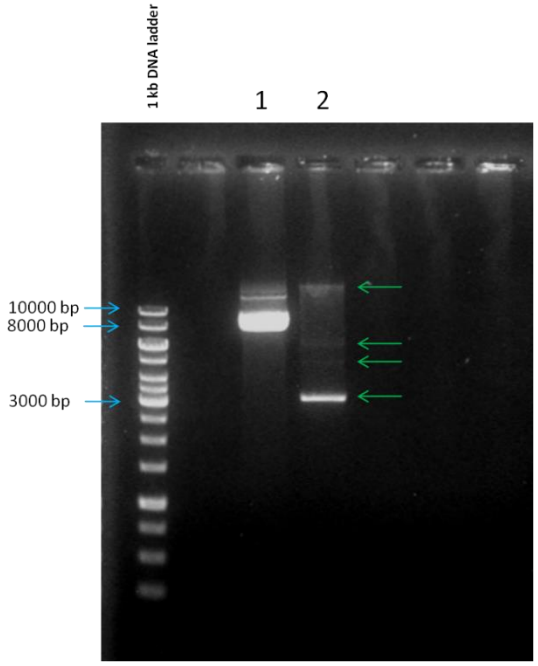


Figure 21: Analytical gel of isolated pIN 29 vector (5-alpha *E.coli*) and pJH-vector. 1 = 6 µl of purified pJH (115.2 ng μl^{-1}), 2 = 6 µl of purified pIN 29 (40.2 ng μl^{-1}).

3.2.2.1 Double digest (blunting method)

PBAH8 (out of *Burkholderia terricola* ZR2-12) and pIN 29 (isolated out of *E.coli*) were restricted by applying a double digest reaction. The pBAH8 vector was restricted in 4 different fragments by using SpHI and HindIII (see figure 22, sample 2). The restriction of pIN 29 delivered two fragments by using NdeI and XbaI (see figure 23, sample 2).

The pBAH8 and pIN 29 vectors were extracted and purified out of the gels by using the Wizard®SV Gel and PCR Clean-Up System (Promega). In case of pBAH8 the band between 5 and 6 kb (figure 22, sample 2) was purified in 2 approaches resulting in DNA concentration of 8.6 and 52.7 ng μl^{-1}. The pIN 29 vector was also purified in two approaches resulting in a DNA concentration of 15.6 and 19.2 ng μl^{-1}. The purified products were dephosphorylated and ligated to the blunted GFP amplicon (62.2 ng μl^{-1}). Several *E.coli* cells were transformed using the ligation product. No transformants were obtained.

5-alpha *E.coli* cells were transformed with pIN 29 and pBAH8 (isolated out of *Burkholderia terricola* ZR2-12) the plasmids were isolated out of a 20 ml overnight cultures (see table 15). Much higher DNA yields were gained in comparison to the first isolation approaches of pIN 29 and pBAH8 isolated from different strains (table 13 and 14).

Only the isolated pIN 29 vectors (125 and 165 ng μl^{-1}) were restricted and blunted again by using NdeI and XbaI. PBAH8 delivered more than one restricted fragment and therefore it wasn't used for further approaches. The whole reaction products were taken for a preparative gel (see figure 24 A and B). The restricted bands at ~ 4.5 kb of pIN 29 (figure 24, samples 2 and 3) were purified by using the Wizard®SV Gel and PCR Clean-Up System (Promega). 8 purifications were done in total (see table 16).

The purified vectors out of table 16 were pooled, meaning 2.1 together with 2.2 and 3.1 with 3.2 in a total amount of 97 µl. Four samples were gained in total. The pooled reactions were dephosphorylated and completely evaporated in a speed vac approach. All reactions were pooled in one reaction tube with 25 µl of nuclease free H_2O. The resulting DNA concentration was at 248.3 ng μg^{-1} (showing contaminations).

The GFP fragment was amplified out of pSM1880 using two different primer amounts. The purified reactions were analyzed in an analytical gel (see figure 25).

Different ligation approaches of the restricted and blunted vectors and the GFP amplicon were done and used for the transformation of 5-alpha *E.coli* cells. No transformants were obtained.

Preparation of plasmids

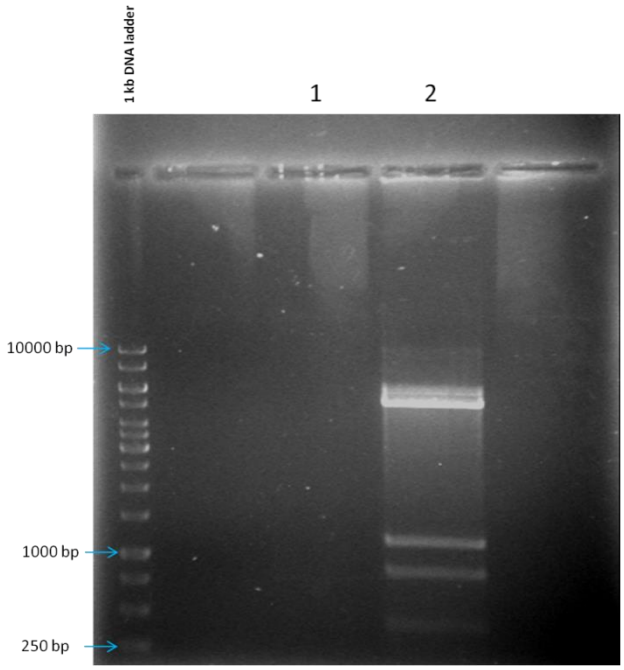


Figure 22: Preparative gel of restricted and blunted pBAH8 (*Burkholderia terricola* ZR2-12) vector. 1 = negative control, 2 = 23 µl of restricted pBAH8 (85.5 ng µl^{-1}).

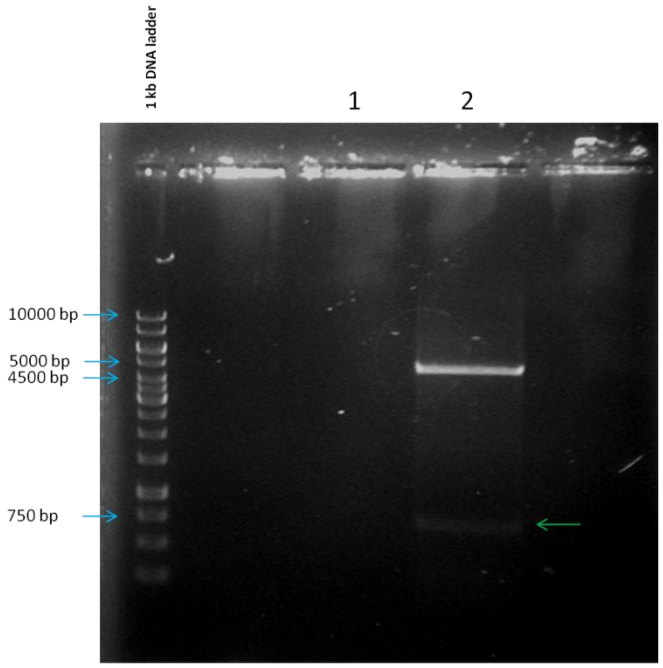


Figure 23: Preparative gel of restricted and blunted pIN 29 vector. 1 = negative control, 2 = 23 µl of restricted (5-alpha *E.coli* isolated*)* pIN 29 (40.2 ng µl^{-1}).

Table 15: Isolated pIN 29 and pBAH8 out of 5-alpha *E.coli* using QIAprep® Spin Miniprep Kit (QIAGEN)

Vector	DNA concentration (ng µl^{-1})
pIN 29 a	125.0
pIN 29 b	165.0
pBAH8 a	128.3
pBAH8 b	135.6

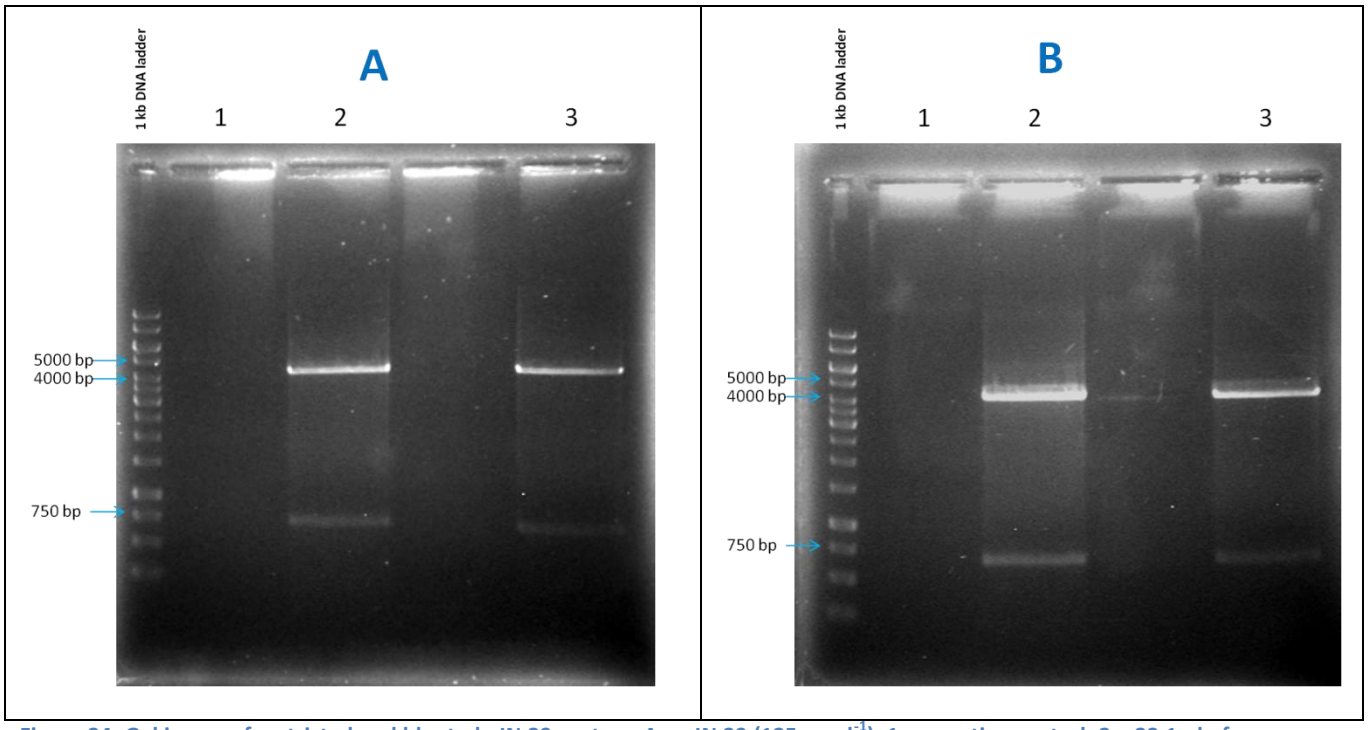


Figure 24: Gel image of restricted and blunted pIN 29 vectors. A = pIN 29 (125 ng µl^{-1}), 1 = negative control. 2 = 23.1 µl of restricted and blunted vector, 3 = same as 2; B = pIN 29 (165 ng µl^{-1}), 1 = negative control, 2 = 23.1 µl of restricted and blunted vector, 3 = same as 2.

Table 16: DNA concentrations of purified, restricted and blunted pIN 29 vectors (out of figure 24 A and B)

Sample		DNA concentration (ng µl^{-1})
A	2.1	7.8
	2.2	7.2
	3.1	6.0
	3.2	6.0
B	2.1	7.6
	2.2	6.3
	3.1	5.5
	3.2	4.7

Preparation of insert

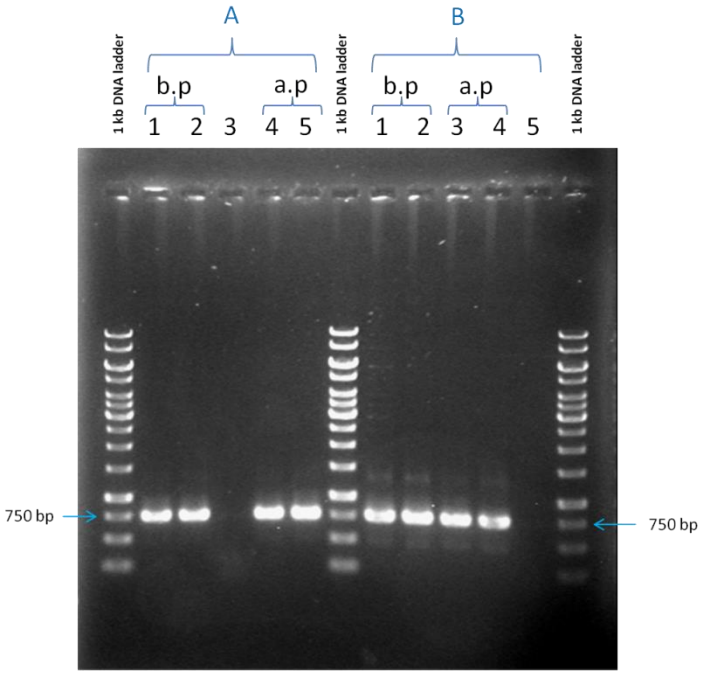


Figure 25: GFP amplification out of pSM1880. A = GFP amplification with 1 µl of each primer. b.p = PCR using material before it was purified, a.p = PCR using material after it was purified, 1 = 76.4 ng µl^{-1}, 2 = 76.2 ng µl^{-1}, 3 = negative control, 4 = 76.4 ng µl^{-1}, 5 = 76.2 ng µl^{-1}; B = GFP amplification with 2 µl of primer. 1 = 87.6 ng µl^{-1}, 2 = 99.3 ng µl^{-1}, 3 = 87.6 ng µl^{-1}, 4 = 99.3 ng µl^{-1}, 5 = negative control.

3.2.3 TA cloning

The pIN 29 vector (142.4 ng µl^{-1}) was restricted and blunted by using NdeI and XbaI (see figure 26, sample 2 and 3). Although no restriction fragments were detectable purification was done by using the Wizard®SV Gel and PCR Clean-Up System (Promega). A DNA concentration of 23.2 ng µl^{-1} was obtained (using 40 µl of elution amount). The vector was t-tailed in a Terminal Transferase Method using ddTTP and the terminal transferase enzyme. The reaction was purified using the Wizard®SV Gel and PCR Clean-Up System (Promega). The resulting DNA concentration was at 10.2 ng µl^{-1}.

The A-tailed GFP amplicon was generated by PCR in a total volume of 50 µl (see figure 27). The amplicon was purified (50 µl nuclease free H_2O) directly after the amplification using the Wizard®SV Gel and PCR Clean-Up System (Promega). The DNA concentration of the purified fragment was 72.8 ng µl^{-1}.

The T-tailed vector was ligated to the A-tailed GFP amplicon. Several transformations of 5-alpha *E.coli* cells using the ligation product were done (see table 17). In addition to the fluorescence check of the transformed colonies a colony PCR was done. All colonies showed a fragment in the range of GFP (figure 28, sample 1 to 8). This accorded to the amplification of the pSM1880 vector (figure 28, sample 10). The pIN 29 sample (figure 28, sample 11) was showing the same amplification band (meaning in range of GFP) as all other ligation

approaches expect of sample number 3. Obviously the pIN 29 vector had complementary binding sites for the GFP AleI and GFP PspXI primer which led to an unspecific amplification. These primers weren't used for further GFP identifications using the modified pIN 29 vector.

Generation of T-vector

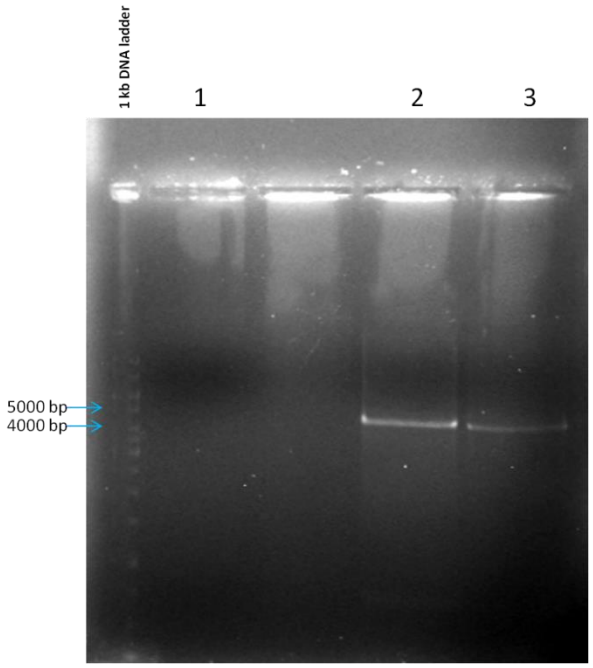


Figure 26: Gel image of restricted and blunted pIN 29 (142.4 ng μl^{-1}). 2 and 3 = 28 µl of pIN 29.

Generation of A-tailed PCR Product

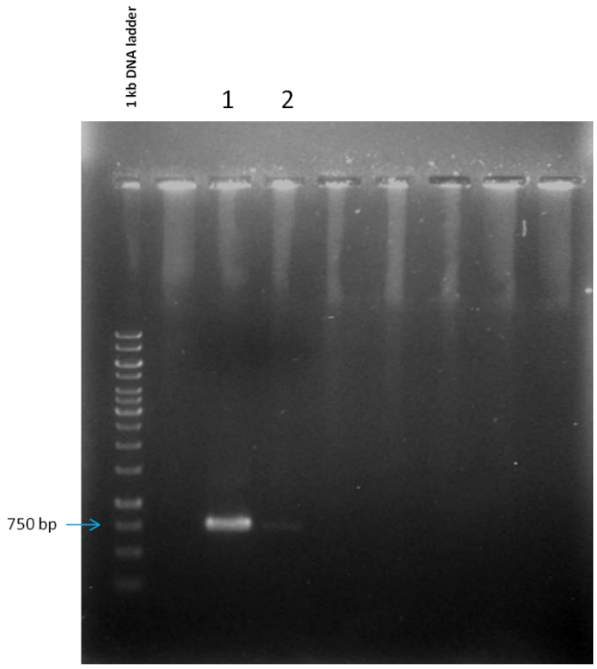


Figure 27: Analytical gel of the amplified GFP out of pSM1880. 1 = 5 µl of PCR reaction, 2 = negative control.

Ligation of A-tailed DNA fragment to the T-vector

Table 17: Number of outgrowing colonies after transformation of *E.coli* with TA-ligation product and fluorescence check

Ligation reaction	Approach number/Dilution	Colonies after 37°C incubation	Fluorescence (GFP and DsRed setting)
2 µl β-mercaptoethanol + 2 µl ligation approach	1 U	0	no
	1 C	0	no
2 µl β-mercaptoethanol + 2 µl ligation approach	2 U	1	no
	2 C	5	no
2 µl ligation approach	3 U	3	no
	3 C	5	no
2 µl ligation approach	4 U	0	no
	4 C	8	no
µl β-mercaptoethanol + 2 µl control ligation approach	5 U	1	no
	5 C	5	no
µl control ligation approach	6 U	0	no
	6 C	3	no

U = undiluted = directly after transformation reaction taken suspension for plate out, C = concentrated = transformation reaction was centrifuged at 2500 rpm for 2 min, resuspended in 100 µl medium and plate out.

Transformation check

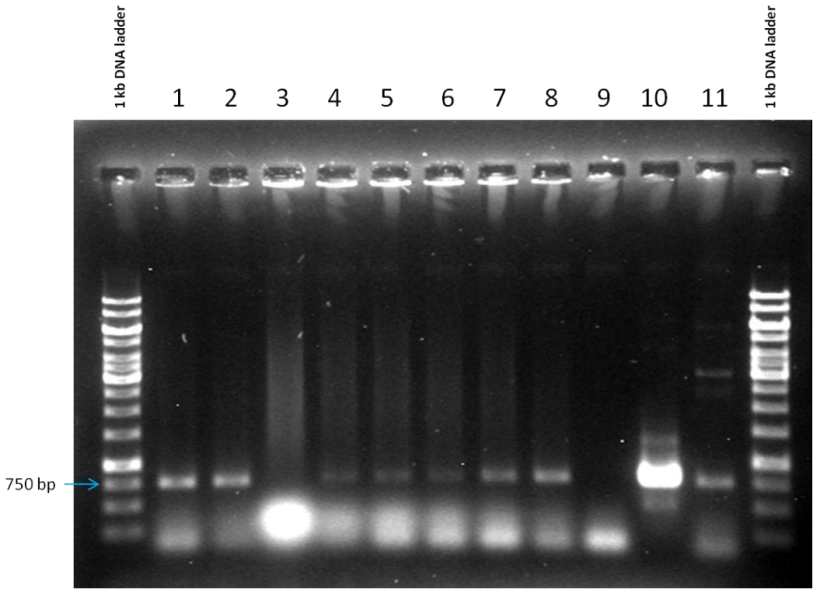


Figure 28: Analytical gel of the redone colony PCR approach. 5 µl of DNA ladder and also 5 µl for each sample were taken for the analysis. 1 = 2 U, 2 = 2 C, 3 = 3 U, 4 = 3 C, 5 = 4 C, 6 = 5 U, 7 = 5 C, 8 = 6 C, 9 = negative control, 10 = pSM1880 vector as positive control, 11 = pIN 29 (141.4 ng μl^{-1}); The description of the abbreviations were taken from table 22.

3.2.3.1 Proofing amplification of AleI/PspXI amplified GFP with Blue/White Screening

GFP out of pSM1880 was amplified and eluted in 30 µl nuclease free water (DNA concentration = 116.5 ng μl^{-1}). The amplicon was ligated with the ready to use pGEM vector. A gel electrophoresis of the purified GFP amplicon and the GFP-pGEM ligation was done (see figure 34).GFP showed a band at ~750 bp (figure 29, sample 1). The ligation product (sample 3) showed 2 bands at approximate 3.5 and 4 kb. The unrestricted pGEM vector (figure 29, sample 4) showed no band.

The GFP-pGEM ligation product was transformed into 5-alpha *E. coli*. The transformation approaches and the number of transformed colonies were listed in table 18.

The concentrated blue and white colonies (table 18, C labeled samples) were analyzed in a PCR reaction using pGEM specific primers (see figure 30). One blue colony (figure 30, sample 1) and two white colonies (figure 30, sample 2 and 4) were showing a band at 1000 bp. Sample 3 showed a band at 250 bp. The 0.1 ng unrestricted pGEM vector and the negative control were not amplified (figure 30, sample 5 and 6).

The white and blue colonies were checked by epifluorescence microscopy for GFP fluorescence (see table 19). Only blue and no white colonies were tested positive for the GFP fluorescence.

Colony material of the transformants (see table 19) were additionally checked in a further PCR reaction by using PspXI and AleI primers (see figure 31). One blue colony (figure 31, sample 1) and two white colonies (figure 31, sample 2 and 4) were showing the same band at approximate 750 bp as the positive control (sample 7). Sample 3, the 0.1 ng unrestricted pGEM vector and the negative control had no detectable product (figure 31, sample 5 and 6).

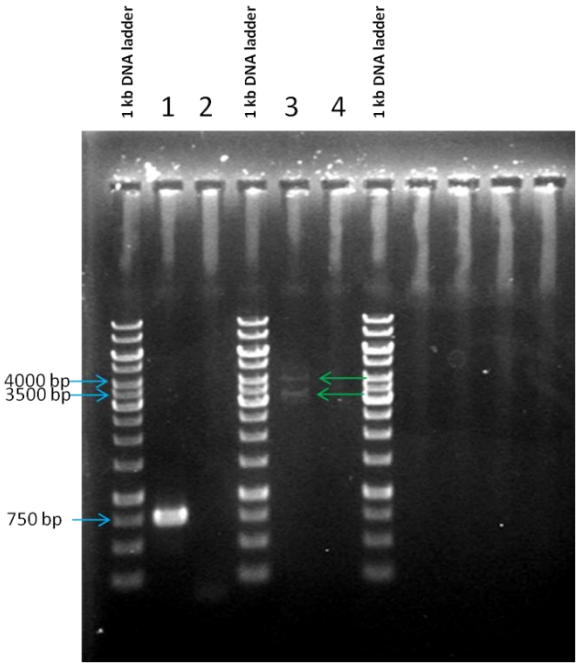


Figure 29: Analytical gel of GFP amplicon and the GFP-pGEM ligation approach. 1 = 5 µl of GFP amplicon. 2 = negative control of GFP amplicon, 3 = 5 µl of GFP-pGEM ligation approach, 4 = 5 µl of unrestricted (0.1 ng μl^{-1}) pGEM vector.

Table 18: Number of blue and white colonies after transformation of 5-alpha *E. coli* cells with GFP-pGEM ligation approach

Transformation	Substances used for transformation	Blue colonies	White colonies
1 U	2 µl ligation product	0	0
2 C	2 µl ligation product	7	5
3 U	2 µl ligation product	2	0
4 C	2 µl ligation product	18	9
5	2 µl 0.1 ng unrestricted pGEM vector	0	0

U = 100 µl of transformation reaction was directly plate out after the transformation reaction, C = The transformation approach was centrifuged after transformation, resuspended in 100 µl and plate out.

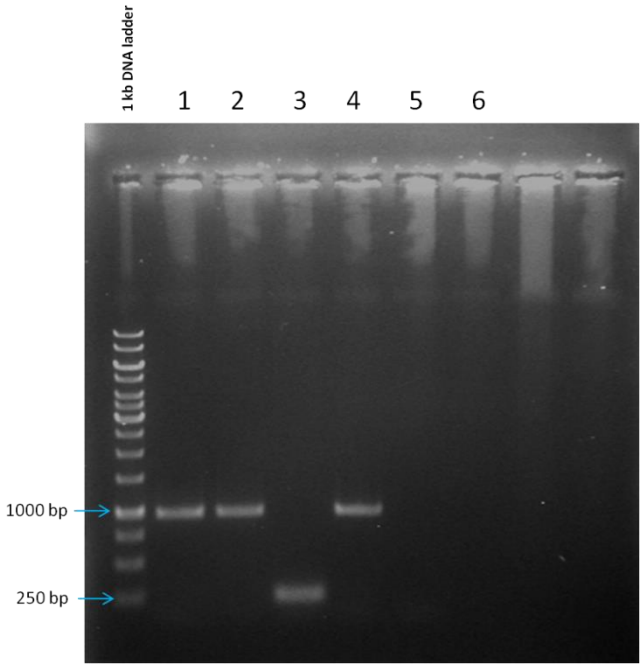


Figure 30: GFP insert check of blue and white colonies (transformants were taken out of table 22), 1 = 2 C blue colony, 2 = 2 C white colony, 3 = 4 C blue colony, 4 = 4 C white colony, 5 = 0.1 ng unrestricted pGEM vector, 6 = negative control.

Table 19: GFP (fluorescence) check of blue and white colonies

Transformation approach	Colonies	Number of fluorescenting colonies
2 C	Blue	3
	White	0
4 C	Blue	6
	White	0

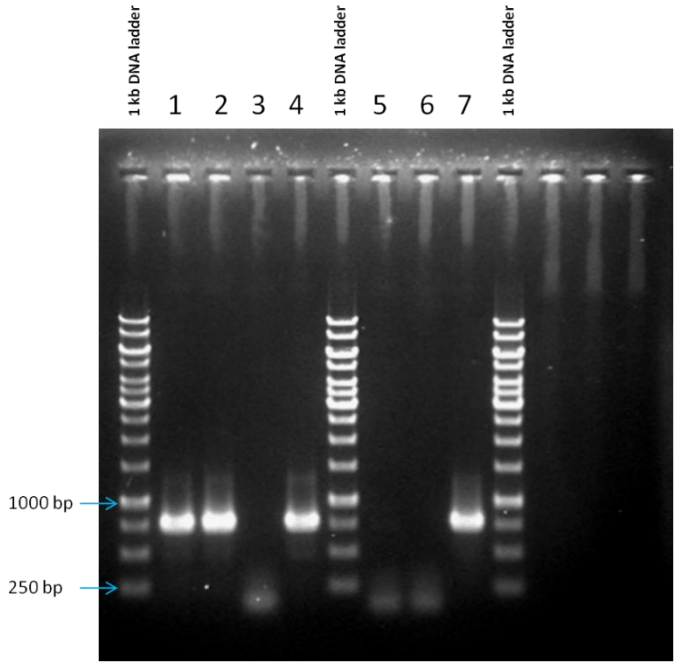


Figure 31: GFP insert check of blue and white colonies (transformants were taken out of table 23), 1 = 2 C blue colony, 2 = 2 C white colony, 3 = 4 C blue colony, 4 = 4 C white colony, 5 = 0.1 ng unrestricted pGEM vector, 6 = negative control, 7 = pSM1880 vector as positive control.

3.2.3.2 Double digest (NdeI and Xbal) and blunting of pIN 29 and ligation to (wild primers) amplified GFP

The GFP gene was amplified out of the pSM1880 vector using the wild primers. The GFP amplicon showed a band at approximate 1.5 kb (see figure 32, sample 1).

The pIN 29 vector was restricted by using NdeI and Xbal and directly blunted. A preparative gel with the whole reaction was done (see figure 33). The restricted and blunted pIN 29 vector showed a restricted fragment at approximate 750 bp (see figure 33, sample 1 and 2 green arrows). The vector was dephosphorylated, purified (11.9 ng μl^{-1}) and ligated with GFP.

A further ligation was done using the same setting as before. Both approaches were designed with a 1:1 molar ratio. An analytical gel of the ligation approaches was done (see figure 34). No ligation product could be identified (see figure 34, sample 1 and 2). The transformation of 5-alpha *E.coli* cells with the ligation products delivered no colonies.

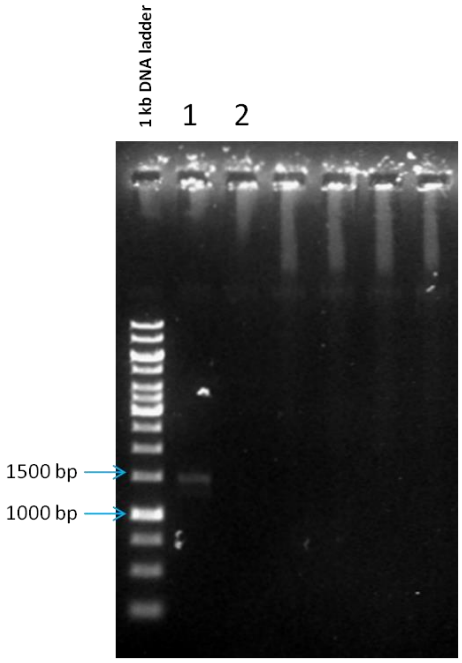


Figure 32: Analytical gel of amplified GFP out of pSM1880 with new Psp XI primer. 1 = 5 µl of PCR product, 2 = 5 µl of negative control.

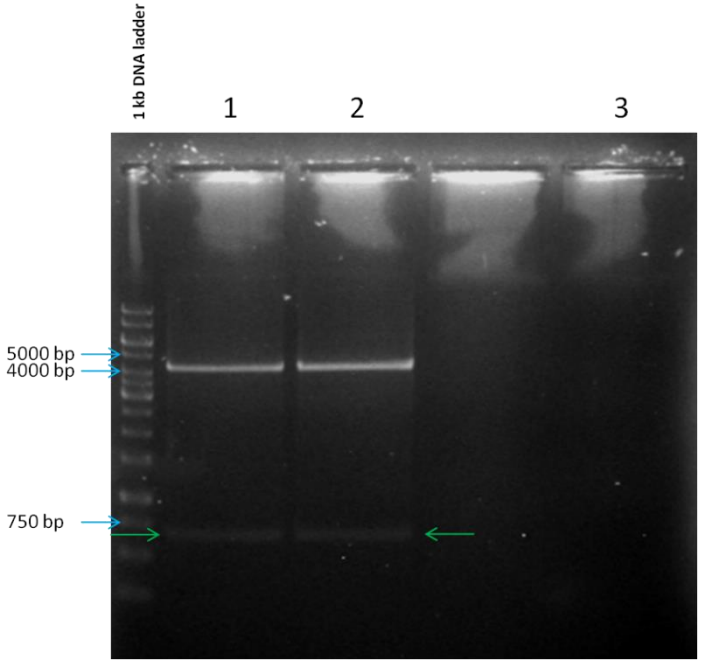


Figure 33: Preparative gel of NdeI/XbaI restricted and blunted pIN 29 (128.4 ng µl^{-1}) vector. 1 and 2 = ~33 µl of restricted and blunted pIN 29 vector. 3 = negative control.

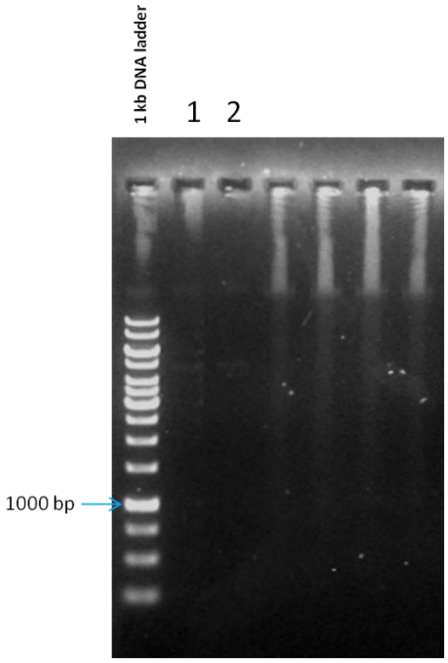


Figure 34: Analytical gel of restricted, blunted and dephosphorylated pIN 29 (11.9 ng µl^{-1}) ligated to GFP. 1 = 5 µl pIN 29 – GFP (37.5 ng µl^{-1}), 2 = 5 µl pIN 29 – GFP (116.5 ng µl^{-1}).

3.2.3.3 Double digest (NdeI and XbaI) pIN 29 and GFP1_NdeI_Vergunst/XbaI amplified GFP

The GFP insert was amplified twice out of pSM1880 by using GFP1_NdeI_Vergunst and GFP1_XbaI_Vergunst primers. A hot start and a standard PCR approach were done. The PCR products of both approaches were analyzed by gel electrophoresis (see figure 35 and 36). The GFP amplicon generated by hot start PCR (see figure 35, sample 1) wasn't amplified in high amount (87.9 ng µl^{-1} after purification) in comparison to the amplified product in the standard PCR (see figure 36, sample 1 = 97.3 ng µl^{-1} after purification). The amplified GFP out of the pBAH vector showed a low amplification product (see figure 36, sample 2 = 59.3 ng µl^{-1} after purification).

Both GFP amplicons were restricted and purified (see figure 37 and 38). The same restriction approach was done for pIN 29 (113.8 ng µl^{-1}) vector (see figure 39).

The restricted GFP amplicon out of the Hot Start PCR were showing 3 different bands at 750, 500 and 250 bp (see figure 37, sample 1 and 2 = 9.3 and 16.6 ng µl^{-1} after purification). The restricted GFP amplicon generated by standard PCR were also showing 3 different fragments at 750, 500 and 250 bp (see figure 38, sample 1).

The restricted pIN 29 didn't show an additional fragment after digestion (see figure 39, sample 1 and 2 = 21.4 and 14.0 ng µl^{-1} after purification).

The purified GFP amplicon out of the Hot Start PCR (16.6 ng µl^{-1}) was used for further ligation approaches with the restricted pIN 29. Two quick ligation reactions with 3:1 and 1:1

molar ratio were done. In addition two T4 DNA ligase ligation approaches using the same molar ratios were generated. The ligation approaches were used to transform several 5-alpha *E.coli* cells. No transformants were obtained. Therefore the different ligation approaches were checked for their insert by using specific primers (see figure 40). Neither the different ligation approaches nor the purified pIN 29 vector used as positive control were showing an amplification by using the pIN 29 specific primers pIN7_insertcheck.

In further PCR reaction the specifity of pIN7_insertcheck primers were tested for the purified pIN 29 (113.5 ng μl^{-1}) (see figure 41). No amplifications were generated by the gradient PCR (see figure 41, sample 1 to 8).

A further PCR was done using also the pIN7_insertcheck primers. A lower annealing temperature was applied and two different Taq polymerases were used for the PCR (see figure 42). No amplifications were obtained (see figure 42, sample 1 and 2).

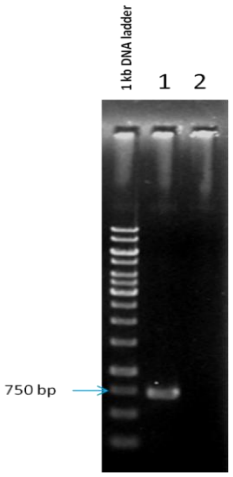


Figure 35: GFP amplicon generated by Hot Start PCR. 1 = 5 µl of amplified GFP out of pSM1880.

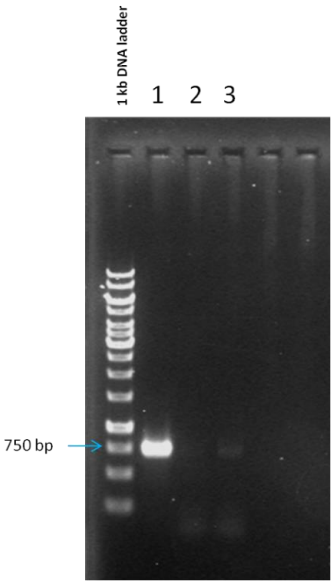


Figure 36: GFP amplicon generated by standard PCR. 1 = 5 µl of amplified GFP out of pSM1880, 2 = 5 µl of amplified GFP out of pBAH8 using a Rp8 colony, 3 = negative control.

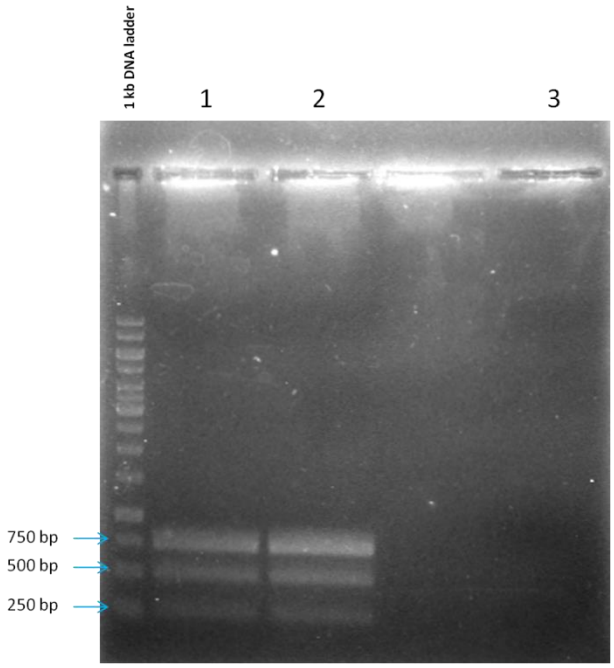


Figure 37: Preparative gel of restricted GFP amplicon generated by Hot Start PCR. 1 = 20 µl of NdeI and XbaI restricted GFP amplicon, 2 = the same as 1, 3 = negative control.

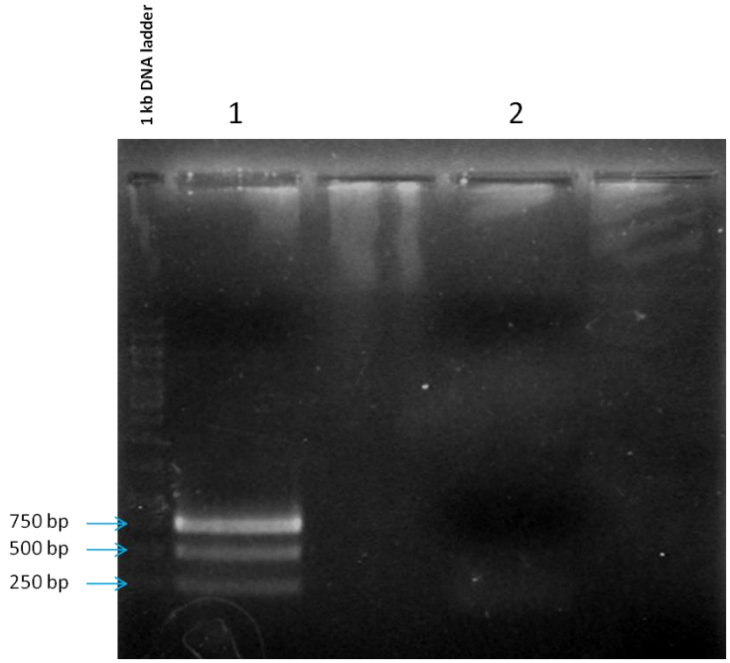


Figure 38: Preparative gel of restricted GFP amplicon generated by standrad PCR. 1 = 40 µl of NdeI and XbaI restricted GFP amplicon, 2 = negative control.

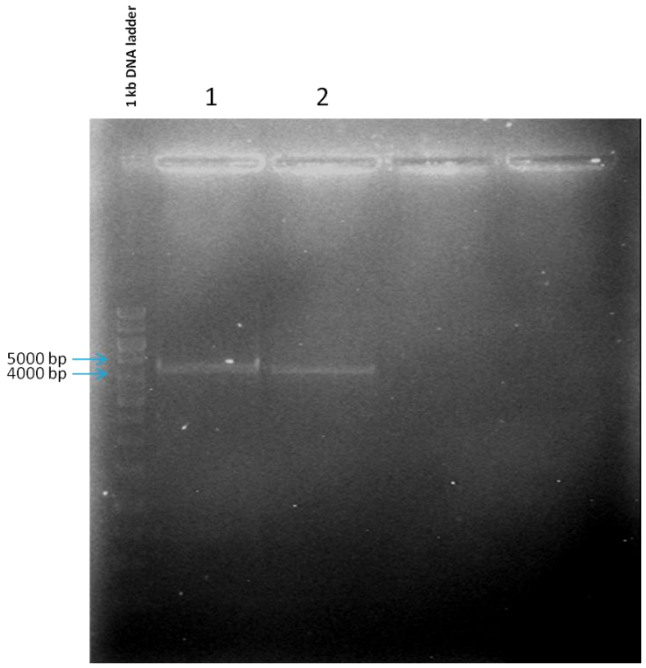


Figure 39: Preparative gel of pIN 29 (113.8 ng µl^{-1}) restriction. 1 = 25 µl of restricted pIN 29 (113.8 ng µl^{-1}), 2 = the same as 1.

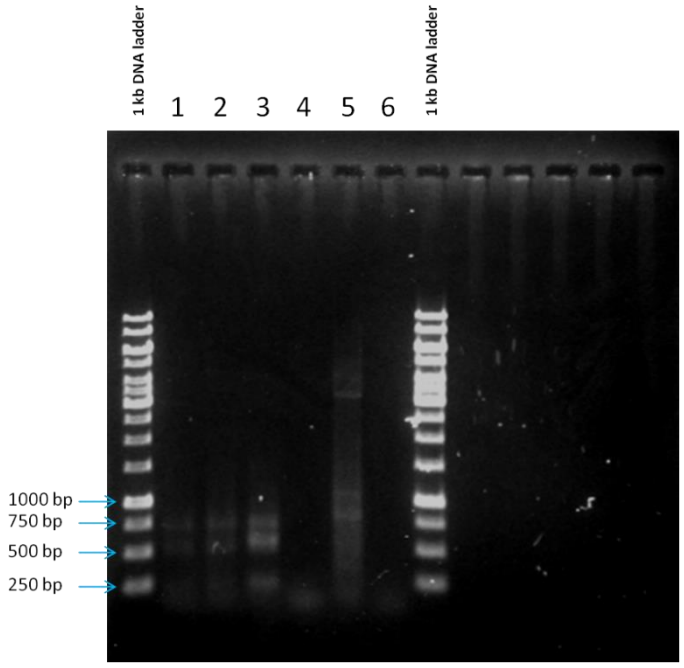


Figure 40: Analytical gel of Quick and T4 ligation approaches. 1 = Quick ligation of restricted pIN 29 (14.0 ng µl^{-1}) and GFP (16.6 ng µl^{-1}) using 3:1 ratio, 2 = same as 1 using a 1:1 ratio, 3 = standard ligation of restricted pIN 29 (14.0 ng µl^{-1}) and GFP (16.6 ng µl^{-1}) using 3:1 ratio, 4 = same as 3 using a 1:1 ratio, 5 = pIN 29 (113.8 ng µl^{-1}), 6 = negative control. For all wells 5 µl of product was used for the analytical gel.

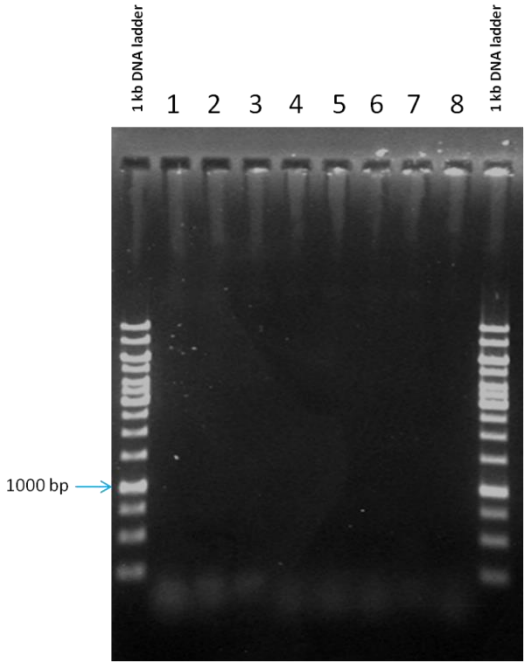


Figure 41: Gradient PCR using pIN 29 (113.5 ng µl^{-1}) as template. 1 – 8 = 10 µl of PCR product.

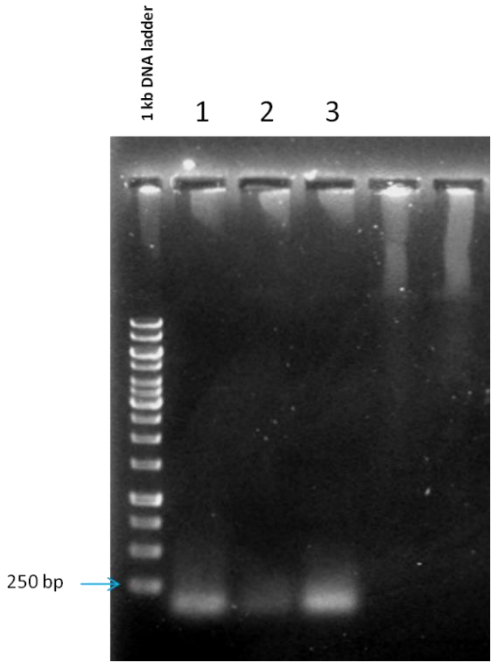


Figure 42: PCR of pIN 29 vector using pIN7_insertcheck primers. 1 = 5 µl pIN 29 (113.5 ng µl^{-1}) of Taq&Go PCR, 2 = 5 µl pIN 29 (113.5 ng µl^{-1}) of Dream Taq PCR, 3 = negative control.

3.2.3.4 Double digest (NdeI and Xbal) pIN 29 and GFP1_BglII_Vergunst /Xbal amplified GFP

The *Nde*I primer for the GFP amplification was replaced by a new including a BglII restriction site instead of NdeI. The GFP insert was amplified out of pSM1880 using two different Vergunst primer pairs for BglII (one standard and a reverse complementary primer = rc). The PCR products were analyzed in an analytical gel (see figure 43). Both GFP amplicons (see figure 43, sample 1 and 2) had almost the same band size. Both amplicons were purified (sample 1 = 90 ng μl^{-1} and sample 2 = 83 ng μl^{-1}). Both amplicons and the pIN 29 (133.8 ng μl^{-1}) vector were restricted (with BglII and Xbal). The restriction reactions were used for preparative gels (see figure 44 and 50). The restricted GFP amplicons showed no additional fragments (see figure 44, sample 1 and 2). Sample 1 (out of figure 44) had a DNA concentration of 27.7 ng μl^{-1} and sample 2 24.2 ng μl^{-1} after purification.

The restricted pIN 29 (133.8 ng μl^{-1}) vector showed a fragment at ~750 bp (see figure 45 sample 3 and 4, green arrows). The pIN 29 fragments at ~4.5 kb were extracted and purified (sample 3 had a DNA concentration of 14.0 ng μl^{-1} and sample 4 of 18.1 ng μl^{-1}).

PIN 29 (14.0 ng μl^{-1}) was ligated to GFP (27.7 ng μl^{-1}) in a first reaction and to GFP (24.2 ng μl^{-1}) in a second one. Both ligations were designed with a 3:1 molar ratio. Additionally the same ligation reactions were done applying a 1:1 and 10:1 molar ratio. In total 6 ligation approaches were generated and used for further transformation of competent *E.coli* cells (see table 20).

None of the transformed colonies were showing GFP fluorescence (see table 20). DNA material of transformed (GFP (27.2 ng μl^{-1})-pIN29 (14.0 ng μl^{-1}) with a molar ratio of 1:1) colonies were checked for DsRed and GFP insert by applying a colony PCR. The PCR products were analyzed in analytical gels (see figure 46 and 47).

The DsRed- as well as the non DsRed fluorescenting- colonies were showing amplifications at ~500 bp using the DsRed_RC primers (see figure 46, sample 3, 5 and 7). None amplifications of the GFP specific fragments were detected.

The same colony material was analyzed with other GFP specific primers (PspXI and AleI). The results of the analytical gel were shown in figure 47. GFP could be amplified out of pSM1880 and colony material of Rp5-pBAH8 (see figure 47, sample 2 and 5). The GFP (27.7 and 24.2 ng μl^{-1}) – pIN 29 (14.0 and 18.1 ng μl^{-1}) transformed colonies showed none GFP specific amplifications (see figure 47, sample 3 and 4).

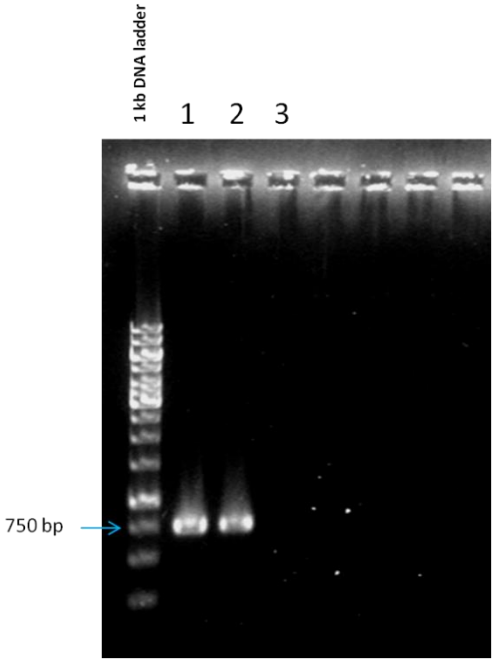


Figure 43: Analytical gel of GFP amplicons (out of pSM1880). 1 = 3 µl GFP amplicon amplified with GFP1_Xbal_Vergunst and GFP1_BglII_Vergunst, 2 = 3 µl GFP amplicon amplified with GFP1_Xbal_Vergunst and GFP1_BglII_rc_Vergunst, 3 = 3 µl of negative control.

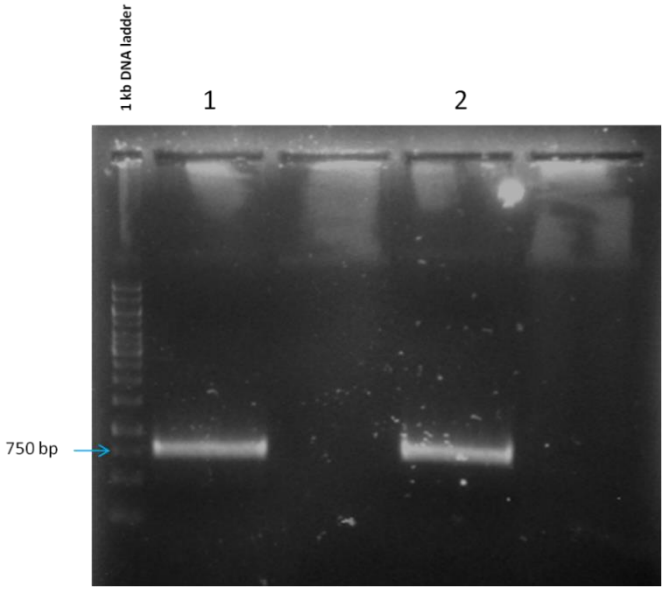


Figure 44: Preparative gel of restricted GFP amplicons. 1 = 40 µl of restricted GFP (GFP1_BglII_Vergunst) amplicon, 2 = 40 µl of restricted GFP (GFP1_BglII_rc_Vergunst).

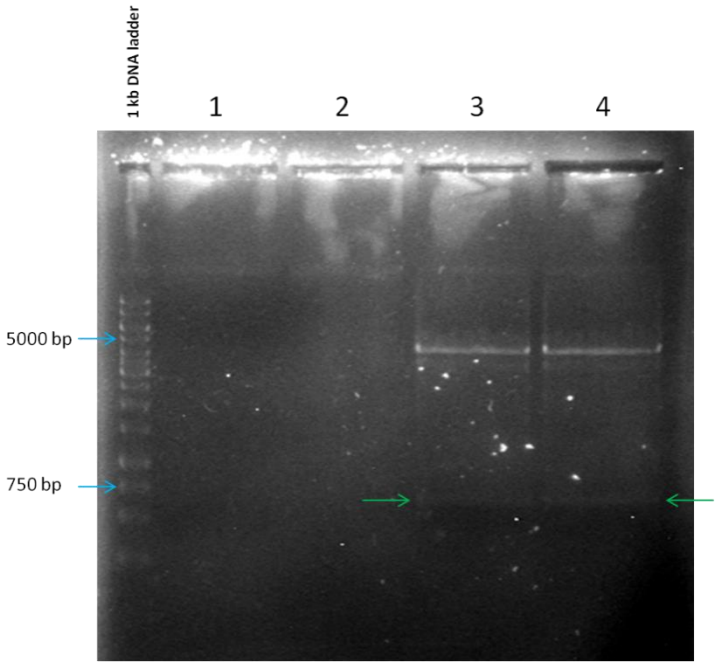


Figure 45: Preparative gel of restricted pIN 29 (133.8 ng µl^{-1}). 1 and 2 = negative control. 3 and 4 = each 25 µl of BglII and XbaI restricted pIN 29 (133.8 ng µl^{-1}).

Table 20: Fluorescence analysis of pIN 29 (14.0 ng µl^{-1}) – GFP transformed 5-alpha *E.coli* cells

Ligation approach		Number of colonies	GFP fluorescence	DsRed fluorescence
3:1	GFP (GFP1_*Bgl*II_Vergunst)	0	No	No
	GFP (GFP1_BglII_rc_Vergunst)	1	No	1
1:1	GFP (GFP1_BglII_Vergunst)	6	No	4
	GFP (GFP1_BglII_rc_Vergunst)	2	No	No
10:1	GFP (GFP1_BglII_Vergunst)	0	No	No
	GFP (GFP1_BglII_rc_Vergunst)	0	No	No

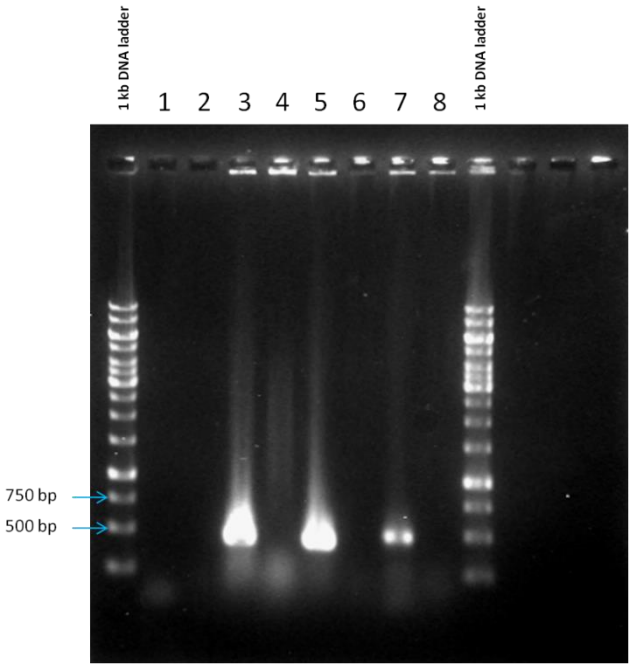


Figure 46: Analytical gel of DsRed- and GFP-insert check of GFP (27.7 and 24.2 ng µl^{-1}) – pIN 29 (14.0 and 18.1 ng µl^{-1}) transformed colonies. 1 = 3 µl of negative control with DsRed_RC primers, 2 = 3µl of negative control with GFP_wild primers, 3 = (positive control for DsRed primers) 3 µl Rp8-pIN 29 PCR product with DsRed_RC primers, 4 = (positive control for GFP primers) Rp5-pBAH8 PCR product with GFP_wild primers, 5 = 3 µl of PCR product (GFP (27.2 ng µl^{-1})-pIN29 (14.0 ng µl^{-1}) DsRed fluorescenting) in a molar ratio of 1:1) with DsRed_RC primers, 6 = the same as 5 with GFP_wild primers, 7 = the same as 5 with non DsRed fluorescenting colony material, 8 = same as 7 with GFP-wild primers.

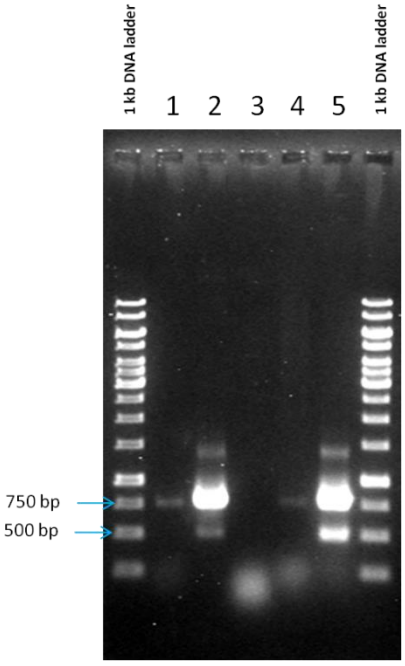


Figure 47: Analytical gel of GFP (27.7 and 24.2 ng µl^{-1}) – pIN 29 (14.0 and 18.1 ng µl^{-1}) transformed colonies using PspXI and AleI primers. 1 = 3 µl of negative control, 2 = 3 µl of pSM1880 (positive control for GFP), 3 = 3 µl of PCR product (GFP (27.2 ng µl^{-1})-pIN29 (14.0 ng µl^{-1}) DsRed fluorescenting) in a molar ratio of 1:1) using PspXI and AleI primers, 4 = the same as 3 with non DsRed fluorescenting colony material, 5 = 3 µl of Rp5-pBAH8 colony material with GFP primers.

3.2.3.5 Double digest (NdeI and XbaI) pIN 29 and GFP1_BglII_Vergunst_ATG /XbaI amplified GFP

Review of the sequence primers for the GFP amplicon had shown a missing "ATG" group at the beginning of the sequence. Therefore the GFP gene was amplified out of pSM1880 by using another primer in 2 identical PCR approaches. Each reaction was purified with a different purification kit. The (GE Healthcare and Promega) purified GFP amplicons were restricted and used for a preparative gel (see figure 54).

The GFP1_BglII_Vergunst_ATG and GFP1_XbaI_Vergunst amplified (Promega kit purified) and restricted GFP (figure 48, sample 1 and 2) shown 2 fragments at 750 and 500 bp. The GE Healthcare purified GFP showed only one fragment at approximate 750 bp (see figure 48, sample 3 and 4).

The restricted and purified GFP amplicon (Promega) was ligated to the restricted pIN 29 (18.1 ng μl^{-1}) vector in a 3:1 molar ratio and transformed into 5-alpha *E. coli* cells. A different number of transformants was achieved (see table 21).

Some of the transformants were shown a green fluorescence by using the epifuorescence microscope.

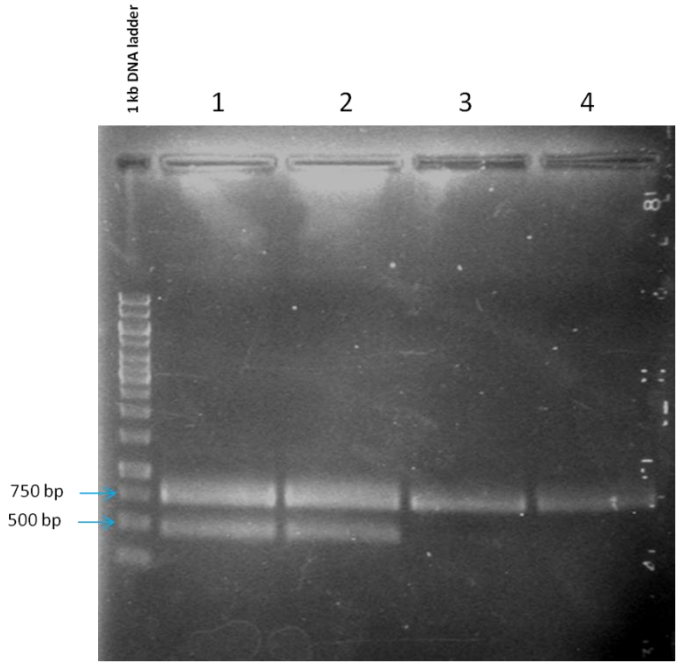


Figure 48: Analytical gel of restricted GFP (GFP1_BglII_Vergunst_ATG) amplicons. 1 and 2 = 25 µl of restricted GFP (95.9 ng/l) purified by Promega kit, 3 and 4 = 25 µl of restricted GFP (37 ng/l) purified by GE Healthcare kit.

Table 21: GFP fluorescence analysis of the transformed 5 – alpha *E. coli* cells

Transformation approach	Number of transformants	Green fluorescent colonies
Standard protocol	> 20	> 5
Additional centrifugation step after transformation	> 40	> 20

3.2.4 Transformation of environmental strains with fluorescent genes

3.2.4.1 Generating pIN 29 vectors including mNeptune and eBFP2 genes

Both Eurofines synthesized vectors (pEX-A-mNeptune_5bglll_3xbal and pEX-A-eBFP2_5bglll_3xbal) containing the mNeptune and the eBFP2 gene were cloned into 5 – alpha *E.coli* cells. Out of each transformation approach more than ~500 colonies were obtained.

Overnight cultures of the transformed *E.coli* colonies were prepared and the vectors isolated by using the QIAprep® Spin Miniprep Kit (QIAGEN) (see table 22).

The isolated vectors (out of table 22) and pIN 29 (119.6 ng μl^{-1}) were restricted and used for preparative gels (see figure 49 and 50).

The mNeptune and eBFP2 fragments (out of figure 49, sample 1,2 and 3,4 fragments at ~750 bp) were extracted and purified out of the preparative gel by applying the Promega kit (mNeptune sample 1 = 22.2 and sample 2 = 21.5 ng μl^{-1}; eBFP2 sample 1 = 10.3 and 2 = 13.5 ng μl^{-1}). The pIN 29 fragment at ~4.5 kb was also extracted and purified by using the Promega kit (out of figure 50, sample 1 = 10.3 ng μl^{-1} and sample 2 = 13.5 ng μl^{-1}).

Two ligation approaches using the mNeptune and eBFP2 fragments together with the pIN 29 vector were done in a 3:1 molar ratio. The ligation reactions were transformed into 5 – alpha *E.coli* cells. The colonies were analyzed for their fluorescence by using the CLSM (see table 23 and 24).

The eBFP2-pIN 29 transformed *E.coli* cells showing an emission at 500-546 nm using an extinction of 405 nm (see table 23). MNeptune-PIN 29 transformed cells had the same emission spectra as pIN 29 (DsRed) transformed cells (see emission spectra of DsRed and mNeptune, table 24) applying the same extinctions settings.

Colony material of each transformed *E.coli* cell was used for a colony PCR approach using the DsRed-RC primers. The PCR products were analyzed in an analytical gel (see figure 51).

The mNeptune-pIN 29 colony material (figure 51, sample 2 and 3) were showing the same amplifications as the pIN 29-DsRed material (figure 51, sample 1 and 7). Also the eBFP2-pIN 29 transformants (figure 51, sample 4 and 5) were showing low amplifications in the same range like DsRed.

A further colony PCR approach using the same settings as before but an additional sample was done (see figure 52).

The purified mNeptune fragments (22.2 and 21.5 ng μl^{-1}) shown low amplifications (see figure 52, sample 2 and 3). The isolated pEX-A-mNeptune_5bglll (out of 5-alpha *E.coli*, 373.4 ng μl^{-1}) vector showed no amplification (see figure 52, sample 4). In comparison to that the mNeptune-pIN 29 transformed colonies (figure 52, sample 5 and 6) were showing the same amplifications as the positive control (figure 52, sample 1).

Different pIN 29-mNeptune transformed colonies were picked and scratched out on new LB selective plates. Colony material was taken from these colonies for further colony PCR reactions (see figure 53 and 54) to check the presence of a mNeptune gene.

With the exception of sample 5 in figure 53 and sample 1 in figure 54 all other reactions were showing amplifications at approximate 500 bp.

Colony materials of both samples were analyzed by CLSM (see table 25).

Both transformants (sample 5 and 1) were showing an different emission spectra in comparison to the pIN 29 transformed cells (see table 24).

Table 22: DNA concentrations of isolated Eurofines vectors out of 5 – alpha *E.coli* cells

Vector	Concentration in ng μl^{-1}
pEX-A-mNeptune_5bglII	373.4 ng μl^{-1}
pEX-A-eBFP2_5bglII_3xbal	397.6 ng μl^{-1}

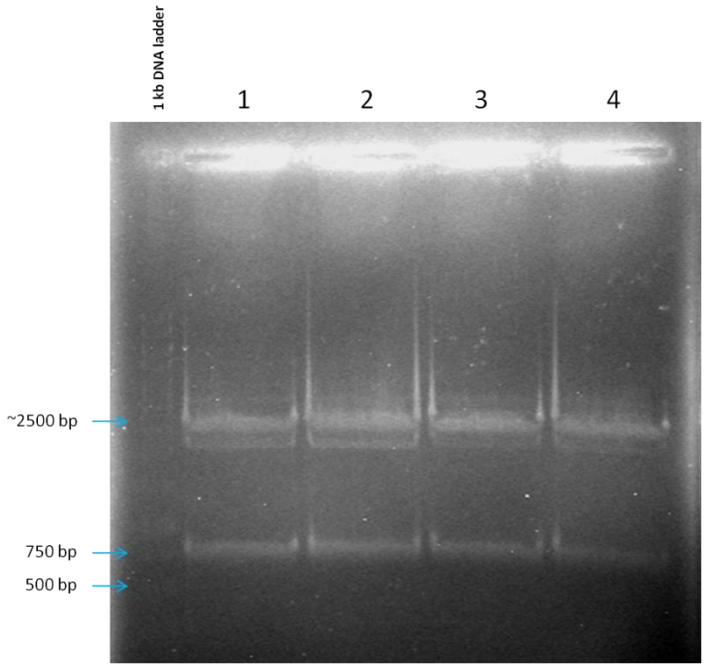


Figure 49: Preparative gel of restricted "Eurofines" vectors. 1 and 2 = 25 µl of *Bgl*II and XbaI restricted pEX-A-eBFP2_5bglII_3xbal vector (397. 6 ng μl^{-1}), 3 and 4 = 25 µl of BglI and XbaI restricted pEX-A-mNeptune_5bglII vector (373.4 ng μl^{-1}).

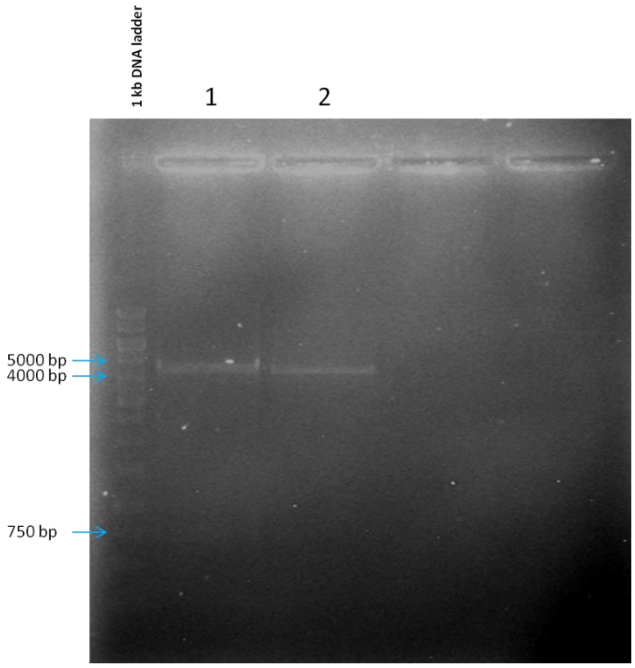


Figure 50: Prepartive gel of restricted pIN 29 vector. 1 and 2 = each 25 µl of BglII and XbaI restricted pIN 29 (119.6 ng µl^{-1}).

Table 23: CLSM settings for identification of eBFP2-pIN29 transformed colonies

Transformation approach	Number of colonies	Extinction (nm) *	Emission (nm)	Gain	Offset (%)
		405	500-546	780	-1
eBFP2 (24.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1})	> 100		+		
eBFP2 (24.2 ng µl^{-1}) – pIN 29 (18. ng µl^{-1})	> 200		+		

*The laser intensity of the channels was 50 %.

Table 24: CLSM settings for identification of mNeptune-pIN29 and pIN 29 transformed colonies

Transformation approach	Number of colonies	Extinction* (nm)	DsRed-Emissionspectra 563-582 (nm)	mNeptune-Emissionspectra 600-650 (nm)	Gain	Offset
mNeptune (22.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1})	> 200	532	+	+	750	-1
		635	-	+	750	-1
mNeptune (22.2 ng µl^{-1}) – pIN 29 (18.1 ng µl^{-1})	> 200	532	+	+	750	-1
		635	-	+	750	-1
pIN29 only^{+}	~10	532	+	+	750	-1
		635	-	+	750	-1

*The laser intensity of the channels was 50 %, $^{+}$ colony material of Rp8 – pIN 29 transformed cells

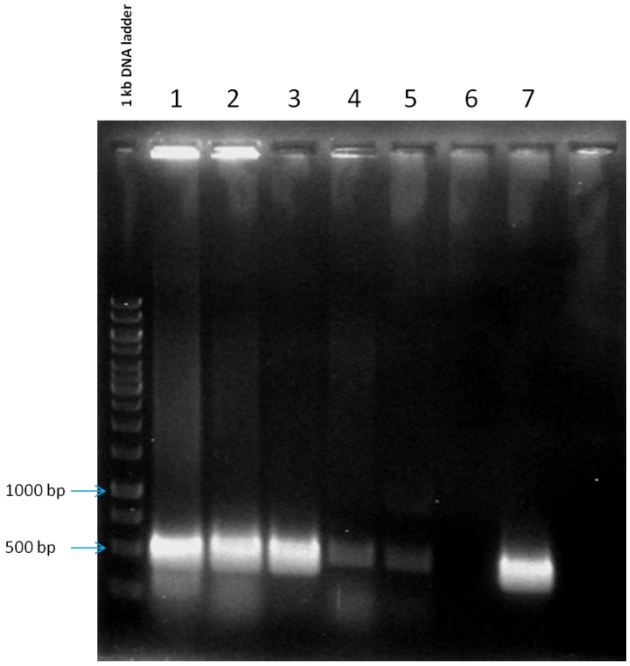


Figure 51: Analytical gel of colony PCR products using DsRed_RC primers. 1 – 7 = 10 µl each well, 1 = colony material of P69-pIN 29 transformant, 2 = mNeptune (22.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1}), 3 = mNeptune (22.2 ng µl^{-1}) – pIN 29 (18.1 ng µl^{-1}), 4 = eBFP2 (24.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1}), 5 = eBFP2 (24.2 ng µl^{-1}) – pIN 29 (18.1 ng µl^{-1}), 6 = negative control, 7 = isolated pIN 29 (124.8 ng µl^{-1}) as positive control.

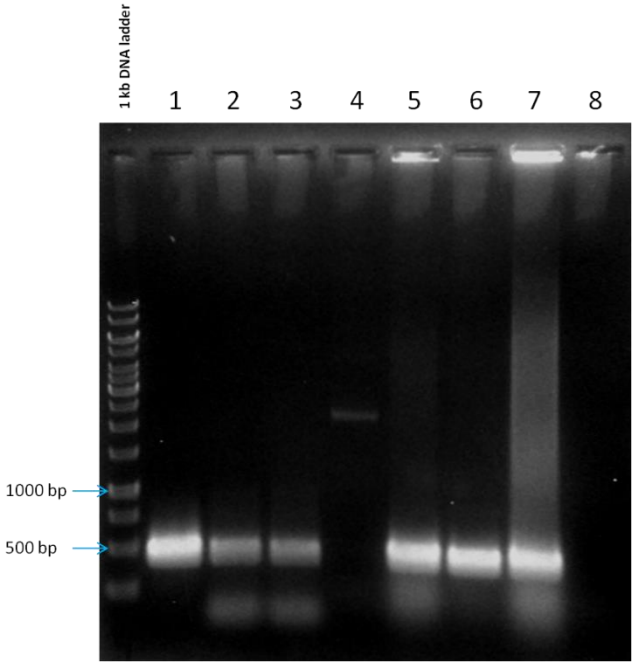


Figure 52: Analytical gel of colony PCR products using DsRed_RC primers. 1 – 8 = 10 µl, 1 = isolated pIN 29 (124.8 ng µl^{-1}, 1:10), 2 = purified mNeptune (22.2 ng µl^{-1}), 3 = purified mNeptune (21.5 ng µl^{-1}), 4 = pEX-A-mNeptune_5bglII (373.4 ng µl^{-1}), 5 = mNeptune (22.2 ng µl^{-1}) – pIN 29 (18.1 ng µl^{-1}) colony, 6 = mNeptune (22.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1}) colony, 7 = P69-pIN 29 colony material, 8 = negative control.

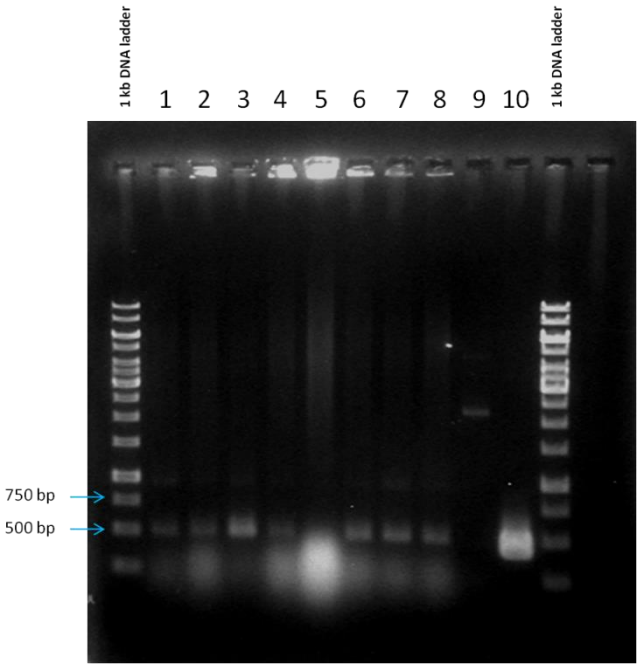


Figure 53: Analytical gel of colony PCR approach using different mNeptune-pIN 29 colony material. 1 – 8 = 5 µl of different mNeptune (22.2 ng µl^{-1}) – pIN 29 (13.5 ng µl^{-1}) transformed 5 – alpha *E.coli* cells, 9 = 5 µl of pEX-A-mNeptune_5bglll (373.4 ng µl^{-1}, 1:10), 10 = 5 µl of purified pIN 29 (124.8 ng µl^{-1}).

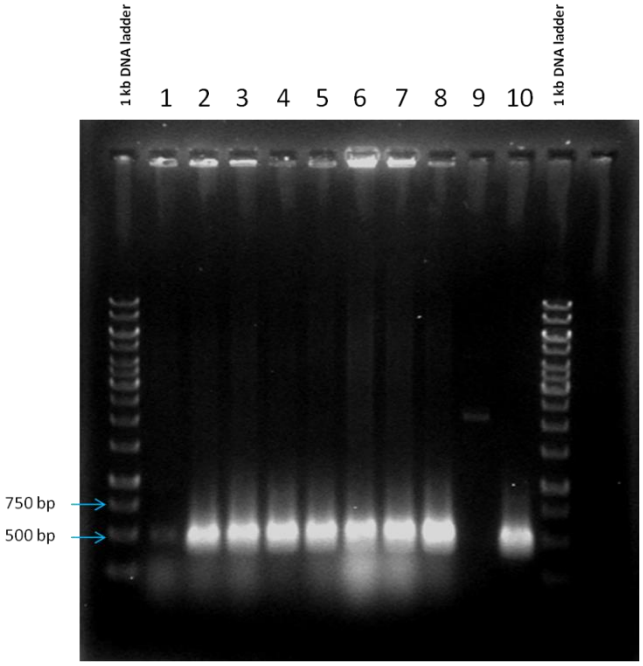


Figure 54: Analytical gel of colony PCR approach using different mNeptune-pIN 29 colony material. 1 – 8 = 5 µl of different mNeptune (22.2 ng µl^{-1}) – pIN 29 (18.1 ng µl^{-1}) transformed 5 – alpha *E.coli* cells, 9 = 5 µl of pEX-A-mNeptune_5bglll (373.4 ng µl^{-1}, 1:10), 10 = 5 µl of purified pIN 29 (124.8 ng µl^{-1}).

Table 25: CLSM settings for identification of mNeptune-pIN 29 and pIN 29 transformed colonies

Transformation approach	Extinction* (nm)	DsRed-Emissionspectra 563-582 (nm)	mNeptune-Emissionspectra 600-650 (nm)	Gain	Offset
mNeptune (22.2 ng μl^{-1}) – pIN 29 (13.5 ng μl^{-1})	532	-	+	750	-1
	635	-	+	750	-1
mNeptune (22.2 ng μl^{-1}) – pIN 29 (18.1 ng μl^{-1})	532	-	+	750	-1
	635	-	+	750	-1
pIN29 only^{+}	532	+	+	750	-1
	635	-	+	750	-1

*The laser intensity of the channels was 50 %, $^{+}$ colony material of Rp8 – pIN 29 transformed cells

Transformation of P69 and ep17 with mNeptune- and eBFP2 – pIN 29

5 – alpha *E. coli* cells containing the mNeptune- and eBFP2 – pIN 29 vector were grown in LB medium overnight. The vectors were isolated out of the onc by using the QIAprep® Spin Miniprep Kit (QIAGEN) (see table 26).

Electro competent ep17 and P69 cells were transformed with the pIN 29- eBFP2 or mNeptune vector (see table 27). It was possible to transform al lot of P69 as well as ep17 competent cells with the mNeptune and eBFP2 containing pIN 29 vector. Material of the mNeptune-pIN 29 transformed cells was used for colony PCR to check if the cloning was successful.

The mNeptune-pIN 29 vector transformed ep17 and P69 cells were showing no DsRed specific amplification by direct comparison to the pIN 29-DsRed containing plasmid (figure 55 sample 1-6, figure 56 sample 3-8). All transformed ep17 and P69 colonies were shown amplifications at approximate 250 bp.

Table 26: DNA Concentrations of isolated mNeptune- and eBFP2- pIN 29 vectors out of 5-alpha *E.coli* cells

Vector definition (ng μl^{-1})	Concentration of isolated vector (ng μl^{-1})
eBFP2 (24.2)– pIN 29 (13.5)	91.2
eBFP2 (24.2) – pIN 29 (18.1)	77.6
mNeptune (22.2) – pIN 29 (13.5)	108.6
mNeptune (22.2) – pIN 29 (18.1)	107.4

Table 27: Results of pIN29- eBFP2- and mNeptune vector transformed ep17 and P69 cells

Strain	Used vector for transformation (ng μl^{-1})	Number of colonies
ep17	mNeptune– pIN 29 (108.6)	> 100
	mNeptune– pIN 29 (108.6)*	> 1000
	mNeptune– pIN 29 (107.4)	> 100
	mNeptune– pIN 29 (107.4)*	> 1000
P69	mNeptune– pIN 29 (108.6)	> 100
	mNeptune– pIN 29 (108.6)*	> 1000
	mNeptune– pIN 29 (107.4)	> 100
	mNeptune– pIN 29 (107.4)*	> 1000

*Cells were centrifuged after transformation

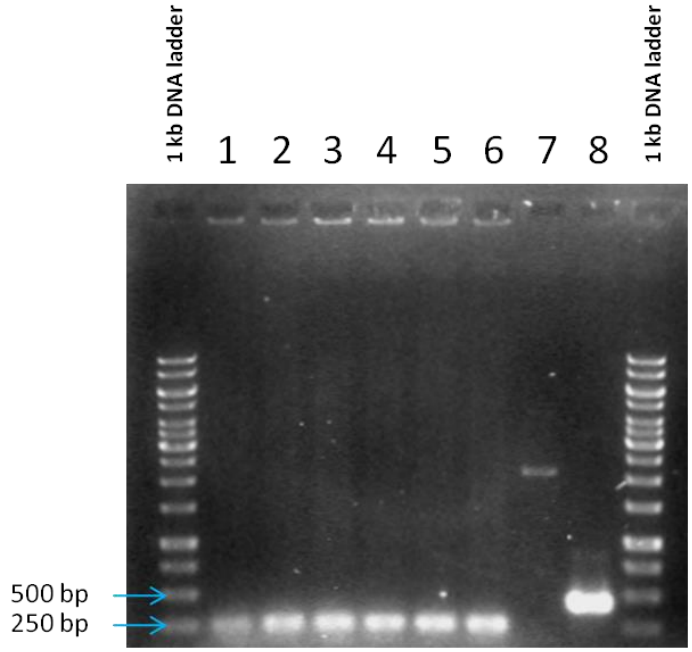


Figure 55: Analytical gel of colony PCR of mNeptune-pIN 29 (108.6 ng µl^{-1}) transformed ep17 cells using DsRed_RC primers. 1 – 6 = 5 µl of mNeptune-pIN29 (108.6 ng µl^{-1}) transformed ep17 cells, 7 = 5 µl of pEX-A-mNeptune_5bglll (373.4 ng µl^{-1}, 1:10), 10 = 5 µl of purified pIN 29 (124.8 ng µl^{-1}, 1:10).

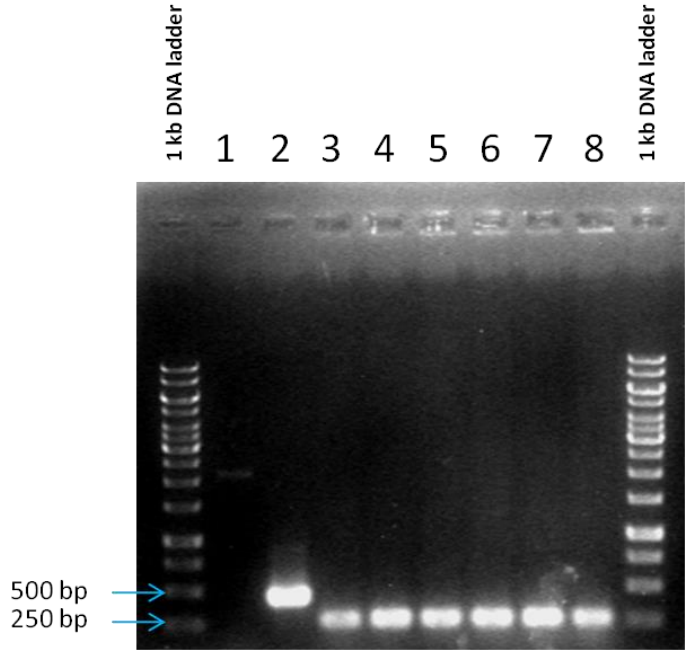


Figure 56: Analytical gel of mNeptune-pIN 29 (108.6 ng µl^{-1}) transformed P69 cells using DsRed_RC primers.1 = 5 µl of pEX-A-mNeptune_5bglll (373.4 ng µl^{-1}, 1:10), 2 = 5 µl of purified pIN 29 (124.8 ng µl^{-1}, 1:10). 3 – 8 = 5 µl of mNeptune-pIN 29 (108.6 ng µl^{-1}) transformed ep17 cells.

3.2.4.2 Transformation of B2g

Two different protocols were used to generate competent B2g cells. Transformed B2g cells could be obtained in both cases (meaning using the “Eppendorf” and “Stanford” protocol). The transformation was done with pIN 29 including the DsRed gene.

Material of the transformed B2g colonies was used for a colony PCR approach. The PCR products were analyzed in an analytical gel (see figure 57 and 64).

B2g cells transformed by using the “Eppendorf” protocol were showing the same amplifications (see figure 63, sample 1 - 4) as the purified pIN 29 vector which was used as positive control for DsRed specific amplification (see figure 63, sample 58).

The same results were also seen for the transforemd B2g cells by using the “Stanford” protocol (see figure 58, sample 1 and 2) and the purified pIN 29 vector (see figure 58, sample 6). Transformed B2g colonies which were grown out on selective LB plates (figure 58, sample 3) showing no amplification. No amplifications were obtained for the H_2O transformed B2g cells (figure 58, sample 4) and for the negative control (figure 58, sample 5).

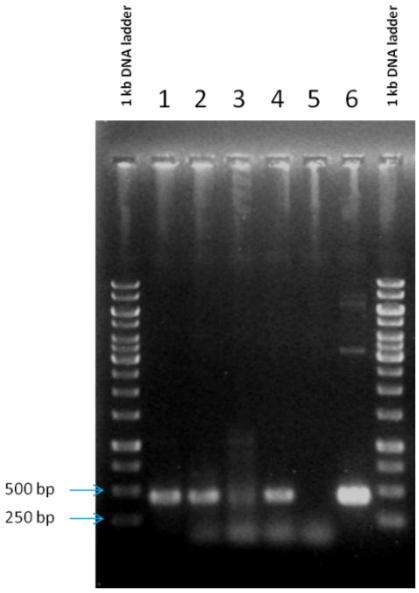


Figure 57: Analytical gel of transformed B2g cells (using the Protocol NO. 4308 915.504, Eppendorf) after PCR amplification with DsRed_RC primers. 1 = 5 µl of B2g - pIN 29 (5 µl pIN19 (141. 4 ng /µl) and 1 mm cuvettes for transformation), 1 = 5 µl of B2G - pIN 29 (1 µl (137. 5 ng /µl) and 2 mm cuvettes for transformation), 3 = 5 µl of of B2G - pIN 29 (1 µl pIN 29 (137. 5 ng /µl) and 2 mm cuvettes from outgrowing colonies of selective plates), 4 = 5 µl of of B2G - pIN 29 (1 µl pIN 29 (141. 1 ng /µl) and 1 mm cuvettes from outgrowing colonies of selective plates), 5 = 5 µl of negative control, 6 = purified pIN 29 (142.4 ng μl^{-1}).

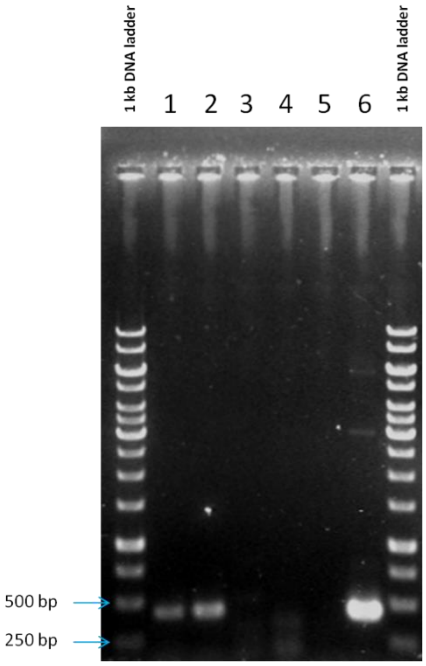


Figure 58: Analytical gel of transformed B2g cells ("Stanford protocol") after PCR amplification with DsRed_RC primers. 1 = 5 µl of B2g - pIN 29 (4 µl pIN 29 (142. 4 ng μl^{-1}) and 1 mm cuvettes after transformation), 1 = 5 µl of B2G - pIN 29 (2 µl pIN 29 (142. 4 ng μl^{-1}) and 1 mm cuvettes after transformation), 3 = 5 µl of of B2g - pIN 29 (4 µl pIN 29 (142. 4 ng μl^{-1}) and 1 mm cuvettes from outgrowing colonies of selective plates), 4 = 5 µl of of B2g - 5 µl H_2O and 1 mm cuvettes from outgrowing colonies of selective plates, 5 = 5 µl of negative control, 6 = purified pIN 29 (142.4 ng μl^{-1}).

3.3 Bacterial Transport vectors

3.3.1 Bacteria-Bacteria-Interactions

"Bacterial LB plates" of all six environmental strains were generated and the interactions between the LB inoculated and the plated strains were analyzed after several days of incubation at 30°C (see table 28-30). First inhibition effects between several strains could be observed already one day after incubation (table 28). Especially the B2g strain showed strong inhibitory effects. B2g seemed to have also inhibitory effects on itself (see table 30 and figure 59 B2g-B2g plate). Inhibitory effects were also detected for both *Serratia* strains (Re4 and Rp8). Both strains were showing the same inhibitory effects after one week of incubation (see table 30).

Table 28: Bacteria – Bacteria Interactions first day after incubation at 30°C

	Smear strain						
Strain in medium	**B2G**	**ep17**	**P69**	**Re4**	**Rp5**	**Rp8**	**H_2O**
B2G	0	0	0	+0	0	0	0
ep17	+0 10 mm	0	0	+0	0	+0	0
P69	+0 7 mm	0	0	0	0	0	0
Re4	+0 15 mm	0	0	0	0	0	0
Rp5	+0 15 mm	0	0	+0	0	+0	0
Rp8	+0 10 mm	0	0	0	0	0	0

0 = no inhibition, +0 = inhibition released by smear strain, ++ = inhibition of both strains, - = no growth of smear strain.

Table 29: Bacteria – Bacteria Interactions second day after incubation at 30°C

	Smear strain						
Strain in medium	**B2G**	**ep17**	**P69**	**Re4**	**Rp5**	**Rp8**	**H_2O**
B2G	+0 10 mm	0	0	+0	0	+0	0
ep17	+0 7 mm	0	0	0	0	0	0
P69	+0 15 mm	0	0	0	0	0	0
Re4	+0 15 mm	0	0	+0	0	+0	0
Rp5	+0 10 mm	0	0	0	0	0	0
Rp8	0	0	0	0	0	0	0

0 = no inhibition, +0 = inhibition released by smear strain, ++ = inhibition of both strains, - = no growth of smear strain.

Table 30: Bacteria – Bacteria Interactions one week after incubation at 30°C

	Smear strain						
Strain in medium	**B2G**	**ep17**	**P69**	**Re4**	**Rp5**	**Rp8**	**H_2O**
B2G	++	-	-	+0	-	+0	0
ep17	+0 10 mm	0	0	+0	0	+0	0
P69	+0 7 mm	0	0	0	0	0	0
Re4	+0 15 mm	0	0	0	0	0	0
Rp5	+0 15 mm	0	0	+0	0	+0	0
Rp8	+0 10 mm	0	0	0	0	0	0

0 = no inhibition, +0 = inhibition released by smear strain, ++ = inhibition of both strains, - = no growth of smear strain.

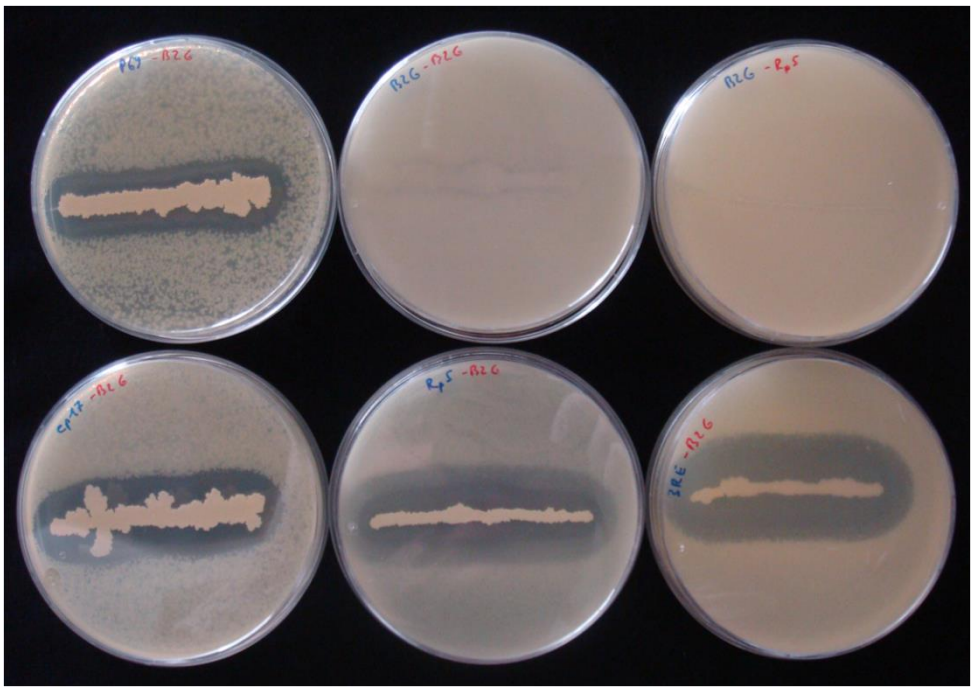


Figure 59: Inhibitory effects of smeared B2g strain on bacteria containing agar; red colored strain on bottom of petri dish indicates the smeared strain.

3.4 Bacteria-fungi interactions

3.4.1.1 Fungi from culture collection SCAM

29 fungi from cryo stocks were cultivated for 1 week on small PDA agar containing petri dishes.

In total, 13 of the 29 stock collection derived fungi were predicted as possible bacterial transportation vectors. The preselecting factors were on one site the fast growing behaviors and on the other the ability to build mycelia which were necessary for bacterial

transportation. The interaction between all 29 fungi and the six environmental strains were tested on normal size 1/5 PDA agar petri dishes. The results were listed in table 31.

Table 31: Interactions between 29 stock collections derived fungi and the 6 environmental strains after 1 week of incubation at RT

Fungi	Identification	Bacterial strain						Fungi as bacterial vector
		3Re4-18	ep-17	Rp8	Rp5	P69	B2g	
Trichoderma	BE1-1-3	++	-, i	++	++	-, i	-, i	suitable
	BR3-1-2	++	-, i	++	++	-, i	++	suitable
	BSE1-1-10	++	-, i	++	++	-, i	-	suitable
	RE2-1-15	++	-, i	++	++	-, i	++	suitable
	RR4-1-11	++	-, i	++	-, i	-, s	-, s	suitable
Penicillium	BR2-1-2	-, i brown	-, i brown	-, i brown	-	-, i brown	-, i	
	BE2-1-1	-,s	-,s	-, i	s, i brown	s, i brown	s, i brown	
	RB1-1-14	+	-, i yellow	+?	s	s	s	
	RE2-1-11	-	-, i brown	-?	-, i	s	+?	
	BSR2-3-4	s, i brown	s, i brown	s, i brown	s	s	s	
	BSE4-3-1	+	-, i brown	+	s	s	s	
Paecillomyces	BE4-1-9	+? s	-, i green	+?, s	-, s	s	s	
	BR4-2-6	+?, i yellow	-, i green	+?	-	-, i green	-?	
	RE2-2-4	+? s	-, i	+?, s	+	-, i	-	
	RR3-1-9	+	-, i yellow	-, i yellow	-	-	-	
Plectosporium	RE1-3-6	+	-, i	+	+?	+?	+?	suitable
Pink fluffy	BE3-1-10	-	-, i red	-	+	-, i	+	suitable
White flat	BR4-1-11	+	-, i	+	+	-, i yellow	-	suitable
Putative *Trichoderma*	BE2-1-12	+	-, i	+	+, i	-, i	+?	suitable
	BR1-1-5	+	-, i	+	-	-, i	-	suitable
Flat fluffy	BE4-3-6	+, i red	-, i red	+, i red	s	s	s	
	RE3-2-6	+, i red	-, i red	+, i red	+	-, i red	+	
Air mycellium	BE4-3-1	+	-?	+	-, i	+?, s	+?, s	
	RE2-1-9	s	-,s	- ,s	s	s	s	
Stinky	RE2-4-3	+?	-, i, s	+?	+?	-, i	-	suitable
	BR1-6-3	+	-, i	+	+	-	-, i	suitable
Till cap	RR1-5-2	+	-, i yellow	+	-	-	-	suitable
	BR1-6-8	+	-, i	+	s	s	-, i	suitable
Epicoccum	RR3-5-16	+	-, i red	-	+	-, i orange	+	suitable

+ to +++ = inhibition strength of fungi by bacterial strain, - = no inhibition of fungi, i = interaction between fungi and bacteria accompanied by changing color of bacteria or agar, s = no or slow growth of fungi, ? = inhibition of fungi or no growth.

3.4.1.2 *Fungi isolated from cropland (maize cultivation)*

Fungi isolated out of 3 different soil types were proofed for their bacteria transporting abilities. The amounts of isolated fungi for each soil type were listed in table 32.

Related to the results in table 32, 50 fungi from SAB and 28 from SNA medium were picked for further analysis. The growth rate of the fungi as well as their ability to produce mycelia were analyzed and summarized in 33. In total, 11 fungi were picked as possible bacterial transport vectors out of the 50 SAB isolated fungi and 9 out of the 28 SNA isolated fungi (see check marks in table 33).

Table 32: Amount of isolated fungi on Sabouraud medium from three different soil types and picked number for further investigations

Media	Soil	Dilution	Fungal colonies	Total picked	Labeling
SAB (V)	PS	10^{-2}	∞	6	1PS V 1 - 6
		10^{-3}	24		
		10^{-4}	3		
		10-5	-		
		10-2	∞		
		10-3	24		
		10-4	5		
		10-5	-		
	Rhizo	10-2	19	22	1Rh V 1-20
		10-3	1		
		10-4	-		
		10-5	-		
		10-2	12		
		10-3	-		
		10-4	-		
		10-5	-		
	DonB	10-2	13	22	1Oc V 1- 20
		10-3	3		
		10-4	-		
		10-5	-		
		10-2	15		
		10-3	4		
		10-4	-		
		10-5	-		
SNA (M)	PS	10-2	24	10	1PS M 1 - 6
		10-3	12		
		10-4	3		
		10-5	-		
		10-2	36		
		10-3	18		
		10-4	7		
		10-5	-		
	Rhizo	10-2	5	6	1Rh M 1-20
		10-3	-		
		10-4	-		
		10-5	-		
		10-2	2		
		10-3	-		

		10-4	-		
		10-5	-		
	DonB	10-2	10	12	1Oc M 1- 20
		10-3	2		
		10-4	-		
		10-5	-		
		10-2	11		
		10-3	4		
		10-4	-		
		10-5	-		

V = nutrients rich medium; M = nutrients low medium; soil definitions: PS = Profisubstrat, Rhizo = Rhizo star, DonB = DonBosco

Table 33: Growth rate and determination of potential bacterial carrying transporter behaviors of the 50 SAB and 28 SNA isolated fungi

Nr of exemplars	Fungi labbeling	Growthrate (after 7 days at RT)	Predicted for good bacteria-fungi interactions
1	**1 PS V-1**	f,wm	✓
2	**1 PS V-2**	f,wm	✓
3	**1 PS V-3**	f,wm	✓
4	**1 PS V-4**	f,wm	✓
5	**1 PS V-5**	f,wm	✓
6	**1 PS V-6**	s,wm	
1	**1 Rh V-1**	s	
2	**1 Rh V-2**	s,wom	
3	**1 Rh V-3**	s	
4	**1 Rh V-4**	s,wom	
5	**1 Rh V-5**	s	
6	**1 Rh V-6**	s	
7	**1 Rh V-7 1**	f,wm	✓
8	**1 Rh V-7 2**	s,wom	
9	**1 Rh V-8**	s,wom	
10	**1 Rh V-9**	s	
11	**1 Rh V-10**	s	
12	**1 Rh V-11**	s,pointed	
13	**1 Rh V-12 1**	f,wm	✓
14	**1 Rh V-12 2**	f,wm	✓
15	**1 Rh V-13**	s,wom	
16	**1 Rh V-14**	s	
17	**1 Rh V-15**	s	
18	**1 Rh V-16**	s,wom	
19	**1 Rh V-17**	s,wm	
20	**1 Rh V-18**	s,wom	
21	**1 Rh V-19**	s,wm	
22	**1 Rh V-20**	s,wom	
1	**1 DonB V-1**	s	
2	**1 DonB V-2 1**	s, pointed	
3	**1 DonB V-2 2**	s, pointed	
4	**1 DonB V-3**	s,wom	
5	**1 DonB V-4**	s,wm	
6	**1 DonB V-5**	s	
7	**1 DonB V-6**	f,wm	✓
8	**1 DonB V-7 1**	f,wm	✓
9	**1 DonB V-7 2**	f,wm	✓

10	**1 DonB V-8**	s	
11	**1 DonB V-9**	s,wm	
12	**1 DonB V-10**	s,wm	
13	**1 DonB V-11**	s	
14	**1 DonB V-12**	s	
15	**1 DonB V-13**	s,wm	
16	**1 DonB V-14**	s	
17	**1 DonB V-15**	s,wm	
18	**1 DonB V-16**	s	
19	**1 DonB V-17**	s,wm	
20	**1 DonB V-18**	s	
21	**1 DonB V-19**	s	
22	**1 DonB V-20**	s	
1	**1 PS M-1**	f, pointed	
2	**1 PS M-2**	f, wm	✓
3	**1 PS M-3**	f, wm	✓
4	**1 PS M-4**	s	
5	**1 PS M-5**	f,wm	✓
6	**1 PS M-6**	s,wm	
7	**1 PS M-7**	f,wm	✓
8	**1 PS M-8**	f,wm	✓
9	**1 PS M-9**	f,wm	✓
10	**1 PS M-10**	f,wm	✓
1	**1 Rh M-1**	s	
2	**1 Rh M-2**	s,wm	
3	**1 Rh M-3**	s	
4	**1 Rh M-4**	s,wm	
5	**1 Rh M-5**	s	
6	**1 Rh M-6**	s,wm	
1	**1 DonB M-1**	s	
2	**1 DonB M-2**	s	
3	**1 DonB M-3**	f,wm	✓
4	**1 DonB M-4**	s	
5	**1 DonB M-5**	s,wm	
6	**1 DonB M-6**	s	
7	**1 DonB M-7**	s	
8	**1 DonB M-8**	s	
9	**1 DonB M-9**	s,wm	
10	**1 DonB M-10**	f,wm	✓
11	**1 DonB M-11**	s	
12	**1 DonB M-12**	s	
Total 78			

V = SAB medium, M = SNA medium, f = Fast growth, s = slow growth, wm = fungi generates mycelium, wom = fungi without generating mycelium

3.4.1.3 Dual culture assay for determining fungi-bacteria interaction

The interactions between all 20 (11 SAB and 9 SNA) maize soil isolated fungi and the six environmental strains were tested on normal size 1/5 PDA agar petri dishes. The results were listed in table 34 and 35. Ep17 had no inhibiting effects on fungi growth. 36 (23 SNA and 13 SAB) fungi were selected as possible transport vectors.

Table 34: Interactions between SAB isolated fungi and the 6 environmental strains after 1 week of incubation at RT

Fungi	Bacterial strain						Fungi as bacterial vector
	Ep17	P69	B2G	Rp5	Rp8	3Re4	
1 PS V-1	-	-	-	-	-	-	suitable
1 PS V-2	-	-	-	-	-	-	Suitable
1 PS V-3	-	-	-	+	+	+	Suitable
1 PS V-4	-	-	+	+	+	+	Suitable
1 PS V-5	-	-	-	+	+	+	Suitable
1 PS V-6	-	-	+	+	+	+	suitable
1 Rh V-1	-	-,s	-,s	-,s	-,s	-,s	–
1 Rh V-2	-,s	-,s	-,s	-,s	-,s	-,s	–
1 Rh V-3	-,s	-,s	-,s	-,s	-,s	-,s	–
1 Rh V-4	-	-	++	+	+	+	–
1 Rh V-5	-,s	-,s	-,s	-,s	-,s	-,s	–
1 Rh V-6							–
1 Rh V-7 1	s	s	s	+	+	+	–
1 Rh V-7 2	s	s	s	s	s	s	–
1 Rh V-8	-	-	+	+	+	+	–
1 Rh V-9	-,s	-,s	-,s	-,s	-,s	-,s	–
1 Rh V-10	-	-	-,i brown	-,i brown	-,i brown	-	suitable
1 Rh V-11	-	-	++	++	-	+	suitable
1 Rh V-12 1	-	-	+++	+	+	+	suitable
1 Rh V-12 2	-	-	++	+	+	+	suitable
1 Rh V-13	-	-	++	+	+	++	suitable
1 Rh V-14	s	s	s	-	-	s	–
1 Rh V-15	s	s	+	-	s	-	–
1 Rh V-16	s	s	s	s	s	s	–
1 Rh V-17	-	-	+	s	s	-	suitable
1 Rh V-18	s	s	+	s	s	s	–
1 Rh V-19	-	-	+	s	s	-	suitable
1 Rh V-20	s	s	s	s	s	s	–
1 OC V-1	-	-	s	s	s	s	–
1 OC V-2 1	-	-	++	s,-	s,-	s,-	–
1 OC V-2 2	-	-	+++	+++	+	+	suitable
1 OC V-3	-	-	+	-	-	s	suitable
1 OC V-4	-	-	+	s	-	s	suitable
1 OC V-5	-	-	s	s	-	s	–
1 OC V-6	-	-	+++	++	++	++	suitable
1 OC V-7 1	-	-	+++	+++	+	+	
1 OC V-7 2	-	-	+++	+++	+	+	suitable
1 OC V-8	-	-	+++	s	s	+	suitable
1 OC V-9	-	+	+++	s	s	-	–

1 OC V-10	s	s	s, i black	s	s	s	–
1 OC V-11	s	s	s	s	s	s	–
1 OC V-12	-	+	+++	-	+	-	suitable
1 OC V-13	-	-	+	s	s	-	suitable
1 OC V-14	s	s	s	s	s	s	–
1 OC V-15	-	-	+++	s	-	-	suitable
1 OC V-16	s	s	s	s	s	s	–
1 OC V-17	-	-	+++	-	-	-	suitable
1 OC V-18	s	s	s	-	s	-	–
1 OC V-19	s ,-	s ,-	s ,-	s	s	-	–
1 OC V-20				s	s	s	–

+ to +++ = inhibition of fungi by bacterial strain, - = no inhibition of fungi, i = interaction between fungi and bacterial strain accompanied by changing color of bacteria or agar, s = no or slow growth of fungi, ? = inhibition of fungi or to slow growth

Table 35: Interactions between SNA isolated fungi and the 6 environmental strains after 1 week of incubation at RT

Fungi	Bacterial strain						Fungi as bacterial vector
	Ep17	P69	B2G	Rp5	Rp8	3Re4	
1 PS M-1	-	-	-	-	-	-	suitable
1 PS M-2	-	-	-	-	-	-	suitable
1 PS M-3	-	-	-	+	+	+	suitable
1 PS M-4	-	-	+	+	+	+	Suitable
1 PS M-5	-	-	-	+	+	+	Suitable
1 PS M-6	-	-	+	+	+	+	suitable
1 PS M-7	-	-,s	-,s	-,s	-,s	-,s	–
1 PS M-8	-,s	-,s	-,s	-,s	-,s	-,s	–
1 PS M-9	-,s	-,s	-,s	-,s	-,s	-,s	–
1 PS M-10	-	-	++	+	+	+	–
1 Rh M-1	-,s	-,s	-,s	-,s	-,s	-,s	–
1 Rh M-2							–
1 Rh M-3	s	s	s	+	+	+	–
1 Rh M-4	s	s	s	s	s	s	–
1 Rh M-5	-	-	+	+	+	+	–
1 Rh M-6	-,s	-,s	-,s	-,s	-,s	-,s	–
1 OC M-1	-	-	-,i brown	-,i brown	-,i brown	-	suitable
1 OC M-2	-	-	++	++	-	+	suitable
1 OC M-3	-	-	+++	+	+	+	suitable
1 OC M-4	-	-	++	+	+	+	suitable
1 OC M-5	-	-	++	+	+	++	suitable
1 OC M-6	s	s	s	-	-	s	–
1 OC M-7	s	s	+	-	s	-	–
1 OC M-8	s	s	s	s	s	s	–
1 OC M-9	-	-	+	s	s	-	suitable
1 OC M-10	s	s	+	s	s	s	–
1 OC M-11	-	-	+	s	s	-	suitable
1 OC M-12	s	s	s	s	s	s	–

+ to +++ = inhibition of fungi by bacterial strain, - = no inhibition of fungi, i = interaction between fungi and bacterial strain accompanied by changing color of bacteria or agar, s = no or slow growth of fungi, ? = inhibition of fungi or to slow growth

3.4.1.4 Fungi-Bacteria Transport assay

A specific fungi-bacteria transport assay was designed. The motility of each bacterial strain was tested on normal size PDA agar plates before applying them on the assay. No movement was detected. The used fungi and bacteria for the assay were listed in table 36 and 37.

Only B2g colonies were transported (stock derived fungi: BE1-1-3, BR4-1-11, BR1-1-5, BR1-6-3; soil derived fungi: 1 PS V-1, 1 PS V-2, 1 PS V-3, 1 PS V-5, 1 PS M-8, 1 PS M-9). BR4-1-11 transported also one ep17 colony. No fluorescence was detectable.

Table 36: List of used fungi and the bacteria ep17, P69, B2g for preparing the Fungi-Bacteria transport assays

Fungi Stock-collection	**Bacterial strain ep17, P69, B2g**
BE 1-1-3	
BSE 1-1-10	
BR 4-1-11	
BR 1-1-5	
RE 2-4-3	
BR 1-6-3	
RR 1-5-2	
Total 7	
Fungi-soil isolated	**Bacterial strain ep17, P69, B2g**
1 PS V-1	
1 PS V-2	
1 PS V-3	
1 PS V-5	
1 Rh V-10	
1 PS M-8	
1 PS M-9	
Total 7	

Table 37: List of used fungi and the bacteria Rp5, Rp8 and 3Re4 for preparing the Fungi-Bacteria transport assays

Fungi Stock-collection	**Bacterial strain Rp5, Rp8, 3Re4-18**
none	
Total 7	
Fungi-soil isolated	**Bacterial strain ep17, P69, B2g**
1 PS V-1	
1 PS V-2	
1 DonB V-17	
1 Rh M-2	
Total 4	

3.4.2 B2g + BR 4-1-11 and BR 1-1-5 – Interactions with maize plants

Related on the results of the fungi-bacteria transport assays three different fungi (BR 4-1-11, BR 1-1-5 and BE 1-1-3) were identified as B2g transporting vectors. The simultaneous application of fungi and B2g were tested on maize plants. The application of B2g and fungi together was compared with the single effects of fungi or bacteria when applying them alone. The roots and leaves weights of the outgrowing maize plants were determined (see table 38).

Untreated maize as well as the one treated with B2g or in combination with fungi was showing a higher yield of root and leave mass compared to the application of fungi alone (see table 38). The root material of each approach was mixed with 5 ml of 0.85 % NaCl. Several dilutions were prepared out of this stock solution and plate on LB plates. The number of outgrowing colonies were counted and listed in table 39.

The combination of B2g with fungi led to a moderate number of outgrowing colonies on maize roots in direct comparison to the B2g application alone. Maize treated with BR 1-1-5 only were showing no outgrow of colonies. In case of BR 4-1-11 treated maize several colonies were growing after incubation.

The CFU of B2g was calculated for selected dilutions and listed in table 45.

The highest CFU amount was shown by maize plats treated only with B2g. A lower CFU was calculated for maize treated simultaneous with B2g in mixture with a fungi. Maize treated with BR4-1-11 shown also a moderate CFU amount. The negative control as well as the BR1-1-5 treated maize were showing no CFU.

Table 38: Weight of roots and leaves of maize treated with different B2g and fungi combinations

Approach	Nr. germinated seeds	weight root (g)	weight leaves (g)
B2g + BR1-1-5			
1	3	0.69286	0.74426
2	4	1.27678	0.08001
3	4	1.55406	0.99102
B2g + BR4-1-11			
1	3	1.31749	0.77380
2	4	1.35393	1.15807
3	3	1.36606	1.12119
Maize only			
1	4	1.30574	0.5908
2	4	1.11966	0.79248
3	4	1.71744	0.63641
maize + B2g			
1	4	2.19124	1.13062
2	4	1.84515	1.12215
3	3	0.83814	0.58459
maize + BR1-1-5			

1	3	0.86742	0.23946
2	2	0.53105	0.33498
3	3	0.52598	0.85438
maize + BR4-1-11			
1	1	0.06100	0.23946
2	3	0.94865	0.33498
3	4	1.34828	0.62425

Table 39: Counted B2G colonies for each maize root derived dilution approach

Approach	Dilution factor											
B2g+BR1-1-5	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	11	10	2	1	0	0	0	0	0	0	0	0
2	122	81	18	16	1	1	1	0	0	0	0	0
3	17	8	1	0	0	0	0	0	0	0	0	0
B2g+BR4-1-11	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	148	96	12	19	3	1	0	0	0	0	0	0
2	10	15	1	0	1	0	0	0	0	0	0	0
3	13	21	2	4	0	0	0	0	0	0	0	0
Maize only (control)	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
maize + B2g	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	∞	∞	81	69	13	6	3	0	0	0	0	0
2	∞	∞	63	75	8	10	0	0	0	0	0	0
3	∞	∞	99	87	13	10	1	1	0	0	0	0
maize + BR1-1-5	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
maize + BR4-1-11	0	0	-1	-1	-2	-2	-3	-3	-4	-4	-5	-5
1	59	24	7	6	3	0	1	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0

∞ = over 1000 bacterial colonies

Table 40: B2g CFU/g root material of maize for selected dilution series

Approach	Dilution	CFU/g	Mean
B2g+BR1-1-5	**0**		
1	-	$7.577*10^3$	$1.712*10^4$
2	-	$3.975*10^4$	
3	-	$4.022*10^3$	
B2G+BR 4-1-11	**0**		
1	-	$4.630*10^4$	$3.77*10^4$
2	-	$4.616*10^3$	
3	-	$6.227*10^3$	
Maize only	**0**		
1	-	0	
2	-	0	
3	-	0	
maize + B2G	**10^{-1}**		
1	-	$1.711*10^5$	$3.03*10^5$
2	-	$1.870*10^5$	
3	-	$5.548*10^5$	
maize + BR 1-1-5	**0**		
1	-	0	
2	-	0	
3	-	0	
maize + BR 4-1-11	**0**		
1	-	$2.392*10^4$	$2.392*10^4$
2	-	0	
3	-	0	

3.4.3 B2G – BR fungi Live/Dead staining approach

The interactions between BR4-1-11 and B2g as well as BR1-1-5 and B2g were tested with a Live/Dead staining approach. The approaches were analyzed for GFP fluorescence by using the Motic epifluorescence microscope (figure 60 - 63).

The red arrows in figure 60 A were indicating cluster of B2g on BR 1-1-5 hyphens under bright field conditions. GFP analysis of the same picture was showing a green fluorescence of the bacteria (see figure 60 B, red arrows).

The unlabeled BR1-1-5 – B2g interaction approach were showing no emission of the B2g colonies (figure 61 A and B, red arrows) during excitation with GFP wavelength. A low background emission of some fungi structures was detectable (see figure 61 B, weak green emitting structures).

The red arrows in figure 62 A were indicating cluster of B2g on BR 4-1-11 hyphens under bright field conditions. GFP analysis of the same picture was showing a green fluorescence of the bacteria (see figure 62 B, red arrows).

The unlabeled BR 4-1-11 – B2g interaction approach were showing no emission of the B2g colonies (figure 63 A and B, red arrows) during excitation with GFP wavelength. A low background emission of some fungi structures was detectable (see figure 63 B, weak green emitting structures).

In general fungi structures of treated approaches were emitting stronger (60 and 62 B, low green fluorescent light) in comparison to the untreated assays (61 and 63 B) when applying GFP extinction.

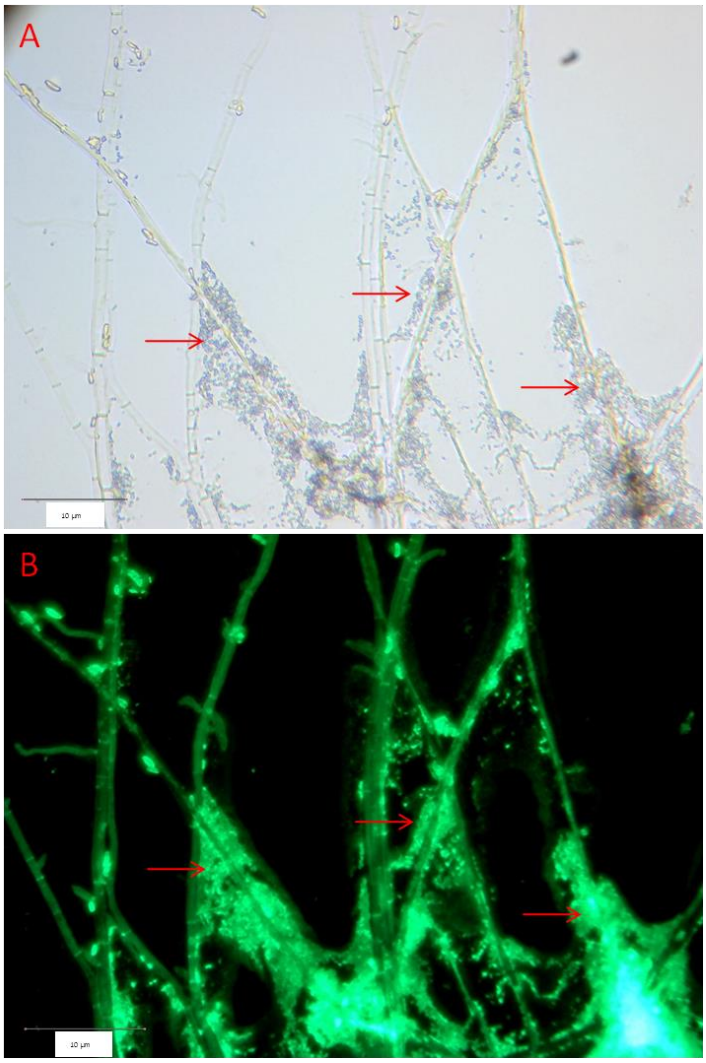


Figure 60: Bright field and GFP analysis of BR 1-1-5 - B2G interactions using the staining Live/dead staining kit. A = Interactions under bright field conditions with a zoom of 200 x, B = Interactions under GFP extinction/emission conditions with a zoom of 200 x.

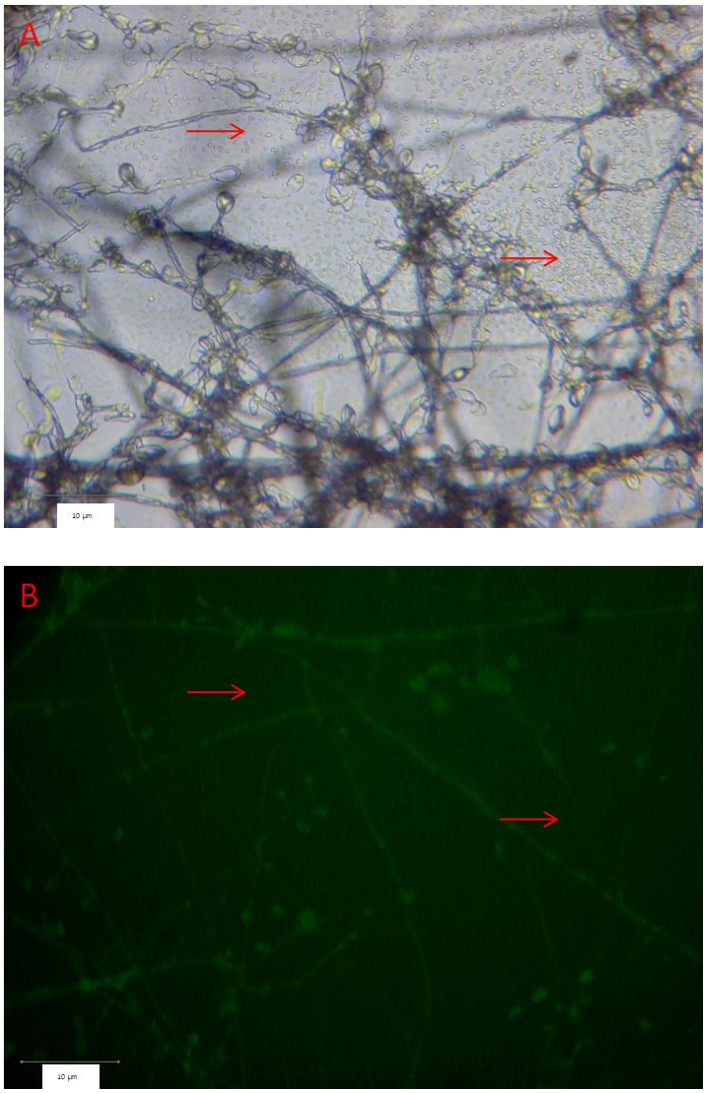
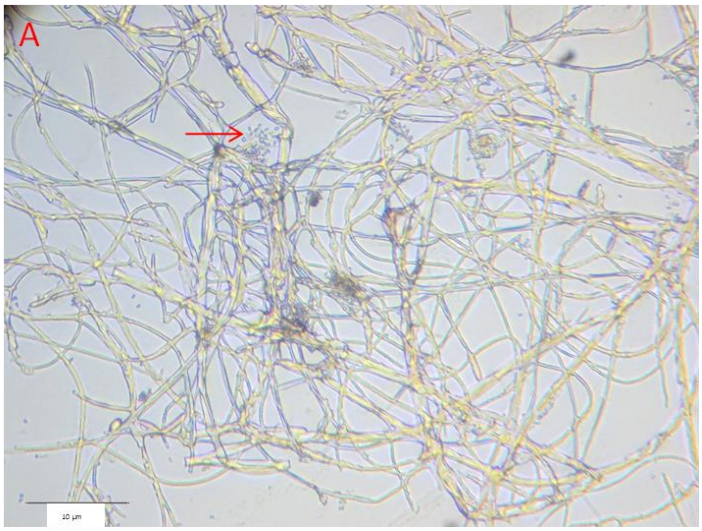


Figure 61: Bright field and GFP analysis of BR 1-1-5 - B2G interactions without staining. A = Interactions under brightfield conditions with a zoom of 200 x, B = Interactions under GFP extinction/emission conditions with an zoom of 200 x.



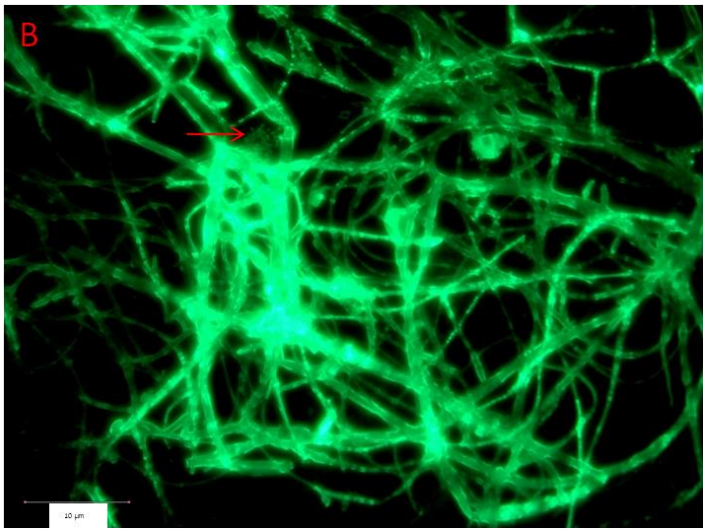
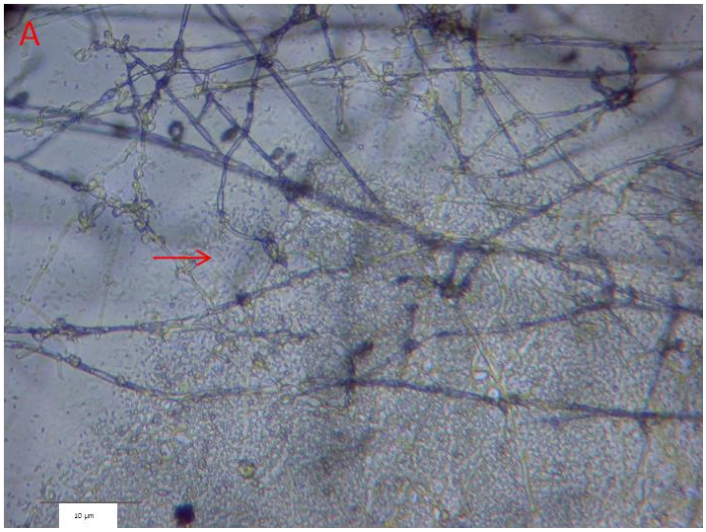


Figure 62: Bright field and GFP analysis of BR4-1-11 - B2g interactions using the Live/dead staining kit. A = Interactions under bright field conditions with a zoom of 200 x, B = Interactions under GFP extinction/emission conditions with a zoom of 200 x.



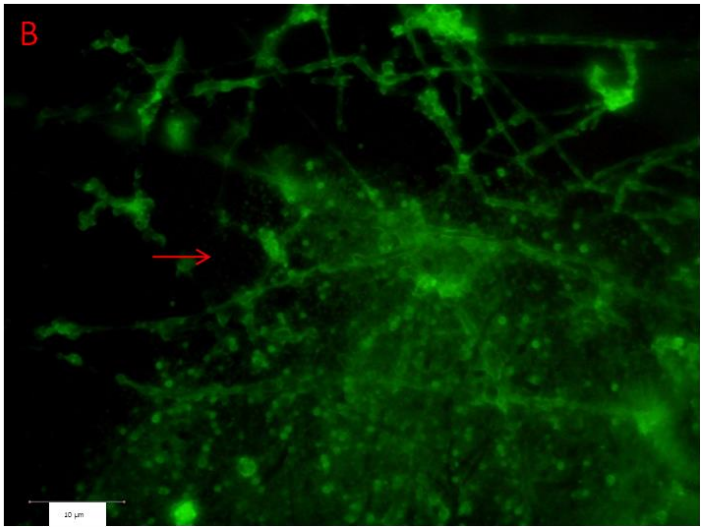


Figure 63: Brightfield and GFP analysis of BR 4-1-11 - B2G interactions without staining. A = Interactions under brightfield conditions with a zoom of 200 x, B = Interactions under GFP extinction/emission conditions with a zoom of 200 x.

3.4.4 B2g – BR fungi spores identification

A further approach was designed to check if the transported B2g colonies were spores.

Both “heated” approaches (figure 64 and 65 B) were showing no outgrowing colonies after incubation on LB plates at 30°C. Otherwise the untreated approaches were showing several outgrowing colonies (figure 64 and 65 A). Therefore, it could be said that the B2g colonies were transported alive.

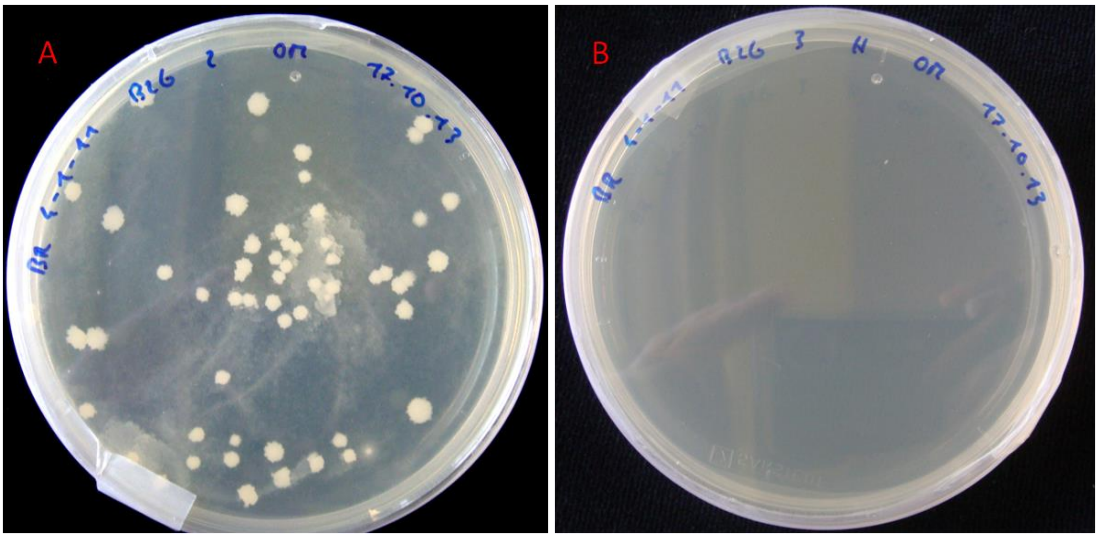


Figure 64: Identification of B2g transportation mode. A = Untreated approach, B = Heat treated approach.

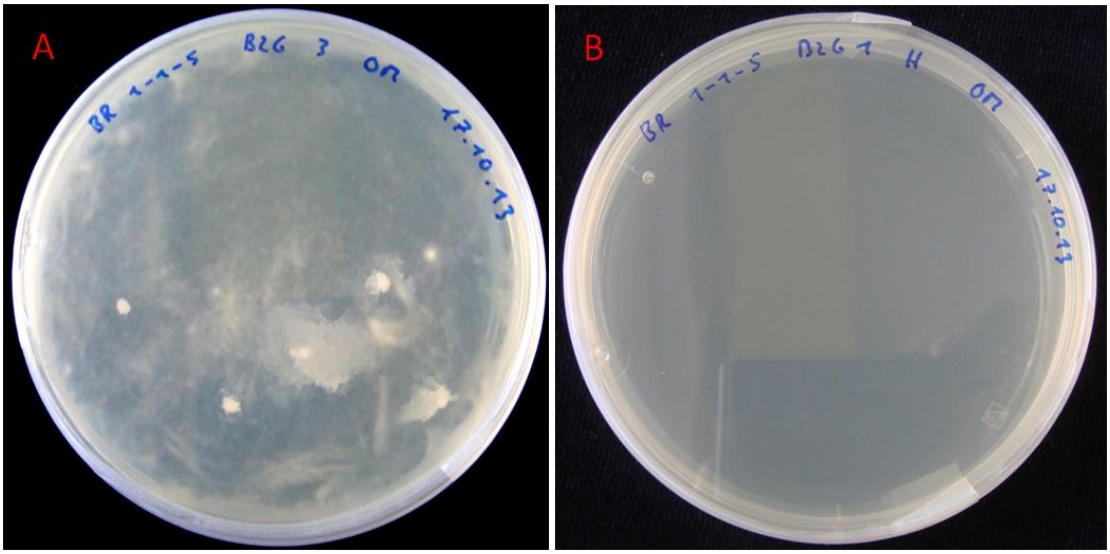


Figure 65: Identification of B2g transportation mode. A = Untreated approach, B = Heat treated approach.

3.4.4.1 BOX PCR of transported B2g colonies

A BOX PCR was done to proof the identity of the transported B2g colonies by using the fungi-bacteria transport assay. The PCR products were analyzed in a gel electrophoresis approach (see figure 66).

The analytical gel in figure 66 showed that the negative control of the BOX PCR (sample 14) had a contamination. Apart from this it could be said that BR4-1-11, BR 1-1-5 and BE 1-1-3 transported B2g (figure 66, samples 2,6) were showing identical bands in comparison to the positive control (samples 1 and 13) which represented the original B2g strain.

A repetition of the BOX PCR was done using the same settings as for the first (see figure 67).

The same results were obtained. The negative control showed again the same contaminations (see figure 67, sample 14).

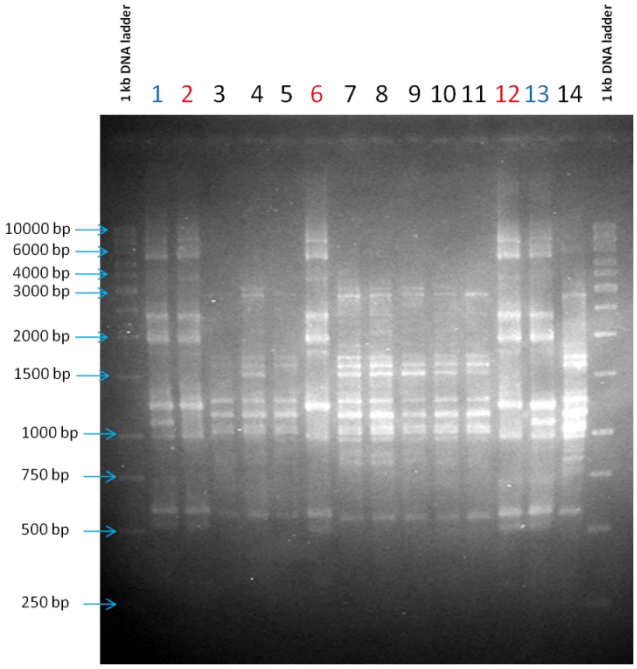


Figure 66: Analytical gel of B2g BOX PCR products. 1 – 14 = 10 µl each chamber, 1 and 13 = DNA material of B2g (cryo stock) as positive control, 2 = DNA material of BR 4-1-11 transported B2g, 3 = DNA material of 1 PS M-8 transported B2g, 4 = DNA material of 1 PS V-2 transported B2g, 5 = DNA material of BR 1-6-3 transported B2g, 6 = DNA material of BR 1-1-5 transported B2g, 7 = DNA material of 1 PS V-2 transported B2g, 8 = DNA material of 1 PS V-1 transported B2g, 9 = DNA material of 1 PS V-5 transported B2g, 10 = DNA material of RE 2-4-3 transported B2g, 11 = DNA material of 1 PS M-9 transported B2g, 12 = DNA material of BE 1-1-3 transported B2g, 14 = negative control.

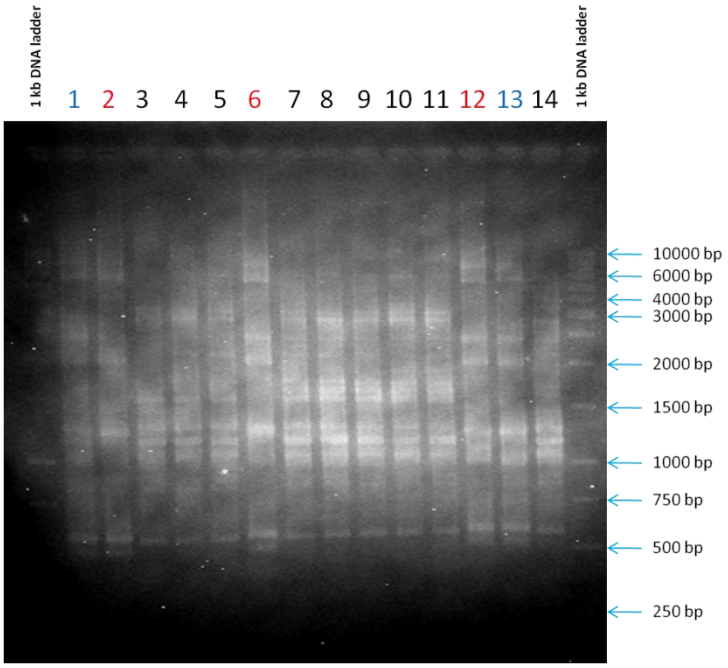


Figure 67: Analytical gel of B2g BOX PCR products. 1 – 14 = 10 µl each chamber, 1 and 13 = DNA material of original B2g (cryo stock) as positive control, 2 = DNA material of BR 4-1-11 transported B2g, 3 = DNA material of 1 PS M-8 transported B2g, 4 = DNA material of 1 PS V-2 transported B2g, 5 = DNA material of BR1-6-3 transported B2g, 6 = DNA material of BR 1-1-5 transported B2g, 7 = DNA material of 1 PS V-2 transported B2g, 8 = DNA material of 1 PS V-1 transported B2g, 9 = DNA material of 1 PS V-5 transported B2g, 10 = DNA material of RE 2-4-3 transported B2g, 11 = DNA material of 1 PS M-9 transported B2g, 12 = DNA material of BE 1-1-3 transported B2g, 14 = negative control.

3.4.5 *Serratia* sp.– *R. solani* – Interactions

The interactions between *R.solani* and the pIN 29 DsRed transformed *Serratia* strains Rp5, Rp8 and 3Re4 were tested in specialized assays. The results were evaluated by CLSM. The interactions between *R.solani* and *Serratia* strains were analyzed in two (“fluid” = figure 68 and ”dry” = figure 69) assays.

In the “fluid” assay (figure 68) the *3Re4* colonies (red dots) were distributed over the whole approach (blue structure = fungus hyphen). In comparison to the “fluid” approach the *Serratia* colonies of the dry approach (figure 69, red dots) were closely attached to the hyphens of the fungi (figure 69, blue structures).

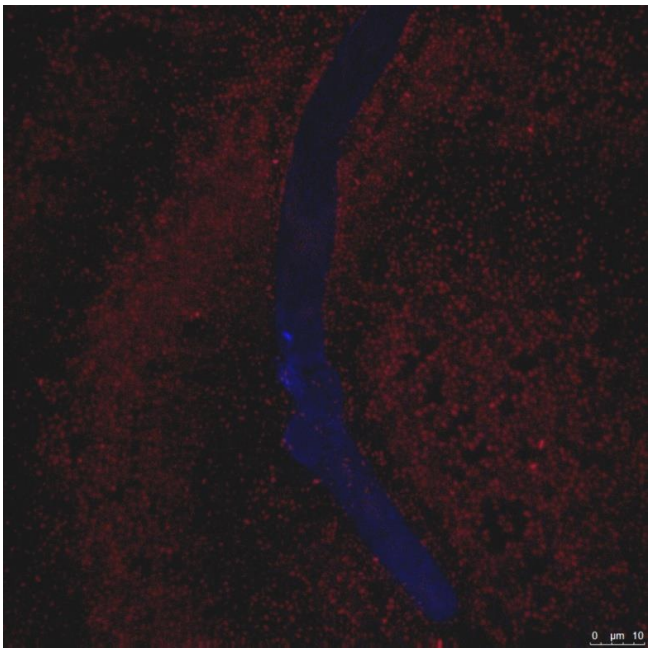


Figure 68: CLSM picture of interactions between *R. solani* and pIN 29 transformed 3Re4 ("fluid" approach).

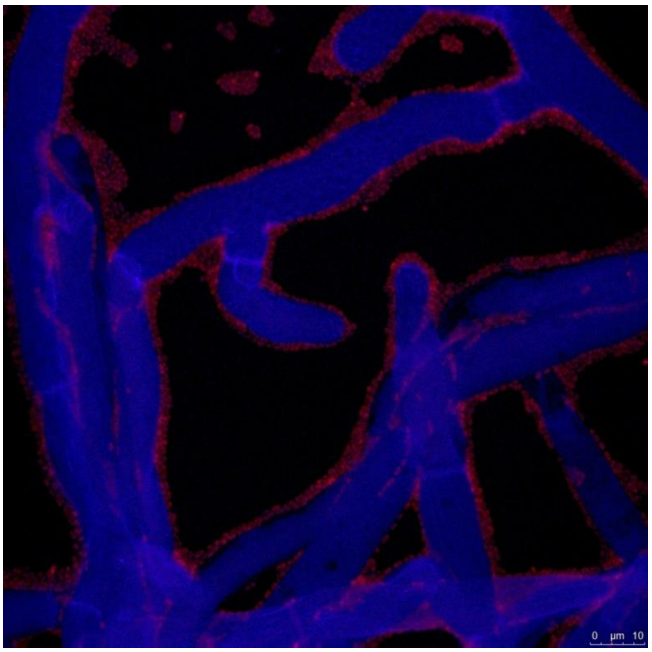


Figure 69: CLSM picture of interactions between *R. solani* and pIN 29 transformed 3Re4 ("dry" approach).

3.5 Bacteria – Plant Interactions

3.5.1 Interactions of *Stenotrophomonas rhizophila* P69, e-p7 with maize and tomato roots

The interactions between ep17- and P69- pIN 29 DsRed transformed cells on tomato and maize seeds were tested in mega pouches approaches. The interactions were visualized by CLSM. Additionally the cell number of bacteria/g of root material of each outgrowing plant was determined.

The root material of tomato and maize plants (see table 40) were mixed with a defined ml amount of 0.85 % NaCl. The suspension was diluted until a factor of 10^{-5}. Each dilution was plate out on selective LB plates. Out of the obtained results the CFU of ep17 and P69 were calculated (see table 41). Ep17 had a low CFU/g on maize and tomato root material in comparison to P69.

The experiment was redone using the same parameters as the first.

In comparison to the first experiment no contamination of the approaches occurred. Each root material was mixed with an adequate amount of 0.85 % NaCl. Several dilutions were done out of this stock solution. The outgrowing colony number of each dilution was detected and listed in table 42.

Out of these results the CFU of ep17 and P69 were calculated (see table 43).

The calculated CFU of P69 of maize and tomato roots seemed to be lower in comparison to experiment 1 (see table 41). Otherwise the CFU of ep17 seemed to be highly increased comparing both approaches of the first and second experiment (table 43).

Related to both experiments the interactions of bacteria on tomato and maize roots were shown in figure 70 to 75.

P69 showed a lower distribution on the tomato roots (figure 70) in comparison to the ep17 strain (figure 71). The colonization of tomato roots with ep17 seemed to be denser compared to P69. These results were correlating to the calculated CFU amounts of bacteria for tomato roots.

The colonization of P69 on maize roots was also not that high (figure 72) in comparison to ep17 (figure 73). The calculated CFU for ep17 and P69 were correlating to these pictures analysis.

The interactions of the first experiment were also analyzed (figure 74 and 75).

No ep17 were detectable neither on maize nor tomato roots (figure 74 and 75). The results corresponded to the calculated CFU's of the first experiment in (see table 41).

Table 41: Number of germinated seeds and weight of tomato and maize root material of each mega pouch approach after several days of incubation

Approach/Plant	Nr. germinated seeds	Weight/Plant (g)
P69 tomato		
1	5	0.0437
2	4	0.03208
3	5	0.01852
4	5	0.00272
ep17 tomato		
1	5	0.01979
2	4	0.04003
3	5	0.06093
4	4	0.06889
P69 maize		
1	4	0.51878
2	4	1.84993
3	4	1.32265
4	5	1.71997
ep17 maize		
1	4	3.92975
2	4	1.85198
3	4	2.21326
4	4	2.3345
Maize control		
1+2	total 8	0.21172
Tomato control		
1+2	total 8	0.04105

Table 42: CFU (ep17 and P69)/g of root material for selected dilution series

Approach/Plant	Dilution	CFU/g	Mean
Tomato P69	**10^{-3}**		
1	-	$5.034*10^{7}$	$3.42*10^{8}$
2	-	$6.045*10^{8}$	
3	-	$3.887*10^{8}$	
4	-	$3.308*10^{8}$	
Tomato ep17	**10^{-0}**		
1	-	$1.970*10^{5}$	$6.50*10^{4}$
2	-	$6.245*10^{4}$	
3	-	0	
4	-	0	

Maize P69	10^{-3}		
1	-	$1.445*10^{8}$	$8.531*10^{7}$
2	-	$1.027*10^{8}$	
3	-	$1.701*10^{7}$	
4	-	$7.703*10^{7}$	
Maize ep17	**no**		
1	0	$2.671*10^{3}$	$1.74*10^{3}$
2	0	$8.099*10^{2}$	
3	10^{-3}	$1.694*10^{6}$	$8.47*10^{5}$
4	10^{-3}	$1.392*10^{3}$	

Table 43: Number of germinated seeds and weight of tomato and maize root material of each mega pouche approach

Approach/Plant	Nr. germinated seeds	Weight/Plant (g)
P69 tomato		
1	3	0.01147
2	3	0.01785
3	3	0.01789
4	4	0.01904
ep-17 tomato		
1	5	0.01742
2	4	0.01355
3	4	0.01147
4	4	0.02890
P69 maize		
1	4	1.8045
2	4	1.52480
3	4	1.80985
4	4	1.55521
ep17 maize		
1	4	1.44209
2	4	1.78314
3	4	1.47607
4	4	2.08563
Maize control		
1+2	total 8	3.39995
Tomato control		
1+2	total 8	0.03987

Table 44: CFU of ep17 and P69/g of root material for selected dilution series

Approach/Plant	Dilution	CFU/g	Mean
Tomato P69	**10^{-2}**		
1	-	$4.708*10^{7}$	$4.06*10^{7}$
2	-	$1.989*10^{7}$	
3	-	$5.366*10^{7}$	
4	-	$4.175*10^{7}$	
Tomato ep17	**10^{-3}**		
1	-	$2.525*10^{8}$	$4.54*10^{8}$
2	-	$2.730*10^{8}$	
3	-	$3.400*10^{8}$	
4	-	$9.515*10^{8}$	
Maize P69	**10^{-2}**		
1	-	$3.325*10^{4}$	$3.03*10^{5}$
2	-	$6.230*10^{5}$	
3	-	$3.232*10^{5}$	
4	-	$2.315*10^{5}$	
Maize ep17	**10^{-4}**		
1	-	$1.872*10^{7}$	$1.20*10^{7}$
2	-	$1.234*10^{7}$	
3	-	$6.775*10^{6}$	
4	-	$1.031*10^{7}$	

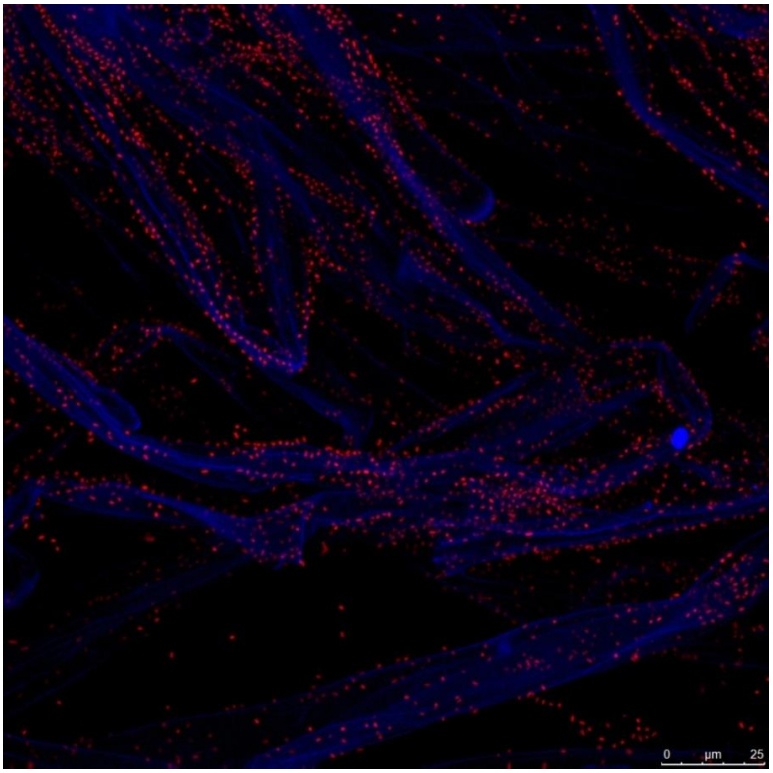


Figure 70: CLSM analysis of P69 colonies on tomato roots

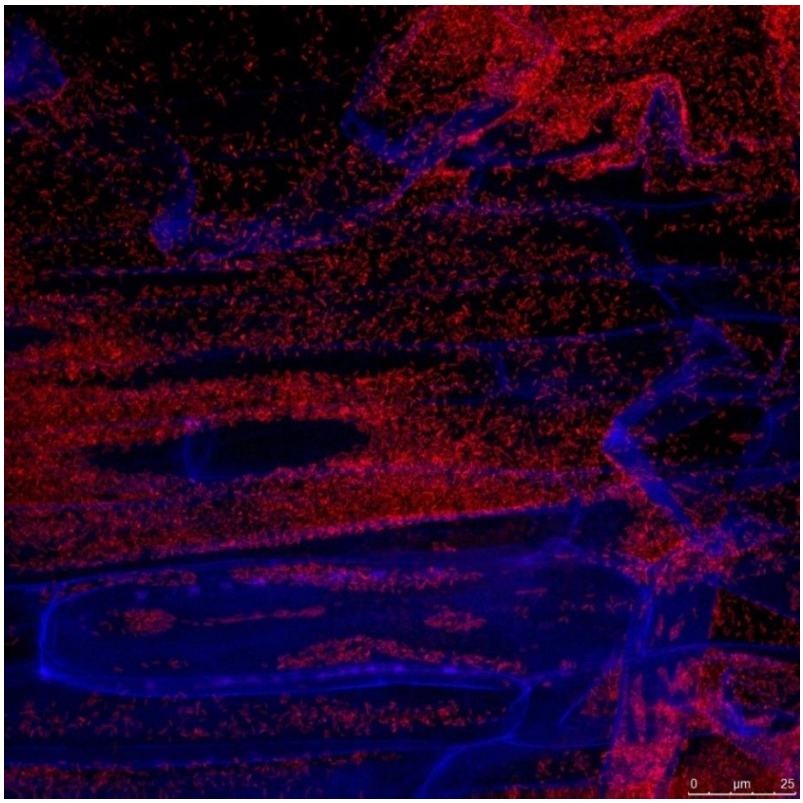


Figure 71: CLSM analysis of ep17 colonies on tomato roots

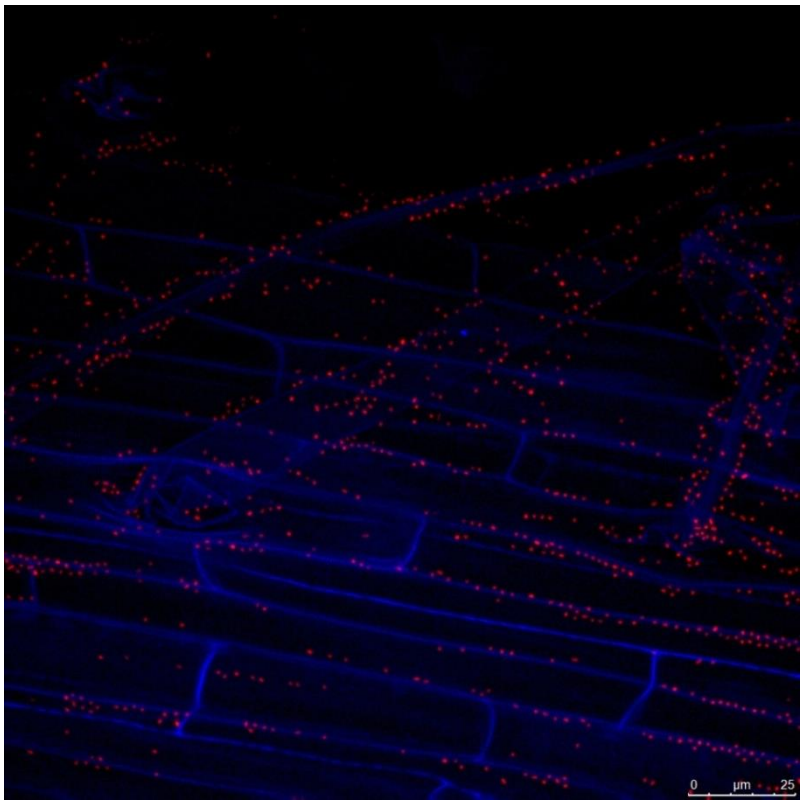


Figure 72: CLSM analysis of P69 colonies on maize roots

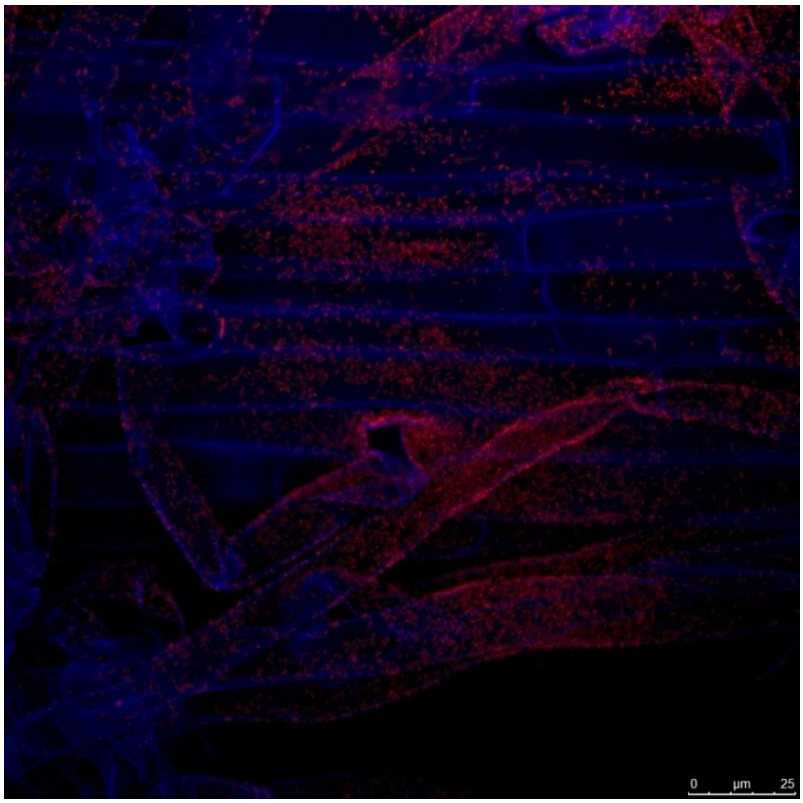


Figure 73: CLSM analysis of ep17 colonies on maize roots

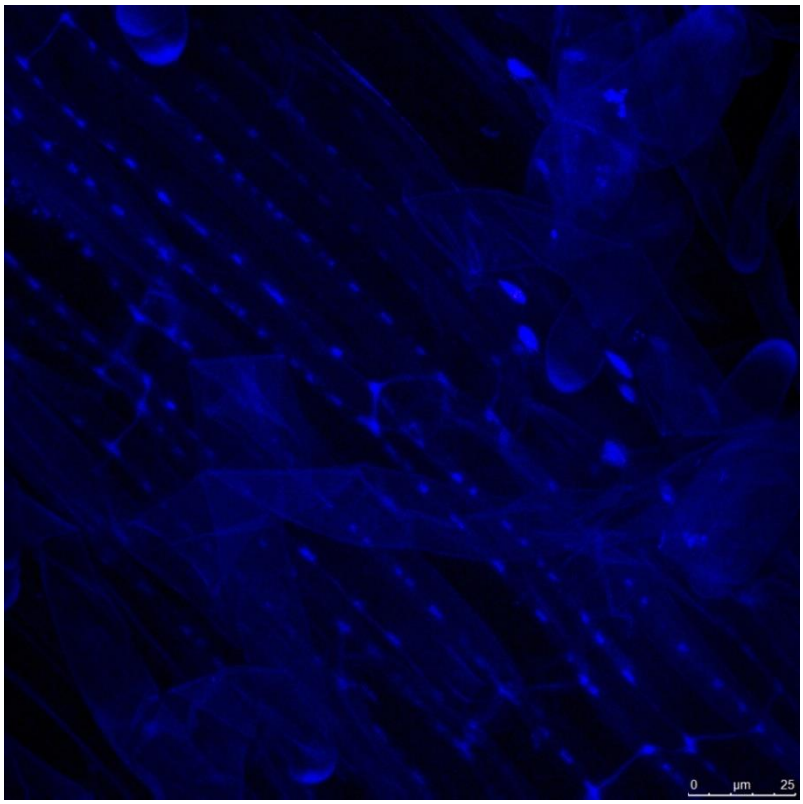


Figure 74: CLSM analysis of ep17 colonies on tomato roots (first experiment)

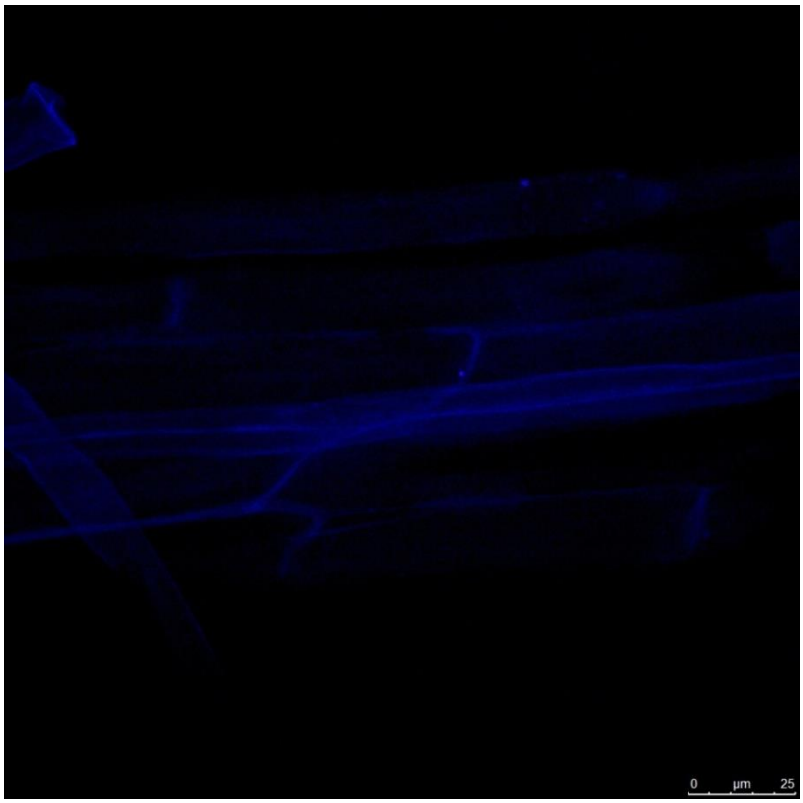


Figure 75: CLSM analysis of ep17 colonies on maize roots (first experiment)

3.5.2 Visualization of ep17 (pIN29 DsRed) and P69 (pBAH8) on maize roots

The distribution amount of ep17 and P69 colonies on maize roots were almost the same (figure 76). No clusters were built either by ep17 (green dots) or P69 (red dots) when applying them together.

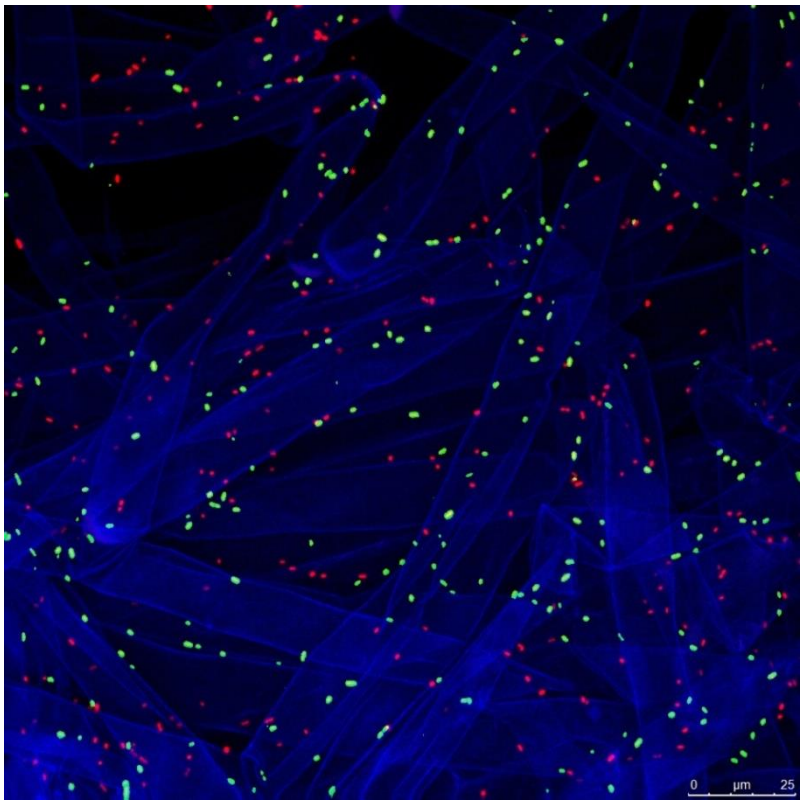


Figure 76: CLSM analysis of maize roots interactions with ep17 (pBAH8) and P69 (pIN29 DsRed) colonies.

3.5.3 Mixture of four different fluorescenting ep17 strains on maize roots

Differentiations of single ep17 transformants were possible by using CLSM (figure 77). The single bacterial structures as well as the distribution seemed to differentiate from each other although the same strain was used (ep17). The red (DsRed) and yellow (mNeptune) fluorescent bacteria were more abundant in comparison to the green (GFP) and blue (eBFP2) colonies. The green and the blue fluorescent bacteria seemed to have a filamentous structure in comparison to the dot-shaped red and yellow bacteria.

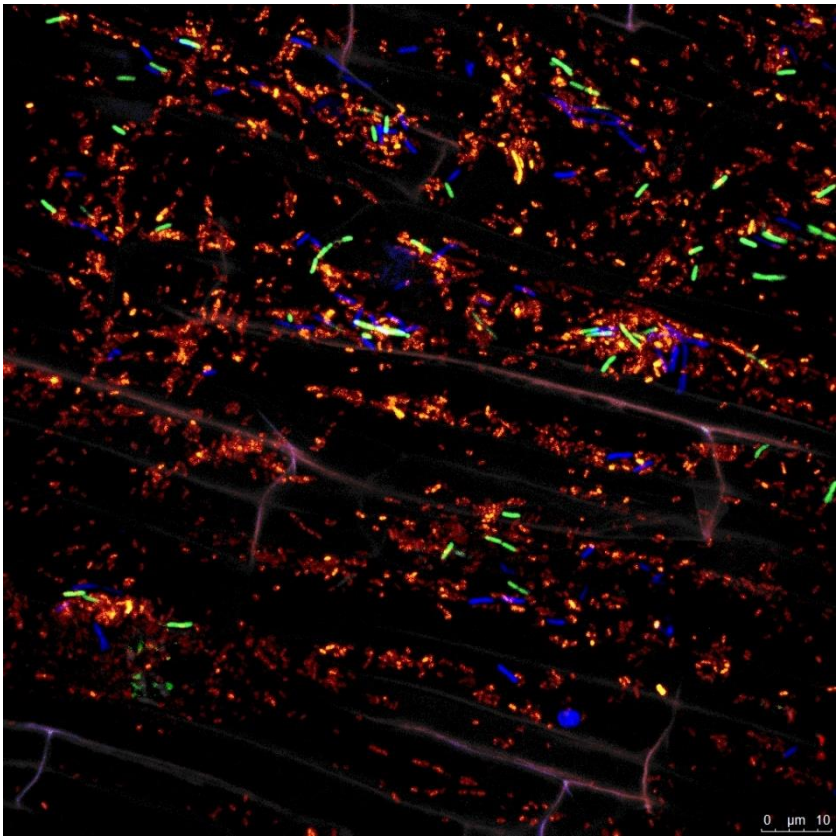


Figure 77: CLSM analysis of 4 different ep17 transformants on maize roots.

4 Discussion

4.1 Plasmid Preparation and fluorescent gene implementation

Confocal laser scanning microscopy (CLSM) represents a valuable tool to monitor microorganisms *ad planta* to study their fade and their interactions with the plant host. One way for targeted monitoring of particular strains is the labelling with fluorochromes. In total, four different fluorescent proteins (GFP, DsRed, eBFP2 and mNeptune) were used in this study. The Leica CLSM which was applied for their detection had four fixed laser sources (= extinction settings). Through this reason the extinction properties of the fluorophores were predefined. Furthermore it was necessary to find fluorescent dyes without overlaying emission spectra. The simultaneous detection of all four dyes was possible based on these two criteria.

The genes for DsRed2 and GFP were already available in different rhizosphere stable vectors (pME6031-DsRed2 and pIN 29 for DsRed; pSM1880 and pBAH8 for GFP). In comparison to that the sequences of mNeptune and eBFP2 had to be determined and synthesized. This was done by using the encoding sequences for both genes available at the NCBI nucleotide database. The genes were synthesized by the Eurofins MWG and delivered in pEX vectors.

A rhizosphere stable plasmid was required as cloning vector for the selected genes. Three different plasmids (pME6031-DsRed2, pBAH8 and pIN 29) served as base material for the cloning approaches. All of the three plasmids were carrying a fluorescent gene. PME6031-DSRED2 and pIN 29 had an implemented DsRed2 gene. The pBAH8 vector was stocked with a GFP gene. These plasmids were already used in earlier studies for studying interactions between bacteria and bacteria and plants (Zachow et al., 2010; Huber et al., 2002; Vergunst et al., 2010).

The first aim was to find a vector and a cloning strategy for the implementation of all four fluorescent genes. The existing genes of the plasmids should be replaced by one of the selected fluorescent genes. Preliminary cloning experiments were performed with the GFP amplicon using pSM1880 as template that carries the pUTTc $P_{A1\text{-}04/03}$ ::gfpmut3-cassette. First cloning experiments were done with the pME6031-DsRed2 plasmid which was isolated out of *Pseudomonas fluorescence* L13-6-12. The vector harbors a DsRed2 sequence and therefore encodes a red fluorescent dye (Zachow et al., 2010). Different cloning methods were tried to clone the amplified GFP sequence out of pSM1880 into the pME6031-DsRed2 vector. Neither the double digest nor the blunting method was successful. By applying the single digest method it was possible to clone the blunted GFP gene into the blunted and dephosphorylated plasmid resulting in a “hybrid vector”, containing both DsRed2 and GFP. Ongoing works were done by using pIN 29 and pBAH8 as base material. The pBAH8 plasmid is a pBBR1MCS-5 derived vector containing a PA1/04/03-gfp mut3-To-T1 cassette and a gentamycin resistance gene (Kovach et al., 1995). This vector was originally built to analyze

the biofilm formation of *Burkholderia cepacia* H111 wildtype and for some specific mutants (Huber et al., 2002). The vector was isolated out of *B. terricola* ZR2-12 in this study. The restriction sites flanking the GFP gene were determined by literature research (Andersen et al., 1998) and by partial sequencing of the vector using the sequencing primers DsRed1_C or DsRed1_N. The restriction of the vector with *Sph*I and *Hind*III was generating multiple fragments instead of two as expected. Further work on this vector was stopped and continued with pIN 29. The rhizosphere-stable pIN 29 plasmid had also an integrated DsRed2 gene. The vector was isolated out of *B. terricola* ZR2-12.

In comparison to the original pIN 29 (Vergunst et al., 2010) the vector used in this study had a trimethoprim resistance gene instead of chloramphenicol. The rest of the plasmid was identical to the original construct. First cloning experiments were done by using the *Nde*I and *Xba*l restriction enzymes to cut out the implemented DsRed gene out of pIN 29. The amplification of GFP out of pSM1880 was done by using the primers GFP_AleI and GFP_PspXI to add the according restriction recognition side. The PCR product was blunted and ligated into the also blunted pIN 29 vector. Different ligations and transformations reactions were tried out. No useful product could be delivered. A reason for that could be explained by the wrong calculation of the insert molar ratio. This was done in the case of the first ligation reactions. The TA cloning approach related to the protocol of Zhou & Gomez didn't work neither (Zhou & Gomez-sanchez, 2000). It was suspected that the amplification of the GFP sequnce out of pSM1880 could deliver a non-functional gene. Therefore the GFP amplicon was checked by Blue/White screening. The GFP_AleI and GFP_PspXI amplified GFP sequence was ligated to the pGEM vector without doing any restriction or blunting steps of the PCR product. Transformants with implemented GFP gene should produce white colonies. Almost equal numbers of blue and white colonies were produced after applying the cloning approach. The fluorescence check was showing that only some of the blue colonies were observed to be green fluorescent. A reason for that could be the in frame cloning of the GFP sequence into to the *lacZ* gene of the pGEM vector. Through this result the functionality of the GFP sequence was ensured.

New primers containing the restrictions sites *Bgl*II and *Xba*l according to the supplemental text of Vergunst et al. (2010) were used for the GFP amplification. The generated amplicons was successfully cloned into the pIN 29. Due to the usage of an instant sticky-end ligase a fast and secure method for … could be established. The ligation product could be used directly for the transformation reaction in comparison to the standard ligation approach. A general cloning protocol was defined for the DsRed2 replacement of the pIN 29 vector by using a gene of choice. This protocol was also used to clone eBFP2 and mNeptune into pIN 29. Therefore, the genes were restricted out of the pEX vectors and cloned into pIN 29. Several *Stentrophomonas* ep17 and P69 strains were transformed by using the mNeptune and eBFP2 containing pIN 29 vectors.

Generally, all environmental strains except the Gram-positive *Bacillus subtilis* B2g were transformable by using the modified pIN 29 vectors. PIN 29 shows a high stability and a

constant expression of the fluorescent genes in all greenhouse experiments. Due to the developed cloning procedure an easy and facilitate handling of the pIN 29 vector could be ensured.

4.2 Bacterial- and Plant-Bacteria-Interactions

In the second part of this study the interactions between selected bacterial control agents (BCAs) and between the BCAs and the host plants were analyzed. The analysis of synergistic or antagonistic interactions of the six environmental strains (*Serratia plymuthica* 3Re4-18, Rp8, Rp5, *Stenotrophomonas rhizophila* P69, ep17 and *Bacillus subtilis* B2g) should deliver insights for a potential BCA mixture. Studies have shown high potential of BCA mixtures against soil borne bacterial pathogens like Anthurium Blight (Fukui et al., 1999). The interactions of all six strains were analyzed *in vitro* by applying plate assays. The *Serratia* strains, Rp8 and 3Re4-18 as well as the *Stenotrophomonas* strain P69 were not inhibited by any of the other strains (except for B2g). In contrast, the *B. subtilis* B2g showed inhibitory effects to all strains. Basing on these results different mixtures of the six BCAs could be tested in further approaches. A recommended combination would be the mixture of ep17, Rp8 and Re4. These three strains were showing no inhibiting effects between each other. This statement could be confirmed by the fact that the non-inhibitory interactions between *Serratia plymuthica* 3Re4-18 and *Stenotrophomonas rhizophila* DSM14405T were already reported by Maurer et al. (2013). According to the results *in vitro*, a mixture of B2g with one of the six BCA's cannot be recommended. One reason for the strong inhibitory effects of B2g could be explained by the production of different substances like antibiotics, lipopeptides and biosurfactants (Nagórska etal., 2007). However, that in vitro antagonists are able to act synergistically on plants was shown by Adam (personal communication). In maize, plant growth promotion by jointly applied *S. rhizophila* P69 and *B. subtilis* B2g was higher than single inoculations.

The interactions between the transformed (modified pIN 29) BCAs and plants were analyzed. GFP and several derivates were already used to study microorganisms in their natural environment (Errampalli et al., 1999). Also the usage of different fluorescent dyes for simultaneous identification of different strains on plant surfaces was done before (Rao et al., 2005). The analysis of plant-bacteria interactions in this study was done by using the fluorescent labeled gram negative BCA's. The interactions between bacteria, tomato and maize plants were done in aseptic systems (germination pouches in closed plastic containers). In the present study, pIN 9-DsRed2 labeled ep17 and P69 strains were used for these interaction studies.

In comparison to P69 ep17 showed a higher density on root surfaces of both tomato and maize. The cell shape between ep17 and P69 appeared also to differ from each other. P69 cells had a globular form in comparison to the prolonged form of ep17. Both *S. rhizophila* strains had a high ability to attach to plant cells. Different substances like hemagglutinin or outer membrane adhesion proteins could be produce by these strains and promote the interactions (Berg et al., 2013a). The mixture of GFP-labeled P69 (pBAH8) and DsRed2-labeled ep17 (pIN 29) on maize roots showed a homogenous distribution of both transformants. No cluster building or separation of one of strains could be identified. An explanation for this could be that both strains prefer the same niche and compete for the similar spectrum of nutrients. Due to the close phylogenetic relationship, it is expected that P69 and ep17 possess comparable mechanisms for root colonization, cell-to-cell communication and nutrient acquisition.

The differentiation of all four fluorescent dyes by using CLSM at once was successful. Four different labeled ep17 strains could be detected simultaneously on maize roots. The differentiation between DsRed2 and mNeptune was also possible. An overlay of the two dyes occurred in some spots. The CLSM (Leica 5500 CTR) in this study had only four fixed laser sources. The optimal extinction maxima of the fluorescent genes are listed in table. The extinction of mNeptune was done by using the 635 nm laser. The maximal extinction is at 600 nm (see table 45). By applying a specific extinction wavelength of 600 nm for mNeptune a better differentiation between DsRed2 would be obtained.

Table 45: Used extinction and emission settings for the fluorescent dyes in this study

Fluorescent dye	Extinction (nm) *	Emission (nm)	Gain	Offset (%)
DsRed	532	532-582	750	-1
eBFP2	405	500-546	780	-1
GFP	475	505-510	750	-1
mNeptune	635	600-650	750	-1

In all transformed bacterial isolates, the four implemented fluorescent genes were shown to be stable and highly expressed. Further studies can be done by adding more fluorescent genes into the pIN 29 vector. Therefore, mixtures containing more than four strains to study more complex interactions studies could be applied, but requires an adequately equipped microscope.

4.3 "Fungi" transporters for BCA's

Interactions between bacteria and fungi occur very often in natural environments. Fungi-derived diseases are often enhanced by synergistic interactions with pathogen bacteria. Bacterial strains was shown to be transported by the fungal hyphen and causes enhance damages to the host plant (Grube et al., 2011). Also plant beneficial effects could be obtained by the synergistic interaction of bacteria and fungi. BCAs are able to attach to hyphen surface of outgrowing fungi and therefore thought to be transported to the plant. The combined effects of fungi and bacteria let to an enhanced uptake of nitrogen or phosphorus by the host plant (Artursson et al., 2006).

Different fungi were tested in this study for their abilities to translocate one of the six BCAs. Fungal isolates from the strain collection of antagonistic microorganisms (SCAM) as well as fungi isolated from maize cropland were used for the interaction studies. In total, 13 fungi from the culture collection and 36 fungi isolated from agricultural soil were chosen as possible transportation vectors. Only B2g colonies were transportable by several fungi. Three of the SCAM-derived fungi namely BR4-1-11, BR1-1-5 and BE1-1-3 were identified as transporters of viable B2g cells. The viability of B2g colonies were tested by the Live/Dead staining kit in a specific designed assay. The analysis of the experiment was done by using the Motic epifluorescence microscope. Due to the specific experimental design a more accurate evaluation by using the CLSM wasn't possible. BR 1-1-5 and BE 1-1-3 belong to the genus *Trichoderma*. Studies of *Trichoderma* species had revealed their antagonistic and mycoparasitic activity against a variety of microorganism (Quelle zB Mukherjee et al. 2012 siehe pdf). The fungi are also able to interact directly with the root surface of plants. The interactions could increase the plants resistance against diseases, their growth potential and stress tolerance. The fungi exhibit so called pathogen- or microbe-associated molecular patterns (PAMP and MAMP). These conserved motifs could be recognized by the plants immune system and let to an expression of different resistance mechanism (Hermos et al., 2012). The interactions between *Trichoderma* BR 1-1-5, BE 1-1-3 and *Bacillus subtilis* B2g in this study would led to the assumption that these fungi were resistant against the antifungal substances produced by the bacteria. Furthermore, the mixture of BR 4-1-11 and BR 1-1-5 with B2g on maize showed the similar or better effects on root and leave weight in comparison to the negative control. The positive interaction of *Trichoderma* and B2g could be used for further studies on different plant species. Different *Trichoderma* strains could be tested for their positive interactions with the *Bacillus* strain. Furthermore the transformation of B2g with a rhizosphere stable plasmid would facilitate the experiments. Studies of GFP tagged *Bacillus subtilis* strains (PGPR) were already done and could be used as source material for ongoing cloning approaches (Krzyzanowska et al., 2012).

References

Ahmad Farah, Ahmad Iqbal, Aqil Farrukh, Khan Saghir Mohammad and Hayat Samsul (2008) Diversity and Potential of Nonsymbiotic Diazotrophic Bacteria in Promoting Plant Growth. In Plant – Bacteria Interactions, eds. Ahmad Iqbal, Pichtel John and Hayat Shamsul, WILEY-VCH Verlag.

Ahmad Iqbal, Aqil Farrukh, Ahmad Farah, Zahin Maryam and Musarrat Javed (2008) Quorum Sensing in Bacteria: Potential in Plant Health Protection. In Plant – Bacteria Interactions, eds. Ahmad Iqbal, Pichtel John and Hayat Shamsul, WILEY-VCH Verlag.

Ai, H., Shaner, N. C., Cheng, Z., Tsien, R. Y., & Campbell, R. E. (2007). Exploration of new chromophore structures leads to the identification of improved blue fluorescent proteins. *Biochemistry*, *46*(20), 5904–10. doi:10.1021/bi700199g

Alavi, P., Starcher, M. R., Zachow, C., Müller, H., & Berg, G. (2013). Root-microbe systems: the effect and mode of interaction of Stress Protecting Agent (SPA) Stenotrophomonas rhizophila DSM14405(T.). *Frontiers in plant science*, *4*(May), 141. doi:10.3389/fpls.2013.00141

Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjørn, S. P., Givskov, M., Molin, S., & Andersen, J. B. O. (1998). New Unstable Variants of Green Fluorescent Protein for Studies of Transient Gene Expression in Bacteria New Unstable Variants of Green Fluorescent Protein for Studies of Transient Gene Expression in Bacteria.

Artursson, V., Finlay, R. D., & Jansson, J. K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental microbiology*, *8*(1), 1–10. doi:10.1111/j.1462-2920.2005.00942.x

Aspray, T. J., Hansen, S. K., & Burns, R. G. (2005). A soil-based microbial biofilm exposed to 2,4-D: bacterial community development and establishment of conjugative plasmid pJP4. *FEMS microbiology ecology*, *54*(2), 317–27. doi:10.1016/j.femsec.2005.04.007

Barriuso Jorge, Solano Ramos Beatriz, Lucas A. José, Lobo Probanza Agustín, García-Villaraco Ana and Mañero Gutiérrez J. Francisco (2008) Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR). In Plant – Bacteria Interactions, eds. Ahmad Iqbal, Pichtel John and Hayat Shamsul, WILEY-VCH Verlag.

Berg Gabriele, Alavi Mohammadali, Schmidt CHristoph S., Zachow Christin, Egamberdieva Dilfuza, Kamilova Faina, Lugtenberg Ben J.J. (2013) Biocontrol and Osmoprotection for Plants under Salinated Conditions. In Molecular Microbial Ecology of the Rhizosphere: Volume 1 & 2, ed. De Bruijn Frans J., John Wiley & Sons.

Berg, G., Krechel, A., Ditz, M., Sikora, R. a, Ulrich, A., & Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic

function against plant pathogenic fungi. FEMS microbiology ecology, 51(2), 215–29. doi:10.1016/j.femsec.2004.08.006

Czaban, J., Gajda, A., & Wróblewska, B. (2007). The motility of bacteria from Rhizosphere and Different Zones of Winter Wheat Roots, *16*(2), 301–308.

Dardanelli Marta S., Carletti S.M., Paulucci N.S., Medeot D.B., Cáceres E.A. Rodriguez, Vita F.A., Bueno M., Fumero M.V. and Garcia M.B. (2010) Benefits of Plant Growth-Promoting Rhizobacteria and Rhizobia in Agriculture. In Plant Growth and Health Promoting Bacteria, Microbiology Monographs Volume 18 ed. Maheshwari Dinesh K. Springer Verlag.

Elia, A., & Conversa, G. (2012). Agronomic and physiological responses of a tomato crop to nitrogen input. *European Journal of Agronomy*, *40*, 64–74. doi:10.1016/j.eja.2012.02.001

Errampalli, D., Leung, K., Cassidy, M. B., Kostrzynska, M., Blears, M., Lee, H., & Trevors, J. T. (1999). Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. *Journal of microbiological methods*, *35*(3), 187–99. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10333070

Fukui, R., Fukui, H., & Alvarez, a M. (1999). Comparisons of single versus multiple bacterial species on biological control of anthurium blight. *Phytopathology*, *89*(5), 366–73. doi:10.1094/PHYTO.1999.89.5.366

Grosch, R., Scherwinski, K., Lottmann, J., & Berg, G. (2006). Fungal antagonists of the plant pathogen Rhizoctonia solani: selection, control efficacy and influence on the indigenous microbial community. *Mycological research*, *110*(Pt 12), 1464–74. doi:10.1016/j.mycres.2006.09.014

Grube, M., Fürnkranz, M., Zitzenbacher, S., Huss, H., & Berg, G. (2011). Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin. *European Journal of Plant Pathology*, *131*(3), 539–548. doi:10.1007/s10658-011-9829-8

Guy Jean L., M. S., & Michel, P. (1993). Centrifugal evaporator-concentrator for concentrating specimens by evaporation of the solvent. USA. und weiter?

Hagemann, M., Ribbeck-Busch, K., Klähn, S., Hasse, D., Steinbruch, R., & Berg, G. (2008). The plant-associated bacterium Stenotrophomonas rhizophila expresses a new enzyme for the synthesis of the compatible solute glucosylglycerol. *Journal of bacteriology*, *190*(17), 5898–906. doi:10.1128/JB.00643-08

Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., … Haas, D. (2000). Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. *Molecular plant-microbe interactions : MPMI*, *13*(2), 232–7. doi:10.1094/MPMI.2000.13.2.232

Hermosa, R., Viterbo, A., Chet, I., & Monte, E. (2012). Plant-beneficial effects of Trichoderma and of its genes. *Microbiology (Reading, England)*, *158*(Pt 1), 17–25. doi:10.1099/mic.0.052274-0

Holzinger, a., Nagendra-Prasad, D., & Huys, G. (2011). Plant protection potential and ultrastructure of Bacillus subtilis strain 3A25. *Crop Protection*, *30*(6), 739–744. doi:10.1016/j.cropro.2011.02.014

Huber, B., Riedel, K., Köthe, M., Givskov, M., Molin, S., & Eberl, L. (2002). Genetic analysis of functions involved in the late stages of biofilm development in Burkholderia cepacia H111. *Molecular microbiology*, *46*(2), 411–26. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12406218

Jeppsson J.-O., Laurell C.-B., and F. B. (1979). Thxi, *25*(4), 629–638.

Kohlmeier, S., Smits, T. H. M., Ford, R. M., Keel, C., Harms, H., & Wick, L. Y. (2005). Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi. *Environmental science & technology*, *39*(12), 4640–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16047804

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. a, Roop, R. M., & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, *166*(1), 175–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8529885

Krzyzanowska, D., Obuchowski, M., Bikowski, M., Rychlowski, M., & Jafra, S. (2012). Colonization of potato rhizosphere by GFP-tagged Bacillus subtilis MB73/2, Pseudomonas sp. P482 and Ochrobactrum sp. A44 shown on large sections of roots using enrichment sample preparation and confocal laser scanning microscopy. *Sensors (Basel, Switzerland)*, *12*(12), 17608–19. doi:10.3390/s121217608

Lin, M. Z., McKeown, M. R., Ng, H.-L., Aguilera, T. a, Shaner, N. C., Campbell, R. E., … Tsien, R. Y. (2009). Autofluorescent proteins with excitation in the optical window for intravital imaging in mammals. *Chemistry & biology*, *16*(11), 1169–79. doi:10.1016/j.chembiol.2009.10.009

Liu, X., Jia, J., Popat, R., Ortori, C. a, Li, J., Diggle, S. P., … Cámara, M. (2011). Characterisation of two quorum sensing systems in the endophytic Serratia plymuthica strain G3: differential control of motility and biofilm formation according to life-style. *BMC microbiology*, *11*(1), 26. doi:10.1186/1471-2180-11-26

Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual review of microbiology*, *63*, 541–56. doi:10.1146/annurev.micro.62.081307.162918

Maheshwari D.K., Aeron Abhinav and Pandey Piyush (2010) Sustainable Approaches for Biological Control of Fusarium Wilt in Pigeon Pea (*Cajanus cajan* L. Millspaugh). In Plant Growth and Health Promoting Bacteria, Microbiology Monographs Volume 18 ed. Maheshwari Dinesh K. Springer Verlag.

Marten, P., Smalla, K., & Berg, G. (2000). Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to Bacillus subtilis. *Journal of applied microbiology*, *89*(3), 463–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11021578

Maurer, K., Zachow, C., Seefelder, S., & Berg, G. (2013). Initial Steps towards Biocontrol in Hops: Successful Colonization and Plant Growth Promotion by Four Bacterial Biocontrol Agents. *Agronomy*, *3*(4), 583–594. doi:10.3390/agronomy3040583

Mayak, S., Tirosh, T., & Glick, B. R. (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant physiology and biochemistry : PPB / Société française de physiologie végétale*, *42*(6), 565–72. doi:10.1016/j.plaphy.2004.05.009

Mohd. Sayeed Akhtar and Zaki A. Siddiqui (2010) Role of Plant Growth Promoting Rhizobacteria in Biocontrol of Plant Diseases and Sustainable Agriculture. In Plant Growth and Health Promoting Bacteria, Microbiology Monographs Volume 18 ed. Maheshwari Dinesh K. Springer Verlag.

Montañez, A., Blanco, A. R., Barlocco, C., Beracochea, M., & Sicardi, M. (2012). Characterization of cultivable putative endophytic plant growth promoting bacteria associated with maize cultivars (Zea mays L.) and their inoculation effects in vitro. *Applied Soil Ecology*, *58*, 21–28. doi:10.1016/j.apsoil.2012.02.009

Müller, H., Westendorf, C., Leitner, E., Chernin, L., Riedel, K., Schmidt, S., … Berg, G. (2009). Quorum-sensing effects in the antagonistic rhizosphere bacterium Serratia plymuthica HRO-C48. *FEMS microbiology ecology*, *67*(3), 468–78. doi:10.1111/j.1574-6941.2008.00635.x

Nagórska, K., Bikowski, M., & Obuchowski, M. (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of Bacillus subtilis as a powerful biocontrol agent. *Acta biochimica Polonica*, *54*(3), 495–508. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17882321

Neveu, B., Labbé, C., & Bélanger, R. R. (2007). GFP technology for the study of biocontrol agents in tritrophic interactions: a case study with Pseudozyma flocculosa. *Journal of microbiological methods*, *68*(2), 275–81. doi:10.1016/j.mimet.2006.08.012

Pérez-García, A., Romero, D., & de Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. *Current opinion in biotechnology*, *22*(2), 187–93. doi:10.1016/j.copbio.2010.12.003

Pimentel, D., & Pimentel, M. (2006). Global environmental resources versus world population growth. *Ecological Economics*, *59*(2), 195–198. doi:10.1016/j.ecolecon.2005.11.034

Rao, D., Webb, J. S., & Kjelleberg, S. (2005). Competitive Interactions in Mixed-Species Biofilms Containing the Marine Bacterium Pseudoalteromonas tunicata Competitive

Interactions in Mixed-Species Biofilms Containing the Marine Bacterium Pseudoalteromonas tunicata, *71*(4). doi:10.1128/AEM.71.4.1729

Vacheron, J., Desbrosses, G., Bouffaud, M.-L., Touraine, B., Moënne-Loccoz, Y., Muller, D., … Prigent-Combaret, C. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Frontiers in plant science*, *4*(September), 356. doi:10.3389/fpls.2013.00356

Vergunst, A. C., Meijer, A. H., Renshaw, S. a, & O'Callaghan, D. (2010). Burkholderia cenocepacia creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. *Infection and immunity*, *78*(4), 1495–508. doi:10.1128/IAI.00743-09

Witzany Guenther (2011) Introduction: Communicative Competences of Bacteria. In Biocummunication in Soil Microorganisms, ed. Witzany Guenther, Springer Verlag.

Yang, J., Kloepper, J. W., & Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in plant science*, *14*(1), 1–4. doi:10.1016/j.tplants.2008.10.004

Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R., & Berg, G. (2010). Strain-specific colonization pattern of Rhizoctonia antagonists in the root system of sugar beet. *FEMS microbiology ecology*, *74*(1), 124–35. doi:10.1111/j.1574-6941.2010.00930.x

Zhou, M., & Gomez-sanchez, C. E. (2000). Universal TA Cloning, *2*, 1–7.

List of Abbreviations

N	nitrogen
PGPR	plant growth promoting rhizobacteria
BCA	biocontrol agents
ISR	induced systemic resistance
AHL	N-acylhomoserine lactones
QS	quorum sensing
ACC	1-aminocyclopropane-1-carboxylate deaminase
GFP	green fluorescent protein
FISH	fluorescent *in situ* hybridization
CLSM	confocal laser scan microscopy
IAA	indole-3-acetic acid
CFU	colony forming unit
ddH_2O	double distilled water
DMSO	dimethylsulfoxid
dNTP	desoxyribonucleosid-5'-triphosphate
DNA	deoxyribonucleic acid
e. g.	exempli gratia
et. al.	et alteri
SI	supplemental information
H_2O	water
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS	sodiumdodecylsulfate
TAE	tris-acetate-EDTA
Tris	tris-hydroxymethyl-amminomethan

List of Figures

List of Figures

List of Tables

Supplemental Information

Table 1: Accession numbers and base bair length of vectors used for pJH assembling

Plasmid description	Accession Number (ACN)	Base pairs (bp)
pME6031	AF118811	8310
pBBR1 MCS-5	U25061	4768

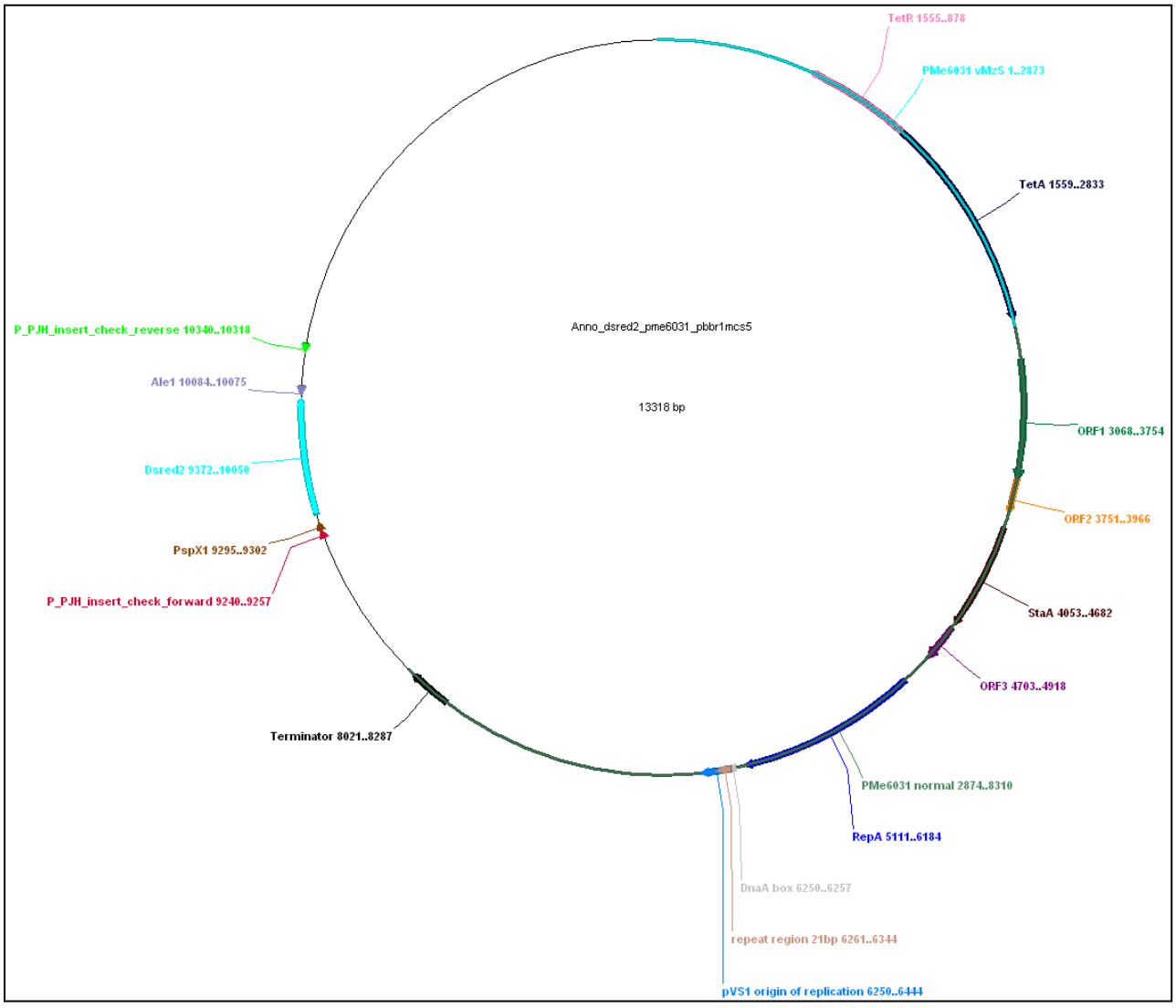


Figure 78: Annotation of the pJH (pBBR1 MCS-5 - pME6031) vector made in ApE - A Plasmid Editor v.2.0.45. The position of DsRed2 and restriction sites for *AleI* and Psp XI enzymes were shown together with DsRed2 gene flanking primers for insert identification.

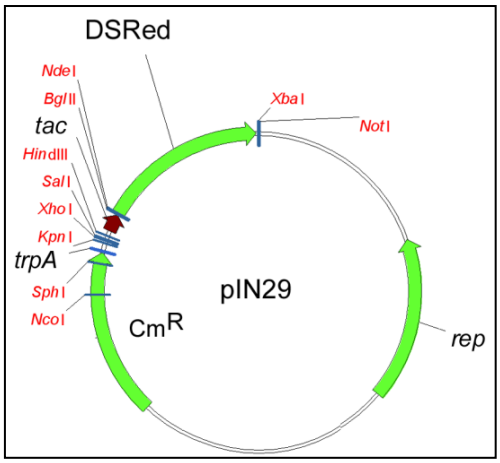


Figure 79: Structure of rhizosphere stable, non-mobile and chloramphenicol resistant pIN 29 vector (Vergunst et al., 2010).

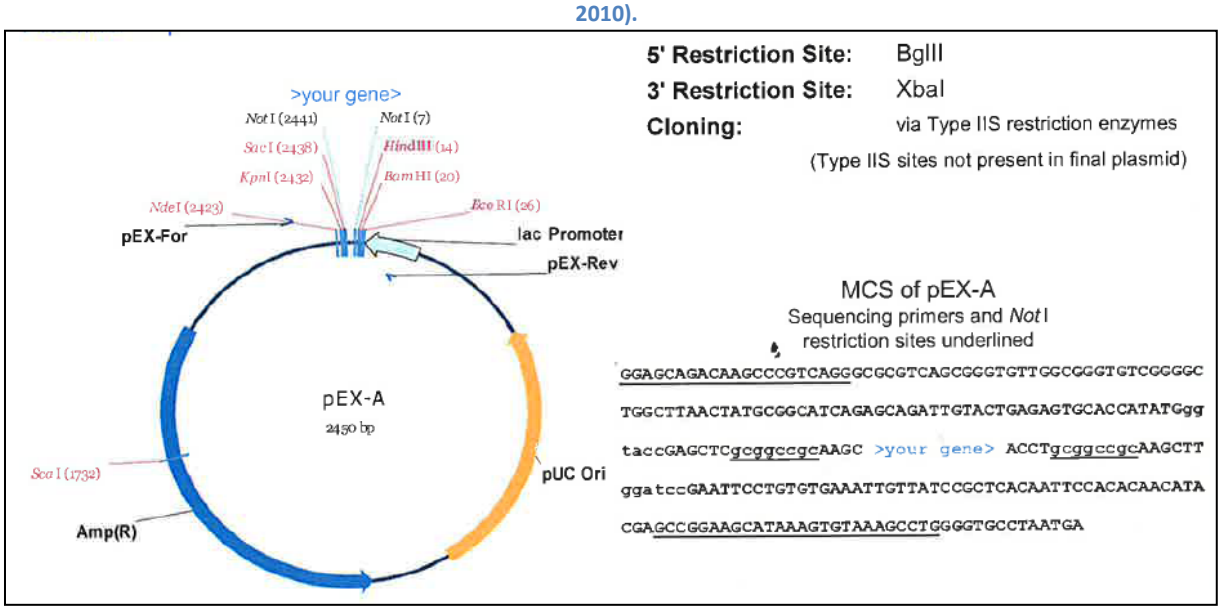


Figure 80: Plasmid map of pEX-A-mNeptune_5bglll_3xbal (2450bp). On the right is the multiple cloning site (MCS) defined. It contains the mNeptune sequence with additional restriction sites for *Bgl*II and XbaI.

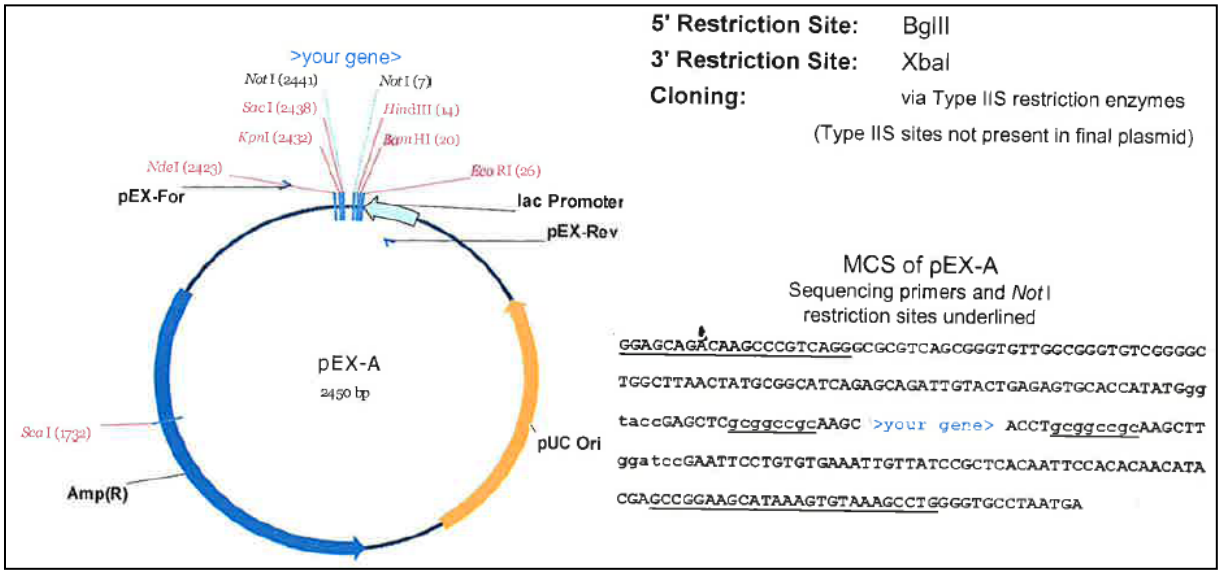


Figure 81: Plasmid map of pEX-A-eBFP2_5bglll_3xbal (2450bp). On the right is the multiple cloning site defined, which contains the eBFP2 sequence with additional restriction sites for Bgl II and XbaI.

Table 2: Sequencing results of pIN 29 and pBAH8.

Vector	Primers	Sequence
pIN 29	**DsRed1_C or DsRed1_N**	AAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGGATCCACGAACCCAGTTGACATAAGCCTG TTCGGTTCGTAAACTGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGA ACGCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTC TATGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCA GCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGCAGCCGTTGTGCTGGTGCT TTCTGATAGTTGTTGTGGGGTAGGCAGTCAGAGCTCGATTTGCTTGTCGCCATAATAGATTCACAA GAAGGATTCGACATGGGTCAAAGTAGCGATGAAGCCAACGCTCCCGTTGCAGGGCAGTTTGCGC TTCCCCTGAGTGCCACCTTTGGCTTAGGGGATCGCGTACGCAAGAAATCTGGTGCCGCTTGGCAG GGTCAAGTCGTCGGTTGGTATTGCACAAAACTCACCCCTGAAGGCTATGCGGTCGAGTCCGAATC CCACCCAGGCTCAGTGCAAATTTATCCTGTGGCTGCACTTGAACGTGTGGCCTAAGCATGCGCCCA ATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAACTAGTAGCCCGCCTAATGAGCGGG CTTTTTTTTGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGC AGGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTC ACACAGGAAGATCTCAT<u>atggcctcctccgagaacgtcatcaccgagttcatgcgcttcaaggtgcgcatggagggcacc gtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagggccacaacaccgtgaagctgaaggtga ccaagggcggccccctgcccttcgcctgggacatcctgtccccccagttccagtacggctccaaggtgtacgtgaagcaccccgc cgacatccccgactacaagaagctg</u>tccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggc gaccgtgacccaggactcctccctgcaggacggctgcttcatctacaaggtgaagttcatcggcgtgaacttcccctccgacggcc ccgtgatgcagaagaagaccatgggctgggaggcctccaccgagcgcctgtaccccgcgacggcgtgctgaagggcgagacc cacaaggccctgaagctgaaggacggcggccactacctggtggagttcaagtccatctacatggccaagaagcccgtgcagctg cccggctactactacgtggacgccaagctggacatcacctcccacaacgaggactacaccatcgtggagcagtacgagcgcacc gagggccgccaccacctgttcctgtTGTAGTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCT ATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG GCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGG CCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGAAATTGTAAGCGTTA ATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGTACTG CGATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTT GCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGAAAGCAAATTCGACCCG GTCGTCGGTTCAGGGCAGGGTCGTTAAATAGCCGCTTATGTCTATTGCTGGTTTACCGGTTTATTG ACTACCGGAAGCAGTGTGACCGTGTGCTTCTCAAATGCCTGAGGCCAGTTTGCTCAGGCTCTCCCC GTGGAGGTAATAATTGACGATATGATCATTTATTCTGCCTCCCAGAGCCTGATAAAAACGGTGAAT CCGTTAGCGAGGTGCCGCCGGCTTCCATTCAGGTCGAGGTGGCCCGGCTCCATGCACCGCGACGC AACGCGGGGAGGCAGACAAGGTATAGGGCGGCGAGGCGGCTACAGCCGATAGTCTGGAACAGC GCACTTACGGGTTGCTGCGCAACCCAAGTGCTACCGGCGCGGCAGCGTGACCCGTGTCGGCGGCT CCAACGGCTCGCCATCGTCCAGAAAACACGGCTCATCGGGCATCGGCAGGCGCTGCTGCCCGCGC CGTTCCCATTCCTCCGTTTCGGTCAAGGCTGGCAAGGTCTGGTTCCATGCCCGGGAATGC
pBAH8	**GFP_C or GFP_N**	ACGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGGTAGAAAAA<u>atgagt aaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaattttctgtcagtgga gagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgttccatggccaacact tgtcactactttc</u>tcttatggtgttcaatgcttttcccgttatccggatcatatgaaacggcatgactttttcaagagtgccatgcccg aaggttatgtacaggaacgcactatatctttcaaagatgacgggaactacaagacgcgtgctgaagtcaagtttgaaggtgata cccttgttaatcgtatcgagttaaaaggtattgattttaaagaagatggaaacattctcggacacaaactcgagtacaactataac tcacacaatgtatacatcacggcagacaaacaaaagaatggaatcaaagctaacttcaaaattcgccacaacattgaagatgg atccgttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtcga cacaatctgcccttttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagtttgtaactgctgctgggattacacat ggcatggatgagctctacaaataaGCTTAATTAGCTGAGCTTGGACTCCTGTTGATAGATCCAGTAATGAC CTCAGAACTCCATCTGGATTTGTTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATTGGTGAGAA TCCAAGCTAGCTTGGCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGG ATATACCACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGC TCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAA TAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTT CGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTC CATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTCTA CACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATT GAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTGGCCA ATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGGCGACAAGGGT GCTGATGCCGCTGACGATTCAGGTTCATCATGCCGTTTGGTGATGGCTTCCATGTCG

Underlined text = original sequence of fluorescent gene