# Manipulation of the plant microbiome: Antimicrobial peptide expression in *Nicotiana attenuata*

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*»To kill an error is as good a service as, and sometimes even better than, the establishing of a new truth or fact.«*

Charles Darwin 1809 - 1882

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# 1 | General Introduction

## 1.1. Plant-Microbe Interactions

PLANTS stand with their roots in soil, a complex environment rich in microbial life. From the emergence of the radicle until the flowering stage, plants come in contact with a highly diverse community of microbial life. Instead of suffering and surrendering to this overwhelming army of microbes, plants thrive (Lelie *et al.* 2009). They do not constantly repel bacteria but rather encourage microbes to inhabit the outer root environment and attract them with secreted root exudates. Plants deposit up to ∼20 % of the photosynthetically fixed carbon into the soil as so-called rhizodeposits[1]. This micro-habitat is called the *rhizosphere*, a term first introduced by Lorenz Hiltner in 1904, and describes the narrow region of soil surrounding the root, which is directly influenced by root secretions and associated soil microorganisms (Hiltner 1904). The *rhizosphere* is one of the most diverse habitats on the planet (Curtis *et al.* 2002; Torsvik *et al.* 2002), and the microbial density is much higher than in the surrounding bulk soil and ranges from $10^8$ to $10^9$ bacteria per gram of soil. This is nearly as many bacteria as

[1] The exact numbers of photosynthetically fixed carbon placed as rhizodeposits are highly variable and should be used with caution. It depends highly on plant age and plant species, and most data come from monocotyledonous plants, showing that young plants secrete more (∼40 %) than older plants (∼10 %) (Jones *et al.* 2009).

humans carry in their guts (Ley *et al.* 2006), or to quote Janzen (1985): »*plants wear their guts on the outside*«. Indeed, just as the human microbiome turned out to be essential for human health, a well-balanced composition of plant microbes promote higher stress tolerance (Yang *et al.* 2009) and healthy growth (Berendsen *et al.* 2012; Mendes *et al.* 2011). However, the human gut is dominated by a »core flora« containing certain abundant bacterial taxa and is relatively species poor (Qin *et al.* 2010). In comparison, soil microbial communities are considered to be the most diverse microbial communities in the world, composite of $10^4$ species per gram of soil (Roesch *et al.* 2007; Weinert *et al.* 2011). Bacteria rarely live as free cells and are commonly organized in a biofilm, which colonizes different surface areas. The plant root surface environment is called *rhizoplane*, consisting of a subgroup of rhizosphere bacteria, which are closely attached to the plant surface.

Although it was long expected that the inner parts of a plant should be sterile, surprisingly many *endophytic* bacteria have been isolated. The word *endophyte* comes from the ancient greek $\varepsilon\nu\delta o\nu$ (endon, for *inner*) and $\phi\upsilon\tau\acute{o}\nu$ (phuton, for *plant*). They are defined as bacteria (and fungi) isolated from surface-sterilized plant tissue, and cause no negative effect on the plant. Since molecular techniques are continuously replacing culturable approaches, the definition of an endophyte was extended to a microbial genome which is located inside a plant organ (Bulgarelli *et al.* 2013; Gaiero *et al.* 2013). Plant endophytic bacteria are less abundant than rhizosphere bacteria and they rarely exceed $10^3$ colony forming units (CFU) per gram root fresh mass (Turner *et al.* 2013b). However, it is still surprising how plants can tolerate bacteria within the intercellular space at all. Pathogenic bacteria (which cause diseases, and are harmful to plants) and mutualistic bacteria (which are beneficial for plants) have similar molecular patterns and can elicit plant immune responses. It is still largely unknown how a plant is able to distinguish friends from foes (Zamioudis and Pieterse 2012). In 1885, Victor Gallipe demonstrated already the presence of bacteria inside of healthy plants, which he postulated must have derived from a subpopulation of the bacterial soil community (Galippe 1885). Plant endophytes do not live intracellularly or in special encapsulated structures surrounded by a membrane: they are usually motile cells which are freely living in the intercellular space of a plant (Reinhold-Hurek and Hurek 2011). Plant endophytic bacteria did not receive much attention, since they are usually symptomless for the plant so that their presence remains unnoticed. Hence we know surprisingly little about *who they are*, *what they do* and *why they are inside plants*. Commonly proposed roles are plant-growth-promotion, protection against pathogens, nutrient acquisition or drought tolerance (Gaiero *et al.* 2013; Hardoim *et al.* 2008; Lundberg *et al.* 2012; Rosenblueth and Martínez-Romero 2006). But although many of these plant-beneficial traits are shown *in vitro* only a minority of endophytes exhibit the same *in planta* under

field conditions. For many endophytes the hypotheses for a mutualistic plant-bacteria relationship are difficult to test since a common obstacle of environmental microbiology is the inability to cultivate most bacteria. The bacterial diversity estimated by molecular techniques far exceeds what could be isolated and classified in pure cultures. James T. Staley termed this the »*the great plate count anomaly*« (Staley and Konopka 1985), and the reasons for microbial unculturability are still largely unknown (Epstein 2013). Endophytic bacteria have been found in a vast number of plant species ranging from tropical to arid environments (Hallmann and Berg 2006). The functional importance of non-culturable endophytic bacteria can only be unraveled if their abundance can be manipulated within a plant growing in its native environment: a challenge that remained unattempted to date.

## 1.2. *Nicotiana attenuata*: an ecological model plant

The wild tobacco, *Nicotiana attenuata* (Torr. ex Watson) is an annual plant native to the Great Basin Desert in southwestern USA. In this arid climate seeds can stay dormant for many decades in long-lasting seed banks[2], and germinate only under favorable conditions of post-fire environments (Fig. 1.1). The germination is triggered by smoke-derived cues, allowing the utilization of the nitrogen-rich soil remaining after the fire (Baldwin and Morse 1994). *N. attenuata* commonly grows in monocultures rapidly inhabiting the poorly vegetated post-fire environment. As a pioneer plant, *N. attenuata* is the first food source for various insects and attacked by a plethora of different herbivores. This highly unpredictable herbivore community has favored the establishment of a sophisticated and adaptive defense response system for the recognition of herbivore feeding and the activation of various defense responses. *N. attenuata* has been extensively used as a model organism to study traits important for survival in nature, in particular the role of jasmonic acid (JA) in plant defense against herbivores (Baldwin 2001; Halitschke and Baldwin 2003; Kallenbach *et al.* 2012; Kessler *et al.* 2004). The activation of jasmonate signaling leads to the accumulation of nitrogen-rich alkaloids like nicotine, the major toxin of *N. attenuata* and the compound the genus *Nicotiana* was named after (Steppuhn *et al.* 2004). The production of nitrogen-rich defense compounds requires a well-balanced growth-defense trade-off for the optimal allocation of the limited resources and the perception of insect attack is a prerequisite which allows the »*on-demand*« production of these compounds. Thus, *N. attenuata* can benefit from the costly investment under heavy herbivore attack (Baldwin 1998; Zavala and Baldwin 2004).

[2]The overall duration of seed dormancy in seed banks is still not completely known, but estimated to last for more than a hundred years (Preston and Baldwin 1999).

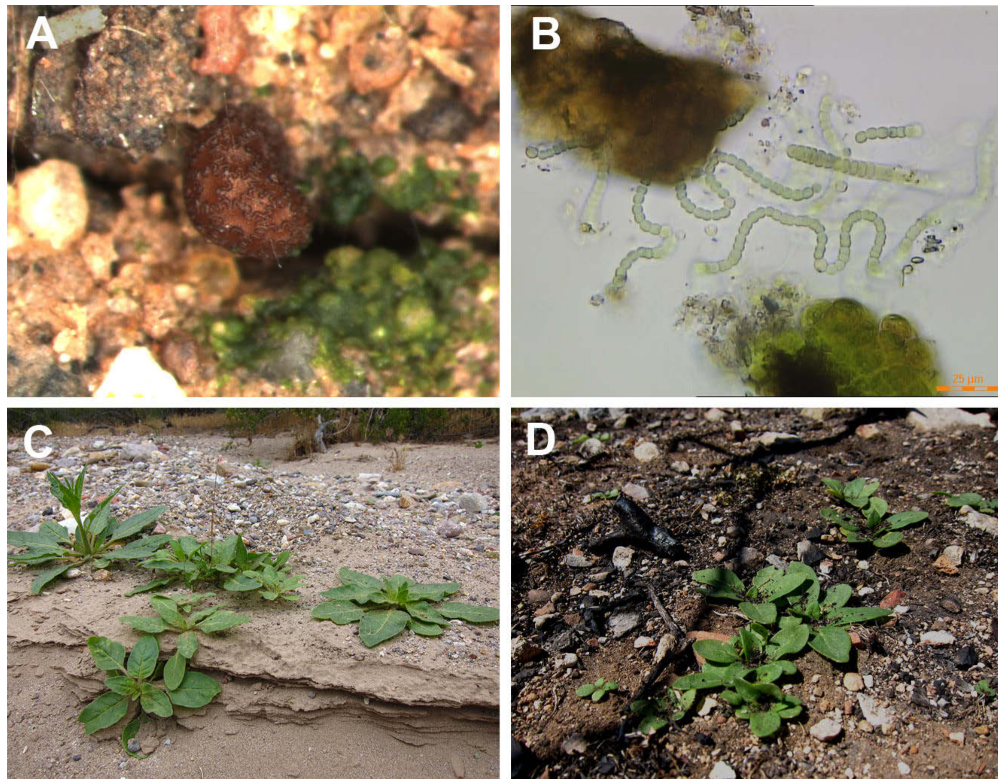



**Figure 1.1.: *N. attenuata* in its native environment, the Great Basin Desert in southwestern Utah (USA).** (A) A single seed of *N. attenuata* under the microscope; the seeds survive in long-lived seed banks for decades in the soil, waiting for favorable conditions to germinate. (B) The addition of water to dry desert soil initiates prospering life between the grains of sand, revealing green algae and cyanobacteria. (C) Native *N. attenuata* populations can be found growing in the sandy soil of dry washes or (D) within the burnt soil of post-fire environments.

The establishment of a plant transformation pipeline for *N. attenuata* allowed the targeted silencing of specific genes using RNAi in a reverse genetic approach. Transformation constructs trigger post-transcriptional gene silencing (PTGS) of target-genes and enable the manipulation of desired traits within the plant. By using constitutive or inducible promoters, genes of interest can also be ectopically over-expressed in transgenic plants, allowing phenotyping within the native environment when released on a field plot. In contrast to well studied plant-herbivore interactions, little is known about mutualistic or pathogenic microbial interactions of *N. attenuata* in nature. *N. attanuata* seems to respond negatively to arbuscular mycorrhizal fungi (AMF) inoculation (Riedel *et al.* 2008), but benefits from the inoculation with a native *Bacillus* strain (Meldau *et al.* 2012). However, experiments with non-culturable bacteria are difficult to perform. Studies currently in progress, address the interaction with natural fungal pathogens, native AMF strains or the composition of *N. attenuata´s* bacterial microbiome (data not

yet published). For instance, in 2011 we took advantage of a fungal outbreak in a wild *N. attenuata* population from the Great Basin Desert (USA) and isolated *Alternaria* and *Fusarium* strains (Schuck S., Weinhold A., Luu V.T. and Baldwin I.T. in review), which have been used in MANUSCRIPT IV to evaluate the potential activity of the transgenic plant lines against native fungal pathogens. *N. attenuata* is a non-domesticated wild plant species, that can be genetically manipulated, which marks *N. attenuata* as an excellent tool to study plant-microbe interactions.

## 1.3. Transgene expression in plants

Since advances in molecular biology allowed for the transformation of plants using *Agrobacterium*-mediated gene transfer (Horsch *et al.* 1985), this technique has developed into a scientific routine and is nowadays even applied in ecological research. However, the unpredictable and stochastic occurrence of transgene silencing and epigenetic alterations, due to *in vitro* regeneration, remain unsolved problems for the transformation of most plant species (Finnegan and McElroy 1994; Graham *et al.* 2011; Stroud *et al.* 2013). An overexpressed transgene can be silenced within a transgenic plant, and might in some cases even trigger the co-silencing of a homologous endogenous gene, as shown in pioneering experiments using transgenic *Petunia* plants (Napoli *et al.* 1990; Van der Krol *et al.* 1990).

When a transgene is inserted into a plant genome, the promoter sequence can be *de novo* methylated, which leads to unwanted transcriptional silencing of the transgene (Dalakouras and Wassenegger 2013; Matzke *et al.* 2009). This has been frequently reported (Dalakouras *et al.* 2011; Gambino *et al.* 2010; Matzke *et al.* 1989; Mishiba *et al.* 2010; Yamasaki *et al.* 2011a) and reflects the role of DNA methylation as a central gene regulatory mechanism for plants. Besides the four ordinary bases (G, A, T and C), plant genomes contain very high amounts of 5-methyl-cytosine ($m^5C$), the methylated form of cytosine. This discovery was made more than 60 years ago (Wyatt 1950), even before the elucidation of the DNA double helix structure by Rosalind Franklin (Franklin and Gosling 1953; Watson and Crick 1953). For a long time $m^5C$ was only considered as a "minor base" in plant genomes, its importance in epigenetic gene regulation (*e.g.* fruit ripening in tomatoes; Zhong *et al.* (2013)) has only recently been recognized (Vanyushin and Ashapkin 2011). Plants have a more complex and sophisticated gene silencing machinery than animals, and do not only methylate cytosines in CG dinucleotides, but also in all other possible sequence contexts at CHG and CHH positions (were H = A,T or C) (Fedoroff 2012; Law and Jacobsen 2010). A transgene can be methylated in the genome with high sequence specificity through a process called RNA-directed DNA

methylation (RdDM) which uses the mRNA as a guide (Dalakouras and Wassenegger 2013). Characteristic symptoms of unwanted transgene silencing by DNA methylation include spatially variegated or transient levels of gene expression within the marker gene (observed as increased sensitivity and non-Mendelian segregation; Mendel (1866)) or the gene of interest (observed as low and variable gene expression). Although most aspects of epigenetic inheritance are understood, principles of methylation establishment during vegetative growth remain unclear (Feng *et al.* 2010; Law and Jacobsen 2010).

A stably transformed plant is only useful for ecological experiments if the transgene-altered phenotype is reliable and remains stable over the entire plant developmental period. In order to better understand and predict the transgene inactivation process, bisulfite sequencing was used in MANUSCRIPT II to investigate the timing of DNA methylation during vegetative development. This allowed the selection of transgenic plant lines showing stable expression patterns of antimicrobial peptides.

## 1.4. Antimicrobial peptides

Antimicrobial peptides[3] (AMPs) are small peptides which can inhibit the growth of a broad range of microbes. They can be found in most multicellular eukaryotes (plants, animals and fungi) and play an important role in defense and innate immunity (Stotz *et al.* 2013; Zasloff 2002). AMPs share common features: They are very small (<10kDa) and contain many cationic and hydrophobic amino acids, resulting in an overall positive net charge and extreme high p*I* values. Typical (and maybe most prominent) members of AMPs are the »defensins«, which have a molecular size of $\sim$5kDa and consist of about 45 to 54 amino acids, structured in three antiparallel $\beta$-sheets and one $\alpha$-helix, stabilized by four disulfide bonds (Fig. 1.2) (Thomma *et al.* 2002). Characteristic for all cysteine-rich AMPs are the even numbers of conserved cysteine residues (4, 6 or 8) connected by intra-molecular disulfide bonds (Pelegrini *et al.* 2011). The first plant AMPs were isolated in 1942 from the endosperm of wheat (*Triticum aestivum*) and named »thionin« or »purothionin« after the high proportion of sulfur-containing cysteine residues (Balls *et al.* 1942). Later in the 1990s another group of cysteine-rich peptides was found and originally grouped as $\gamma$-thionins, but they were later renamed and placed in their own »defensin« family due to their similarity with animal defensins (Broekaert *et al.* 1995). The structural pattern of AMPs are highly conserved across kingdoms, and similar peptides can be found in plants, insects or mammals (Bulet *et al.* 2004). Cysteine-rich

[3]The term »antimicrobial peptide« (AMP) is a very loose description and a purely functional terminology. It usually includes the classical cysteine-rich peptide families, encoded by single genes, as well as glycine or proline-rich peptides and short linear $\alpha$-helical peptides. Other peptides of these classes are also involved in various different cellular processes besides »antimicrobial« activity (Carvalho and Gomes 2009).

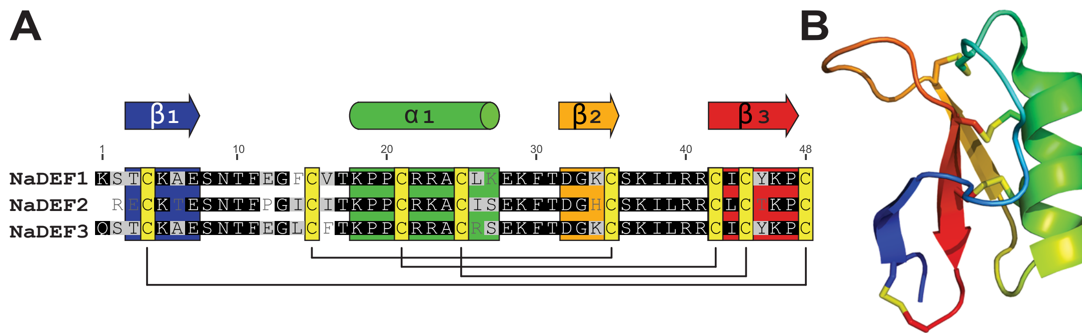



**Figure 1.2.: Alignment of *N. attenuata* defensin 1 - 3.** (A) Sequence alignment of the mature domains of three defensin peptides from *N. attenuata* showing conserved cysteine residues (yellow). (B) Three dimensional structure of *N. alata* floral defensin 1 (NaD1) (Lay *et al.* 2003b), which shows 100 % amino acid sequence homology to *N. attenuata* DEF2.

plant AMPs include the peptide families of thionins, defensins, heveins, lipid-transfer proteins, knottins, snakins and cyclotides (Sampedro and Valdivia 2014). All AMPs have N-terminal signal peptides targeting them to the protein secretion pathway. They receive a couple of post-translational modifications by passage through the endoplasmatic reticulum which involves the removal of the signal peptide and the formation of disulfide bonds (Matsubayashi 2011). Disulfide bonds maintain the tertiary structure of peptides outside of the cytoplasm and are essential for peptide activity[4]. The small sizes and extremely high p*I* values of AMPs makes it difficult to perform standard proteomic methods when working with these peptides. Thus, the development of a universal, gel-free shotgun proteomic method was a great advance allowing the comparison of AMP amounts among different transgenic plants (Manuscript III).

The mode of action of AMPs involves binding or interaction with lipids, since they can destabilized, penetrate and disrupt lipid bilayers of cells (Brogden 2005; Shai 2002). AMPs have been shown to have a broad spectrum of activity against different bacteria (gram-positive and negative) and fungi, which made them promising candidates for the engineering of disease-resistant crop plants (López-García *et al.* 2012; Zeitler *et al.* 2013). Hence, information about the activity spectrum of AMPs or AMP-expressing plants is mainly restricted to studies testing plants for increased resistance against phytopathogenic fungi and oomycetes (Chen *et al.* 2004; Huang *et al.* 2012; Koo *et al.* 2002; Lay *et al.* 2003a; Lee *et al.* 2003; Park *et al.* 2000; Ponti *et al.* 2003; Yang *et al.* 2006, 2008). AMPs have also been considered for application in clinical studies to replace conventional antibiotics (Fox 2013; Zhao *et al.* 2013). Likewise, tests involving bacteria were usually

[4]Except for a rare example in humans, where a $\beta$-defensin showed antimicrobial activity only after disulfide bond reduction (Schroeder *et al.* 2011).

only performed with plant or human pathogens. Only little information is available regarding their potential impact on plant beneficial bacteria.

## 1.5. Reprogramming apoplastic defense to manipulate beneficial bacteria in plants

As a response to pathogen attack, plants can deposit diverse defensive proteins into the intercellular space, such as chitinases, glucanases, proteases or polygalacturonase inhibitors (Hückelhoven 2007; Lee *et al.* 2004). However, the intercellular space is colonized by endophytic bacteria, which in some way evade the plants' defense response (Compant *et al.* 2010; Reinhold-Hurek and Hurek 2011; Sattelmacher 2001; Turner *et al.* 2013b). The artificial overproduction of AMPs, which are constitutively produced and secreted into the extracellular space could target beneficial endophytic bacteria dwelling in the apoplastic space of the plant. This would *in theory* allow the creation of »antimicrobial« plants, which could interfere with colonization by endophytic bacteria. The absence or reduction of endophytes could reveal effects in plants grown under natural conditions. We therefore selected different AMPs for ectopic expression in *N. attenuata*, listed in Table 1.1.

**Table 1.1.:** List of antimicrobial peptides (AMPs) used for ectopic expression in *N. attenuata* (For a structural overview see Fig. 5.1 in MANUSCRIPT III.)

| plant line | peptide name | peptide family | organism of origin | kDa | pI | reference | Phyt AMP ID* |
|---|---|---|---|---|---|---|---|
| **DEF1** | NaDefensin1 | defensin | *Nicotiana attenuata* | 5.5 | 9.3 | Rayapuram and Baldwin 2008 | no entry |
| **DEF2** | NaDefensin2 | defensin | *Nicotiana attenuata* | 5.3 | 9.1 | Lay *et al.* 2003a | no entry |
| **VRD** | VrD1 | defensin | *Vigna radiata* | 5.1 | 9.1 | Chen *et al.* 2002 | PHYT00055 |
| **FAB** | Fabatin-1 | defensin | *Vicia faba* | 5.2 | 9.1 | Zhang and Lewis 1997 | PHYT00022 |
| **THIO** | Thionin 2.1 | thionin | *Arabidopsis thaliana* | 4.8 | 9.4 | Bohlmann *et al.* 1998 | PHYT00086 |
| **ICE** | Mc-AMP1 | knottin | *Mesembryanthemum crystallinum* | 4.2 | 9.3 | Pelegrini *et al.* 2011 | PHYT00272 |
| **PNA** | Pn-AMP2 | hevein | *Ipomoea nil* | 4.2 | 8.5 | Koo *et al.* 2002 | PHYT00234 |
| **ESC** | Esculentin-1 | esculentin | *Rana plancyi fukienensis* | 4.8 | 9.6 | Ponti *et al.* 2003 | n/a |
| **SSP** | Spheniscin-2 | avian defensin | *Aptenodytes patagonicus* | 4.5 | 11.6 | Thouzeau *et al.* 2003 | n/a |
| **LEA** | LJAMP2 | lipid transfer protein | *Leonurus japonicus* | 9.1 | 9.0 | Yang *et al.* 2006 | PHYT00112 |
| **CAP** | sheperin I + sheperin II | glycine rich protein | *Capsella bursa-pastoris* | 2.4<br>3.3 | 7.3<br>7.3 | Park *et al.* 2000 | PHYT00256<br>PHYT00257 |

*`http://phytamp.pfba-lab-tun.org/main.php` (Hammami *et al.* 2009), n/a (not applicable)

In order to increase the chances for successful *in vivo* stability and activity in *N. attenuata*, they derived from a broad range of peptide families. Most of AMPs are plant peptides, chosen from the PhytAMP database (Hammami *et al.* 2009), and were reported to have antibacterial activity. In total, eleven constructs were made for the ectopic expression of eleven different antimicrobial peptides in *N. attenuata*. The genes encoding for these peptides were synthesized in sequential PCR reactions (Tab. A.1), and cloned behind a constitutive 35S promoter (see MANUSCRIPT I). Peptides[5] which had been reported for antibacterial activity only on gram-positive bacteria (*e.g.* SSP, LEA, PNA, FAB and ICE) were of particular interest, since this group of bacteria rarely contains plant pathogens but many plant beneficial bacteria *e.g. Bacilli* (Long *et al.* 2010; Meldau *et al.* 2012). The $\beta$-defensin of the King penguin (*Aptenodytes patagonicus*), for instance, was reported to show strong effects against *Micrococcus luteus* and *Bacillus megaterium* (Thouzeau *et al.* 2003), evidence that made the SSP line of special interest. However, avian defensins have never been expressed in plants and seem to be unstable in *N. attenuata* (MANUSCRIPT III). Plant peptides were generally preferred, since they are likely to remain stable when expressed in another plant. Likewise, particular preference was given to peptides which had been successfully expressed in *N. tabacum* beforehand, *e.g.* PNA (Koo *et al.* 2002), ESC (Ponti *et al.* 2003) or LEA (Yang *et al.* 2007).

The subcellular localization of the peptide and its secretion into the apoplast is a crucial aspect for targeting endophytic bacteria in plants. Accumulation in the vacuole is unlikely to affect extracellular bacteria, unless the plant cells get ruptured. Therefore, the analysis of peptide localization and quantification in the apoplast (MANUSCRIPT III) was a vital step to evaluate the AMP expressing plant lines. The plant AMPs retained their native signal peptide, but the two animal peptides (ESC and SSP) were fused to the signal peptide of the polygalacturonase-inhibiting protein (PGIP) leader sequence from *Phaseolus vulgaris*, which has been reported to target peptides for secretion in *N. tabacum* (Ponti *et al.* 2003). Ecological studies which tried to explore effects of transgenic plants on the rhizosphere or in the plant microbiome miss the prerequisite to convincingly show AMP accumulation and localization in their transgenic plants (Rasche *et al.* 2006a,b; Sessitsch *et al.* 2003). Furthermore, activity of the peptides *in planta* was investigated using native endophytic bacteria in plant infiltration assays (MANUSCRIPT IV).

[5]Throughout the manuscripts the abbreviations used for the plant lines are also used as synonyms for the peptides and the peptide genes. The naming in previous publications or databases was inconsistent, and for instance the »LEA« peptide can be found under three other pseudonyms: La-LTP (*Leonurus artemisia* Lipid transfer protein), LJAFP (*Leonurus japonicus* antifungal peptide) and LJAMP2 (*Leonurus japonicus* antimicrobial peptide2) (Yang *et al.* 2006, 2008).

## 1.6. Scope of this thesis

The overall scope of this thesis was to use transgenic plants to manipulate endophytic bacteria *in planta*, and to establish a toolbox for plant-microbe studies under native conditions. Non-culturable endophytic bacteria are readily missed in experiments where plant growth performance is measured after inoculation with culturable strains. The overall contribution of the »*unseen majority*«[6] can only be evaluated if their abundance is directly manipulated using transgenic plants. The expression of AMPs allows a more targeted and specific manipulation of bacteria, with less possible side-effects in comparison to the manipulation of phytohormone levels (as these result in multiple different phenotypes, unrelated to microbes). In order to address the question, *are endophytic bacteria relevant for the fitness of N. attenuata in nature*, several criteria needed to be met for a transgenic plant; all are addressed and answered in the manuscripts of this thesis.

- MANUSCRIPT I: What is the most efficient way to screen transgenic plants? What order of selection procedures allows a fast selection process with minor effort but highest success rate? Are the screening criteria sufficient to use transgenic plants for ecological experiments?
- MANUSCRIPT II: Can transgene expression instability by epigenetic gene silencing be predicted? What are the reasons for transgene silencing? When does DNA methylation happen, and where in gametes or in somatic cells? At what speed does DNA methylation spread during vegetative growth? Can DNA methylation be avoided? Does the callus regeneration process during plant transformation alter the DNA methylation, and can this be used to recover silenced transgenes?
- MANUSCRIPT III: Are the expressed AMPs localized extracellularly? How can small cysteine-rich peptides be selectively extracted from plant cell walls? Is it possible to quantify AMP accumulation to compare different constructs? Which AMP is highly expressed and accumulated in the apoplast of *N. attenuata*?
- MANUSCRIPT IV: Do AMPs show *in vivo* activity in the plant apoplast? Which bacterial taxa can be influenced by AMP expression? Can a transgenic plant likewise influence »wild« isolates? Is AMP expression sufficient to create aposymbiotic plants?

[6]From Rout and Southworth (2013), referring to the numerical dominance of microorganisms in terrestrial ecosystems and their integral role in ecosystem function.

# 2 Manuscript Overview & Author's Contributions

## Manuscript I:

**»Efficient screening of transgenic plant lines for ecological research«**

**Gase K, Weinhold A, Bozorov T, Schuck S, Baldwin IT**

Published in *Molecular ecology resources* (2011) **11**: 890–902
`http://dx.doi.org/10.1111/j.1755-0998.2011.03017.x`

- In **manuscript I**, the experiences for a faster transgenic plant screening and a more efficient selection process was summarized in a flow chart. The occurrence of polyploidy and the percentage of silenced plant lines was observed for antimicrobial peptide expressing plant lines. The extension of the standard screening protocol by flow cytometry and diagnostic PCRs allowed a quicker exclusion of unsuitable lines and the effective selection of desired plant lines. Furthermore a particular order of the selection steps was suggested.
- The flow chart was developed by AW together with KG. The ploidy measurements, diagnostic PCRs and southern blot analysis on the antimicrobial peptide expressing plant lines were performed by AW. KG designed most of the binary transformation vectors and wrote the manuscript. TB performed diagnostic PCRs and SS screened parts of the transgenic lines. ITB designed research and revised the manuscript.

# Manuscript II:

**»Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants«**

**Weinhold A, Kallenbach M, Baldwin IT**

Published in *BMC Plant Biology* (2013) **13**: 99
http://dx.doi.org/10.1186/1471-2229-13-99

- In **manuscript II**, the occurrence of rapid transcriptional gene silencing was examined in antimicrobial peptide expressing plant lines. The analysis of RNA-directed DNA methylation revealed a sudden promoter methylation increase and epigenetic gene silencing in the $T_3$ generation of certain plant lines. The DNA methylation increased rapidly during vegetative plant development and did not require a generational change for establishment. A secondary callus regeneration process could be applied to rescue gene expression and avoid promoter methylation in sense expression as well as *ir* lines.
- The design of this study, the first draft and all experiments with antimicrobial peptide over-expressing plant lines were planned and performed by AW. MK performed experiments with *ir-acx1* lines. ITB participated in the design of the study and revised the manuscript. AW is corresponding author of this manuscript.

## Manuscript III:

**»Label-free nanoUPLC-MS$^E$ based quantification of antimicrobial peptides from the leaf apoplast of *Nicotiana attenuata*«**

**Weinhold A, Wielsch N, Svatoš A, Baldwin IT**

Submitted to *BMC Plant Biology* (2014)

- In **manuscript III**, a method was developed allowing the quantification of antimicrobial peptides within the apoplast of transgenic *N. attenuata* plants. For this study a selective high throughput peptide extraction process was developed and coupled with an absolute peptide quantification method by data-independent acquisition using nanoUPLC-MS$^E$. From all 10 analyzed transgenic plant lines at least 7 could be confirmed in peptide accumulation whereas 3 antimicrobial peptides showed very high abundances within the apoplast.
- The screening of the transgenic plants, the peptide extraction and the desalting method for the development of the sample preparation protocol was planned and performed by AW. NW performed the nanoUPLC-MS$^E$ measurements and wrote parts of the methods section. The data analysis and the first draft of the manuscript was performed by AW. AS and ITB participated in the design of the study and revised the manuscript. AW is corresponding author of this manuscript.

# Manuscript IV:

**»*In planta* manipulation of native endophytic *Bacillus* spp. in *Nicotiana attenuata*: Engineering an aposymbiotic plant«**

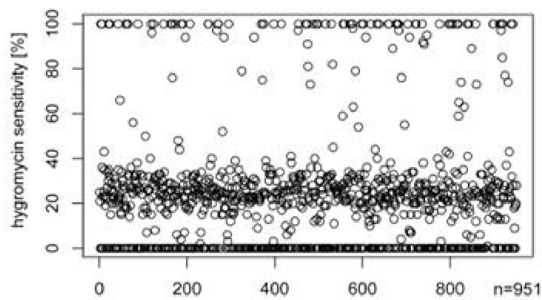
**Weinhold A, Baldwin IT**

Submitted to *New Phytologist* (2014)

- In **manuscript IV**, the *in vivo* activity of a particular antimicrobial peptide (ICE) could be shown, by infiltration of native endophytic bacteria. The ICE lines showed strong effects on *Bacillus spp.* but no effect on actinobacteria or proteobacteria. Phylogenetic highly similar endophytic *Bacillus megaterium* strains showed a varied susceptibility against the peptide, indicating that the soil community might be able to outcompete antimicrobial activity by diversity. The transgenic plants were free of pleiotroic growth effects and showed no increased resistance against phytopathogenic fungi.
- AW planned and performed all experiments and wrote the manuscript. ITB participated in the design of the study and revised the manuscript. AW is corresponding author of this manuscript.

# 3 Manuscript I

## 3.1. Efficient screening of transgenic plant lines for ecological research




Transgenic plants were germinated on antibiotic containing media to select for stable transgene insertion and resistance marker segregation.

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Molecular Ecology Resources (2011) **11**, 890–902 doi: 10.1111/j.1755-0998.2011.03017.x

# Efficient screening of transgenic plant lines for ecological research

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## Abstract

**Plants stably transformed to manipulate the expression of genes mediating ecological performance have profoundly altered research in plant ecology. *Agrobacterium*-mediated transformation remains the most effective method of creating plants harbouring a limited number of transgene integrations of low complexity. For ecological/physiological research, the following requirements must be met: (i) the regenerated plants should have the same ploidy level as the corresponding wild-type plant and (ii) contain a single transgene copy in a homozygous state; (iii) the T-DNA must be completely inserted without vector backbone sequence and all its elements functional; and (iv) the integration should not change the phenotype of the plant by interrupting chromosomal genes or by mutations occurring during the regeneration procedure. The screening process to obtain transformed plants that meet the above criteria is costly and time-consuming, and an optimized screening procedure is presented. We developed a flow chart that optimizes the screening process to efficiently select transformed plants for ecological research. It consists of segregational analyses, which select transgenic $T_1$ and $T_2$ generation plants with single T-DNA copies that are homozygous. Indispensable molecular genetic tests (flow cytometry, diagnostic PCRs and Southern blotting) are performed at the earliest and most effective times in the screening process. qPCR to quantify changes in transcript accumulation to confirm gene silencing or overexpression is the last step in the selection process. Because we routinely transform the wild tobacco, *Nicotiana attenuata,* with constructs that silence or ectopically overexpress ecologically relevant genes, the proposed protocol is supported by examples from this system.**

*Keywords*: ecological genomics, field releases, plant transformation, ploidy, reverse genetics, screening

*Received 14 December 2010; revision received 14 March 2011; accepted 17 March 2011*

## Introduction

Stably transformed plants have proven a powerful tool in ecological research to investigate the ecological relevance of particular genes. For this, the inserted sequence elements are designed either to silence intrinsic or to overexpress intrinsic or heterologous genes of interest. This reverse genetics approach allows for the creation of transgenic lines with either abnormally low or high levels of transcripts for a particular gene in an otherwise isogenic background. These isogenic lines provide a particularly efficient means of studying the fitness consequences of a given gene's expression (Steppuhn *et al.* 2004; Zavala *et al.* 2004; Kang *et al.* 2006; Schwachtje *et al.* 2008).

Two general strategies are used for plant transformation: *Agrobacterium*-based transformation and a group of unrelated techniques collectively referred to as 'direct DNA transfer' (Kohli *et al.* 2003). Direct DNA transfer methods such as particle bombardment (Christou 1992) often result in transgenic loci with a high transgene copy number (often more than 40) (Kohli *et al.* 2003; Latham *et al.* 2006). *Agrobacterium*-based transformation procedures produce lines with less complex transgenic loci, but still, the integration of multiple T-DNA copies into a limited number of loci is common (De Buck *et al.* 2009; Bhat & Srinivasan 2002). The transformation mediated by *Agrobacterium* involves the transfer of the T-DNA molecule to the eukaryotic host cell and its integration into the host genome. The machinery required for this process comprises proteins encoded by bacterial chromosomal genes and Ti-plasmid virulence genes as well as the host intracellular transport and DNA repair machinery (Tzfira & Citovsky 2006; Gelvin 2009; Lacroix & Citovsky 2009). The T-DNA region is defined by its left and right border sequences, two 25-bp inverted repeats, originally present on the Ti plasmid (Tzfira *et al.* 2004). To allow the creation of transgenic plant lines with desired insertions, the

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T-DNA borders were transferred to a binary vector, and the sequences that should be integrated into the plant genome are cloned between these border sequences (Hoekema *et al.* 1983).

T-DNA integration occurs randomly throughout the plant genome (Gelvin & Kim 2007; Kim *et al.* 2007) by non-homologous end-joining (Gheysen *et al.* 1991) and is accompanied by deletions and rearrangements of the T-DNA flanks and of the target DNA near the integration site (Latham *et al.* 2006; Muller *et al.* 2007; Gambino *et al.* 2009). The integration of DNA from binary plant transformation vectors is not always limited to the region between the T-DNA borders. 'Read-through' events can occur and result in the unwanted cotransfer of vector backbone sequences, such as bacterial resistance genes. After *Agrobacterium*-mediated T-DNA transfer, binary vector backbone sequences can be detected in up to 75% of the transgenic plants (Kononov *et al.* 1997; Wenck *et al.* 1997; McCormac *et al.* 2001; Kohli *et al.* 2003; Lange *et al.* 2006; Gambino *et al.* 2009). Regulatory agencies that govern the release of transgenic organisms do normally not permit the release of plants carrying such sequences. *Agrobacterium* infection and, in most cases, the regeneration process necessary to regenerate a transgenic plant via cell culture and callus formation from a single transformed plant cell can lead to somoclonal variations (Bhat & Srinivasan 2002), including genome-wide mutations (Latham *et al.* 2006) and polyploidization (Bubner *et al.* 2006).

*Agrobacterium*-based transformation has proven to be the best-suited approach to produce transgenic plant lines with single-copy T-DNA insertions (De Buck *et al.* 2009; Bhat & Srinivasan 2002; O'Malley & Ecker 2010, Meza *et al.* 2002; Sallaud *et al.* 2003; Olhoft *et al.* 2004; Yu *et al.* 2010). Transformed plant lines may contain two or more independent transgenic loci, but sufficient lines with single T-DNA insertions in a single locus can be expected (De Buck *et al.* 2004). This makes *Agrobacterium*-based transformation the preferred method to create transgenic plants for ecological research.

If the transgenic plants are to be used to answer questions about the organismic-level consequences of a particular gene's expression, then the transformants must fulfil a number of strict criteria: The transgenic line should have the same ploidy level as the plant that was transformed (Schwachtje & Baldwin 2008). The insert should comprise the complete T-DNA originating from the binary vector used for transformation without deletions, rearrangements or other mutations. In each line, only one transgenic locus with a single T-DNA copy should be present, and each line should be homozygous with respect to this locus. The integration site of the transgenic DNA should not disrupt other functional genes, which could confound the analysis of phenotypes associated with the gene of interest. To exclude mutations arising from T-DNA insertion or regeneration, at least two independent lines created with the same T-DNA should be evaluated, and both should exhibit the same phenotype, as the chances that the T-DNA inserted twice into the same functional gene are vanishingly small (Schwachtje *et al.* 2008).

For many plant scientists interested in the function of genes at a whole plant level, the utilization of genetically modified plants silencing or overexpressing a particular gene of interest is the most powerful means of answering functional questions. One important challenge for this approach is the lack of transformation and selection procedures for many plant species of ecological interest. This protocol paper will help ecological researchers to create and select transgenic lines that fulfil the requirements of their research questions. We have developed an *Agrobacterium*-mediated transformation system for the wild tobacco *Nicotiana attenuata* Torrey ex Watson, an ecological model plant (Baldwin 2001). Based on our experiences with this method in producing transgenic lines for use in ecological research, we describe a protocol that optimizes the efficiency of the transformation and selection system and can be applied to other plants of ecological interest.

The transformation procedure is laborious and comprises the construction of appropriate binary plant transformation vectors, *Agrobacterium*-based transformation and the selection and regeneration of the first transgenic generation ($T_0$ plants). Because the $T_0$ generation originates from regenerated calli, growth regulators used during regeneration may have a lasting effect on plant performance (Bhat & Srinivasan 2002). It also cannot be excluded that $T_0$ plants are chimeras regenerated from two or more transformed plant cells, or have increased ploidy levels (Bubner *et al.* 2006). Because of these properties, $T_0$ plants should not be used for experiments. The first non-chimeric transgenic plant generation that did not undergo the regeneration process ($T_1$) is produced after self-pollination of the $T_0$ flowers, and the resulting $T_1$ seeds should be germinated on medium containing the selective antibiotic. This allows the identification of individuals carrying T-DNA insertions and indicates possible silencing problems because of promoter methylation that may interfere with the transcription of the transgene (Stam *et al.* 1997).

The $T_1$ generation represents an important intermediate stage in the selection of transgenic lines that fulfil the criteria for ecological research. In most cases, this generation contains homozygous lines with a single T-DNA insertion locus. The ploidy level, the arrangement of the T-DNA insertion and often the level of transcription of the transgene will—if no further chromosomal rearrangements occur—remain unchanged in subsequent inbred generations.

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Further inheritance studies with the $T_2$ generation, obtained by self-pollination of the $T_1$ generation and germination, are necessary to identify candidate transgenic $T_1$ lines that harbour single transgene insertions in a homozygous state. Each of the selected lines should be evaluated by a number of molecular genetic analyses to confirm that the above criteria are met. These analyses include flow cytometry to determine the ploidy level of the transgenic lines, appropriate diagnostic polymerase chain reactions (PCR) to ensure the insertion of the complete T-DNA into the plant chromosome and to exclude vector backbone integrations, Southern blotting to confirm single T-DNA insertions into the plant chromosome and qPCR to evaluate silencing efficiency or the level of transcript accumulation of the transgene.

The transformation procedure and the inheritance studies necessary to produce and identify the required transgenic lines consume an enormous amount of human and material resources. The time to grow one generation from germination to seed ripening can take, depending on the species, several months, in the case of *N. attenuata* 3–4 months. It is therefore important to limit the screening effort to what is absolutely necessary. In this study, we present a workflow protocol that enables researchers to produce and select transgenic lines in an optimized screening process. We analyse each step of the selection process using examples from the screening of *N. attenuata* lines and discuss possible variations.

## Materials and methods

### *Construction of plasmids for the transformation of* N. attenuata

The initial vector for the construction of the pSOL8/pSOL9 plasmid series was pSOL3LOX (10.6 kb) (Bubner *et al.* 2006). After replacing the *nptIII* (aminoglycoside phosphotransferase class III) gene with *nptII* from pAcGFP1-1 (http://www.clontech.com) and replacing the inverted repeat gene fragments with *N. attenuata DCL2* cDNA (HQ698849) fragments, thereby providing optimized cloning sites, cloning vector pSOL8DCL2 (10.2 kb; HQ698851) was created.

The pSOL8 series inverted repeat gene silencing plasmids (Fig. 1a) were created by replacing the *Xho*I-*Sac*I and *Pst*I-*Hin*dIII inverted repeat fragments of pSOL8DCL2 with inverted repeat PCR fragments (0.3–0.6 kb) of the following *N. attenuata* genes: pSOL8DC3 [10.8 kb; RNA-dependent RNA polymerase 1 gene (DQ988990) combined with WRKY6 gene (AY456272)], pSOL8PNRP [10.2 kb; gene similar to *Arabidopsis thaliana* putative nematode resistance protein mRNA (AY080778)], pSOL8AEP65 [10.2 kb; gene similar to *N. tabacum* Avr9/Cf-9 rapidly elicited protein 65 mRNA (AF211539)] and pSOL8AEP150 [10.1 kb; gene similar to *N. tabacum* Avr9/Cf-9 rapidly elicited protein 150 mRNA (AY775041)].

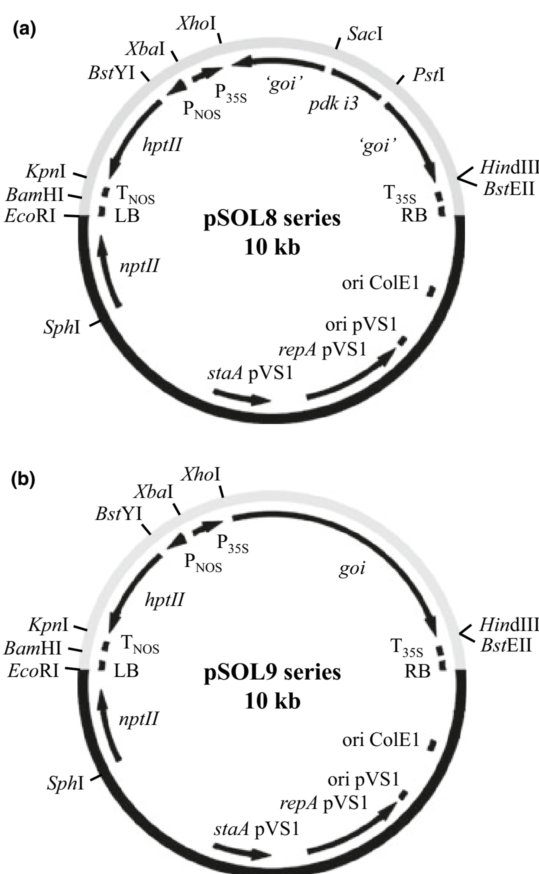


**Fig. 1** The pSOL8 series (a) and pSOL9 series (b) binary plant transformation vectors. Abbreviations: LB/RB, left/right border of T-DNA; $P_{NOS}$/$T_{NOS}$, promoter/terminator of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*; $P_{35S}$/$T_{35S}$, 35S promoter/terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); *goi*, gene of interest; i, intron 3 of *Flaveria trinervia pdk* gene for pyruvate, orthophosphate dikinase; *nptII*, aminoglycoside phosphotransferase class II; ori, origin of replication.

The following pSOL9 series gene expression plasmids (Fig. 1b) were obtained by replacing the 1.4 kb *Xho*I-*Hin*dIII-fragment of pSOL8DCL2 with appropriate PCR fragments (0.2–0.4 kb) allowing the overexpression of the following antimicrobial protein genes: pSOL9CAP [9.2 kb; *Capsella bursa-pastoris* antimicrobial peptide mRNA (HQ698850)], pSOL9ESC [9.0 kb; synthetic gene similar to *Rana plancyi fukienensis* mRNA for esculentin-1P precursor protein (AJ968397)], pSOL9FAB [9.0 kb; synthetic gene similar to *Vicia faba* fabatin precursor

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mRNA (EU920043)], pSOL9ICE [9.0 kb; *Mesembryanthemum crystallinum* antimicrobial peptide 1 precursor mRNA (AF069321)], pSOL9LEA [9.1 kb; synthetic gene similar to *Leonurus japonicus* antimicrobial protein mRNA; (AY971513)], pSOL9PNA [9.1 kb; synthetic gene similar to *Ipomoea nil* antifungal protein mRNA (U40076)], pSOL9SSP [9.0 kb; synthetic gene for *Aptenodytes patagonicus* Spheniscin-2 (P83430)] and pSOL9VRD [9.0 kb; synthetic gene similar to *Vigna nakashimae* defensin-like protein gene (AY856095)]. As reference examples, the sequences of pSOL8DC3 and pSOL9CAP have been submitted to GenBank (HQ698853 and HQ698852).

### *Plant transformation, regeneration and cultivation*

Transformation of *N. attenuata* was performed as described in Kruegel *et al*. 2002. In brief, hypocotyls from 8- to 10-day-old seedlings were cut into 3-mm-long pieces with a scalpel that previously had been dipped into a culture of *A. tumefaciens* LBA4404 (Invitrogen, http://www.invitrogen.com) carrying the binary plant transformation vector. After 3 days of cocultivation with *Agrobacterium*, the transgenic tissue went through the following regeneration steps on specific phytagel-based media, containing the selective antibiotic hygromycin B (20 g/l) from Duchefa, http://www.duchefa.com (H0192) and the antibacterial antibiotic ticarcillin disodium/clavulanate potassium (125 mg/l) (Duchefa T0190): callus induction (14–21 days), shoot regeneration (14–21 days) and shoot maturation (14–21 days). Subsequently, plantlets were cultured for at least 21 days on rooting medium without both antibiotics. After root regeneration, plants were grown on soil, first in Magenta boxes (http://www.bio-world.com), and finally in 2-litre pots for flowering, self-pollination and seed production in the glasshouse.

### *Flow cytometry*

Flow cytometry was performed with leaf material from *N. attenuata* on a flow cytometer CCA-II (Partec, http://www.partec.com) as described in Bubner *et al*. 2006.

### *Germination of* N. attenuata *and screening for individuals with T-DNA insertions*

Germination of *N. attenuata* was performed as described in Kruegel *et al.* 2002 with the exception that 60 seeds were germinated per plate. If screening for individuals with T-DNA insertions should be performed, the selective antibiotic hygromycin B (Duchefa H0192) was added at a concentration of 35 mg/l to the germination medium. After 10 days, the ratio of seedlings surviving the antibiotic selection was determined.

### *Diagnostic PCRs for integrity of T-DNA insertions*

Genomic DNA (gDNA) was isolated from leaves or seedlings of *N. attenuata* by a modified cetyltrimethylammonium bromide method (Bubner *et al.* 2004). PCR was performed with DreamTaq™ DNA Polymerase (Fermentas, http://www.fermentas.com) according to the instructions of the manufacturer with 1–100 ng of gDNA per sample. The following primer pairs were used: PROM FOR/INT REV and INT FOR/TER REV for the inverted repeat gene silencing constructs; PROM FOR/TER REV for the gene overexpression constructs; and DCL2GF1/DCL2GR1 or GGPP22-22/GGPP23-21 as positive controls amplifying a 334-bp or a 241-bp fragment of *DCL2* (GenBank GU479998) or *ggpps* (GenBank EF382626). Cycles were 5 min 95 °C (30s 95 °C, 30s 55–60 °C, 1 min 72 °C), repeated 30 times, 5 min 72 °C, hold 20 °C. Primer sequences are given in Table S1 (Supporting Information).

### *Southern blotting*

Southern blotting was performed as described in Jassbi *et al.* (2008), with the exception that a 287-bp hygromycin phosphotransferase gene (*hptII*) probe obtained by PCR with primer pair HYG1-18/HYG2-18 (Table S1, Supporting Information) was used. Labelling was performed with the GE Healthcare (http://www.gehealthcare.com) Readyprime DNA labelling system and ProbeQuant g-50 microcolumns according to the instructions of the manufacturer; 7 μg of genomic DNA was digested with restriction enzymes from New England Biolabs (http://www.neb.com) and blotted onto a nylon membrane (GeneScreenPlus; PerkinElmer, http://www.perkinelmer.com) according to the manufacturer's protocol.

### *qPCR*

Plant material was ground in liquid nitrogen with mortar and pestle. Total RNA was extracted with TRI reagent™ (SIGMA, http://www.sigmaaldrich.com) according to the manufacturer's instructions. RNA quality was checked on a 1% agarose gel, and concentration was measured spectrophotometrically at 260 nm.

For qPCR analysis, at least three replicated biological samples were used. One microgram of total RNA obtained from each sample was reverse transcribed using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen) for a total volume of 20 μl according to the instructions of the manufacturer. cDNA samples were diluted 1:10 and used for SYBR®Green-based qPCR, carried out on a Stratagene MX3005P™ using qPCR™ Core Kits for SYBR®Green No ROX (Eurogentec, http://www.eurogentec.com) according to the instructions of

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the manufacturer. Analysis of data was carried out according to the comparative Ct ($2^{-\Delta\Delta Ct}$) (Bubner & Baldwin 2004) method or by standard curves (Jassbi *et al.* 2008). The actin cDNA was amplified with primer pair Actin-F1/Actin-R1 (Table S1, Supporting Information) and used as an internal standard for normalizing cDNA concentration variations. For the determination of transcript abundances of the genes of interest, appropriate gene-specific primers were used.

## Results and discussion

Here, we discuss the most efficient protocol to produce genetically modified plants utilizable in ecological research; the workflow that we describe is summarized in Fig. 2.

### *Construction of binary plant transformation vectors*

Binary plant transformation vectors consist of two general regions—one representing the T-DNA, defined by the left and right border repeats, and one with the regions and genes necessary for replication in *Escherichia coli* and *Agrobacterium tumefaciens*. To avoid unwanted side effects and to achieve a high stability of the T-DNA inserts in the plant genome, the T-DNA should only contain the elements necessary for the intended function of the transgene. These elements are the two expression cassettes for

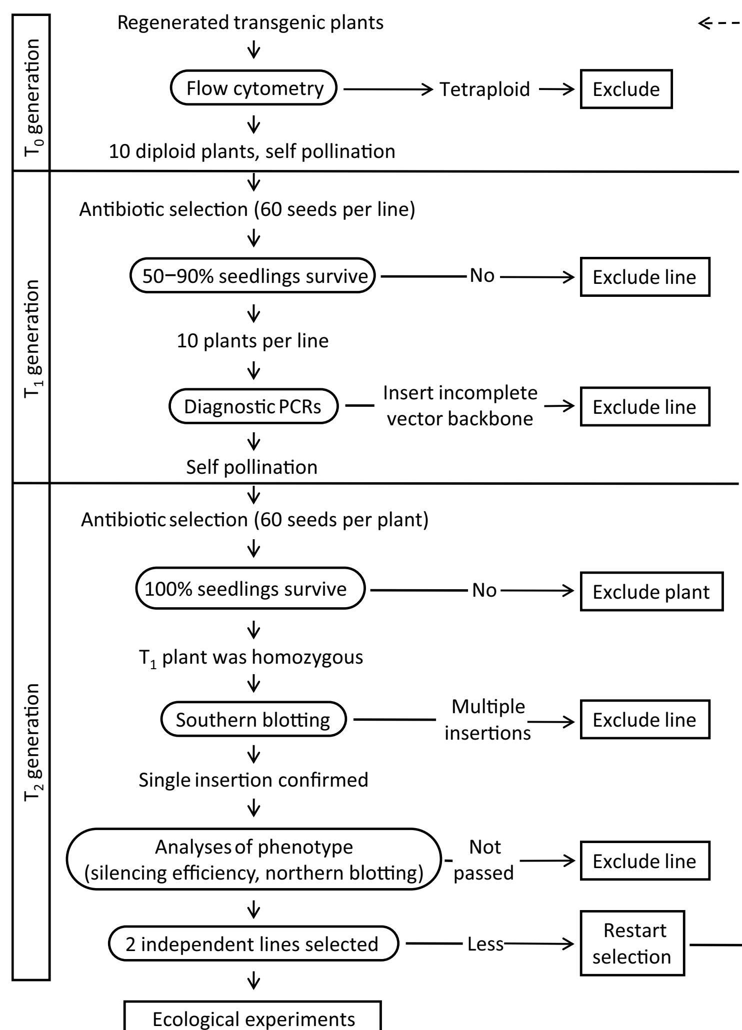


**Fig. 2** Workflow for efficient screening of transgenic lines for ecological research. Each test is performed as early as possible during plant screening to obtain a fast and reliable selection.

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the transgene and the plant selectable marker gene, both containing promoter, transgene sequence and terminator. In gene silencing vectors, the transgene region should consist of an inverted repeat of a partial sequence of the gene to be silenced, separated by a functional intron larger than 100 bp. The presence of this non-repeated sequence allows replication of the plasmid in bacteria despite the long inverted repeat (Warren & Green 1985). Splicing of the intron from the mRNA in the host plant greatly increases the probability that a dsRNA molecule is formed, which in turn efficiently initiates the silencing of the target gene. In our experience, target gene fragments with sizes ranging from 150 bp to about 1 kb can be used for efficient gene silencing. Routinely, our silencing constructs carry inverted repeat fragments of about 300 bp. If a member of a gene family or a certain allele should be silenced, the choice of the gene sequence requires special consideration. Nucleic acid homology of 23 nt is sufficient to direct post-transcriptional silencing of a gene (Thomas *et al.* 2001). To silence a single gene, sequence homology of more than 22 nt should be avoided. To silence a gene family, a sequence with homologies of more than 22 nt should be chosen. To enhance transgene stability, the use of different promoters and terminators, e.g. promoter/terminator of the nopaline synthase gene from the Ti plasmid of *A. tumefaciens* and 35S promoter/terminator from the cauliflower mosaic virus, on the same T-DNA is advisable.

The bacterial part of the binary transformation vector should contain an origin of replication functional in *E. coli*, e.g. from the ColE1 plasmid (construction of the binary vectors is performed in *E. coli*), an origin of replication and the genes that are necessary for plasmid replication in *A. tumefaciens*, e.g. from plasmid pVS1 and an antibiotic resistance marker both selectable in *E. coli* and *A. tumefaciens*. Because T-DNA border 'read-through' events are quite common during T-DNA integration, the use of a bacterial resistance gene that is already widely spread in nature, e.g. the *nptII* kanamycin resistance gene, is advisable.

Examples for binary plant transformation vectors constructed according to the principles described above are the pSOL8 gene silencing series (Fig. 1a) and the pSOL9 gene overexpression series (Fig. 1b), both extensively used to transform *N. attenuata*.

### *Transformation and regeneration*

*Agrobacterium*-based transformation is the preferred method to create transgenic plants for ecological research. After transformation, the regeneration of differentiated plants from the transformed cells is necessary. Establishing transformation and regeneration procedures is probably the most challenging step in the utilization of transgenic approaches for non-model plants. Published transformation and regeneration procedures [as examples see *N. tabacum* (Horsch *et al.* 1985; Gallois & Marinho 1995), *Beta vulgaris* (Lindsey & Gallois 1990), *A. thaliana* (Valvekens *et al.* 1988; Clough & Bent 1998) and *Hordeum vulgare* (Tingay *et al.* 1997)] suggest that protocols specifically adapted for each species, and sometimes for each cultivar of each species (Valvekens *et al.* 1988) to be transformed, need to be worked out. With considerable effort, including as many as 10 people-years, we developed a transformation and regeneration procedure for *N. attenuata* (described in the 'Materials and methods' section). Because of different media and hormone requirements of this organism, established procedures from other closely related species like *N. tabacum* could not be applied.

### *Determination of ploidy level*

One of the heritable somoclonal variations that may occur during tissue culture is autopolyploidy (Bubner *et al.* 2006). The extent of polyploidization can be substantial: In diploid tomato, 24.5–80% of transformants were found to be tetraploid [depending on cultivar and method; (Ellul *et al.* 2003)], and up to 92% of originally triploid bermuda grass *Cynodon dactylon* × *transvaalensis* cv. Tif Eagle transformants were found to be hexaploid (Goldman *et al.* 2004). To ensure the comparability and the relevance of the results obtained from experiments with transgenic plants and control plants, it is essential that only transgenic plants with the same ploidy level as the plant that has been transformed are selected. The ploidy level of the first transgenic generation ($T_0$) is preserved in all following generations produced by self-pollination. Ploidy-level determination of the lines of this generation should be the first step in selecting transgenic lines. This approach allows the early elimination of unwanted lines and saves resources.

The most efficient and conclusive method of determining ploidy levels is by flow cytometry. Because $T_0$ plants may be chimeric, material from leaves close to the flowers is determined to obtain results indicative of the ploidy level of the $T_1$ generation. Ideally, 10 independent transgenic lines with the correct ploidy level should be identified from this step in the screening process. According to our experience with *N. attenuata*, plants with doubled sets of chromosomes can often be visually distinguished from plants with the original set of chromosomes, as these plants commonly have 10% broader leaves and larger trichomes, stomates, flowers and seeds. They produce fewer seeds (20–40 vs. 100–200 seeds per capsule in diploid individuals) and have abnormal growth forms. Critical assessment of the phenotypes of the $T_0$ plants with respect to measured ploidy values has

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in our experience greatly facilitated reliable elimination of tetraploid plants.

The ploidy analyses performed with transgenic *N. attenuata* lines from transformation experiments with more than 200 different constructs document that the frequency of occurrence of tetraploid plants substantially varies depending on the construct used for transformation. This effect occurred with all inbred lines (6th to the 30th inbred generation) used as starting material for the transformation. An example for the frequency of occurrence of tetraploid plants is shown in Fig. 3. Plants from the 30th inbred generation of a genotype collected from Utah (Glawe *et al.* 2003) were transformed with a series of binary vectors (pSOL9CAP, pSOL9ESC, pSOL9FAB, pSOL9ICE, pSOL9LEA, pSOL9PNA, pSOL9SSP and pSOL9VRD), all ectopically overexpressing heterologous genes coding for proteins with antimicrobial activities. The ploidy levels of plants from 11 to 25 independent $T_0$ lines per construct were measured using flow cytometry. Depending on the transgene, 0–57% of the transgenic plants were tetraploid. All together, 159 plants were analysed, 37% were tetraploid and 63% diploid. The increase in the portion of tetraploid plant lines occurring after transformation with particular constructs may require that a larger number of transgenic lines need to be tested to obtain the desired number of diploid lines.

### *Screening for homozygous lines with single-copy T-DNA*

Transgenic plant lines used for ecological research should carry a single T-DNA copy in a single transgenic locus. We have found that a single copy is sufficient for the expected phenotypic effect (gene silencing or overexpression). To avoid segregation, these lines should be homozygous with respect to the T-DNA insertion. Multiple independent transgenic loci would dramatically increase the effort required to generate and identify non-segregating, homozygous lines. In the case of overexpression, multiple T-DNA copies or even strong promoters of a single T-DNA copy may lead to transgene mRNA concentrations above a critical level and elicitation of silencing of the overexpressed gene (Lechtenberg *et al.* 2003; Eamens *et al.* 2008; Hirai *et al.* 2010).

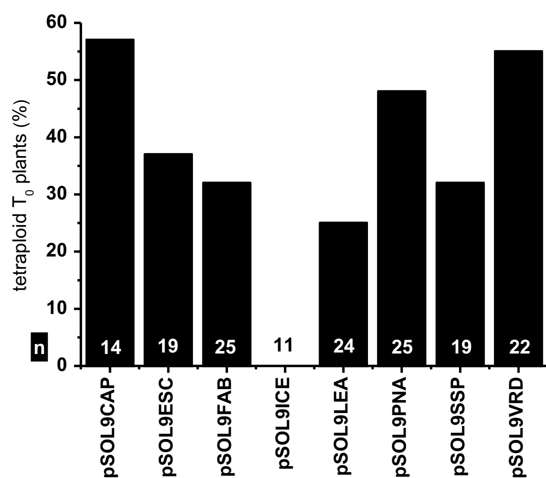


**Fig. 3** The fraction of tetraploid *Nicotiana attenuata* plants after transformation of the 30th Utah inbred line with pSOL9 vectors. The numbers of tested plants are indicated.

Transgenic plants with two or more independent transgenic loci can generally be identified by inheritance studies (Vain & Thole 2009). Non-independent transgenic loci (T-DNA insertions at a distance smaller than 50 cM on the same chromosome) and complex insertions into one locus need to be identified by Southern analysis (protocol section 7) at later stages in the screening process. Any T-DNA insertion into $T_0$ plants will usually be hemizygous, because the probability of simultaneous insertions into the same locus of two homologous chromosomes is very low.

To produce homozygous lines with respect to a transgenic locus, self-pollination of $T_0$ plants is required. Screening for T-DNA insertions can be performed most efficiently by growing seedlings on medium containing the selective antibiotic. Only plants carrying the T-DNA and expressing the resistance marker gene will grow. The expected Mendelian inheritance ratio for a single transgenic locus in the $T_1$ offspring should be 1 (homozygous) to 2 (hemizygous) to 1 (wild type); thus, 75% of the offspring would carry the T-DNA. A second independent transgenic locus would lead to a ratio of 15 (any transgenic locus) to 1 (wild type), and 93.75% of the offspring would carry the T-DNA. Additional transgenic loci would further increase this portion.

If the ratio of transgenic vs. non-transgenic seedlings is lower than expected (<50%), silencing of the selectable marker gene may be the reason. In this case, and only if a simultaneous silencing of the transgene can be excluded, screening for the selectable marker gene could be performed by PCR genotyping of seedlings grown on non-selective medium. To assess the extent to which silencing of the selectable marker gene occurs during the screening process, the $T_1$, $T_2$ and $T_3$ inbred generations of the *N. attenuata* pSOL9 overexpression lines were analysed (Fig. 4). The initial number of $T_1$ lines was 10–12 depending on the construct. Because according to our observations (data not shown) silencing of the selectable marker gene is associated with silencing of the transgene and will, once initiated, reoccur in the following generations, all plant lines exhibiting signs of silencing were excluded from further screening. Silencing of the resistance gene

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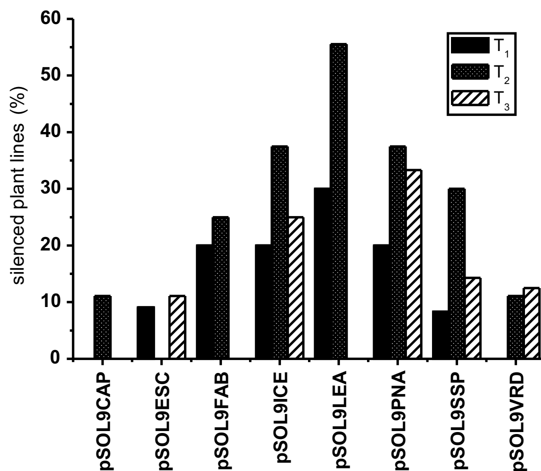


**Fig. 4** Portion of lines in which silencing of the *hpt*II gene occurred during different stages of screening ($T_1$, $T_2$ or $T_3$ generation) as detected by hygromycin sensitivity. The initial number of $T_1$ lines was 10–12 depending on the construct. Plants in $T_1$ and $T_2$ stage were considered as silenced if more than 50% of seedlings were hygromycin sensitive. Homozygous $T_3$ plants were considered as silenced if segregation occured.

occurred unpredictably and irregularly. The mean portion of silenced lines was 13.4% for $T_1$, 26.0% for $T_2$ and 12.0% for $T_3$ generations. Gene silencing in sense overexpression lines is most likely caused by promoter methylation (Weber *et al.* 1990; Stam *et al.* 1997). This form of epigenetic regulation can occur in each offspring generation of a previously unsilenced line. To guarantee a high expression level of the transgene, the functionality of the resistance marker gene should be examined for each new generation by germination on medium containing the selective antibiotic.

T-DNA insertions may interrupt genes essential for embryo or gametophyte development, thus leading to embryo- (Errampalli *et al.* 1991) or gametophyte-lethal lines (Feldmann *et al.* 1997; Howden *et al.* 1998). This will result in exceptional segregation, characterized by progeny segregating for fewer T-DNA carrying seedlings than predicted by Mendelian principles. Exceptional segregation may occur in about 9% of the transgenic lines (Feldmann *et al.* 1997) and should be accounted for in the segregation analyses.

The inheritance-based screening of transgenic plant lines is always performed via self-pollination and germination of 60 seedlings per plant. In the first step, the $T_1$ generation seeds from 10 independent $T_0$ lines with the correct ploidy level are germinated. A $T_0$ plant is considered an appropriate candidate line for carrying a single T-DNA insertion if 50–90% (75% calculated) of the $T_1$ offspring are transgenic. More transgenic seedlings point to multiple T-DNA integrations, while fewer indicate gene silencing or exceptional segregation.

In the second screening step, $T_2$ seeds from 10 $T_1$ plants of each single T-DNA insertion candidate line are germinated. If all $T_2$ seedlings from a $T_1$ plant carry the T-DNA, this plant represents the desired homozygous genotype of the respective line and can be used for further screening (Fig. 2). Plants with extreme, unexpected phenotypes should not be used for seed production.

At this point, it should be mentioned that because of extremely long generation times or self-incompatibility in some ecologically interesting systems (e.g. trees), self-pollination cannot be applied in the screening process. Each system may present its specific challenges, which have to be overcome in a specific way, for instance, by deeper genetic analyses of the $T_0$ generation.

### *Confirmation of complete T-DNA integrations using PCR*

Strand breaks followed by deletions, inversions and rearrangements at the T-DNA flanking regions are some of the events during *Agrobacterium*-mediated T-DNA integration that may result in non-functional transgenes (Latham *et al.* 2006; Muller *et al.* 2007; Gambino *et al.* 2009). Selection for non-functional integrations of the transgene occurs when a plant is transformed with a transgene the product of which interferes with plant regeneration. This process leads to a dramatically reduced transformation efficiency. When this occurs, the regenerated lines often carry large deletions of the T-DNA flank containing the transgene, in our experience with *N. attenuata*. In contrast, lines that carry no functional resistance marker are eliminated by selection for antibiotic resistance during tissue culture.

It is essential to demonstrate that each candidate line that should be used in further screening carries a complete, functional T-DNA. Antibiotic selection guarantees the intactness of the T-DNA flank harbouring the selectable marker gene, but the integrity of the T-DNA transgene flank still has to be demonstrated. This is efficiently carried out with a diagnostic PCR-based analysis of the $T_1$ genome from which selected fragments of the transgene are amplified. The presence of a PCR product at the expected size provides strong evidence of a complete T-DNA insertion. Lines that do not yield the expected PCR product carry an incorrect transgene, most likely a deletion of the region to be amplified, and should be excluded from further screening. The results of this analysis are only informative for plants with single T-DNA insertions, because in plants with multiple insertions, a positive result does not exclude the presence of an additional defective transgene. If appropriate, this test can already be performed on $T_0$ plants to exclude lines

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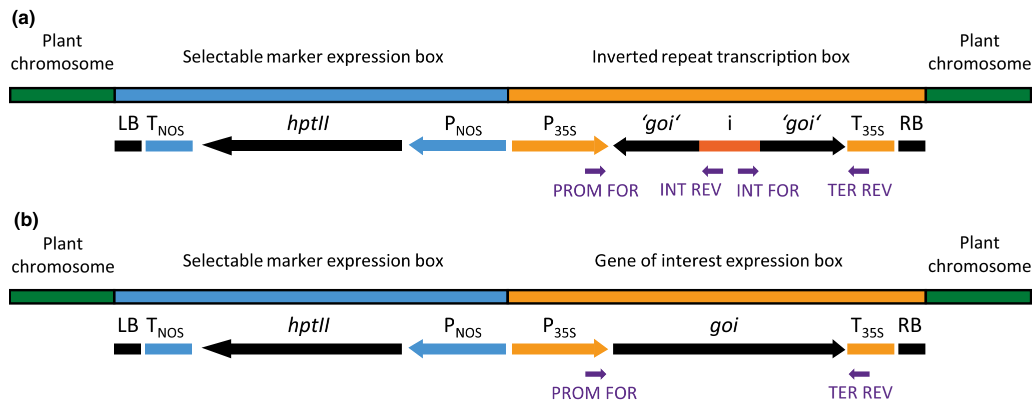


**Fig. 5** The positions of annealing sites of the diagnostic PCR primers at the T-DNA integrated into the plant genome: (a) inverted repeat silencing vectors, (b) overexpression vectors. Abbreviations: see Fig. 1.

without functional T-DNA insertions or on plants from later generations to confirm the presence of a correctly inserted transgene.

To test whether the transgene expression cassettes of different *N. attenuata* lines transformed with pSOL8 or pSOL9 constructs were integrated completely, we designed four universal primers that bind to the cassettes' functional elements (Fig. 5): PROM FOR (35S promoter), INT REV (*pdk*-intron 5′-end) and INT FOR (*pdk*-intron 3′-end) TER REV (35S terminator). The *N. attenuata* silencing lines were analysed with primer pairs PROM FOR/INT REV (amplifying the transgene 5′ copy) and INT FOR/TER REV (amplifying the transgene 3′ copy adjacent to the right T-DNA border). These allow for the amplification of separate fragments for each transgene copy, thus avoiding low PCR efficiency because of amplification of an inverted repeat. For the analyses of the transgenic *N. attenuata* lines that were transformed with pSOL9 overexpression constructs, we used primer pair PROM FOR/TER REV, amplifying the DNA between promoter and terminator of the transgene expression box.

Examples for diagnostic PCR are shown in Fig. 6. The results of all diagnostic PCR performed with 24 transgenic *N. attenuata* lines transformed with the inverted repeat silencing constructs pSOL8DC3, pSOL8PNRP, pSOL8AEP65 and pSOL8AEP150 are summarized in Table S2 (Supporting Information). The rate of incomplete insertions varied between 0 and 60% depending on the transgene construct. Any transgenic line yielding a negative PCR result for the amplification of the 5′ transgene copy always yielded the same result for the 3′ transgene copy, whereas lines with the combination negative PCR result for the 3′ transgene copy and positive result for the 5′ transgene copy were found. These results demonstrate that deletions at the left border T-DNA flank

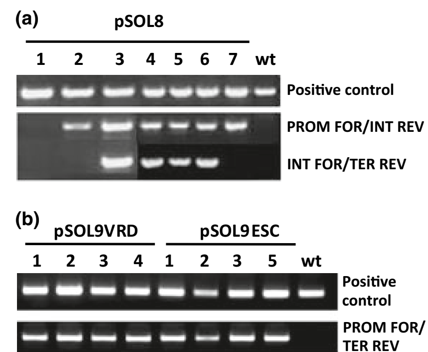


**Fig. 6** Diagnostic PCRs with chromosomal DNA from transgenic *Nicotiana attenuata* plants transformed with (a) pSOL8 inverted repeat vectors (1–3: pSOL8DC3; 4: pSOL8PNRP; 5: pSOL8AEP65; 6: pSOL8AEP150: 7 pSOL8PNRP; wt: wild type) and (b) pSOL9 overexpression vectors (lines indicated). Primer pairs are shown.

are common in *N. attenuata*. The highest rates of incomplete insertions were found in plants transformed with pSOL8PNRP and pSOL8DC3 for the silencing of the PNRP gene and for the combined silencing of the RdR1 and WRKY3 genes. We interpret this result as being consistent with selection for incomplete insertions when the product of a transgene is detrimental to the plant.

### *Confirming the lack of vector backbone*

The absence of binary vector backbone sequences in the genome is an important quality criterion established by many regulatory agencies for plants that are to be used in field releases. Demonstrating that a transgenic line does not contain vector sequences outside the T-DNA can be done by a PCR-based approach or—parallel to the

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determination of T-DNA copy number described in the following chapter—by Southern analysis. Because vector backbone integration often occurs after a T-DNA border 'read-through', the lack of PCR products from primer pairs designed for the amplification of the vector backbone adjacent to the T-DNA borders demonstrates the absence of vector backbone. For Southern analysis, these primer pairs are used to amplify probes from the transformation vector. Genomic DNA of plants, which does not allow the detection of a DNA fragment with these probes, is considered to contain no vector backbone. If the regulatory agencies require that the absence of critical vector regions, such as the bacterial antibiotic resistance gene or plasmid origins of replication, is to be explicitly demonstrated, primers for these regions can be designed and used in the PCR analyses accordingly. All transgenic lines that contain unwanted vector sequences should be eliminated.

A recent study (Oltmanns *et al.* 2010) demonstrated that starting plant transformation from the *A. tumefaciens* chromosome could be a way to reduce the portion of transgenic plant lines with vector backbone integrations dramatically, but so far, this procedure to produce *Agrobacterium* plant transformation strains is laborious and not applicable to the widely used strain *A. tumefaciens* LBA4404.

### *Determination of T-DNA copy number by Southern analysis*

Multiple transgenic loci and complex T-DNA insertions into one locus present in the genome of transgenic plant lines can, in most cases, be reliably detected by Southern analysis. To confirm single T-DNA insertions in lines selected so far, the probe should be identical to a part of the antibiotic resistance marker gene. This gene does not exist in the wild-type plant genome, but should be present in the genome of all transgenic plants as a consequence of antibiotic selection during regeneration. Moreover, this allows the same probe for the screening of different transgenic plant lines generated with T-DNA from different binary vectors to be used, as long as the selectable marker gene is the same.

For Southern analysis, chromosomal DNA from two or more homozygous $T_2$ generation individuals of each transgenic line that passed all previous screening tests is completely digested in separate reactions with at least two different restriction enzymes. These enzymes must not cut the T-DNA on both sides or inside the probe sequence, but in order to detect multiple insertions into a single transgenic locus and to reduce the expected size of the genomic fragments carrying this sequence, the T-DNA should be cut once. Under these conditions, all transgenic lines that yield in the Southern analysis multiple bands with any of the restriction enzymes harbour multiple transgenic loci or multiple T-DNA insertions at one locus. Incomplete T-DNA insertions or unwanted sequence rearrangements of the transgenic locus are indicated when fragments smaller than the minimal possible T-DNA size calculated from T-DNA borders and the restriction sites on it are detected. Single bands equal to or larger than the calculated minimal size obtained with all used restriction enzymes are indicative of a single T-DNA insertion. Transgenic lines yielding this pattern are chosen for further screening. An example for a Southern blot of each four independent lines from three different constructs is shown in Fig. 7. However, the existence of additional T-DNA fragments in the genome of the chosen lines, not detectable with the selected probe, cannot be definitively excluded.

### *Confirmation of transgene function*

Before a transgenic line that has been demonstrated to harbour a correct single T-DNA insertion in homozygous stage can be used in ecological experiments, the function of the transgene should be confirmed on the level of RNA. For overexpression lines, the mRNA abundance of the transgene is quantified by qPCR. Choosing an amplicon comprising the stop codon will provide an additional control that the full-length gene is expressed. For gene silencing lines, the silencing efficiency is determined. The

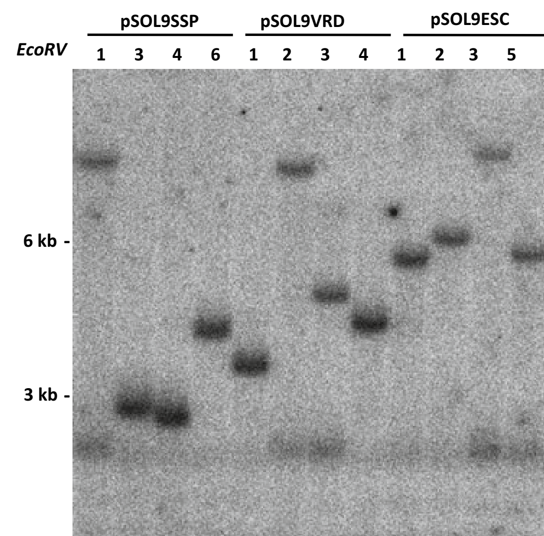


**Fig. 7** Southern blotting with nucleic acid from each four independent transgenic *Nicotiana attenuata* $T_2$ lines transformed with vectors pSOL9SSP, pSOL9VRD or pSOL9ESC. The DNA for the Southern blot was digested with *Eco*RV. A fragment of the marker gene (*hpt*II) served as probe.

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relative transcript abundance of the gene of interest both for the transgenic line and wild type plants is measured by qPCR using an amplicon originating from the target gene outside the inverted repeat. Silencing efficiency is then calculated as 100% minus the ratio (in percentage) of relative transcript abundance in the transgenic line and in wild type plants grown under the same conditions and treated in the same way. In our experience with *N. attenuata*, only lines that allow efficient silencing of the target gene (>20%) are valuable for ecological experiments.

qPCR is a very robust means of determining how efficiently an endogenous plant gene is silenced. Nevertheless, the choice of the intrinsic plant gene that shows constant transcript abundance in all the experiments performed and serves as standard gene for normalization is essential for the reliability of the results obtained with this method (Czechowski *et al*. 2005; Gutierrez *et al*. 2008).

If appropriate, transgene function should be confirmed on a phenotypic level by studying the phenotypes associated with silencing or overexpression of the targeted gene. Depending on the insertion site, the strength of expression of the transgene can vary considerably because of 'position effects' (Prols & Meyer 1992; Matzke & Matzke 1998; *Qin et al*. 2003). Different lines with the same T-DNA insertion may thus have modulated phenotypes with different strengths. This titration of phenotypes can be a powerful means of examining the fitness consequences of a gene. In certain cases, the functional transgene will lead to morphological changes, which may allow a pre-selection during the screening process.

## Conclusions

Reverse genetics is a powerful tool in plant ecology. To take advantage of this tool, transgenic plants that fulfil the requirements for the structure and stable inheritance of the transgene need to be created and selected. The screening of transgenic plants is a costly and time-consuming procedure. We developed a flow chart protocol (Fig. 2) that allows for the efficient production and selection of transgenic plants for ecological research. We encourage groups working in the field of ecology to make use of the resources described in this study, and the authors will be happy to share plasmids, plant lines and experience with all interested groups.

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## Data accessibility

Tables S1 and S2 (Supporting Information): Dryad repository http://dx.doi.org/10.5061/dryad.8951.

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DNA sequences: GenBank accessions GU479998; HQ698849-HQ698853.

## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** Sequences of the primers used for diagnostic PCRs, Southern blotting, and qPCR.

**Table S2** Results of diagnostic PCRs with 24 different transgenic *N. attenuata* lines.

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**Supplemental Table S1** Sequences of the primers used for diagnostic PCRs, Southern blotting, and qPCR.

| Primer Name | Sequence |
|---|---|
| Actin-F1 | 5'-GGTCGTACCACCGGTATTGTG-3' |
| Actin-R1 | 5'-GTCAAGACGGAGAATGGCATG-3' |
| DCL2GF1 | 5'-AAGGATGGCTCATTCCTGGTG-3' |
| DCL2GR1 | 5'-AGAGCTTCAACAAGCAGAGAAGG-3' |
| GGPP22-22 | 5'-GAAGATTCGCGAGGTGTATTGG-3' |
| GGPP23-21 | 5'-CAAGGCAACCAACGGAGCAGC-3' |
| HYG1-18 | 5'-CCGGATCGGACGATTGCG-3' |
| HYG2-18 | 5'-CTGACGGACAATGGCCGC-3' |
| INT FOR | 5'-GGTAACATGATAGATCATGTC-3' |
| INT REV | 5'-CATACTAATTAACATCACTTAAC-3' |
| PROM FOR | 5'-GGAAGTTCATTTCATTTGGAG-3' |
| TER REV | 5'-GCGAAACCCTATAGGAACCC-3' |

**Supplemental Table S2** Results of diagnostic PCRs with 24 different transgenic *N. attenuata* lines.

| Construct | Type | Generation | Number of lines tested | PCR1 negative | PCR2 negative | PCR1 + PCR2 negative* | % Incomplete insertions |
|---|---|---|---|---|---|---|---|
| pSOL8DC3 | ir | $T_1$ | 9 | 2 | 5 | 2 | 56 |
| pSOL8PNRP | ir | $T_3$ | 10 | 2 | 6 | 2 | 60 |
| pSOL8AEP65 | ir | $T_3$ | 2 | 0 | 0 | 0 | 0 |
| pSOL8AEP150 | ir | $T_3$ | 3 | 0 | 0 | 0 | 0 |

PCR1: PROM FOR/INT REV (35S promoter to 5’ intron)

PCR2: INT FOR/TER REV (3’ intron to 35S terminator)

*a negative PCR1 occurred only in lines with negative PCR2

ir: inverted repeat silencing

# 4 Manuscript II

## 4.1. Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants

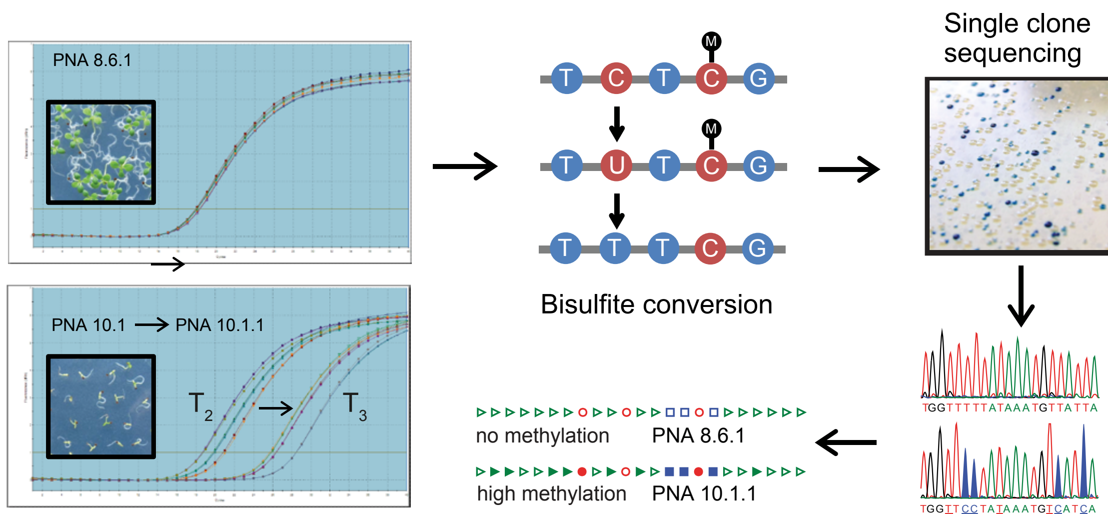


Illustration of the separate steps during bisulfite sequencing for DNA methylation analysis.

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# Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants

Arne Weinhold*, Mario Kallenbach and Ian Thomas Baldwin

## Abstract

**Background:** Genetically modified plants are widely used in agriculture and increasingly in ecological research to enable the selective manipulation of plant traits in the field. Despite their broad usage, many aspects of unwanted transgene silencing throughout plant development are still poorly understood. A transgene can be epigenetically silenced by a process called RNA directed DNA methylation (RdDM), which can be seen as a heritable loss of gene expression. The spontaneous nature of transgene silencing has been widely reported, but patterns of acquirement remain still unclear.

**Results:** Transgenic wild tobacco plants (*Nicotiana attenuata*) expressing heterologous genes coding for antimicrobial peptides displayed an erratic and variable occurrence of transgene silencing. We focused on three independently transformed lines (PNA 1.2, PNA 10.1 and ICE 4.4) as they rapidly lost the expression of the resistance marker and down-regulated transgene expression by more than 200 fold after only one plant generation. Bisulfite sequencing indicated hypermethylation within the 35S and NOS promoters of these lines. To shed light on the progress of methylation establishment, we successively sampled leaf tissues from different stages during plant development and found a rapid increase in 35S promoter methylation during vegetative growth (up to 77% absolute increase within 45 days of growth). The levels of *de novo* methylation were inherited by the offspring without any visible discontinuation. A secondary callus regeneration step could interfere with the establishment of gene silencing and we found successfully restored transgene expression in the offspring of several regenerants.

**Conclusions:** The unpredictability of the gene silencing process requires a thorough selection and early detection of unstable plant lines. *De novo* methylation of the transgenes was acquired solely during vegetative development and did not require a generational change for its establishment or enhancement. A secondary callus regeneration step provides a convenient way to rescue transgene expression without causing undesirable morphological effects, which is essential for experiments that use transformed plants in the analysis of ecologically important traits.

## Background

Transgenic plants have become an essential component in ecological research, allowing the precise study of gene functions under field conditions [1-3]. Despite progress in the development of more efficient transformation techniques, the unpredictable and stochastic occurrence of transgene silencing and epigenetic alternations after the tissue culture step remain unsolved problems for most plant species [4-7]. Basically two forms of gene silencing have been described, transcriptional gene silencing (TGS), in which gene expression is directly blocked, and posttranscriptional gene silencing (PTGS) in which mRNA is degraded [8]. PTGS has been exploited as a very powerful tool for reverse genetic studies and is revolutionizing plant ecology, particularly for non-model plants, where the introduction of "silencing-constructs" in self-compatible inverted repeat (IR) or antisense (AS) orientations enables the targeted silencing of endogenous genes *in trans* [9-12]. Unfortunately, this process can be undermined by unwanted TGS, if the promoter of the transgene is *de novo* methylated, thereby diminishing the expression of the silencing-construct [13-17]. *De novo* DNA methylation can be highly sequence-specific for a transgene, as a result

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of the process called RNA-directed DNA methylation (RdDM) [17-20]. However, the pattern of establishment and prerequisites for the methylation process remain elusive [21]. Characteristic symptoms of unwanted transgene silencing are spatially variegated or transient gene expression levels, patterns which have been observed in several different plant taxa including *Nicotiana tabacum* [22-24], *Petunia hybrida* [25,26], *Arabidopsis thaliana* [27,28], *Gentiana triflora* X *G. scabra* [29] and even in some transgenic woody plants such as grapevine (*Vitis* spp.) [30] and birch trees (*Betula platyphylla*) [31].

The wild tobacco (*N. attenuata* Torr. ex S. Watson) is an annual plant, native to the Great Basin Desert in the western United States and is used as a model organism to study traits important for survival under real world conditions, in particular the role of jasmonic acid (JA) in plant defense against herbivores [32]. *N. attenuata* has been frequently transformed with many different sense-expression, inverted repeat (IR) or antisense (AS) silencing-constructs to manipulate different layers of plant defense for field studies of gene function [1,33-37]. A stably transformed plant is only useful for ecological experiments if the transgene-altered phenotype remains stable over the entire period of plant development. In the glasshouse the life cycle of *N. attenuata* takes about 70–80 days until the plant produces seeds and develops from a vegetative rosette-stage, through stalk elongation, into the generative flowering phase. Over the course of development the plant reconfigures its defense strategy from largely inducible to constitutive deployment of various jasmonate-mediated chemical defenses [38]. Transgenerational phenotypic stability is also essential if different lines are to be crossed to combine traits so that parental phenotypes can be faithfully transmitted in a hemizygous state to the subsequent hybrid generations. The *N. attenuata* line ir-ACX1 was created to suppress a particular step in the JA biosynthesis pathway due to the silencing of the endogenous *acetyl-CoA-transferase* 1 (*acx*1), but as recently shown the ability to suppress JA accumulation was lost when $T_3$ generation plants were used during a field experiment [37]. Similar findings of leaky or lost phenotypes in *N. attenuata* lines have been reported in other studies [34,36] highlighting the importance of the early detection of "unstable" plant lines.

The methylated form of cytosine was discovered more than 60 years ago [39], but despite the very high amounts found in wheat seedlings, it was long considered only as a "minor base" in plant genomes [40]. Its importance in epigenetic gene regulation is increasingly being recognized, but the overall process remains poorly understood [41-44]. If a genomic sequence functions as a promoter, *de novo* methylation can lead to transcriptional silencing of the downstream gene [45,46]. Cytosine methylation plays an important role in many cellular processes such as tissue-specific gene expression, embryogenesis or genomic imprinting [47]. Nevertheless, its generally accepted main function in plants is in the control of "invasive elements" such as transposons or viral sequences [48-50]. In contrast to mammals, plants not only methylate cytosines in CG dinucleotides, but also in all other possible sequence contexts at CHG and CHH positions (where H = A,T or C) [46,51]. The symmetric DNA methylation patterns at CG and CHG sites can be sustained during semiconservative DNA replication and are therefore somatically and even meiotically stable [52,53]. A methylation at the CHH position is called asymmetric, because it has no mirror position on the complementary DNA strand and hence will be lost during the DNA replication process. For maintenance during mitosis, an asymmetric site needs therefore a constant signal as a permanent *de novo* methylation trigger [18,45,46,54]. Although most aspects of epigenetic inheritance are understood, somatic cells are considered to be relative static and the principles of methylation establishment in vegetative grown plants remain unclear [21,46,55-57].

The aim of this study was to illuminate the timing of the transgene inactivation process and to summarize our strategy for an optimized selection of *N. attenuata* plants with desired, stable phenotypes in a set of antimicrobial peptide expressing lines. Since a combination of TGS and PTGS effects can lead to a progressive shutdown of transgene expression [15,58-60], we were interested in finding early indicators and methods to avoid or even predict unwanted transgene silencing in the wild tobacco *N. attenuata.*

## Results

### Non-Mendelian segregation of the resistance marker as the first indicator of transgene silencing

To be able to work with *N. attenuata* lines that constitutively express antimicrobial peptides under a 35S promoter, we created eleven different transformation vectors containing eleven different antimicrobial peptide genes (see Methods section for details). From each construct more than 10 independent transformed plant lines were created and in total the segregation data of 113 plant lines were observed over three generations of inbreeding. For a high probability in selecting stable expressing plant lines, we used the optimized screening protocol described in Gase *et al.* [61]. This includes the use of flow cytometry (for ploidy analysis), diagnostic PCRs (to confirm completeness of the insert), qRT-PCR (for gene expression analysis) and southern blotting (for detection of insertion number). The segregation analysis of the resistant marker provides not only information about zygosity but can additionally reveal independent

segregating loci and the occurrence of unwanted transgene silencing very early in the screening process. A regenerated $T_0$ plant should ideally harbor only one T-DNA copy in a single locus, which is usually inherited as a simple, dominant Mendelian trait. According to Mendel's law of independent assortment [62], offspring derived from self-pollination would show an expected 3:1 ratio, with 25% of the seedlings sensitive to hygromycin. From our segregation data of 113 independently transformed *N. attenuata* lines most of the seedlings showed hygromycin sensitivity in the calculated range (Figure 1). We considered all seedlings with 10–50% sensitivity as being offspring from a hemizygous mother plant and selected only these for further inbreeding (Figure 1A indicated in black). Epigenetic mechanisms could lead to deviations from Mendelian segregation ratios and all seedlings with unusual high numbers of sensitivity (>50%) were therefore excluded from further screening [61]. In the second generation ($T_2$), we usually seek seedlings with 0% sensitivity, indicating that they originated from a homozygous plant (Figure 1B). As a selection criterion, all sibling plants of the same line should not deviate from any of the expected ratios and show also 0% or 10–50% sensitivity. The occurrence of a single plant with non-Mendelian segregation (>50%) would lead to an exclusion of the entire transgenic line. In the $T_3$ generation, the progenies from the homozygous plants were again tested for stability and any newly occurring segregation led to the exclusion of the line (Figure 1C).

The majority of seedlings of the more than 1200 analyzed plants segregated within the expected ranges, nevertheless 12 of 113 lines (11%) were excluded in the $T_1$ stage, 22 of 94 (23%) in the $T_2$ stage and 15 of 70 (21%) in the $T_3$ stage, due to non-Mendelian segregation patterns. Altogether 43% of the antimicrobial peptide expressing *N. attenuata* lines were excluded for this reason. The $T_3$ seedlings from three independent lines (PNA 1.2, PNA 10.1 and ICE 4.4.) indicated nearly a complete loss of resistance, with sensitivity rates comparable to wild-type seedlings. Because of this drastic and uniform switch within only one plant generation, these three lines provided the opportunity to further investigate the otherwise unpredictable occurrence of gene silencing.

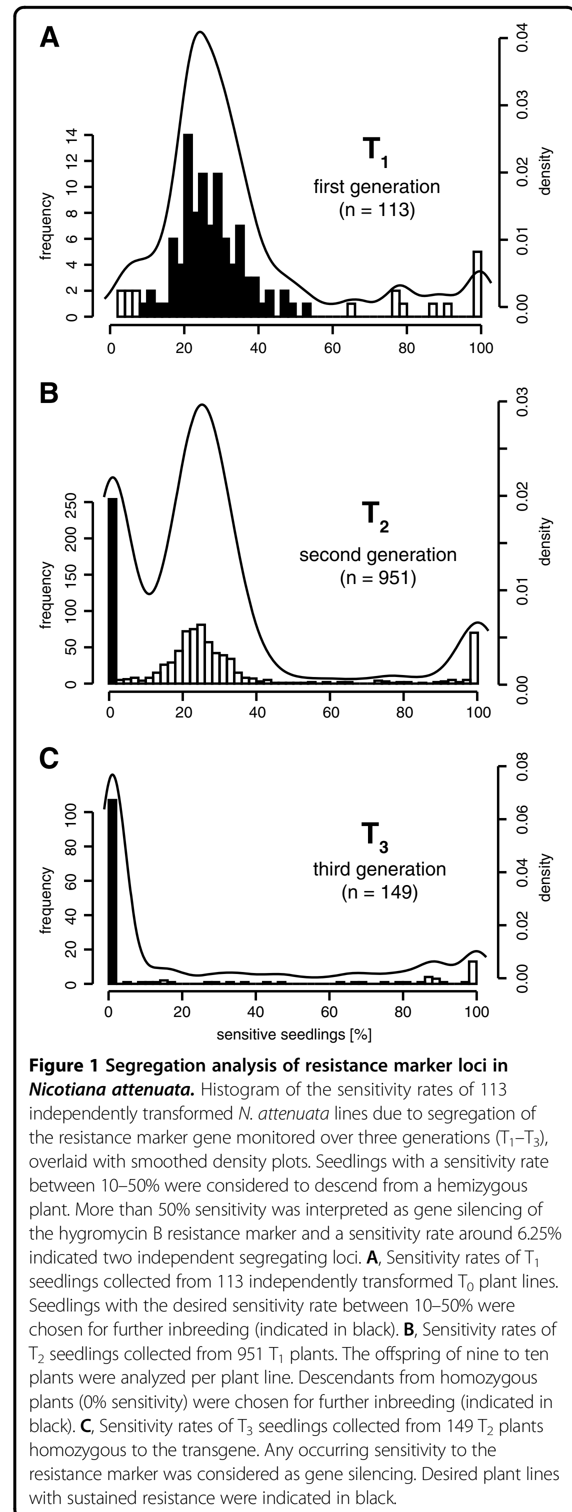


**Figure 1 Segregation analysis of resistance marker loci in *Nicotiana attenuata*.** Histogram of the sensitivity rates of 113 independently transformed *N. attenuata* lines due to segregation of the resistance marker gene monitored over three generations ($T_1$–$T_3$), overlaid with smoothed density plots. Seedlings with a sensitivity rate between 10–50% were considered to descend from a hemizygous plant. More than 50% sensitivity was interpreted as gene silencing of the hygromycin B resistance marker and a sensitivity rate around 6.25% indicated two independent segregating loci. **A**, Sensitivity rates of $T_1$ seedlings collected from 113 independently transformed $T_0$ plant lines. Seedlings with the desired sensitivity rate between 10–50% were chosen for further inbreeding (indicated in black). **B**, Sensitivity rates of $T_2$ seedlings collected from 951 $T_1$ plants. The offspring of nine to ten plants were analyzed per plant line. Descendants from homozygous plants (0% sensitivity) were chosen for further inbreeding (indicated in black). **C**, Sensitivity rates of $T_3$ seedlings collected from 149 $T_2$ plants homozygous to the transgene. Any occurring sensitivity to the resistance marker was considered as gene silencing. Desired plant lines with sustained resistance were indicated in black.

### Variability in transgene expression precedes loss of resistance

To select appropriate transgenic lines with high levels of transgene expression, we routinely analyze homozygous $T_2$ plants by qRT-PCR during the screening process. As an example, we show the transgene expression profiles for three antimicrobial peptides (PNA, ICE and FAB) in 14 independently transformed *N. attenuata* lines (see

Methods section for details). Several plant lines showed the desired high and uniform levels of gene expression, whereas others showed low or variable gene expression levels among independently transformed lines expressing the same constructs (Figure 2A). The offspring of these plants was tested on hygromycin containing media, to confirm enduring resistance within the $T_3$ seedlings (Figure 2B). Of particular interest were lines ICE 4.4, PNA 1.2 and PNA 10.1, because they nearly completely lost hygromycin resistance in the $T_3$ generation and before this, showed even variable expression of the antimicrobial peptide genes in the $T_2$ (Figure 2).

To analyze how much a complete loss of resistance correlates with the downregulation of the neighboring transgene, we compared expression levels in both generations ($T_2$ and $T_3$) from lines PNA 1.2, PNA 10.1 and ICE 4.4. To ensure similar growth conditions, the germination was performed on hygromycin-free media and lines showing stable transgene expression (PNA 8.6.1 and ICE 1.1.1) were included as positive controls. The expression analysis again revealed a very high plant-to-plant variability in the $T_2$ stage (Figure 3A), but also a very strong down-regulation of gene expression in the $T_3$ generation. This was consistent with the observed loss of hygromycin resistance (Figure 3B). Comparing the $T_2$ and $T_3$ stage, plants of line ICE 4.4.1 showed a 41 fold (10–73), lines PNA 1.2.1 a 268 fold (63–472) and lines PNA 10.1.1 a 210 fold (51–370) reduced transgene expression, respectively (Figure 3A, Additional file 1). Compared to the stable expressing control lines (PNA 8.6.1 and ICE 1.1.1), the results of the transgene

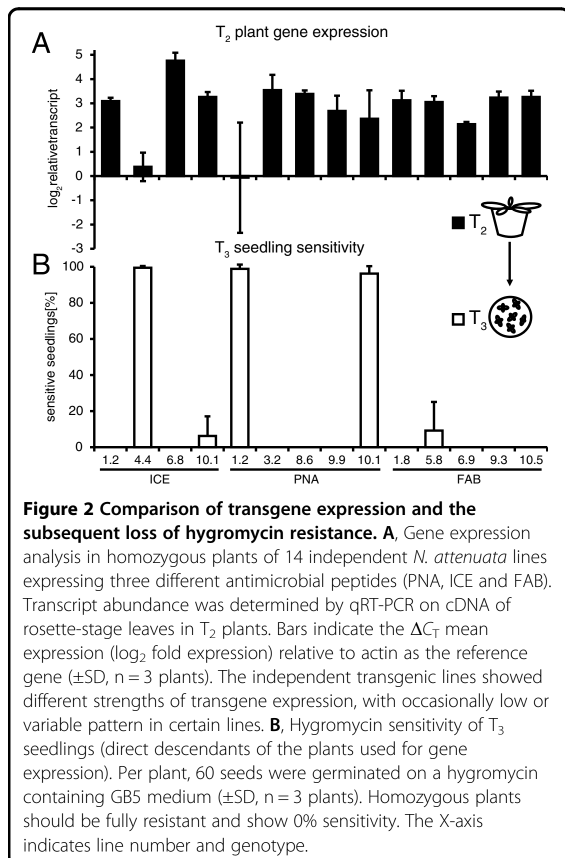


**Figure 2 Comparison of transgene expression and the subsequent loss of hygromycin resistance. A**, Gene expression analysis in homozygous plants of 14 independent *N. attenuata* lines expressing three different antimicrobial peptides (PNA, ICE and FAB). Transcript abundance was determined by qRT-PCR on cDNA of rosette-stage leaves in $T_2$ plants. Bars indicate the $\Delta C_T$ mean expression ($log_2$ fold expression) relative to actin as the reference gene (±SD, n = 3 plants). The independent transgenic lines showed different strengths of transgene expression, with occasionally low or variable pattern in certain lines. **B**, Hygromycin sensitivity of $T_3$ seedlings (direct descendants of the plants used for gene expression). Per plant, 60 seeds were germinated on a hygromycin containing GB5 medium (±SD, n = 3 plants). Homozygous plants should be fully resistant and show 0% sensitivity. The X-axis indicates line number and genotype.

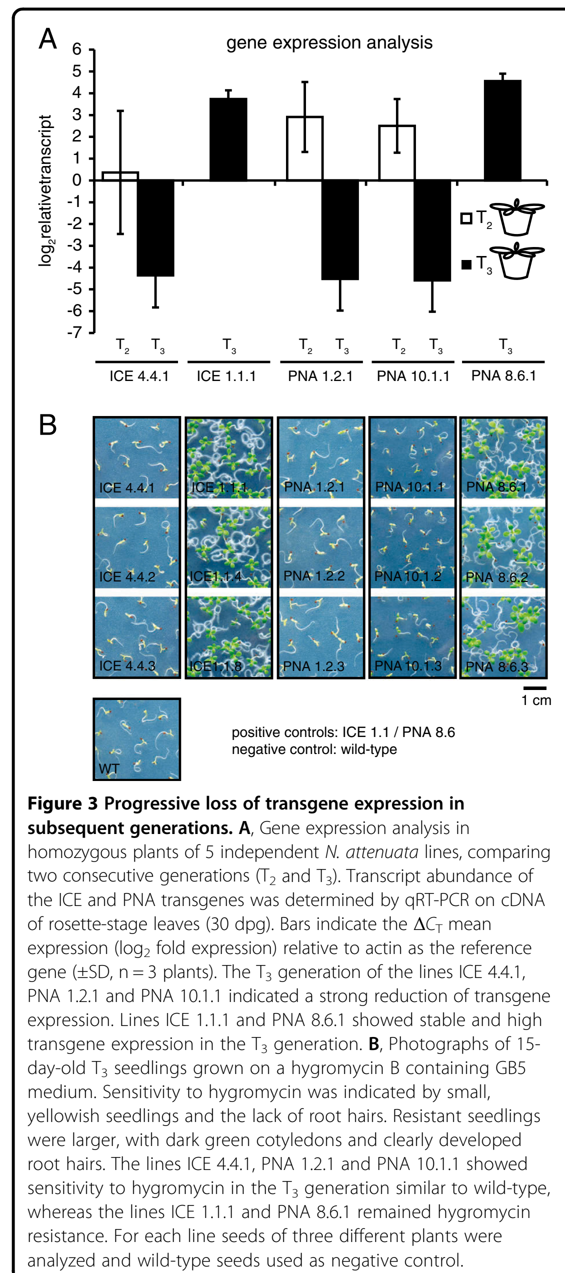


**Figure 3 Progressive loss of transgene expression in subsequent generations. A**, Gene expression analysis in homozygous plants of 5 independent *N. attenuata* lines, comparing two consecutive generations ($T_2$ and $T_3$). Transcript abundance of the ICE and PNA transgenes was determined by qRT-PCR on cDNA of rosette-stage leaves (30 dpg). Bars indicate the $\Delta C_T$ mean expression ($log_2$ fold expression) relative to actin as the reference gene (±SD, n = 3 plants). The $T_3$ generation of the lines ICE 4.4.1, PNA 1.2.1 and PNA 10.1.1 indicated a strong reduction of transgene expression. Lines ICE 1.1.1 and PNA 8.6.1 showed stable and high transgene expression in the $T_3$ generation. **B**, Photographs of 15-day-old $T_3$ seedlings grown on a hygromycin B containing GB5 medium. Sensitivity to hygromycin was indicated by small, yellowish seedlings and the lack of root hairs. Resistant seedlings were larger, with dark green cotyledons and clearly developed root hairs. The lines ICE 4.4.1, PNA 1.2.1 and PNA 10.1.1 showed sensitivity to hygromycin in the $T_3$ generation similar to wild-type, whereas the lines ICE 1.1.1 and PNA 8.6.1 remained hygromycin resistance. For each line seeds of three different plants were analyzed and wild-type seeds used as negative control.

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silencing were much more apparent and line ICE 4.4.1 showed 428 fold (99–757), line PNA 1.2.1 836 fold (197–1476) and PNA 10.1.1 872 fold (210–1534) lower gene expression levels, respectively (Additional file 1). In summary, transgenic lines that indicated a loss of hygromycin resistance had an at least 100 fold lower transgene expression, compared to a stable lines expressing the same constructs.

#### Multiple T-DNA insertions in silenced lines

Several reports describe a correlation between the incidence of unwanted gene silencing and high transgene copy number, making the selection of single copy T-DNA insertions by Southern blotting a very important part of the screening process [61]. The Southern blot analysis of the silencing affected lines ICE 4.4.1, PNA 1.2.1 and PNA 10.1.1 indicated in the *Xba*I digest no evidence for abnormalities, but the digest with *Eco*RV indicated two T-DNA insertions for all three lines (Figure 4). Unusually, the second T-DNA fragment showed nearly the same size in all three independently transformed lines. Since the fragment size resembles the size of the entire transgenic cassette from left to right border (2.84 kb for pSOL9PNA and 2.76 kb for pSOL9ICE respectively) this indicates the integration of two T-DNA copies adjacent to each other, which could be responsible for the observed transgene silencing in these lines. However, multiple T-DNA copies at two independent loci can be also identified much earlier in the screening process by their unusual segregation rate (6.25% sensitive seedlings instead of 25% for a single locus). In our dataset, only a very small portion of lines (6 out of 113) showed a segregation rate around 6.25% in the $T_1$ stage and were considered as harboring transgenes at two independent loci (Figure 1A). This enables an early exclusion of these lines from the further screening process. The Southern blot indicated for most of the analyzed lines only single T-DNA insertions, including the stable control lines (ICE 1.1.1 and PNA 8.6) (Figure 4).

#### Sensitive seedlings showed increased NOS promoter methylation

Unwanted or unintended transgene silencing was commonly associated with an increase in methylation within the promoter region of the transgene [24,26,29,63]. Since we found evidence for epigenetic gene silencing (intermediate stages of sensitivity and high variability among replicates), we analyzed promoter methylation levels in the transgenic cassette by bisulfite sequencing. Seedlings from line ICE 10.1 showed a transitional loss of hygromycin resistance and we separated hygromycin sensitive (yellow) and hygromycin resistant (green) seedlings to compare NOS promoter methylation levels within a 294 bp fragment (Figure 5A, Additional file 2). Among these isogenic seedlings, the resistant phenotypes were consistent with the methylation levels and sensitive seedlings had increased methylation levels, particular in the CHG and CHH sites (Additional file 2). Interestingly, the CTG at the 84th position (123 bp before translation start site) was entirely methylation free in

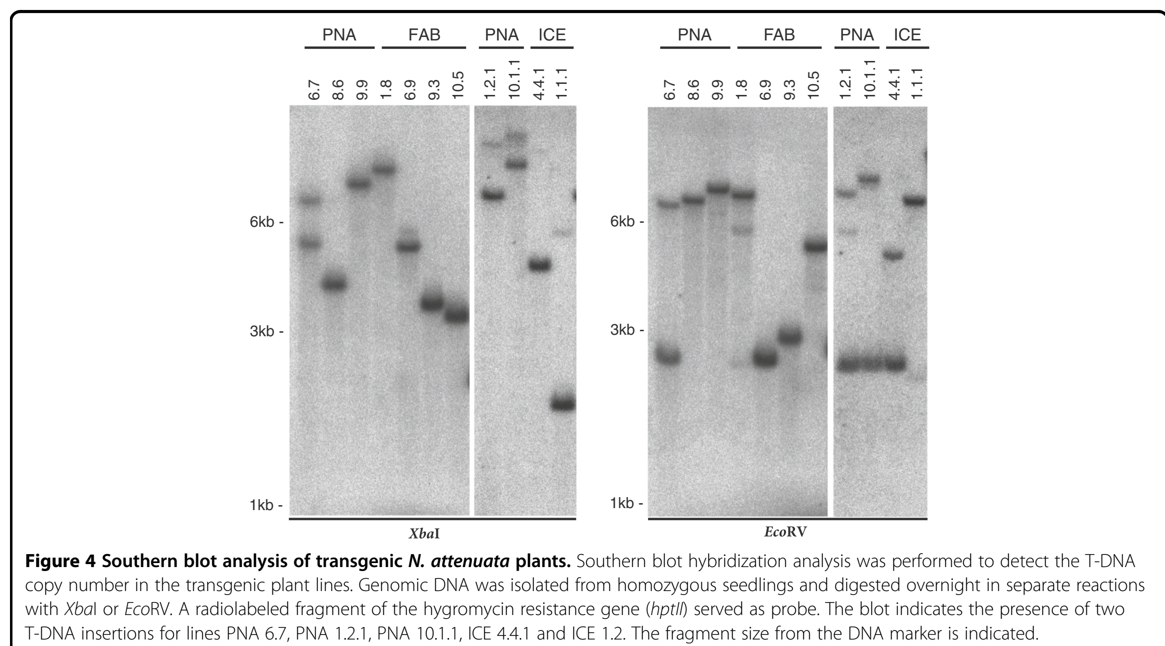


**Figure 4 Southern blot analysis of transgenic *N. attenuata* plants.** Southern blot hybridization analysis was performed to detect the T-DNA copy number in the transgenic plant lines. Genomic DNA was isolated from homozygous seedlings and digested overnight in separate reactions with *Xba*I or *Eco*RV. A radiolabeled fragment of the hygromycin resistance gene (*hptII*) served as probe. The blot indicates the presence of two T-DNA insertions for lines PNA 6.7, PNA 1.2.1, PNA 10.1.1, ICE 4.4.1 and ICE 1.2. The fragment size from the DNA marker is indicated.

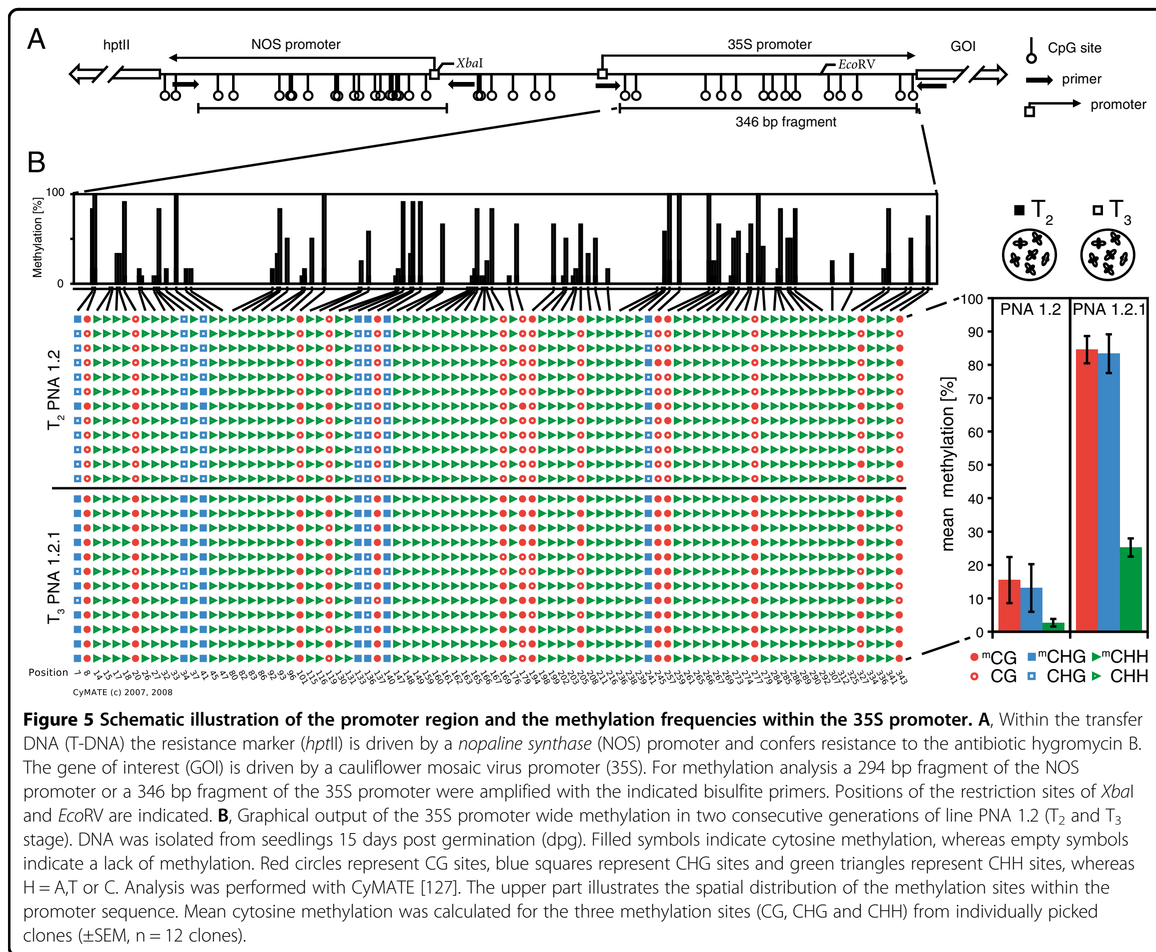


**Figure 5 Schematic illustration of the promoter region and the methylation frequencies within the 35S promoter. A**, Within the transfer DNA (T-DNA) the resistance marker (*hpt*II) is driven by a *nopaline synthase* (NOS) promoter and confers resistance to the antibiotic hygromycin B. The gene of interest (GOI) is driven by a cauliflower mosaic virus promoter (35S). For methylation analysis a 294 bp fragment of the NOS promoter or a 346 bp fragment of the 35S promoter were amplified with the indicated bisulfite primers. Positions of the restriction sites of *Xba*I and *Eco*RV are indicated. **B**, Graphical output of the 35S promoter wide methylation in two consecutive generations of line PNA 1.2 ($T_2$ and $T_3$ stage). DNA was isolated from seedlings 15 days post germination (dpg). Filled symbols indicate cytosine methylation, whereas empty symbols indicate a lack of methylation. Red circles represent CG sites, blue squares represent CHG sites and green triangles represent CHH sites, whereas H = A,T or C. Analysis was performed with CyMATE [127]. The upper part illustrates the spatial distribution of the methylation sites within the promoter sequence. Mean cytosine methylation was calculated for the three methylation sites (CG, CHG and CHH) from individually picked clones (±SEM, n = 12 clones).

resistant seedlings, but to 100% methylated in sensitive seedlings (Additional file 2A). Since this site is located directly downstream of a CCAAT box [64] it appears to be particularly important for the transcription process.

#### Hypermethylation of the 35S promoter

For the methylation analysis of the 35S promoter, individual reverse primers were designed for the two different expression cassettes (pSOL9PNA, pSOL9ICE) which allowed amplification of nearly the entire 35S promoter sequence (Figure 5A). Within the 346 bp fragment a total of 14 CG, 7 CHG and 65 CHH sites were found as potential targets for methylation. To allow the direct comparison of promoter methylation differences in (resistant) $T_2$ and (sensitive) $T_3$ seedlings, all seeds were germinated on hygromycin-free media. The analysis of line PNA 1.2 indicated in the $T_2$ stage seedlings methylation levels of 15.5% (±6.9%) CG and 13.1% (±7.1%) CHG methylation, respectively (Figure 5B). In comparison, the methylation rates of $T_3$ stage seedlings (PNA 1.2.1) were more than 5 fold increased with 84.5% (±4.1%) CG and 83.3% (±5.8%) CHG methylation. At the asymmetric sites, the CHH methylations levels were 9 fold increased from 2.7% (±1.2%) in $T_2$ to 25.3% (±2.7%) in $T_3$ seedlings. The clearly increased levels of 35S promoter methylation were consistent with the observed loss of gene expression in this generation (Figure 3A).

#### *De novo* cytosine methylation is only acquired during vegetative growth

To trace methylation changes of the 35S promoter at different times during plant growth, we sequentially sampled leaf material 30, 45 and 60 days post germination (dpg) (Figure 6A). The three silencing affected lines (PNA 1.2.1, PNA 10.1.1 and ICE 4.4.1) showed in both generations ($T_2$ and $T_3$) much higher 35S promoter methylation rates compared to the control lines (ICE 1.1.1 and PNA 8.6.1) (Figure 6). The line PNA 8.6.1 indicated the lowest methylation levels and showed throughout the sampling period over both generations mean

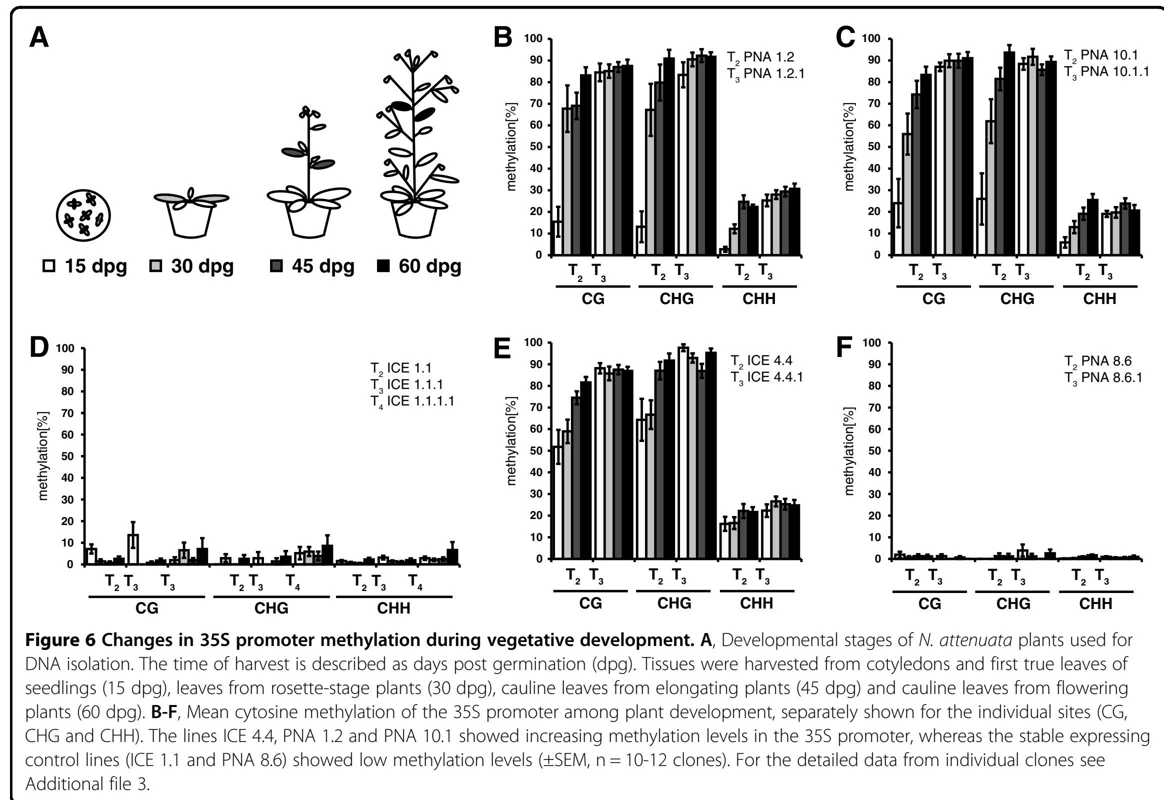


**Figure 6 Changes in 35S promoter methylation during vegetative development. A**, Developmental stages of *N. attenuata* plants used for DNA isolation. The time of harvest is described as days post germination (dpg). Tissues were harvested from cotyledons and first true leaves of seedlings (15 dpg), leaves from rosette-stage plants (30 dpg), cauline leaves from elongating plants (45 dpg) and cauline leaves from flowering plants (60 dpg). **B-F**, Mean cytosine methylation of the 35S promoter among plant development, separately shown for the individual sites (CG, CHG and CHH). The lines ICE 4.4, PNA 1.2 and PNA 10.1 showed increasing methylation levels in the 35S promoter, whereas the stable expressing control lines (ICE 1.1 and PNA 8.6) showed low methylation levels (±SEM, n = 10-12 clones). For the detailed data from individual clones see Additional file 3.

rates of only 0.9% (±0.7%) in CG, 1.3% (±1.0%) in CHG and 0.8% (±0.3%) in CHH methylation (Figure 6F, Additional file 3). These extremely low methylation levels indicate a complete DNA conversion during the bisulfite treatment and therefore a negligible false positive signal due to incomplete conversion. The second control was investigated until the $T_4$ generation (ICE 1.1.1.1) and showed in all three generations consistent low rates of 35S promoter methylation (Figure 6D). These two stable expressing lines indicated no tendency for an increase in 35S promoter methylation after a generational change or during vegetative growth (Figure 6, Figure 7A).

In contrast, the unstable lines ICE 4.4, PNA 1.2 and PNA 10.1 all showed increasing levels of 35S promoter methylation during growth (Figure 6B,C,E; Figure 7A). As a consequence the methylation levels deviated strongly between seedlings and flowering plants within the same generation. For instance, the CHG methylation levels of line PNA 1.2 indicated only 13.1% (±7.1) in seedlings but 90.9% (±4.0%) in flowering plants. This resembles an absolute methylation increase during plant development of more than 77% within only 45 days. The most rapid cytosine methylation increase was observed between seedlings and rosette-stage plants, where the CG and CHG levels changed within 15 days with a velocity of more than 3% per day (Figure 7A). Although the ICE 4.4 line started initially with higher methylation levels in seedling stage, it followed a similar trend and all three independent lines showed a similarly dramatic increase in methylation over time (Figure 7A). During the growth of $T_3$ plants, the promoter methylation levels increased only slightly and reached a plateau-like level at around 90% for CG and CHG sites and ca. 30% for CHH sites.

Surprisingly, at the generational transition low differences could be observed between the $T_2$ and $T_3$ plants. The mean methylation levels of the $T_3$ seedlings were highly similar to the levels found in the flowering $T_2$ plants (Figure 7). Even the comparison of the individual frequencies at the individual positions indicated no methylation resetting or enhancement across the generational change (Figure 7B). It should be explicitly mentioned that the $T_3$ generation seeds were not collected from exactly the same plants used here as $T_2$ generation. Both seed generations had been collected beforehand and both generations were grown simultaneously adjacent to each other in the glasshouse. Regardless, the intensity of the methylation increase was highly reproducible and the patterns from both generations matched perfectly (Figure 7A). Among all analyzed

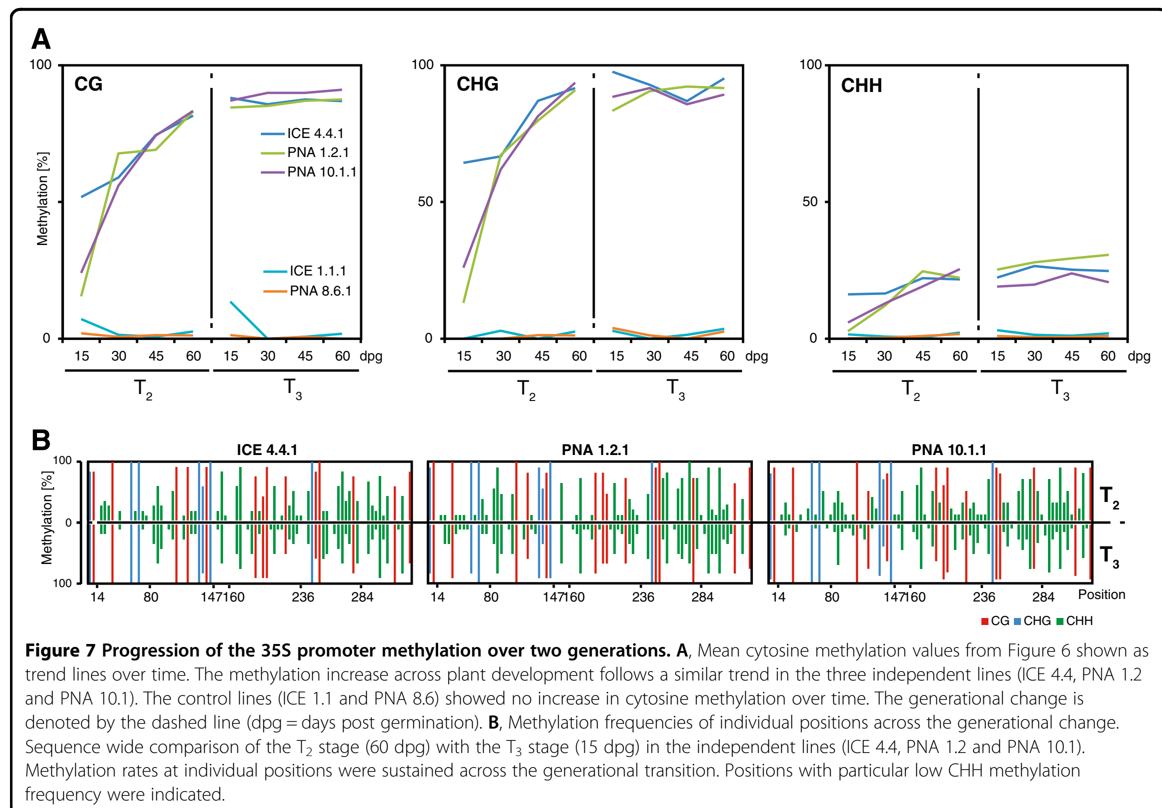


**Figure 7 Progression of the 35S promoter methylation over two generations. A**, Mean cytosine methylation values from Figure 6 shown as trend lines over time. The methylation increase across plant development follows a similar trend in the three independent lines (ICE 4.4, PNA 1.2 and PNA 10.1). The control lines (ICE 1.1 and PNA 8.6) showed no increase in cytosine methylation over time. The generational change is denoted by the dashed line (dpg = days post germination). **B**, Methylation frequencies of individual positions across the generational change. Sequence wide comparison of the $T_2$ stage (60 dpg) with the $T_3$ stage (15 dpg) in the independent lines (ICE 4.4, PNA 1.2 and PNA 10.1). Methylation rates at individual positions were sustained across the generational transition. Positions with particular low CHH methylation frequency were indicated.

clones certain asymmetric positions were only methylated at very low frequencies. In particular, the cytosines at the 14th and 160th position showed, for instance, 0% methylation in both generations (Figure 7B). We grouped the asymmetric CHH sites into low, medium and high methylated positions and found that the overall methylation preference was nucleotide-specific with higher probability at certain positions (e. g. CAA) compared to others (e. g. CCC) (Additional file 4). These findings were similar to the site-specific preferences of asymmetric positions found in a genome-wide analysis of the epigenome in Arabidopsis [65].

### The epigenetic status of the transgene was equally inherited by parental lines

Since we commonly combine phenotypes of transgenic plants by crossing, we wanted to determine whether the heredity of a silenced transgene might be parent-of-origin-specific. We performed reciprocal crosses between wild-type and transgenic lines and tested the hemizygous offspring for hygromycin resistance. The crosses with the silencing affected lines (PNA 1.2, PNA 10.1 and ICE 4.4) all showed high levels of hygromycin sensitivity, independent of the direction of the cross revealing equal inheritance of the silenced allele through both female and male gametes (Additional file 5). The crossings with the stable expressing control lines (PNA 8.6.1 and ICE 1.1.1) always retained their hygromycin resistance. Although crossings had, in certain cases, the potential to reduce silencing [28,66], we did not observe a reduction compared to plants produced from self-pollinations.

### Equivalent transgene inactivation in IR-lines

Unwanted transgene inactivation is not restricted to sense expression lines and has been reported frequently for inverted repeat (IR) constructs, which can also lose their *in trans* silencing ability [15,67]. In the process of producing several hundred IR-lines for the targeted silencing of endogenous *N. attenuata* genes involved in plant defense against herbivores, we have observed several incidents of resistance marker loss in several IR-lines over the past decade. Most recently, this was observed in the $T_3$ generation of the ir-ACX1 line, which normally shows a reduced ability to accumulate jasmonic acid (JA) after wounding due to the *in trans* silencing of the endogenous *acx*1 gene [37]. Consistently with our previous observations, the $T_3$ seedlings of ir-ACX1 also lost the ability to grow on hygromycin containing media (Additional file 6AB). To test the general applicability of a cell-culture induced transgene reactivation

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we included this IR-line as a candidate for the secondary regeneration process.

**Restored gene expression after secondary regeneration**

Most methods used to arrest the progress of transgene silencing include the use of cytidine analogs or viral methylation inhibitors, but these substances can cause severe growth and developmental abnormalities [68]. The cell culture step of the plant transformation procedure itself has been shown to be a significant source of methylation changes [4,69,70] and we evaluated whether the addition of a secondary cell culture step could interfere with the somatic acquirement of *de novo* methylation for the recovery of phenotypes in transformed but epigenetically silenced *N. attenuata* lines. Explant cultures were created from hypocotyls of transgenic homozygous $T_2$ seedlings of lines PNA 1.2, ICE 4.4 and ir-ACX1 and were called "secondary regenerants". The offspring of the secondary regenerants showed a large variability in hygromycin resistance. Most strikingly 41% of the regenerated plants produced offspring with full resistance to hygromycin and only 24% showed a resistance loss as seen after conventional propagation of these lines (Figure 8A, Additional file 7A).

To test if these phenotypically "recovered" plants also were restored in the expression of the transgene, we isolated RNA from rosette-stage plants. The gene expression analysis indicated much higher gene expression levels (about 200–300 fold increased) after the secondary regeneration, compared to conventionally propagated plants of the same line (Figure 8B). Most of the regenerated lines now showed gene expression levels very similar to those of the stable expressing lines (PNA 8.6.1 and ICE 1.1.1). The transgene activity of the ir-ACX1 line was not tested by gene expression analysis, but instead we determined the ability to suppress JA accumulation after simulated herbivory, as it would be performed during an experiment. All offspring from the tested ir-ACX1 regenerants showed suppressed JA accumulation, compared to wild-type plants (Additional file 6C). This

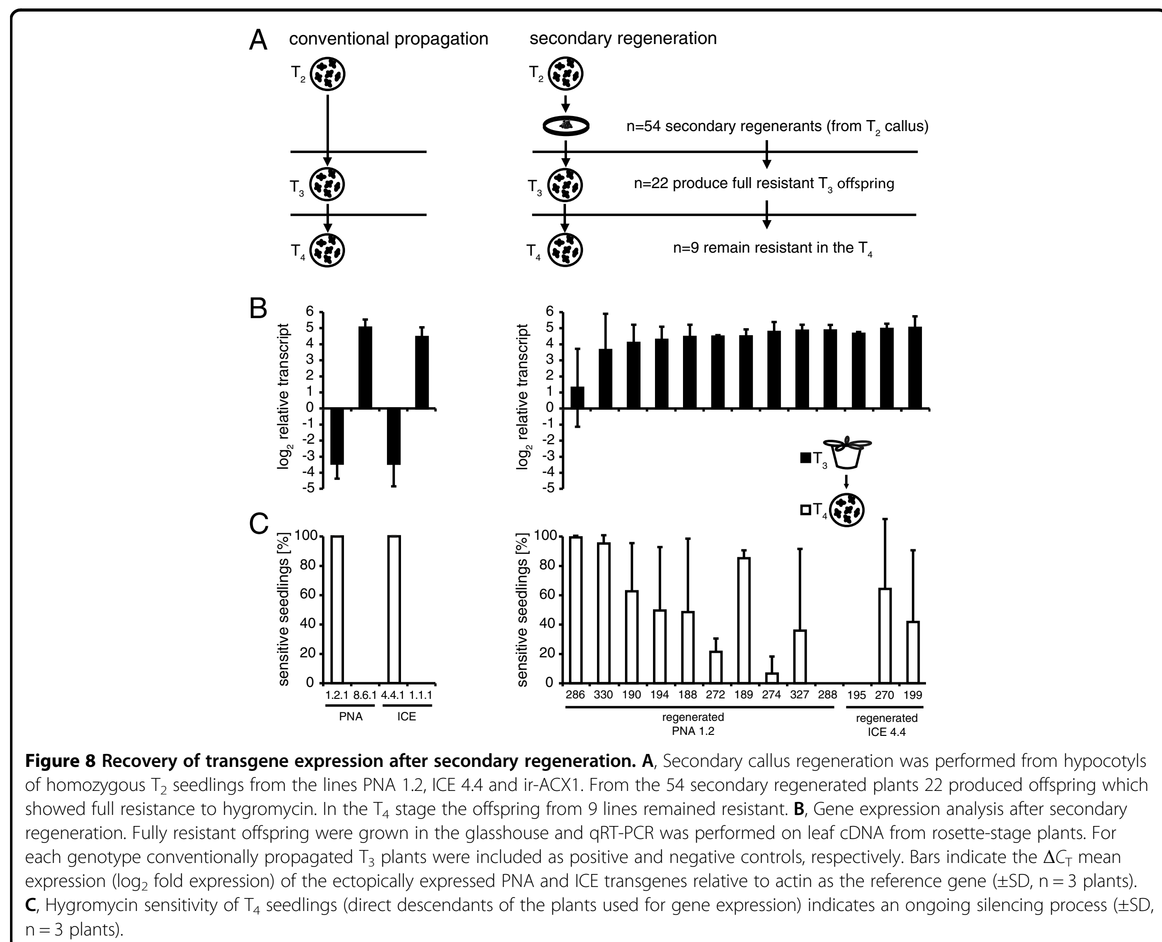


**Figure 8 Recovery of transgene expression after secondary regeneration.** **A**, Secondary callus regeneration was performed from hypocotyls of homozygous $T_2$ seedlings from the lines PNA 1.2, ICE 4.4 and ir-ACX1. From the 54 secondary regenerated plants 22 produced offspring which showed full resistance to hygromycin. In the $T_4$ stage the offspring from 9 lines remained resistant. **B**, Gene expression analysis after secondary regeneration. Fully resistant offspring were grown in the glasshouse and qRT-PCR was performed on leaf cDNA from rosette-stage plants. For each genotype conventionally propagated $T_3$ plants were included as positive and negative controls, respectively. Bars indicate the $\Delta C_T$ mean expression ($log_2$ fold expression) of the ectopically expressed PNA and ICE transgenes relative to actin as the reference gene (±SD, n = 3 plants). **C**, Hygromycin sensitivity of $T_4$ seedlings (direct descendants of the plants used for gene expression) indicates an ongoing silencing process (±SD, n = 3 plants).

indicated an actively expressed IR-construct and a recovered *in trans* silencing ability of the endogenous *acx1* gene after secondary regeneration.

To explore the durability of this recovery, we germinated the subsequent generation ($T_4$) on hygromycin containing media. Here the progression of marker gene silencing returned with the characteristic highly variable plant-to-plant pattern of hygromycin sensitivity (Figure 8C, Additional file 6D, Additional file 7B). Lines with low or variable gene expression levels had the highest probability of losing the resistance in the subsequent generation indicating a negative correlation between strength of transgene expression and the subsequent loss of the resistance marker. Finally, at least one line from each of the PNA and ICE regenerants (No. 288 and 195), but seven of the ir-ACX1 regenerants (No. 170, 174, 176, 185, 263, 264 and 265) showed enduring resistance up to the $T_4$ generation.

## Discussion

### Erratic occurrence of unwanted transgene silencing

This study summarizes our experience in the overall occurrence of transgene silencing during the screening of *N. attenuata* plants and provides guidance in identifying and avoiding unstable plant lines. Erratic occurrence and variegated phenotypes are commonly reported phenomena of transgene silencing and have been shown in many different plant species [22,25,27,29,71]. This was recently illustrated for *N. benthamiana* plants, transformed with a 35S:GFP construct [58,59]. These plants showed erratic and non-uniform *gfp* expression phenotypes, which differed strongly among isogenic sibling plants, but also among tissues from the same plant. If no visual marker is used, as in our case, the accurate selection based on the resistance marker turns out to be extremely important. Here, the miscellaneous inactivation pattern could be found in the intermediate resistance stages of seedlings or so called “gradual silencing” [27,71,72]. We frequently found intermediate resistant seedlings together with a non-Mendelian distribution (which could also strongly differ among sibling plants). We hypothesize that the gene silencing starts in the 35S promoter and then gradually spreads into the NOS promoter of the resistance marker, as discussed in Mishiba *et al.* [63]. Here the advantage of a head-to-head orientation of both promoters becomes clear, as it places them in close vicinity and a loss of the resistance marker would provide an accurate harbinger of the forthcoming silencing within the expression cassette.

The following three indicators were our major criteria for the early detection of plant lines affected by unwanted gene silencing: (A) unusual segregation rates with >50% of sensitive seedlings, (B) intermediate phenotypes of seedlings with unclear levels of resistance and (C) large differences in gene expression among isogenic plants. We suggest from our experience that testing the subsequent generations for resistance would be the easiest way to ensure stable transgene expression in *N. attenuata*. It is generally advisable to keep the number of generations as small as possible in transgenic plants, since with each new generation the probability of silencing increases. These selection criteria are independent of the mechanism responsible for the transgene silencing process, whether it be by TGS or PTGS [73-75]. As long as the selected plant lines show uniform levels of gene expression and Mendelian pattern of inheritance for the resistance marker, they could be considered as “stable” and used for further experiments (Figure 9).

### Sense transgene silencing in *Nicotiana attenuata*

The intensity of transgene silencing can vary greatly among different plant species. In transgenic gentian plants (*Gentiana triflora* X *G. scabra*) the 35S enhancer

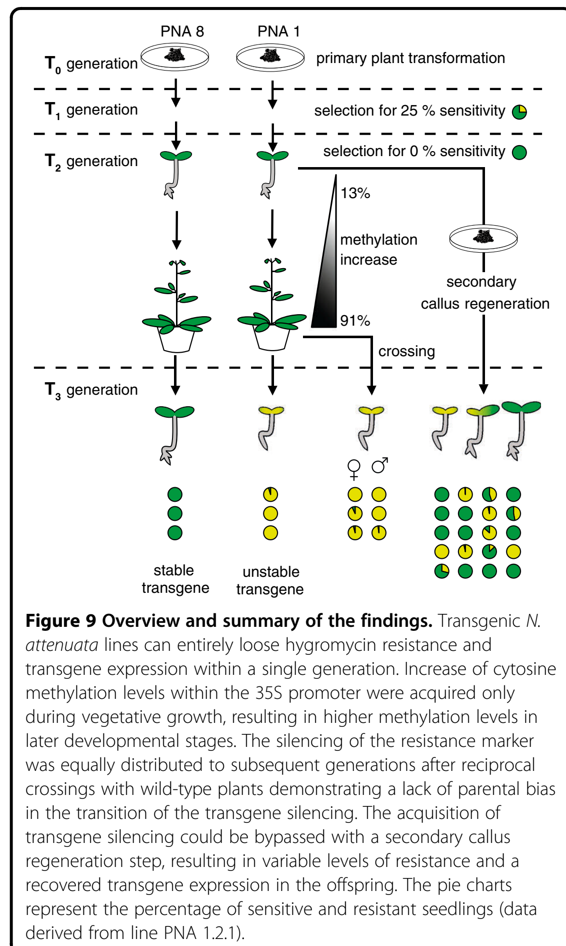


**Figure 9 Overview and summary of the findings.** Transgenic *N. attenuata* lines can entirely loose hygromycin resistance and transgene expression within a single generation. Increase of cytosine methylation levels within the 35S promoter were acquired only during vegetative growth, resulting in higher methylation levels in later developmental stages. The silencing of the resistance marker was equally distributed to subsequent generations after reciprocal crossings with wild-type plants demonstrating a lack of parental bias in the transition of the transgene silencing. The acquisition of transgene silencing could be bypassed with a secondary callus regeneration step, resulting in variable levels of resistance and a recovered transgene expression in the offspring. The pie charts represent the percentage of sensitive and resistant seedlings (data derived from line PNA 1.2.1).

sequence showed progressive methylation, independently of copy number and position of insertion [63]. All tested gentian lines showed strong *de novo* methylation, whereas the same construct was methylated with much lower rates in *N. tabacum* [63]. Even among closely related *Nicotiana* species the spontaneous silencing of a transgene was associated with higher methylation levels in *N. benthamiana* than in *N. tabacum* [58]. Similar observations were made with unstable transgene expression in *N. plumbaginifolia* [76]. These reports are consistent with the hypothesis of a more rigorous gene silencing machinery in wild diploid plant species, than in the cultivated tetraploid crop. Gene silencing by DNA methylation is a natural defense mechanism against viruses, transposons and other form of "invasive elements" [47,48,50]. Plants have a more complex and sophisticated gene silencing apparatus than animals do and make use of cytosine methylation at multiple sites in combination with histone modifications and harbor a vast variety of small RNAs [44,46]. Plants even have a signal transmission pathway for small RNAs, which can act as mobile signals to direct RdDM systemically [77]. The very active systemic spreading of the silencing signal through the phloem was first observed in the solanaceous plants, tobacco and tomato [78,79] and later demonstrated also for Arabidopsis [80]. From our initial 113 independent sense expression *N. attenuata* lines we omitted 43% after three generations due to indications of gene silencing. *N. attenuata* has a highly sophisticated suite of defenses against herbivores [32] and it might be, that this plant also has an active methylation apparatus to protect its genome against genetic manipulations [81], which Michael Wassenegger once aptly called a "gene silencing-based resistance against transgene overexpression" [82].

#### Factors influencing transgene silencing – the gene dosage effect

Factors which have often been shown to increase the probability of transgene silencing are the transgene copy number and the strength of expression [83,84]. In addition, T-DNA rearrangements, read-through transcripts or improperly terminated or non-polyadenylated mRNA are also associated with transgene silencing [85-87]. Certainly position effects and integration into heterochromatin have been frequently reported in association with local gene silencing [26,88], but integrations into euchromatin can be similarly silenced and more recent studies suggest that overall, the insertion position plays only a minor role [89,90]. Strong viral promoters, such as the 35S cauliflower mosaic virus promoter, were thought to produce "aberrant RNA" after exceeding a certain threshold of expression [16,85]. The progressive methylation of the 35S promoter and the observed downregulation of the transgene in the lines ICE 4.4, PNA 1.2 and PNA 10.1 might be mainly induced by the presence of two T-DNA copies in close proximity to each other (Figure 4). These complex insertions at a single locus can trigger transgene silencing as shown in earlier studies [91]. Any form of repeated T-DNA arrangement appears to increase the overall silencing probability [28,92]. But despite the intensity, the methylation increase occurred relatively late in these lines and the loss of the resistance marker was not revealed until the $T_3$ generation (Figure 2B). We hypothesize that the expression of the two T-DNA copies remains below a threshold level when plants are hemizygous. Once homozygous in the $T_2$, these thresholds are exceeded and the sum of the four T-DNA copies likely initiate the silencing process. This scenario would explain why also all hemizygous $T_2$ sibling plants of these lines were inconspicuous and showed no abnormal segregation.

The attempt to reactivate a silenced transgene by crossing with wild-type plants, to create hemizygous offspring with reduced T-DNA copy number, was partly successful in Arabidopsis and petunia [28,66]. In our case the sensitivity in crossings did not differ from self-pollination, probably because the methylation levels had already accumulated past the silencing threshold in flowering $T_2$ plants (Figure 9). Similar as reported for *N. tabacum* hybrids, we found no evidence of a specifically maternal or paternal contribution to the inactivation process [71]. Further monitoring of the crosses could be still interesting if after ongoing propagation, demethylation might occur, as has been seen in other backcrosses with wild-type plants [24].

#### Successive increase of *de novo* methylation during development

Usually, epigenetic modifications were considered to be stable in somatic cells and during normal plant development [46,56]. Most substantial epigenetic changes have been reported during gamete formation and embryogenesis in plants [93-95]. Progressive demethylation events that could be observed in endosperm tissue were interpreted as a way to reinforce transposon methylation in the embryo [96-98]. Since transgene silencing has been often described as a sudden switch of the phenotype between plant generations, a similar mechanism might have been responsible for enhancing transgene methylation during the reproductive phase. Our observation of a high variability in rosette-stage plants (line PNA 1.2 showed 23 fold difference in gene expression among biological replicates; Figure 2A) lead to the hypothesis that epigenetic changes might start already early during vegetative growth and increase with different velocities amongst individual plants. Other studies suggested a somatic inactivation as well, pointing to

evidence of diminishing expression of a reporter gene during development [71,99,100]. However, in these studies, methylation levels were not analyzed in different stages of plant development. Our methylation kinetic showed a strong somatic increase during growth, but nearly no changes between the generations, resembling a continuous inheritance of the methylation status to the offspring (Figure 7). The recent model of a methylation reinforcement during the reproductive stage, as seen for transposons [98], seems to be not applicable to the *de novo* methylation of transgenes.

Successive analysis of methylation changes have largely been restricted to tissue cultures or micropropagated plants [101]. In a long-term callus cultures of pearl millet (*Pennisetum glaucum*), a gradual decrease in GUS activity could be associated with increased methylation levels, 18 month after transformation [102]. In potato, a successive increase of gene silencing could be shown during a 5 year period of vegetative propagation [84]. In contrast, we found within only 15 days of normal plant development an absolute increase of 50% in total CG methylation. Developmental methylation increases reported in flax and Arabidopsis were only observed after treatment with DNA demethylating agents and therefore more a remethylation to the former status [103,104]. Likewise, the demethylated genome of Arabidopsis *ddm1* mutants showed remethylation after complementation with the wild-type allele [105]. However, it required multiple generations to reach approximately wild-type levels and methylation changes in different plant stages were not compared. A plant stage dependent transgene expression is particular problematic if certain phenotypes (e.g. flower movement) can be only observed in late developmental stages. For ecological field experiments in which plant fitness measurements play a central role, it is important to ensure transgene functionality over the entire plant life during a field season. Indeed, the strong transgene silencing effects we saw in our lines can be the result of an orchestrated combination of different transcriptional and posttranscriptional effects, which together contribute to the downregulation of the transgene. Since gene expression levels might not be comparable among different (particularly, senescing) plant stages, the survey of the cytosine methylation levels was the more appropriate method to visualize changes during development. Comparable analysis of the timing of gene silencing in chicken cells indicated that histone hypoacetylation and transcriptional shutdown occurs even before the promoter shows hypermethylation [106].

#### Inhibition of transgene silencing

Cytidine analogs and methytransferase blockers are commonly used treatments to prevent gene silencing in cell cultures. These chemicals can inhibit the transgene methylation process and have been successfully applied in plant, as well as in animal cell cultures [23,91,107]. However, a treatment of cell cultures differs substantially from that of an intact organism. The stable co-expression of silencing inhibitors in *N. benthamiana* and *N. tabacum* plants resulted in much higher transgene expression levels, but both plant species suffered from abnormal growth and altered leaf morphologies, which would invalidate their use in ecological experiments [68,108]. Although plants are surprisingly able to tolerate even mutations in genes of the DNA methylation pathway (e.g. *methyltransferase*1 mutants are embryolethal in mammals), the knockdown of the expression of these genes leads to the accumulation of developmental abnormalities [109,110]. The gene silencing machinery is an important part of the gene regulatory mechanism and their disturbance has global negative effects on development [111]. To date, there is no nuanced method available of selectively recovering only a single silenced transgene without causing substantial collateral damage to genome-wide methylation patterns.

#### Reactivation of transgene expression through cell culture to rescue phenotypes for ecological research

The cell culture step of the plant transformation process is a common source of unintended side effects [70,112]. The somaclonal variations that result from the de- and re-differentiation steps of cell culturing can be of genetic or epigenetic origin. Since DNA methylation patterns were highly variable among regenerated plants, an altered DNA methylation machinery during cell culture conditions had been suggested [69,112,113]. Most studies in different plant species found a genome-wide trend towards hypomethylation after a tissue culture step with even the possibility of restoring the activity of a former deactivated transgene [101,114,115]. Recently, an epigenome analyses in rice (*Oryza sativa*) revealed the details of the genome-wide loss of DNA methylation after regeneration [4]. We demonstrated for transgenic *N. attenuata* plants, that a secondary callus regeneration step could be used to recover transgene expression in the offspring of the regenerated plants. In this way, the desired gene expression levels could be achieved, even from plants with progressively silenced constructs (Figure 8, Figure 9). However, the transgene was re-silenced within most of the regenerants after two generations, highlighting the temporary character of the recovery. Regardless, the onset of gene silencing was successfully deferred for one generation with plants that produce many seeds, which provides a long-term source of material for further experiments. Similar attempts in gentian plants failed and the gene suppression persisted, probably because already silenced leaf tissue was used for the secondary regeneration [116].

Therefore we used hypocotyl tissue of $T_2$ seedlings, which were still resistant and indicated a relative low methylation rate (Figure 6). We hypothesize, that a cell culture induced transgene recovery mainly functions by interfering with the somatic *de novo* methylation process, rather than actively demethylating a transgene. The offspring of the regenerants were phenotypically normal, making this method suitable for ecological research.

## Conclusions

There is considerable interest in the creation of transformed plant lines with stable and heritable phenotypes, but the dynamics of epigenetic mechanisms during plant development can lead to gradual changes within a single generation and "transgene half-life" could compromise long-term experiments. Overall, the regulation of cytosine methylation in vegetative tissue seems be more dynamic than previously thought. Unlike in animals in which the germline is sequestered, plants develop germ cells directly from somatic cells relative late in their life cycle. Any vegetative acquired change of the genome could therefore be potentially submitted to the offspring, giving plants the potential to flexibly adapt to a rapid changing environment [117,118]. Apparently epigenetic processes can play a much greater role in driving plant evolution than previously thought [43,44,119].

## Methods

### Construction of transformation vectors

For heterologous expression of antimicrobial peptides in *N. attenuata* altogether 11 different vectors were constructed [61]. Plants transformed with the vectors pSOL9PNA, pSOL9ICE and pSOL9FAB were analyzed here in more detail. The different antimicrobial peptide coding genes were selected from the PhytAMP database (http://phytamp.pfba-lab-tun.org/) [120] and were synthesized in sequential PCR reactions with overlapping 40 bp primers. Full length synthesized genes were cloned in pSOL9 binary plant transformation vectors consisting of a *hygromycin phosphotransferase II* (*hpt*II) gene as a selectable marker under a *nopaline synthase* promoter (NOS) and the gene of interest (GOI) under a cauliflower mosaic virus promoter (35S) [61]. Transgenic "PNA" plants expressed an antifungal peptide (hevein) from the Japanese morning glory *Ipomoea nil* (synthetic gene similar to the Pn-AFP2 precursor [GenBank: U40076]). Transgenic "ICE" plants expressed an antimicrobial peptide (knottin) from the common ice plant *Mesembryanthemum crystallinum* (synthetic gene identical to the Mc-AMP1 precursor [GenBank:AF069321]) and transgenic "FAB" plants expressed an antimicrobial peptide (fabatin) from the broad bean *Vicia faba* (synthetic gene similar to the fabatin-1 precursor [GenBank: EU920043]). The sequences of the PNA and FAB constructs were manually adapted to the codon usage table of *N. tabacum* (http://gcua.schoedl.de/).

### Plant transformation and line screening

*N. attenuata* Torr. ex S. Watson seeds were originally collected in 1988 from a natural population at the DI Ranch in Southwestern Utah. Wild-type seeds from the 30th inbreed generation were used for the construction of transgenic plants and as WT controls in all experiments. Plant transformation was performed by *Agrobacterium tumefaciens*-mediated gene transfer as previously described [121]. Explant cultures were regenerated from elongated hypocotyl tissue and the selection for correct T-DNA integrations was performed on phytagel-based media supplemented with 20 mg/L hygromycin B (Duchefa). For germination seeds were sterilized for 5 min with a 2% (w/v) aqueous solution of sodium dichloroisocyanuric acid (DCCS) and treated for 1 h with 0.1 M gibberelic acid ($GA_3$) in 50 × diluted liquid smoke solution (House of Herbs). At least 60 seedlings per plant were germinated on Gamborg's B5 (GB5) Medium (Duchefa) supplemented with 35 mg/L hygromycin B (Duchefa) and incubated in a growth chamber (Percival, day 16 h 26°C, night 8 h 24°C). After 10 days the segregation rate (% of sensitive seedlings) was determined and resistant seedlings transferred to the glasshouse under constant temperature and light conditions (day 16 h 26-28° C, night 8 h 22-24°C). Since *N. attenuata* is self-compatible , the collected seeds result generally from self-pollination, except if crossings with different lines are indicated. For crossings, the flowers were antherectomized before opening and hand-pollinated using pollen from either homozygous transgenic or wild-type plants. Independent overexpression plant lines used in this study were: PNA 1 (A-09-678), PNA 3 (A-09-768), PNA 6 (A-09-792), PNA 8 (A-09-823), PNA 9 (A-09-825), PNA 10 (A-09-826), ICE 1 (A-09-653), ICE 4 (A-09-702), ICE 6 (A-09-748), ICE 10 (A-09-807), FAB 1 (A-09-662), FAB 5 (A-09-855), FAB 6 (A-09-857), FAB 9 (A-09-865), FAB 10 (A-09-866). The plant generations were indicated within the line number as follows: $T_1$ seeds or plants have only the line number (e.g. PNA 8), $T_2$ seeds were indicated by an extra number to identify the plant from which seeds were collected from (e.g. PNA 8.6), $T_3$ seeds were additionally numbered (e.g. PNA 8.6.1 etc.). Two lines harboring an inverted repeat construct for silencing the expression of *N. attenuata acetyl-CoA-transferase* 1 (*acx*1), ir-ACX1 (A-07–466-1) and ir-ACX1 (A-07-468-3), were described in [37].

### Genomic DNA isolation

Genomic DNA was isolated with a modified hexadecyltrimethylammonium bromide (CTAB) method described in [122]. For Southern blotting 15 day old seedlings were ground in liquid nitrogen to a fine powder and

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300 mg used for DNA isolation. The quality and concentration was estimated by agarose gel electrophoresis. For bisulfite sequencing gDNA was isolated from cotyledons and first true leaves of seedlings 15 days post germination (15 dpg), leaves of rosette-stage plants (30 dpg), cauline leaves of elongating plants (45 dpg) and cauline leaves of flowering plants (60 dpg) (see Figure 6A for illustration). The last three time points were successively sampled from the same plants. Materials from 5 biological replicates were pooled, ground in liquid nitrogen to a fine powder and 300 mg used for DNA isolation. For the isolation of DNA from elongated plants (45 and 60 dpg) a modified buffer with higher salt concentration was used (2% CTAB, 100 mM Tris–HCl [pH 8.0], 20 mM EDTA [pH 8.0], 2.2 M NaCl, 2% PVPP [Mr 40.000], 10 mM ascorbic acid). The amount and quality of DNA was estimated on a Nanodrop spectrophotometer (Thermo Scientific).

### Southern blot analysis

A total amount of 6 μg gDNA was digested overnight at 37°C with 140 U *Eco*RV and *Xba*I (New England Biolabs) in independent reactions, each enzyme providing only one restriction site within the T-DNA of the binary vector (indicated in Figure 5A). The digested DNA was separated on a 1% (w/v) agarose gel for 17 h at 23 Volt. DNA was blotted overnight onto a Gene Screen Plus Hybridization Transfer Membrane (Perkin-Elmer) using the capillary transfer method. A gene specific probe for the *hpt*II gene was amplified with the primer pair HYG1-18 (5′-CCGGATCGGACGATTGCG-3′) and HYG2-18 (5′-CTGACGGACAATGGCCGC-3′) [61] and radiolabeled with [α-$^{32}$P] dCTP (Perkin-Elmer) using the Rediprime II DNA Labeling System (GE Healthcare) according to the manufacturer′s instructions.

### RNA isolation and qRT-PCR

Tissue was harvested from rosette-stage leaves and ground in liquid nitrogen to a fine powder. RNA isolation was performed with a salt precipitation method modified from the US patent of Gentra Systems, Inc. publication No. 5973137 [123] and adapted for *N. attenuata* tissue. Approximately 150–300 mg ground and frozen tissue was dissolved in 900 μL cell lysis buffer (2% [w/v] sodium dodecyl sulfate, 77 mM [tri-] sodium citrate, 132 mM citric acid, 10 mM ethylenediaminetetraacetic acid) and shortly mixed. Per sample 300 μL protein precipitation buffer (4 M NaCl, 19 mM [tri-] sodium citrate, 33 mM citric acid) was added and the tubes inverted ten times. Samples were incubated on ice for 5 min and centrifuged at room temperature in a table top centrifuge (5 min at 16.100 g). The supernatant was collected and extracted with 500 μL chloroform:isoamylalcohol mix (24:1 v/v). After centrifugation (3 min at 16.100 g) the upper aqueous phase was collected and nucleic acids precipitated with 1 volume isopropanol for 15 min at room temperature. Nucleic acids were pelleted in a table top centrifuge (3 min at 16.100 g), washed twice with 400 μL 70% (v/v) ethanol and air dried for 5 min. The final pellet was dissolved in 50 μL nuclease free water (Ambion / Life technologies). The nucleic acid was DNAse-treated using the TURBO DNA-free kit (Ambion / Life Technologies) according to the manufacturer's instructions. Quality and amount of the remaining RNA was determined using a 1% (w/v) agarose gel and a Nanodrop spectrophotometer (Thermo Scientific). The absence of genomic DNA was tested with 20 ng RNA in a 35 cycle PCR programm (94°C for 1 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s) with the same primers as for qRT-PCR. 4 μg of total RNA was reverse transcribed with oligo(dT)$_{18}$ primers (Fermentas) and the SuperScript II reverse transcriptase enzyme (Invitrogen / Life Technologies). Quantitative Real Time-PCR (qRT-PCR) was performed with 1:10 diluted cDNA (20 ng) on a Mx3005P QPCR System (Stratagene) with either a SYBR Green based PCR Master Mix (Applied Biosystems / Life Technologies) or a qPCR Core kit for SYBR Green (Eurogentec). For amplification the following primers were used: ICE-94F (5′-AATGGAAAAGGATGTCGAGAGG-3′), ICE-167R (5′-CATCCAACCTGACGGTAACAGAA-3′), PNA-86F (5′-GGAGACAAGCTAGTGGGAGGC-3′), PNA-154R (5′-TGGAGCCACAGTAGCCCC-3′), FAB-111F (5′-CAGGTTTAATGGACCATGCTTG-3′), FAB-184R (5′-CACCACCTTTGTAACCTTCTCCC-3′). The used program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 1 cycle of 95°C for 15 s, 60°C for 30 s, 95°C for 15 s as dissociation curve. For relative gene expression analysis the comparative Δ cycle threshold method ($\Delta C_T$ ) was used. Gene expression was shown as $\log_2$ ($\Delta C_T$) relative to *N. attenuata* actin as the reference gene (Actin-F1 5′-GGTCGTA CCACCGGTATTGTG-3′ and Actin-R1 5′-GTCAAGA CGGAGAATGGCATG-3′) [61].

### Bisulfite genomic sequencing

DNA methylation analysis was performed by the bisulfite sequencing method [124]. The bisulfite conversion was performed using the EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. A total of 1 μg gDNA was converted for 5 h with the following program 95°C for 5 min, 60°C for 25 min, 95°C for 5 min, 60°C for 85 min, 95°C for 5 min, 60°C for 175 min. The target sequences were amplified from the converted DNA with 0,05 U/μL JumpStart Taq DNA Polymerase with the provided reaction buffer (Sigma-Aldrich), 200 μM dNTP Mix (Fermentas) and 0,5 μM of the following primer: MetCNOSR5 (5′-AGATYYGGTGYAGATTA TTTGGATTGA-3′) and MetCNOSF6 (5′-TTARRTCCT CTATTTRAATCTTTRACTCC-3′) for a 294 bp fragment of the NOS promoter (−40 to −333 bp before the start

codon) and MetC35SF2 (5′-AGGGYAATTGAGAYTT TTTAATAAAGGG-3′) and MetC35SRPNA2 (5′-CAAR ARAACAATAAACATAATACARTATTTCATCTC-3′) or MetC35SRICE2 (5′-ATTTCARCAAAAAARATRAAACC TTAACCATCTC-3′) for a 346 bp fragment of the CaMV 35S promoter (−1 to −346 bp before the start codon). Primers were designed using Methprimer software (http://www.urogene.org/methprimer/) and Kismeth [110] and manually adapted according to [125] and [126], to avoid amplification-bias of non-converted DNA. Cycle parameters used were 94°C for 1 min followed by 35 cycles with 94°C for 30 s, 53°C for 30 s, 72°C for 30 s and a final step with 72°C for 5 min. PCR products were gel excised and purified with the NucleoSpin Extract II kit (Macherey-Nagel) and cloned into pGEM-T Easy vector system (Promega). Plasmids of individual picked clones were isolated with NucleoSpin Plasmid Kit (Macherey-Nagel). Sequencing was performed with the BigDye Terminator mix v3.1 (Applied Biosystems) supplemented with 5% dimethyl sulfoxide (DMSO). Sequences were manually trimmed and the data analysis performed with the online tools CyMATE (http://cymate.org/) [127] and MethTools 2.0 (http://methdb.igh.cnrs.fr/methtools/) [128]. Nucleotide frequencies at CHH positions were graphical illustrated with WebLogo 3 (http://weblogo.threeplusone.com/) [129]. For the 35S promoter methylation kinetic a minimum of 10–12 individual clones per sample were analyzed.

### Secondary callus regeneration

Homozygous seedlings of the lines PNA 1.2, ICE 4.4 and ir-ACX1 (A-07-466-1 and A-07-468-3) were chosen for secondary callus regeneration. $T_2$ stage seedlings (still resistant) were grown for 10 days on GB5 media supplemented with hygromycin B (35 mg/L). The hypocotyls were cut in small pieces as done for the normal plant transformation procedure but without dipping the scalpel in *Agrobacterium* suspension. The explant cultures were grown into a callus and regenerated as previously described [121]. Fully regenerated plants were grown in pots in the glasshouse for self-pollination and seed production. Secondary regenerated lines originating from PNA 1.2 seedlings were A-11-xxx (188, 189, 190, 191, 193, 194, 272, 274, 275, 276, 277, 278, 286, 288, 308, 327, 329 and 330). Secondary regenerated plants originating from ICE 4.4 seedlings were A-11-xxx (195, 196, 199, 200, 201, 202, 268, 269, 270, 271, 307 and 328). Secondary regenerated lines originating from ir-ACX1 (A-07-466-1) seedlings were: A-11-xxx (170, 171, 172, 173, 174, 175, 176, 177, 178, 263, 264, 265, 281 and 283) and from ir-ACX1 (A-07-468-3) A-11-xxx (179, 180, 181, 183, 184, 185, 266 and 282). The first seed generation ($T_3$) from the regenerants were germinated on hygromycin containing media and seedlings with 0% sensitivity were brought to the glasshouse for RNA isolation and further propagation to test the subsequent generation ($T_4$) for resistance.

### Jasmonic acid extraction and analysis

Leaves at nodes +1 [130] from rosette-stage (30 days old) plants were wounded by rolling a fabric pattern wheel three times on each side of the midvein and the wounds were supplied immediately with 20 μL of 1:5 (v/v) diluted oral secretion of *Manduca sexta*. Leaf tissue was collected 60 min after the treatment and was frozen immediately in liquid nitrogen for subsequent analysis. Jasmonic acid was extracted and analyzed as described in [35].

## Additional files

**Additional file 1: Fold difference of transgene expression in consecutive generations.**

**Additional file 2: NOS promoter methylation between sensitive and resistant seedlings of line ICE 10.1. A**, Methylation status of the NOS promoter among isogenic seedlings from line ICE 10.1. Different methylation sites (CG, CHG and CHH) were indicated by different colors. Analysis was performed by CyMATE [127]. **B**. Phenotypes of 10-day-old seedlings used for DNA isolation and bisulfite conversion. Isogenic seedlings of line ICE 10.1.2 were divided into sensitive and resistant seedlings and analyzed separately. Mean methylation rate from five clones is shown for the individual methylation sites (CG, CHG and CHH). (± SEM, n = 5 clones).

**Additional file 3: Detail of 35S promoter methylation analysis of individual clones.** Tissue harvested 15, 30, 45 and 60 days post germination of lines ICE 4.4 ($T_2$) and ICE 4.4.1 ($T_3$); ICE 1.1 ($T_2$), ICE 1.1.1 ($T_3$) and ICE 1.1.1.1 ($T_4$); PNA 1.2 ($T_2$) and PNA 1.2.1 ($T_3$); PNA 10.1 ($T_2$) and PNA 10.1.1 ($T_3$); PNA 8.6 ($T_2$) and PNA 8.6.1 ($T_3$).

**Additional file 4: Sequence preference in CHH methylation sites.** The nucleotide composition of 8-mer sequences around the CHH sites (methylated cytosine in the fifths position) divided in groups with low methylation (0–9%), medium methylation (10–49%) and high methylation (50–100%) frequencies. The pooled frequency data of lines ICE 4.4.1, PNA 1.2.1 and PNA 10.1.1 derived from one time point (60 dpg $T_3$). The logo graphically illustrates the sequence enrichment at particular positions around the methylation site. Maximum sequence conservation is 2 bit, no nucleotide preference is 0 bit. Figures were made with WebLogo 3 [129].

**Additional file 5: Inheritance of the silenced allele after reciprocal crossing with wild-type.** The hybrid offspring (hemizygous to the transgene) should be theoretically fully resistant to hygromycin B. The silenced state of the transgene was equally distributed to subsequent generations. **A**, Percentage of sensitive seedlings after crossing (± SD, n = 3 plants). **B**, Phenotypes of seedlings on hygromycin B containing GB5 media.

**Additional file 6: Transgene silencing in line ir-ACX1. A**, Jasmonic acid accumulation 1 h after wound and oral secretion treatment in rosette leaves of ir-ACX1 and wild-type plants. The $T_3$ generation of ir-ACX1 lost their capacity to suppress jasmonic acid accumulation [37]. **B**, The $T_3$ seedlings from line ir-ACX1 (A-07-468) developed sensitivity to hygromycin B. **C**, Transgene activity indicated by jasmonic acid accumulation determined in wound and oral secretion treated leaves of secondary regenerated ir-ACX1 lines. A reduced accumulation of jasmonic acid after wounding compared to wild-type (WT) indicated a functional IR-construct. **D**, Hygromycin sensitivity of $T_4$ seedlings (direct descendants of the plants used for wound treatment) indicates an ongoing silencing process (±SD, n = 3 plants).

**Additional file 7: Phenotypes after secondary regeneration. A**, Photographs of $T_3$ seedlings collected from secondary regenerants of line

ICE 4.4 and PNA 1.2. Cell culture-induced variations resulted in variegated pattern of sensitivity on hygromycin B containing GB5 media. **B**, Photographs of $T_4$ generation seedlings collected from fully resistant secondary regenerated plants. As positive and negative controls conventional propagated $T_4$ seedlings are shown.

**Competing interests**
The authors declare that they have no competing interests.

**Authors' contributions**
AW planned and performed the experiments, analyzed the data and wrote the manuscript. MK performed all experiments with ir-ACX1 lines. ITB participated in the design of the study and revised the manuscript. All authors read and approved the final manuscript.

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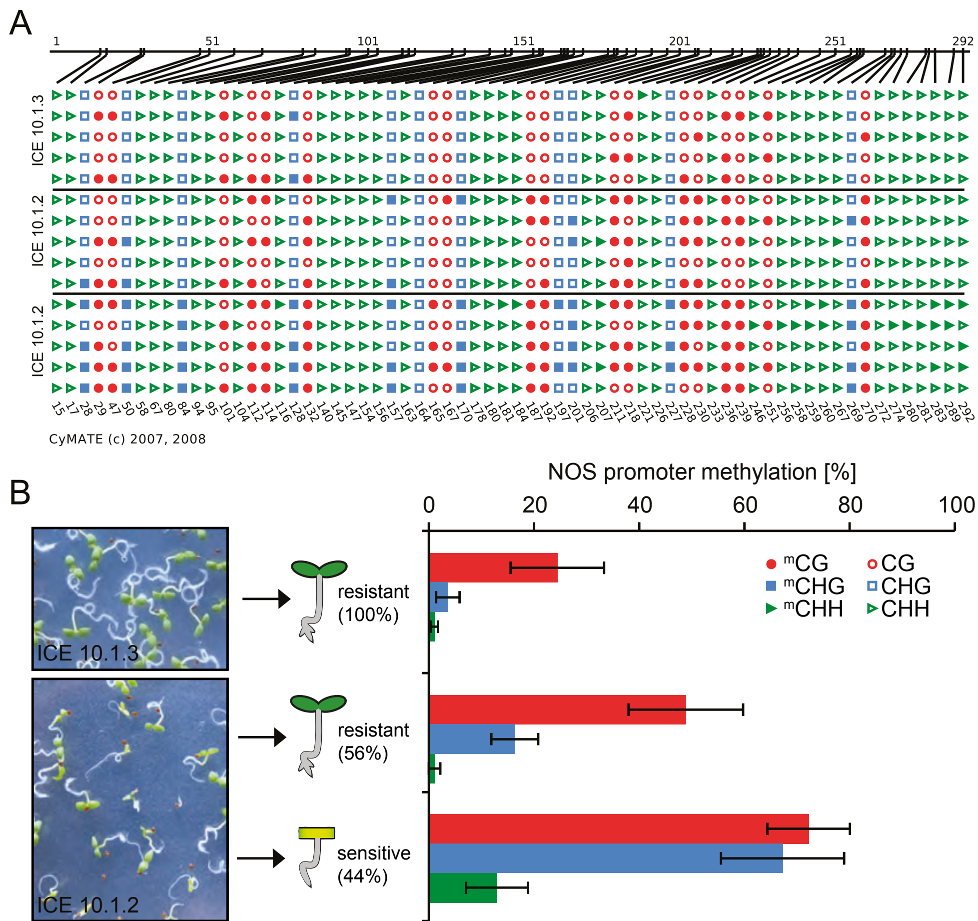
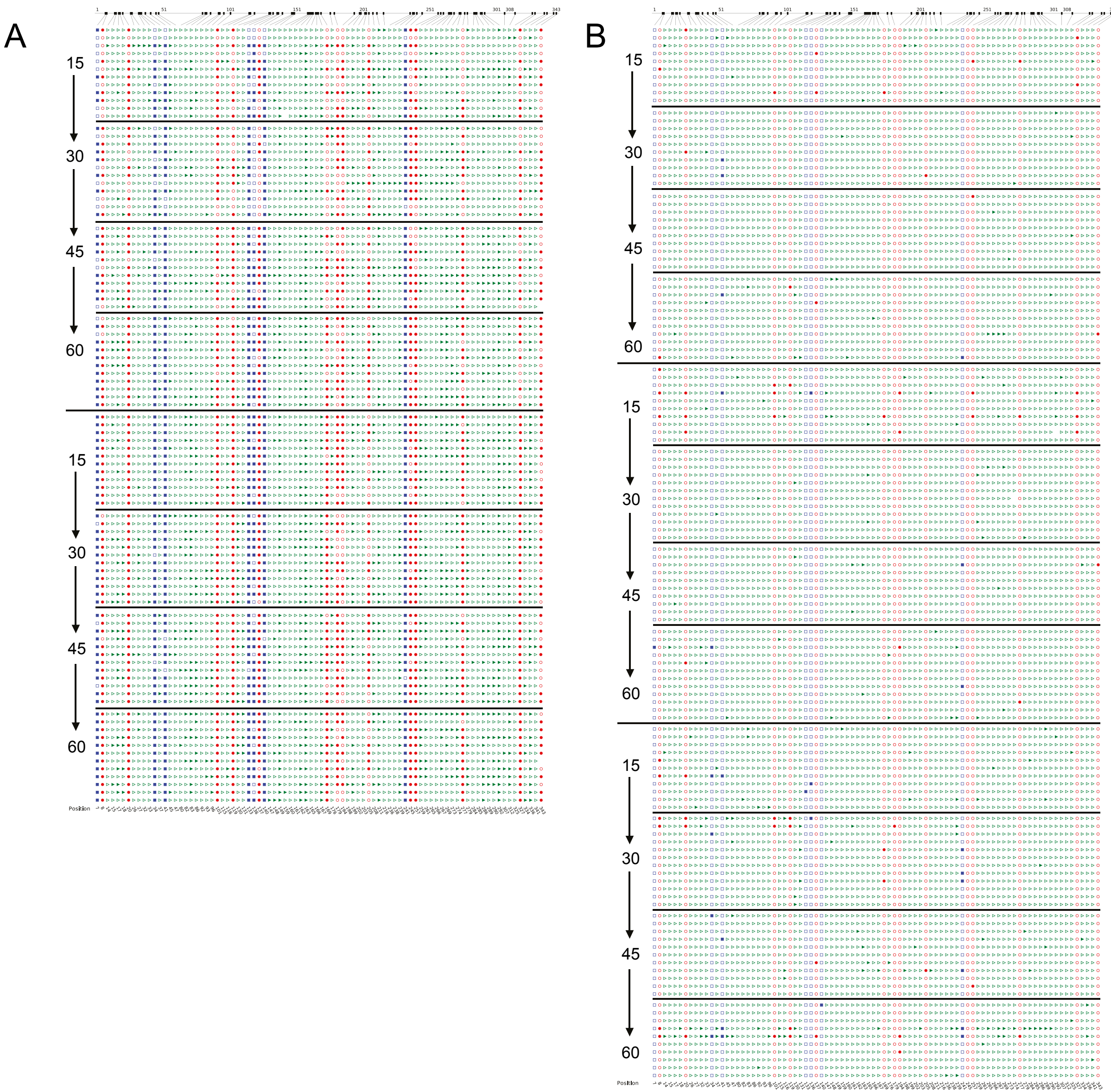
**Additional file 1: Fold difference of transgene expression in consecutive generations**

| | | $\Delta C_T$ ($C_T$ actin – $C_T$ *goi*) | $\Delta\Delta C_T$ ($T_3$– $T_{2/control}$) | Fold difference ($2^{-\Delta\Delta Ct}$) |
|---|---|---|---|---|
| $T_2$ | PNA 1.2 | 2.91 ± 1.60 | 0.00 ± 1.60 | 1.7 (0.3 – 3.0) |
| $T_3$ | PNA 1.2.1 | -4.52 ± 1.45 | -7.43 ± 1.45 | 267.6 (62.9 – 472.2) |
| | | | | |
| $T_2$ | PNA 10.1 | 2.51 ± 1.23 | 0.00 ± 1.23 | 1.4 (0.4 – 2.3) |
| $T_3$ | PNA 10.1.1 | -4.59 ± 1.44 | -7.10 ± 1.44 | 210.4 (50.6 – 370.1) |
| | | | | |
| $T_3$ | PNA 8.6.1 | 4.56 ± 0.34 | 0.00 ± 0.34 | 1.0 (0.8 – 1.3) |
| $T_3$ | PNA 1.2.1 | | -9.07 ± 1.45 | 836.4 (196.6 – 1476.3) |
| $T_3$ | PNA 10.1.1 | | -9.15 ± 1.44 | 871.8 (209.8 – 1533.8) |
| | | | | |
| $T_2$ | ICE4.4 | 0.37 ± 2.82 | 0.00 ± 2.82 | 3.6 (0.1 – 7.1) |
| $T_3$ | ICE4.4.1 | -4.36 ± 1.47 | -4.73 ± 1.47 | 41.4 (9.6 – 73.2) |
| | | | | |
| $T_3$ | ICE 1.1.1 | 3.74 ± 0.40 | 0.00 ± 0.40 | 1.0 (0.8 – 1.3) |
| $T_3$ | ICE4.4.1 | | -8.10 ± 1.47 | 427.7 (98.8 – 756.6) |




**Additional file 2: NOS promoter methylation between sensitive and resistant seedlings of line ICE 10.1 A,** Methylation status of the NOS promoter among isogenic seedlings from line ICE 10.1. Different methylation sites (CG, CHG and CHH) were indicated by different colors. Analysis was performed by CyMATE [127]. **B,** Phenotypes of 10-day-old seedlings used for DNA isolation and bisulfite conversion. Isogenic seedlings of line ICE 10.1.2 were divided into sensitive and resistant seedlings and analyzed separately. Mean methylation rate from five clones is shown for the individual methylation sites (CG, CHG and CHH). (± SEM, n=5 clones)




**Additional file 3: Detail of 35S promoter methylation analysis of individual clones.** Tissue harvested 15, 30, 45 and 60 days post germination of **A**, lines ICE 4.4 ($T_2$) and ICE 4.4.1 ($T_3$); **B**, ICE 1.1 ($T_2$), ICE 1.1.1 ($T_3$) and ICE 1.1.1.1 ($T_4$).

C

15
30
45
60
15
30
45
60

Position

D

15
30
45
60
15
30
45
60

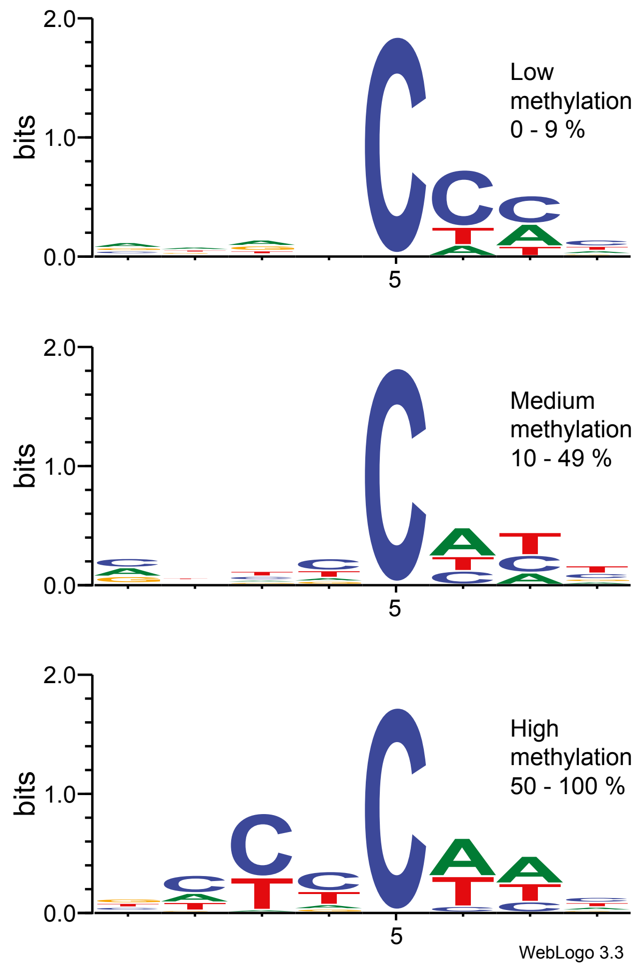
Position

E

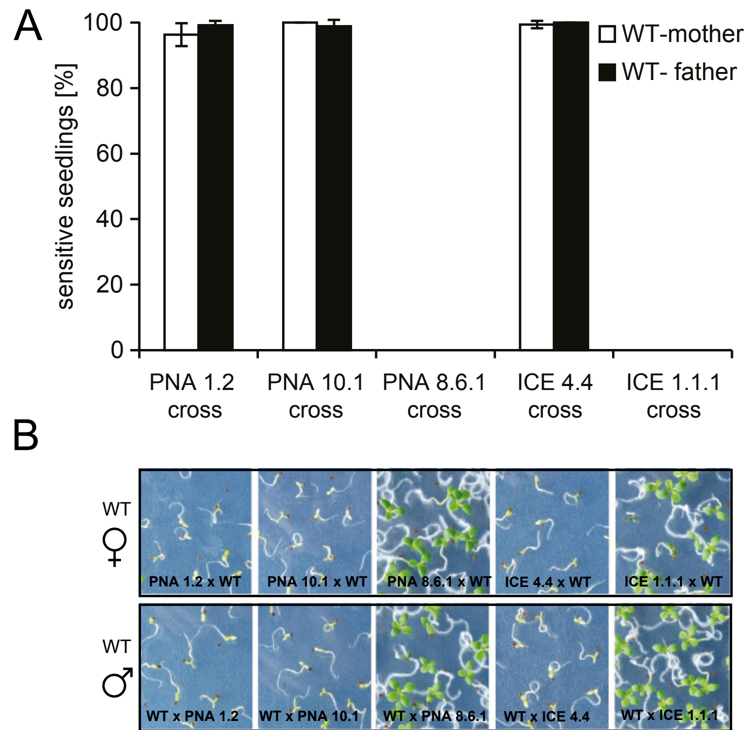
15
30
45
60
15
30
45
60

Position

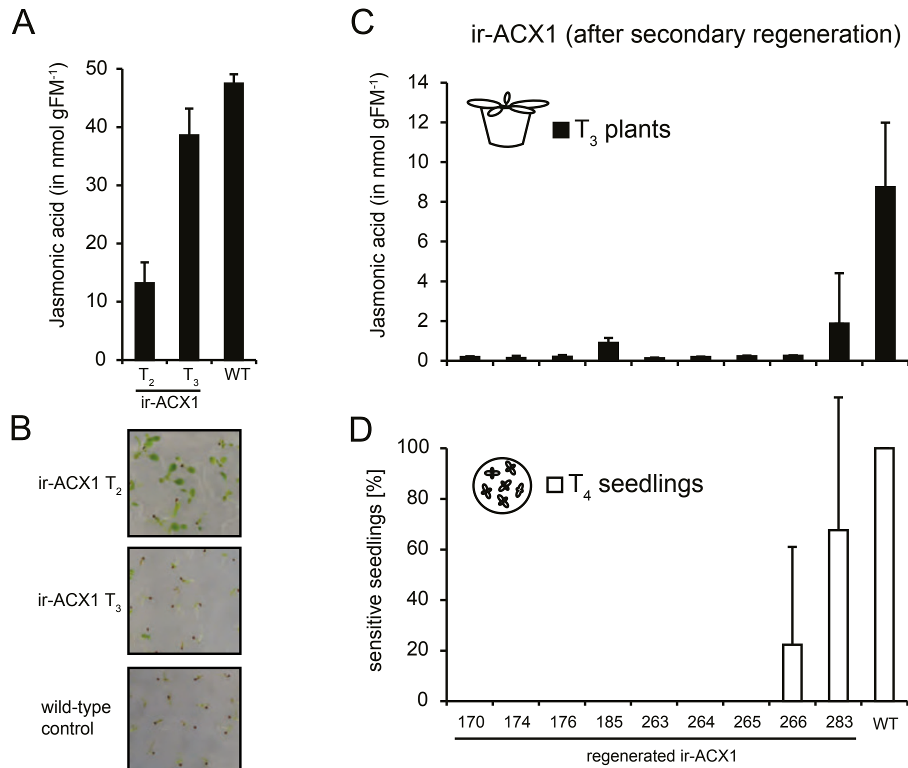
**Additional file 3: Detail of 35S promoter methylation analysis of individual clones.** Tissue harvested 15, 30, 45 and 60 days post germination of **C**, lines PNA 1.2 ($T_2$) and PNA 1.2.1 ($T_3$); **D**, PNA 10.1 ($T_2$) and PNA 10.1.1 ($T_3$); **E**, PNA 8.6 ($T_2$) and PNA 8.6.1 ($T_3$).




**Additional file 4: Sequence preference in CHH methylation sites**
The nucleotide composition of 8-mer sequences around the CHH sites (methylated cytosine in the fifths position) divided in groups with low methylation (0-9 %), medium methylation (10-49 %) and high methylation (50-100 %) frequencies. The pooled frequency data of lines ICE 4.4.1, PNA 1.2.1 and PNA 10.1.1 derived from one time point (60 dpg T3). The logo graphically illustrates the sequence enrichment at particular positions around the methylation site. Maximum sequence conservation is 2 bit, no nucleotide preference is 0 bit. Figures were made with WebLogo 3 (http://weblogo.threeplusone.com/) [129].



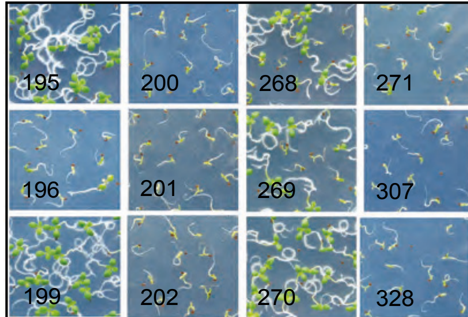

**Additional file 5: Inheritance of the silenced allele after reciprocal crossing with wild-type** Reciprocal crossings of homozygous transgenic plants with wild-type (WT). The hybrid offspring (hemizygous to the transgene) should be theoretically fully resistant to hygromycin B. The silenced state of the transgene was equally distributed to subsequent generations. **A,** Percentage of sensitive seedlings after crossing (± SD, n=3 plants). **B,** Phenotypes of seedlings on hygromycin B containing GB5 media.



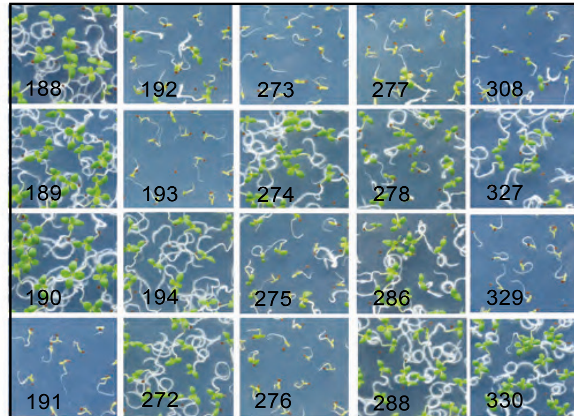

**Additional file 6: Transgene silencing in line ir-ACX1 A,** Jasmonic acid accumulation 1 h after wound and oral secretion treatment in rosette leaves of ir-ACX1 and wild-type plants. The $T_3$ generation of ir-ACX1 lost their capacity to suppress jasmonic acid accumulation [37]. **B,** The $T_3$ seedlings from line ir-ACX1 (A-07-468) developed sensitivity to hygromycin B. **C,** Transgene activity indicated by jasmonic acid accumulation determined in wound and oral secretion treated leaves of secondary regenerated ir-ACX1 lines. A reduced accumulation of jasmonic acid after wounding compared to wild-type (WT) indicated a functional IR-construct. **D,** Hygromycin sensitivity of $T_4$ seedlings (direct descendants of the plants used for wound treatment) indicates an ongoing silencing process (±SD, n=3 plants).

A

$T_3$ offpring from ICE 4.4 regenerants

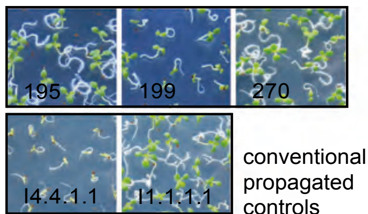



$T_3$ offpring from PNA 1.2 regenerants

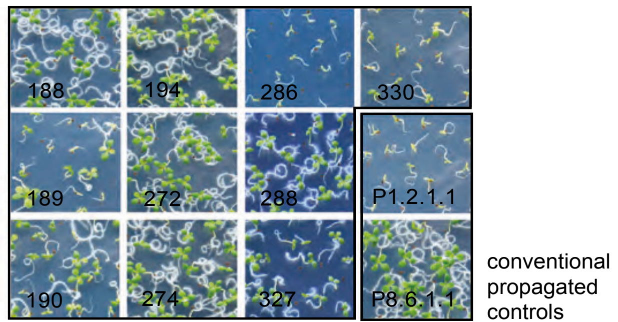



B

$T_4$ offpring from ICE 4.4 regenerants



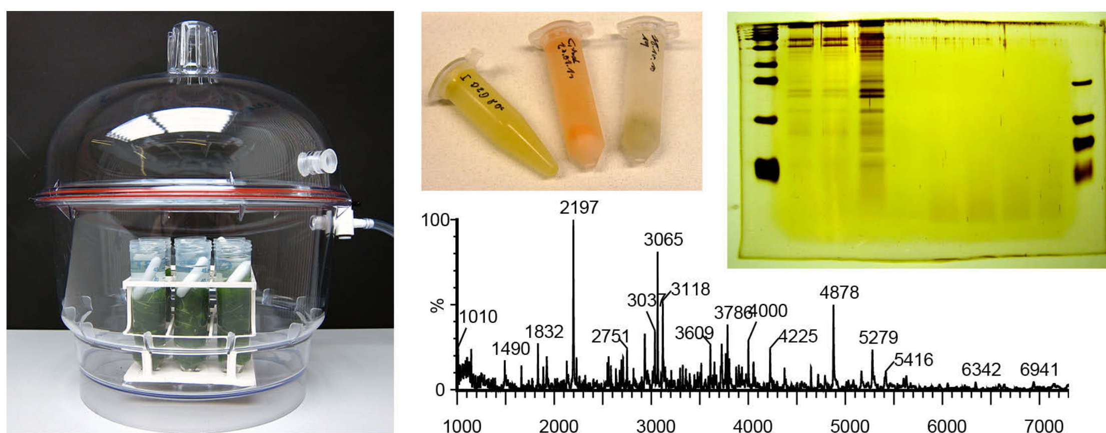

$T_4$ offpring from PNA 1.2 regenerants




**Additional file 7: Phenotypes after secondary regeneration A,** Photographs of $T_3$ seedlings collected from secondary regenerants of line ICE 4.4 and PNA 1.2. Cell culture-induced variations resulted in variegated pattern of sensitivity on hygromycin B containing GB5 media. **B,** Photographs of $T_4$ generation seedlings collected from fully resistant secondary regenerated plants. As positive and negative controls conventional propagated $T_4$ seedlings are shown.

# 5 | Manuscript III

## 5.1. Label-free nanoUPLC-MSE based quantification of antimicrobial peptides from the leaf apoplast of *Nicotiana attenuata*




Vacuum infiltration of leaves was perform in a desiccator at -80kPa. Each peptide extraction yielded about 1.5 - 2 mL intercellular fluid which was further separated on a Tris-Tricine gel or by MALDI/TOF MS.

# Label-free nanoUPLC-MS$^E$ based quantification of antimicrobial peptides from the leaf apoplast of *Nicotiana attenuata*

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## Summary

The over-expression of antimicrobial peptides (AMPs) in plants is a promising approach for crop disease resistance engineering, but apart from achieving high transgene expression, the accumulation and *in planta* stability within the apoplast should be validated for each new AMP candidate. The small size and extreme p*I* range of AMPs limits the utility of gel-based methods for plant protection screens. Despite recent advances in quantitative shotgun proteomics, high throughput methods for AMPs are lacking. Here we present a rapid, versatile and label-free quantitative method that allows researchers to compare the amounts of various heterologously expressed AMPs from the leaf apoplast of *Nicotiana attenuata*. The sizes of the 10 separately expressed AMPs were ranged from 28 to 91 amino acids in length. We coupled a rapid apoplastic peptide extraction method with the label-free nanoUPLC-MS$^E$ methodology and identified and quantified 7 of 10 ectopic expressed AMPs in the intercellular fluid of transgenic plants. This LC-MS approach proved to be high sensitivity in the detection of AMPs and exhibited the high level of analytical reproducibility required for label-free quantitative measurements. The quantitative comparisons revealed high accumulations of three particular peptides, belonging to the defensin, knottin and lipid-transfer protein families, which attained concentrations of 91 to 254 pmol per g leaf fresh mass. The method allows for the rapid quantification of apoplastic peptides without cumbersome and time- consuming purification or chromatographic steps and can be easily adapted to other plant species.

### 5.1.1. Introduction

Antimicrobial peptides (AMPs) are a diverse group of small, cationic peptides that can inhibit the growth of a broad range of microbes. They can be found in plants as well as in

animals and have been shown to play an important role in defense and innate immunity (Stotz *et al.* 2013; Zasloff 2002). The stable ectopic expression of AMPs has great potential to protect crops against a wide range of pathogens (López-García *et al.* 2012; Zhou *et al.* 2011), but several AMPs have been shown to be unsuitable for agricultural practice. A universal screening procedure which could show accumulation levels within the plant would allow researchers to rapidly screen amongst the hundreds of different AMPs to choose new candidates for plant protection.

One of the first animal-peptides heterologously expressed in plants was cecropin B, a small AMP from the giant silk moth *Hyalophora cecropia*. Attempts to detect the peptide in transgenic tobacco and potato plants failed, indicating *in planta* instability (Allefs *et al.* 1995; Florack *et al.* 1995). Cecropin B has been shown to be extremely susceptible to endogenous plant peptidases and even modified versions of the peptide had half-lives of only a couple of minutes when exposed to various plant extracts (Mills *et al.* 1994; Owens and Heutte 1997). Finally, peptidases identified within the intercellular fluid of *Nicotiana tabacum* plants (Delannoy *et al.* 2008), were found to be responsible for peptide degradation, and remain a festering problem for the heterologous protein production in plants (Doran 2006). Recent studies repeatedly report about peptide instabilities (Zhou *et al.* 2011), which are the main focus for the *de-novo* design of peptides for plant protection (Marcos *et al.* 2008; Zeitler *et al.* 2013).

Most AMPs share a number of features: they are very small (<10kDa), have a net positive charge and an even number of conserved cysteine residues (4, 6 or 8), which are connected by intra-molecular disulfide bridges (Pelegrini *et al.* 2011). Cysteine-free AMPs are rarely described in plants, and among these, mainly glycine-rich peptides showed a similar antimicrobial activity (Odintsova *et al.* 2013; Park *et al.* 2000). AMPs are typically produced as pre-proteins containing N-terminal signal peptides, essential for successful heterologous expression, as they avoid an undesired intracellular accumulation and all of the associated collateral damage (Delannoy *et al.* 2008). The secretion of AMPs is also a natural prerequisite for a plant to protect the intercellular space against invasion by microbial pathogens by "poisoning the apoplast" Hückelhoven 2007. The secretion also allows the formation of disulfide bridges when passing through the endoplasmatic reticulum.

The plant cell wall proteome (or secretome) is insufficiently studied, as the extraction of cell wall proteins is challenging (Isaacson and Rose 2007; Lee *et al.* 2004). Secreted proteins can be tightly bound to the polysaccharide matrix or other cell wall components, requiring specific methods for their release and simultaneously minimizing contaminations with intracellular proteins (Jamet *et al.* 2006). Destructive procedures are commonly performed releasing AMPs from the cell wall of ground kernels, using

buffers with high concentrations of salts (Feiz *et al.* 2006). Weakly bound proteins can be released with non-destructive vacuum infiltrations, in which proteins are washed out of the apoplast from an intact leaf (Jamet *et al.* 2008).

Recent progress and developments in mass spectrometry have expanded the field of proteomics from merely protein profiling to the accurate quantification of proteins. The shift from gel-based to gel-free shotgun proteomics allows high throughput and label-free quantitative comparison of biological samples, opening new possibilities in plant sciences (Helm *et al.* 2013; Schulze and Usadel 2010; Wong and Cagney 2010). Small, cysteine rich peptides could especially benefit from this development, as their peculiar molecular features make them ineligible for most classic gel-based procedures. However, high throughput methods for the analysis of AMPs from plant tissue are still lacking.

The wild tobacco (*Nicotiana attenuata*) has been widely used as an ecological model plant and for field studies of gene function. The development of a stable transformation procedure for this species (Krügel *et al.* 2002) allowed for the manipulation of different layers of plant defenses and revealed genes important for defense against herbivores under natural field conditions e.g. (Kallenbach *et al.* 2012). We transformed wild tobacco plants with constructs for the ectopic expression of various AMPs to increase the plant´s resistance against microbes. To increase the probability of a successful expression, we chose 10 different AMPs from plants as well as animals (Table 5.1). Here we describe the

**Table 5.1.:** Abbreviations of the transgenic *Nicotiana attenuata* lines and molecular properties of the ectopic expressed antimicrobial peptides.

| plant line | peptide name | peptide family | organism of origin | monoisotopic mass [Da] | pI | GeneBank (NCBI) |
|---|---|---|---|---|---|---|
| DEF1 | NaDefensin1 | defensin | *Nicotiana attenuata* | 5475.68 | 9.33 | KF939593 |
| DEF2 | NaDefensin2 | defensin | *Nicotiana attenuata* | 5300.58 | 9.08 | KF939594 |
| VRD | VrD1 | defensin | *Vigna radiata* | 5118.33 | 9.06 | AY437639 |
| FAB | Fabatin-1 | defensin | *Vicia faba* | 5236.40 | 9.12 | EU920043 |
| ICE | Mc-AMP1 | knottin | *Mesembryanthemum crystallinum* | 4213.92 | 9.30 | AF069321 |
| PNA | Pn-AMP2 | hevein | *Ipomoea nil* | 4179.68 | 8.52 | U40076 |
| ESC | Esculentin-1 | esculentin | *Rana plancyi fukienensis* | 4781.74 | 9.63 | AJ968397 |
| SSP | Spheniscin-2 | avian defensin | *Aptenodytes patagonicus* | 4504.29 | 11.63 | P83430 |
| LEA | LJAMP2 | lipid transfer protein | *Leonurus japonicus* | 9119.53 | 9.02 | AY971513 |
| CAP | sheperin I + | glycine rich protein | *Capsella bursa-pastoris* | 2360.95 | 7.28 | HQ698850 |
| | sheperin II | | | 3257.29 | 7.28 | HQ698850 |

development of a peptide extraction method, capable of supporting high throughput plant screenings to confirm stable expression of a variety of different AMPs (with molecular masses ranging from 2.3 to 9.1 kDa and isoelectric points between 7.3 and 11.6). Our goal was to develop a method that allows for the rapid processing of many samples with relatively small volumes without requiring complex purification or chromatographic steps. The direct analysis of the crude intercellular fluid by nanoUPLC-MS$^E$ allows for the (qualitative) detection and even the (quantitative) comparison of peptide amounts among the different transgenic lines. Furthermore, this method does not rely on the availability of antibodies and can be easily adapted to other plant species.

### 5.1.2. Results

#### Ectopic expression of AMPs in transgenic *N. attenuata* plants

For the ectopic expression of AMPs in the wild tobacco (*N. attenuata*), ten different transformation constructs harboring ten different antimicrobial peptides (AMPs) were constructed. Two of the peptides (DEF1 and DEF2) were endogenous AMPs from *N. attenuata* and were ectopically expressed in all plant tissues. Most of the other peptides were derived from plants (see **Table 1**) and selected to span the range of diversity found in the various AMP families (e.g. defensins, heveins, knottins, lipid-transfer proteins and glycin-rich peptides). Additionally, two peptides from animals were tested for their suitability in *N. attenuata*. The stable transformation of *N. attenuata* was performed by *Agrobacterium* mediated gene transfer (Krügel *et al.* 2002) and all peptides were expressed under the control of a constitutive 35S promoter. To direct their channeling into the protein secretion pathway, all peptides contained their native signal peptide (Fig.5.1). Only the animal derived ESC and SSP constructs were fused to a plant signal peptide of the polygalacturonase-inhibiting protein (PGIP) leader sequence from *Phaseolus vulgaris*, which has been shown to target peptides for secretion in *N. tabacum* (Ponti *et al.* 2003). The complete sequences of the pre-peptides and the composition of the disulfide bridges are illustrated in Fig.5.1. Due to inconsistent naming of the peptides in the literature we use the names of the plant lines from Table 5.1 also as a synonym for the expressed peptides and the peptide genes. All transformed plants were thoroughly screened following the optimized protocol described in Gase *et al.* 2011. Homozygous, single copy lines were tested for stable transgene expression by qRT-PCR and all epigenetically silenced plant lines excluded (Weinhold *et al.* 2013). Although gene expression analysis confirms the functional expression of a transgene, it provides no information about actual protein levels or stability of the ectopically expressed peptide

**Figure 5.1.: Abbreviations of the transgenic *N. attenuata* lines and the amino acid sequences of the ectopically expressed antimicrobial peptides.** The N-terminal signal peptides are indicated in red, the mature peptide sequences are shown in blue and C-terminal or other domains in grey. Cysteine residues which are connected by disulfide bridges are indicated. The simulated 3D structures of the DEF2, LEA and ICE peptides were retrieved from SWISS-MODEL (`http://swissmodel.expasy.org/`) and drawn with PYMOL softwarepackage 0.99rc6 (2006 DeLano Scientific).

within a plant. Therefore we extend the screening procedure with a method that allows for the comparison of peptide abundances.

### Selective peptide isolation by intercellular fluid extraction

The subcellular localization of the AMPs requires specific methods for a selective extraction (Fig. 5.2). We modified a vacuum infiltration/centrifugation protocol (Dani *et al.* 2005), for the extraction of the apoplastic or intercellular fluid (ICF) from *N. attenuata* leaves (Fig. S5.1). ICF samples should theoretically contain only proteins and peptides from the apoplast and loosely bound cell wall proteins, as the cytoplasmic membrane remains undamaged during processing. To specifically enhance the solubility of basic peptides we used two different infiltration buffers, both containing high concentrations of salt and both with acidic pH (MES buffer pH 5.5 and citric acid buffer pH 3.0). The infiltration of about 5 - 6 leaves per plant allowed the recovery of 2.5 - 3 mL yellowish ICF. The overall yield among all plants was relatively homogenous with a mean value of 320 $\mu$L ICF/gFM ($\pm$30 $\mu$L, n=33 plants). By using a gentle centrifugation force (300 $\times$ g) tissue damage and intracellular protein contamination could be avoided, which would

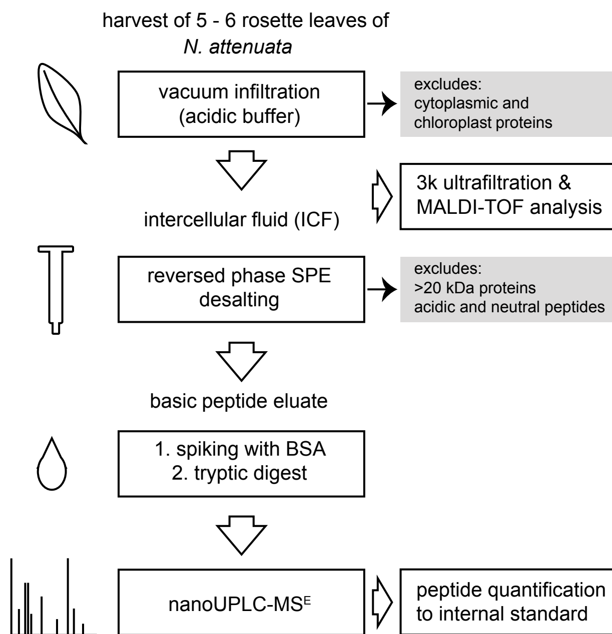



**Figure 5.2: Schematic representation of the workflow used for sample preparation of antimicrobial peptides (AMPs).** Intercellular fluid (ICF) was extracted by vacuum infiltration and desalted using reversed phase solid phase extraction cartridges (SPE). The samples were spiked with bovine serum albumin (BSA) which served as internal standard, tryptically digested and analyzed by nanoUPLC-MS$^E$. Final peptide quantity was calculated and expressed as pmol per g fresh mass (FM).

be indicated by a greenish color of the ICF. For all downstream MS based applications a rigorous desalting of the ICF samples was necessary. We initially used small volume (500 $\mu$L) ultrafiltration devices with a 3K cut-off and analyzed samples by MALDI-TOF mass spectrometry. To target also extremely small <3kDa peptides, we switched to reversed phase SPE cartridges for desalting and used a three-step elution to sequentially elute peptides by their charge for a higher purification and enrichment of basic peptides. With this procedure small volume samples could be rapidly desalted, reducing handling time, the probability of protein loss and allowing for the processing of multiple samples in parallel for nanoUPLC-MS$^E$ analysis (for a sample preparation flowchart, see Fig. 5.2).

### AMP mass mapping by MALDI-TOF mass spectrometry

To compare the peptide mass pattern of transgenic with those of WT plants, the desalted crude ICF extracts were subjected to analysis by Matrix-Assisted Laser Desorption/Ionization – Time-of-Flight Mass Spectrometry (MALDI-TOF/MS). This approach was chosen as it is well suited for the rapid screening of peptide samples of low complexity due to its simplicity. Samples were analyzed in linear ion mode in the *m/z* range of 1,000 – 10,000 to cover the expected masses of all peptides (2.3 to 9.1 kDa). Only in two of the transgenic lines, we found a peak within the expected mass range of the expressed peptides for ICE – 4,215.85 Da (calculated monoisotopic mass 4,213.92 Da) and LEA – 9,122.71 Da (calculated monoisotopic mass 9,119.53 Da) (Fig. 5.3). This

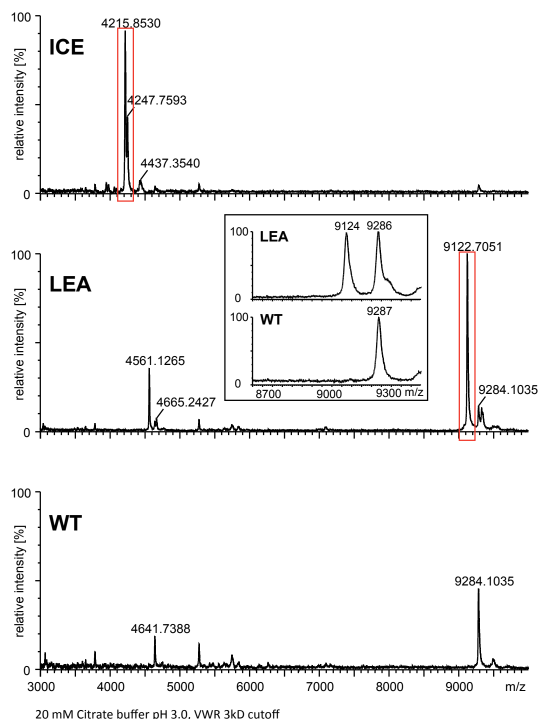



**Figure 5.3: Comparison of the MALDI-TOF mass spectra acquired from the intercellular fluid (ICF) of WT and transgenic ICE and LEA lines in the mass range 1–10 kDa.** ICF was extracted with citrate buffer (pH 3.0), desalted by ultrafiltration (VWR 3K columns) and analyzed in linear ion mode. Peaks within the mass ranges of the expressed peptides are highlighted. The inset shows the MALDI-TOF/MS analysis of the supernatant from WT and LEA lines (35 mL concentrated by Amicon 3K columns).

was a strong indication for AMP accumulation and successful localization within the apoplast. The peak masses indicated full mature peptide length without evidence for truncations or proteolytic loss. However, with this method most of the other transgenic lines showed no difference from WT plants, regardless of types of ultrafiltration devices used (Fig. S5.2), most probably because of the limited resolution and sensitivity of MALDI-TOF/MS analysis for peptides at molecular weights above 3 kDa. To test for an eventual leakage of the peptides during ICF processing, we concentrated and analyzed also the used infiltration buffer (hereafter called supernatant) which remains after leaf removal following the vacuum infiltration (Fig. S5.1). The analysis of the supernatant revealed a peak in the LEA line, indicating the partial release of this peptide into the supernatant during the vacuum infiltration process (Fig. 5.3, inset).

#### AMP identification and quantification by nanoUPLC–MS$^E$

As most of AMP could not be detected by MALDI-TOF/MS, the samples were subjected to nanoUPLC-MS$^E$ analysis. This method, known as data-independent acquisition, relies on chromatographic separation of peptides that can be achieved with high reproducibility on an ultra-performance nanoUPLC system combined with global tandem mass spectrometry acquisition at alternating low/high collision energy (Ullmann-Zeunert *et al.* 2012). The high sampling rate in MS$^E$ data acquisition enables collection of sufficient data points

to quantify peak ion intensities and was implemented in the label-free quantification of proteins where the intensity of three most intense or most efficiently ionized tryptic peptides of a protein, called universe response factor, is used as a measure of its abundance (Silva *et al.* 2006). For nanoUPLC-MS$^E$ analysis, ICF samples were desalted by reversed phase SPE cartridges (Fig. 5.2) and 5 $\mu$L of the final eluted fraction was spiked with 1 pmol bovine serum albumin (BSA), which functioned as an internal standard for quantification, followed by digestion with trypsin. To assess the applied quantification method, linear response and analytical reproducibility were considered. To this end serial dilutions were injected, corresponding to 2.5-25 $\mu$L ICF sample containing BSA amounts ranging from 50-500 fmol. Among the detected tryptic peptides, several could be reliable matched to the sequences of the overexpressed AMPs (Tab. 5.3). Overall 7 of

**Table 5.3.:** Tryptic peptides of overexpressed AMPs detected by nanoUPLC-MS$^E$ in the intercellular fluid of *N. attenuata* plants.

| line | pep score | calc. [MH]+ | exp. [MH]+ | Rt [min] | Δppm | sequence | sequence coverage |
|---|---|---|---|---|---|---|---|
| DEF1 | 8.41 | 1999.9077 | 1999.9001 | 36.36 | 3.77 | AESNTFEGFC*VTKPPC*R | 35.4% |
| | 8.08 | 1000.4028 | 1000.4050 | 24.83 | -2.21 | C*IC*YKPC* | 14.6% |
| DEF2 | 8.87 | 1977.9619 | 1977.9522 | 39.11 | 4.93 | TESNTFPGIC*ITKPPC*R | 36.2% |
| | 7.39 | 707.3444 | 707.3393 | 36.28 | 7.17 | AC*ISEK | 12.8% |
| | 7.79 | 938.3905 | 938.3894 | 19.83 | 1.24 | C*LC*TKPC* | 14.9% |
| VRD | 8.49 | 1465.6243 | 1465.6233 | 25.76 | 0.65 | C*LIDTTC*AHSC*K | 26.1% |
| | 8.44 | 1089.4137 | 1089.4163 | 33.56 | -2.42 | TC*YC*LVNC* | 17.4% |
| | 7.03 | 1534.6249 | 1534.6270 | 23.58 | -1.41 | GMTRTC*YC*LVNC* | 26.1% |
| LEA | 10.27 | 1518.7911 | 1518.7911 | 39.09 | 0.01 | SYSGINLGNAAGLPGK | 17.6% |
| | 9.91 | 1925.8779 | 1925.8732 | 38.39 | 2.39 | C*GVSIPYQISPNTDC*SK | 18.7% |
| | 8.35 | 1236.6117 | 1236.6116 | 37.25 | 0.14 | MAPC*LPYVTGK | 12.1% |
| | 9.32 | 1061.4906 | 1061.4867 | 28.46 | 3.66 | GPLGGC*C*GGVK | 12.1% |
| | 9.64 | 1020.5135 | 1020.5143 | 20.98 | -0.74 | AIGC*NTVASK | 11.0% |
| | 9.52 | 992.4647 | 992.4653 | 22.49 | -0.56 | QAVC*NC*LK | 8.8% |
| | 9.16 | 715.4095 | 715.4097 | 28.81 | -0.31 | GLIDAAR | 7.7% |
| PNA | 6.37 | 3421.3268 | 3421.3042 | 37.93 | 6.60 | LC*GNGLC*C*SQWGYC* GSTAAYC* GAGC*QSQC*K | 73.2% |
| FAB | 7.58 | 1924.8133 | 1924.8100 | 31.21 | 1.74 | FNGPC*LTDTHC*STVC*R | 34.0% |
| ICE | 9.26 | 1879.7229 | 1879.7198 | 39.08 | 1.68 | EDQGPPFC*C*SGFC*YR | 40.5% |
| | 8.62 | 716.3829 | 716.3838 | 25.60 | -1.27 | QVGWAR | 16.2% |
| | 7.25 | 2252.8720 | 2252.8730 | 43.86 | -0.43 | GC*REDQGPPFC*C*SGFC*YR | 48.6% |

Carbamidomethylated cysteine indicated as C*; Δ ppm = $10^6(M_{tn} - M_{exp}){M_{tn}}^{-1}$

10 transgenic *N. attenuata* lines (DEF1, DEF2, VRD, FAB, ICE, PNA, and LEA) were tested positive and confirmed for AMP expression. From the LEA peptide up to 7 tryptic peptides could be identified, resembling 88% of the mature peptide sequence. Although most AMPs result only in a small number of tryptic peptides (Table S5.1), due to their small sizes, the sum of all detectable peptides resulted in more than 50% sequence coverage (except for FAB, with only 34%) (Table 5.3). The defined amount of BSA, that was spiked into the samples, allowed for the calculation of the molar concentration of each AMP per mL ICF or per g fresh mass (FM), based on the relationship between the intensity

of the internal standard to the peptides of interest (Silva *et al.* 2006). The quantitative comparison among all plant lines indicated relatively low peptide amounts expressed within the PNA, FAB, DEF1 and VRD lines with only 0.2 – 11 pmoL $g^{-1}$ FM (Fig. 5.4). In particular the low abundant PNA peptide was only detected in 1 out of 3 biological replicates. In contrast, three other plant lines (DEF2, ICE and LEA) indicated very high peptide amounts with 92 – 254 pmoL $g^{-1}$ FM (Fig. 5.4). This confirmed the desired

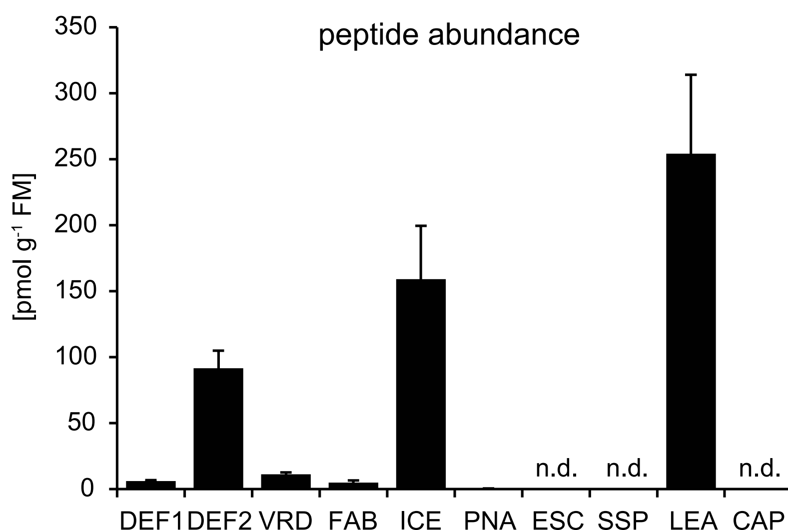



**Figure 5.4.: Comparison of peptide abundance calculated from LC-MS$^E$ data of different transgenic *N. attenuata* lines.** Intercellular fluid (ICF) was extracted with MES buffer (pH 5.5) and desalted using reversed phase cartridges. The samples were analyzed using nanoUPLC-MS$^E$ and the peptide abundance was calculated based on the relation between the averages of the intensity of the three most intense peptides of the internal standard (BSA) to the peptides of interest (Silva *et al.* 2006). The peptide abundances were calculated and expressed as pmol per g fresh mass (FM), showing the standard error among three biological replicates (for DEF2, ICE and LEA n=6); n.d. = not detected.

high accumulation within the apoplast, as it would be required for these transgenic plants. To estimate the accuracy of the quantification method, the linear response of AMPs to the internal standard BSA (which was assessed for linear responses within the used concentrations) was determined by analyzing serially diluted samples. For highly abundant peptides (DEF2, ICE and LEA) MS$^E$ based quantification revealed a wide linear dynamic range among the injected concentrations for up to 8000 fmol (Fig. 5.5a). Since we worked with native concentrations from biological samples we could not further exceed these values to reach possible saturation limits. The amounts of these three highly abundant AMPs were analyzed in 3 additional plants, confirming high accumulation in all 6 biological replicates and among all individual quantifications a small technical

error with an average relative standard deviation of 21.1% (Fig. S5.3). Also the low abundant AMPs showed linearity with BSA in serial dilutions (Fig. 5.5b). As the DEF1

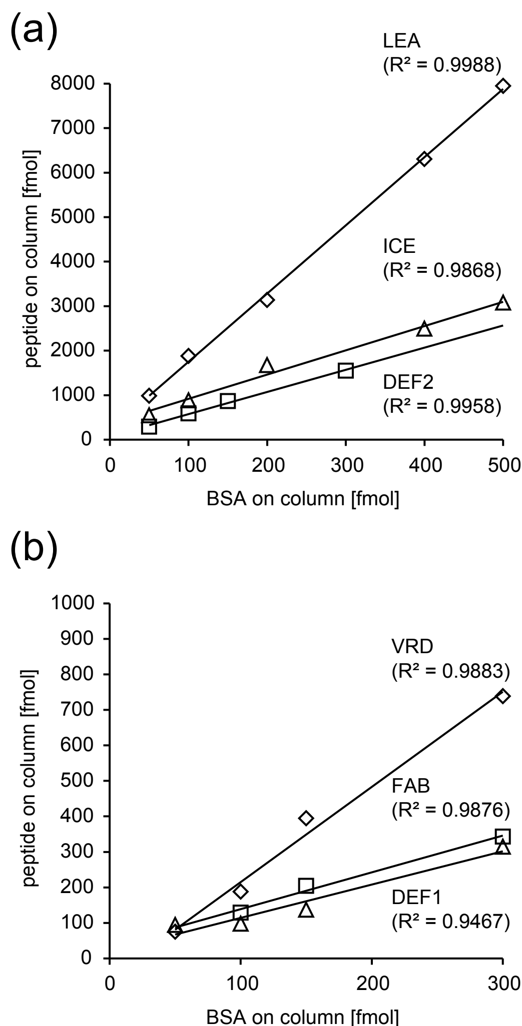
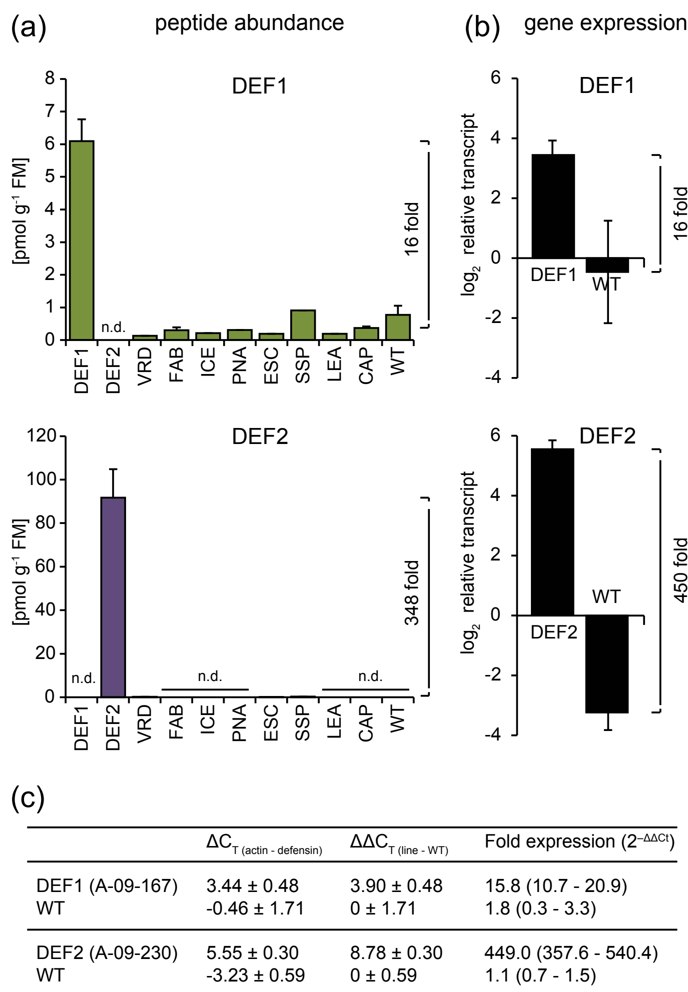



**Figure 5.5: Linear dynamic range of nanoUPLC−MS$^E$ measurements of AMPs.** To determine the linear dynamic range of quantification the calculated peptide amounts [fmol/column] were plotted against the corresponding amount of BSA in the sample (50-500 fmol); BSA was linear in the full range tested. (a) Linear regression ($R^2$) shown for the high abundant AMPs (LEA, ICE and DEF2). (b) Linear regression ($R^2$) shown for the low abundant AMPs (VRD, FAB and DEF1).

and DEF2 peptides were endogenous defensins of *N. attenuata*, peptide levels could be directly compared to native levels within an untransformed plant. The DEF1 peptide could indeed be detected in the ICF of WT plants and in most of the other transgenic lines as well (Fig. 5.6a). The DEF1 over-expression line showed the highest peptide amounts, which was about 16-fold higher than the average found in all other lines. This correlated with the expectations from gene expression data, which showed on average a 16-fold increase in transcript level compared to WT (Fig. 5.6b). The DEF2 plants showed much higher transcript levels, which were on average 450-fold higher compared to WT. This was as well consistent with the observed peptide amounts, which were 348-fold elevated compared to the basal amount found in some transgenic lines. As we had evidence of




| | $\Delta C_{T\,(actin - defensin)}$ | $\Delta\Delta C_{T\,(line - WT)}$ | Fold expression ($2^{-\Delta\Delta Ct}$) |
|---|---|---|---|
| DEF1 (A-09-167) | 3.44 ± 0.48 | 3.90 ± 0.48 | 15.8 (10.7 - 20.9) |
| WT | -0.46 ± 1.71 | 0 ± 1.71 | 1.8 (0.3 - 3.3) |
| DEF2 (A-09-230) | 5.55 ± 0.30 | 8.78 ± 0.30 | 449.0 (357.6 - 540.4) |
| WT | -3.23 ± 0.59 | 0 ± 0.59 | 1.1 (0.7 - 1.5) |

**Figure 5.6: Comparison of peptide abundance with strength of gene expression.** (a) Abundance of endogenous antimicrobial peptides (DEF1 and DEF2) in WT and transgenic plants. The DEF1 overexpressing lines showed about 16 fold higher amounts compared to the average found in all other lines. The DEF2 overexpressing lines showed about 348 fold higher amounts compared to the average found in all other lines. (b) Gene expression from the DEF1 and DEF2 lines compared to WT. Transgene expression is shown as $\log_2$ relative expression compared to actin as reference gene for line DEF1 and DEF2 (± SD, n = 4 plants). (c) Calculation of fold differences in gene expression compared to WT using the comparative $C_T$ method.

peptide release during ICF processing, we analyzed also the supernatants from each plant line by nanoUPLC-MS$^E$. We concentrated 15 mL supernatant using SPE cartridges and analyzed 5% of the eluted fraction (equivalent to 750$\mu$L supernatant). The quantitative comparison of the concentrated supernatant revealed a similar pattern as observed from the ICF samples. The highest peptide amounts were found as well in the DEF2, ICE and LEA lines (Fig. S5.4), indicating that peptides might be released into the buffer nearly proportional to the overall peptide amount found in the apoplast. However, the active secretion of the peptides from the roots could not be confirmed. Plants were grown in hydroponic solutions from which 50 mL was concentrated using SPE cartridges. From the eluted fractions 10% were analyzed (equivalent to 1.7 mL root exudate), showing no match for any of the expressed AMPs.

### 5.1.3. Discussion

The facile absolute quantification of plant proteins has the potential to substantially advance many research areas, however sample complexity still thwarts robust quantifications, particularly for cationic AMPs. In this study, we developed a high throughput method for extracting and processing intercellular fluid from leaf tissue, generating samples suitable for mass spectrometric analysis and allowing the detection and quantification of different ectopically expressed AMPs in transgenic *N. attenuata* plants. We adapted a vacuum infiltration method for *N. attenuata* and tested different desalting procedures to analyze peptide abundances with nanoUPLC-MS$^E$ in a high throughput fashion (Fig. 5.2). The results of the quantification could confirm the accumulation of heterologously expressed peptides in the apoplast and could be easily adapted to the analysis and quantification of other apoplastic peptides.

#### AMPs require specific extraction methods

Many purification methods make use of the unique biochemical properties of AMPs, such as their small size, their positive charge, their tolerance to acids and heat or even the presence of disulfide bridges, as done recently by Hussain *et al.* 2013. We took advantage of the subcellular localization within the apoplast and the selectivity of extraction during vacuum infiltration. The obtained intercellular fluid (ICF), also commonly called apoplastic wash fluid (AWF) or intercellular washing fluid (IWF), shows a tremendously reduced complexity compared to crude, whole cell fractions, containing cytoplasmic and chloroplast proteins. To achieve an optimized infiltration process, the ICF extraction protocol needs to be adapted to each plant species (Lohaus *et al.* 2001). The salt concentrations and the pH of the infiltration buffer have also a large influence on the protein extraction efficiency (Boudart *et al.* 2005). In general, mild acids are commonly used for the extraction of AMPs as done for the isolation of floral defensins from the ornamental tobacco, *N. alata* (Lay *et al.* 2003a). In addition, the use of acidic buffers has the advantage of reducing phenolic browning of the extracts, which is a common problem for other protein extraction buffers used in *N. attenuata* and other tobacco species, e.g. for trypsin protease inhibitor extraction (Jongsma *et al.* 1994). Ultrafiltration can separate proteins by size, but allows no further purification. We tested the pre-cleaning of large proteins with a 30K cut-off ultrafiltration device and a heat clearance step prior to desalting (10 min at 80°C) and could confirm the heat stability of the ICE and LEA peptides (Fig. S5.2). But we generally omitted these steps as they did not improve the overall sample quality. Desalting with reversed phase SPE cartridges allows not only size exclusion, but also separation by charge, which resulted in a further

reduction of the ICF sample complexity. As the sequentially elution steps during SPE processing removed contaminants and could enrich basic peptides in the final fraction, it was the preferred method for all nanoUPLC-MS$^E$ measurements. The whole method was developed as a universal extraction of cationic peptides. Since there is no all-round method which could cover conditions of all AMPs, it was not surprising that the method was not optimal for the CAP peptides. These glycine-rich peptides were not cleavable by trypsin and likely need specific modifications regarding the desalting process or the use of different digestion enzyme to increase the chances of later detection.

### nanoUPLC-MS$^E$ based AMP quantification

Due to their small size, AMPs are commonly overlooked and underrepresented in genome annotations in plants (Lease and Walker 2006; Manners 2007; Silverstein *et al.* 2007). Similarly, AMPs are also underrepresented in conventional, gel-based proteome studies, due to difficulties in detecting basic peptides with high p*I* level and small molecular sizes (<10 kDa) (McNulty and Slemmon 2004). Small cysteine-rich peptides are not amenable for most methods routinely used for large proteins and even AMPs that accumulate to high levels have been shown to be barely detectable on immunoblots (Lee *et al.* 2011). In the past, the production of efficient antibodies with affinity to the mature peptide has been shown to be problematic (Lay *et al.* 2003a; Zhou *et al.* 2011). Their small size does not usually allow any tagging without having a negative impact on the *in vivo* activity and certainly a misleading influence on protein stability. *In vitro* test have shown potential for the use of RP-HPLC and NMR based methods for the quantification of pure fractions of cyclotides, but showed also limitations for spectrophotometric methods (Conibear *et al.* 2012). Through technical advances in high-performance LC separation of peptides and development of modern mass spectrometer with high resolution and scanning rates, label-free quantification of proteins has been implemented in proteomic routine (Neilson *et al.* 2011; Shen *et al.* 2009). This simple and cost-efficient method enables simultaneous protein quantification across many samples. NanoUPLC-MS$^E$ based quantification of proteins, used in this study, combined advantages of ultra-performance liquid chromatography that provides high reproducibility in nanoUPLC runs with sufficient sensitivity of MS$^E$ acquisition required for accurate quantitative analysis. Instead of analyzing secreted proteins from cell culture media (Blackburn *et al.* 2010; Cheng *et al.* 2009), we directly injected desalted and tryptically digested ICF samples derived from plant tissue for quantification by nanoUPLC-MS$^E$.

Despite the achieved *in vitro* precisions, variability among samples prepared from complex tissues is the major limitation in the application of quantitative proteomics (Levin *et al.* 2011; Silva *et al.* 2006), which is particularly true for cell wall bound

peptides. Despite the variability among biological replicates resulting from separate infiltration procedures (Fig. S5.3), we found consistent patterns of peptide abundance and, among the highly abundant peptides, a remarkable large linear dynamic range. It should be noted that the small size of most AMPs strongly limits the options in selecting best ionizable tryptic peptides for quantification measures (Silva *et al.* 2006), in contrast to very large and abundant plant proteins, which yield a much broader variety of tryptic peptides and allow more precision in quantification (Ullmann-Zeunert *et al.* 2012). When necessary, we included also miss-cleaved or fragmented tryptic peptides to be able to perform the top 3 matched peptide quantification for all AMPs. This seemed to be the most appropriate method as it resulted in good linear ranges for all AMPs compared to BSA. Only in the analysis of the defensins (DEF1, DEF2 and VRD) was the linearity greater if the sum of the intensity of all matched peptides was used for quantification. However, as this procedure decreased accuracy for the LEA and ICE peptides, we used the top 3 matched peptides for quantification to maintain comparability among all the different AMPs. Another possible way improving further accuracy could be achieved by using a peptide standard of a similar size as the AMPs.

**AMP localization and expression in plants**

In the ornamental tobacco (*N. alata*) two floral defensins had been previously reported to be localized only in the vacuole, suggesting that their carboxyl-terminal pro-domains have a protein trafficking function (Lay and Anderson 2005), which could recently be confirmed Lay *et al.* 2014. The orthologous DEF2 peptide of *N. attenuata* has 100% amino acid similarity to *N. alata* NaD1 and we expected an accumulation within the vacuole. However, large amounts of the over-expressed peptide were detectable within the ICF of *N. attenuata*, consistent with their secretion into the apoplast (Fig. 5.4). Although the DEF1 peptide shared 86% protein sequence similarity with DEF2, their expression strength and the amount of accumulated peptide differed dramatically between these lines. DEF2 was much more over-expressed than DEF1, an observation that strongly calls into question the ability to predict suitable candidates for over-expression studies based merely on sequence data. The overall tremendous differences in AMP accumulation amongst all plant lines emphasize the value of a direct assessment of peptide amounts. In fact, the PNA and ESC lines were initially among our most promising candidates, as these peptides have been successfully expressed previously in *N. tabacum* (Koo *et al.* 2002; Ponti *et al.* 2003). But in *N. tabacum* the esculentin-1 peptide showed also signs of degradation by exopeptidases (Ponti *et al.* 2003), a result consistent with our observation. However, the lack of AMP detectability could either indicate instability or amounts below the detection limit, both leading to an exclusion of the plant lines from further studies.

AMPs usually need to accumulate to large amounts, as was found in the DEF2, ICE and LEA lines, to exert a biological function. Interestingly, most of the peptides could also be found within the supernatant, that was left over after vacuum infiltration (Fig. S5.4). More strikingly, the overall pattern of peptide abundance was very similar among ICF and supernatant samples. This suggests that either the peptides readily diffuse out of the apoplast during the infiltration process, or are washed from the leaf surface. The analysis of a pure leaf surface wash would be a promising future experiment, which could further clarify this hypothesis. A leaf surface deposition is in particularly likely for the DEF1 and DEF2 peptides as the concentrations (per mL) were only 10 – 19 fold lower in the supernatant than the concentrations (per mL) from the ICF samples. In contrast, the concentrations of the other peptides were 44 – 143 time lower in the supernatant.

### Further application and conclusion

Bio-analytical technology has recently made tremendous progress in the development of peptide quantification techniques (Helm *et al.* 2013) and opens a wide field for applications. The analyses of peptide fluctuations within the plant cell wall, after wounding or infection, are possible further applications. The most limiting factor for peptide quantification is perhaps the bias resulting from sampling and sample preparation. As the various chemical properties of different peptides result in diverging affinity for extraction and/or purification it is challenging estimating the "real" *in vivo* concentrations. Further improvement is expected if other digestion methods then trypsine-assisted proteolysis will be tested for small polypeptides with a limited number of Lys and Arg in the chain. We could show that even a simple extraction procedure can be used for the efficient release of a diverse set of antimicrobial peptides from leaf tissue as a universal method to achieve reliable peptide quantification results by nanoUPLC-MS$^E$.

## Experimental procedures

### Construction of plant transformation vectors

The sequences of different genes coding for antimicrobial peptides were selected from the PhytAMP database (http://phytamp.pfba-lab-tun.org/main.php) and from NCBI (Table 5.1. The animal peptides SSP and ESC were fused to the signal peptide of the polygalacturonase-inhibiting protein (PGIP) leader sequence from *Phaseolus vulgaris* as described in (Ponti *et al.* 2003). All AMP sequences were tested for the presence of a signal peptide using the SignalP 3.0 Server (`http://www.cbs.dtu.dk/services/SignalP/`). The sequences for the SSP, ESC, PNA, VRD and FAB constructs were manually adapted

to the codon usage table of *N. tabacum* (`http://gcua.schoedl.de/`). Genes from *N. attanuata* were directly PCR amplified from leaf cDNA. Most of the other constructs were synthesized in sequential PCR reactions with overlapping 40 bp primers and did not require the availability of cDNA from the organism of origin. Only the glycine-rich CAP peptide gene could not be synthesized and was amplified from root cDNA of a wild *Capsella bursa-pastoris* plant collected in front of the Institute for Chemical Ecology (Jena, Germany) using primers with *Xho*I and *Hind*III restriction sites (underlined) CAP15F (5'-GCGGCGCTCGAGATGGCTTCCAAGACTTTGATAC-3') and CAP16R (5'-GCGGCGAAGCTTAAACACCGGGCTGAGTC-3'). The amplified gene (HQ698850) coded for 7 additional amino acids, but had still 92% identity to shep-GRP (AF180444), previously described in (Park *et al.* 2000). All genes were cloned in pSOL9 binary plant transformation vectors under a cauliflower mosaic virus promoter (35S) described in Gase *et al.* 2011. Two peptides had amino acid substitutions compared to their native sequence DEF2 (Ile102Met) and Esc (Met28Leu).

**Plant transformation and growth conditions**

*Nicotiana attenuata* Torr. ex S. Watson seeds were originally collected in 1988 from a natural population at the DI Ranch in Southwestern Utah. Wild-type seeds from the $30^{th}$ inbreed generation were used for the construction of transgenic plants and as WT controls in all experiments. Plant transformation was performed by *Agrobacterium tumefaciens*-mediated gene transfer as previously described (Krügel *et al.* 2002). Transgenic plant lines were screened as described in Gase *et al.* 2011 and Weinhold *et al.* 2013. Homozygous, single insertion $T_3$ plant lines used in $MS^E$ quantification were: LEA 1.7.1 (A-09-721), PNA 8.6.1 (A-09-823), FAB 9.3.1 (A-09-865), ICE 6.4.2 (A-09-748), CAP 6.4.1 (A-09-949), DEF1 F.3.1 (A-09-167), DEF2 C.7.1 (A-09-230), SSP 6.5.1 (A-09-671), ESC 1.3.1 (A-09-693) and VRD 4.7.1 (A-09-668). Additional lines used for MALDI analysis were: ICE 1.1.9 (A-09-653), SSP 4.6.1 (A-09-775), ESC 2.7.1 (A-09-778) and VRD 1.9.1 (A-09-652). Seeds were germinated as described in Krügel *et al.* 2002 and incubated in a growth chamber (Percival, day 16 h 26 °C, night 8 h 24 °C). Ten-days-old seedlings were transferred to communal Teku pots and ten days later into individual 1 L pots and cultivated in the glasshouse under constant temperature and light conditions (day 16 h 26 - 28 °C, night 8 h 22 - 24 °C). For collection of root exudates, plants were grown in hydroponic culture in individual 1L pots containing 0.292 g/L Peter's Hydrosol (Everri, Geldermalsen, the Netherlands). After 25 days of growth the hydroponic solution from 5 plants was pooled and 50 mL sterile filtered using a Minisart sterile filter 0.2 $\mu$m (Sartorius). The solution was concentrated using reversed phase SPE cartridges (see below).

### Vacuum infiltration

The Intercellular fluid (ICF) was extracted from 35 – 45 days old *N. attenuata* plants using a modified vacuum infiltration method (Dani *et al.* 2005). Per plant 6 – 8 fully expanded leaves were detached and, if necessary, the midrib excised with a scissor (Fig. S5.1. The leaves were rolled with forceps and submerged in 40 mL chilled (4°C) infiltration buffer, either MES buffer pH 5.5 (20 mM MES/KOH pH 5.5, 1M NaCl, 200 mM KCl, 1 mM Thiourea) or a citrate buffer pH 3.0 (20 mM citric acid/sodium citrate pH 3.0, 200 mM $CaCl_2$, 1 mM Thiourea). The submerged leaves were placed into a desiccator and a vacuum of -80 kPa applied for 5 minutes. Air bubbles were dislodged from the leaves with gentle agitation. By slowly releasing the vacuum the apoplastic spaces were filled with infiltration buffer, which was visually checked indicated by darken of the leaves. Leaves were surface dried with paper towels and placed into a barrel of a 20 mL syringe, stuffed with glass wool at the tip and hung in a 50 mL centrifuge tube. ICF was released by slow centrifugation (300 × g) in a swing bucket rotor for 15 min at 4°C supplemented with 10 $\mu$L protease inhibitor cocktail for plant cell and tissue extracts (Sigma, P9599). After two rounds of infiltration the used infiltration buffer was clarified by centrifugation (20 min at 400 g) and 15 mL saved as "supernatant". Samples were frozen at -20°C until further processing.

### Peptide desalting

The peptide fractions of the ICF samples were desalted and concentrated either by ultrafiltration or reversed phase SPE cartridges. Prior ultrafiltration some ICF samples were also heat cleared at 80°C for 10 min in a heating block and the heat sensitive proteins removed by centrifugation in a table top centrifuge (16,000 × g, 10 min). The supernatant was desalted and concentrated with either Amicon Ultra-0.5 columns (Ultracel 3K Membrane) or with the VWR Centrifugal Filter (modified PES 3K), both with a loading capacity of 500 $\mu$L and a 3 kDa size cutoff. Samples were re-loaded and centrifuged for 15 min at 14,000 × g at room temperature in a table top centrifuge, washed 3× with 450$\mu$L Milli-Q water and stored at -20°C until further analysis. Solid phase extraction was performed using Phenomenex Strata[2122] X 33 $\mu$m Polymeric Reversed Phase columns (30 mg/mL), conditioned prior use with 1 mL acetonitrile (ACN) and equilibrated with 1 mL Milli-Q water. From each sample 1 mL was consecutively applied until the whole sample was loaded. The column was washed 3× with 1 mL Milli-Q water. Elution was performed in three steps, eluting first the acidic peptides in 500 $\mu$L 40% ACN / water (v/v), second the neutral peptides in 500 $\mu$L 70% ACN / water (v/v) and finally the basic peptides in 500 $\mu$L 70% ACN / 0.3% formic acid (v/v). AMPs

could be only detected in the final fraction. Samples were stored in the freezer at -20°C until further analysis.

### Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

Crude samples desalted by ultrafiltration were analyzed using a MALDI Micro MX mass spectrometer (Waters). All measurements were performed in the *m/z* range of 1,000 – 10,000 in linear ion mode. The lyophilized samples were reconstituted in 10 $\mu$L aqueous 0.1% TFA. One $\mu$L of sample was mixed with 1$\mu$L aliquot of $\alpha$-cyano-4-hydroxycinnamic acid ($\alpha$-matrix, 10 mg/mL in ethanol/acetonitrile, 1:1, v/v), and 1$\mu$L of the solution was spotted onto a metal 96-spot MALDI target plate. The instrument was operated in positive ion mode, with 3.5 kV set on the sample plate, and 12 kV on the extraction grid. A nitrogen laser (337 nm, 5 Hz) was used for ionization/desorption and the extraction of ions was delayed by 500 ns. The pulse voltage was 1100 V, and the detector voltage was set to 2.15 kV. MassLynx v4.1 software was used for data acquisition (Waters). Each spectrum was combined from 15 laser pulses. Angiotensin II, bradykinin, ACTH, insulin, cytochrome C, and myoglobin (all Sigma) at 1 to 10 pmol on target were used to calibrate the mass spectrometer.

### Sample Preparation for nanoUPLC-MS$^E$ Analysis

Following SPE, 5$\mu$L per sample were vacuum-dried (up to 50$\mu$L were tested) and reconstructed in 50 $\mu$L of 50 mM ammonium bicarbonate buffer containing 1 pmol BSA used as internal standard. The proteins were reduced by addition of DTT to a final concentration of 10 mM, incubated for 30 min at 60 °C and alkylated with 15 mM iodoacetamide in the dark for 30 min at room temperature. Proteolysis was carried out by adding 100 ng of sequencing grade porcine trypsin (Promega) at 37 °C overnight. The samples were vacuum-dried and kept at -20 °C. Prior analysis, the samples were re-dissolved in 20 $\mu$L 3% ACN/ 0.1% formic acid (v/v) solution.

### NanoUPLC-MS$^E$

The peptide amounts were quantified using a nanoAcquity UPLC system on-line connected to a Q-ToF Synapt HDMS mass spectrometer (Waters). To test linearity to the internal standard different volumes of the samples (1 to10 $\mu$L) were injected containing final concentrations of BSA ranging from 50 - 500 fmol (on column). Up to six technical and

biological replicates were measured to estimate the biological and analytical reproducibility of the method. Samples were concentrated on a Symmetry C18 trap-column (20 $\times$ 0.18 mm, 5 $\mu$m particle size, Waters) at a flow rate of 15 $\mu$L/ min. The trap-column was on-line connected to on a nanoAcquity C18 analytical column (200 mm $\times$75 $\mu$m ID, C18 BEH 130 material, 1.7 $\mu$m particle size, Waters) and the peptides were separated at a flow rate of 350 nL/ min using following LC-gradient: 1 – 30% B (13 min), 30 – 50% B (5 min), 50 – 95% B (5 min), 95% B (4 min), 95% – 1% B (1 min) [Solvent (A): 0.1% formic acid in ultra-pure water; solvent (B) 100 % acetonitrile]. The eluted peptides were transferred through a NanoLockSpray ion source into the mass spectrometer operated in V-mode at a resolution of at least 10 000 (FWHM). LC-MS data were acquired under data-independent acquisition at constant collision energy of 4 eV in low energy (MS) mode, ramped in elevated energy ($MS^E$) mode from 15 to 40 eV. The mass range (*m/z*) for both scans was 300 – 1,900 and 50 – 1,700 Da, respectively. The scan time was set at 1.5 sec for both modes of acquisition with an inter-scan delay of 0.2 sec. A reference compound, human Glu-Fibrinopeptide B [650 fmol/mL in 0.1% formic acid/acetonitrile (v/v, 1:1)], was infused through a reference sprayer at 30 s intervals for external calibration. The data acquisition was controlled by MassLynx v4.1 software (Waters).

**Data Processing and Protein Identification**

The acquired continuum LC-$MS^E$ data were processed using ProteinLynx Global Server (PLGS) version 2.5.2 (Waters) to generate product ion spectra for database searching according to Ion Accounting algorithm described by Li *et al.* 2009. The thresholds for low/ high energy scan ions and peptide intensity were set at 150, 30 and 750 counts, respectively. Database searches were carried out against Swissprot database (downloaded on Juli 27, 2011 `http://www.uniprot.org/`) combined with protein sequences of AMPs at a False Discovery Rate (FDR) of 2%, following searching parameter were applied for the minimum numbers of: product ion matches per peptide (3), product ion matches per protein (5), peptide matches (1), and maximum number of missed tryptic cleavage sites (1). Searches were restricted to tryptic peptides with a fixed carbamidomethyl modification for Cys residues. For quantification a universal response factor was calculated from BSA (the averaged intensity of the three most intense peptides) as described by (Silva *et al.* 2006).

**Gene expression analysis**

The isolation of RNA and the qRT-PCR were performed as previously described (Weinhold *et al.* 2013). For amplification of the endogenous *N. attenuata* defensin genes

following primers were used: Def1-7F (5'- CGCTCCTTGTGCTTCATGG-3'), Def1-83R (5'-GTACTCTTAGCTTGCACCTCATAGGC-3'), Def2-21F (5'-CATGGCATTTGCTATCTTGGC-3'), Def2-98R (5'- TTGCTTTCTGTTTTGCATTCTCTAG-3').

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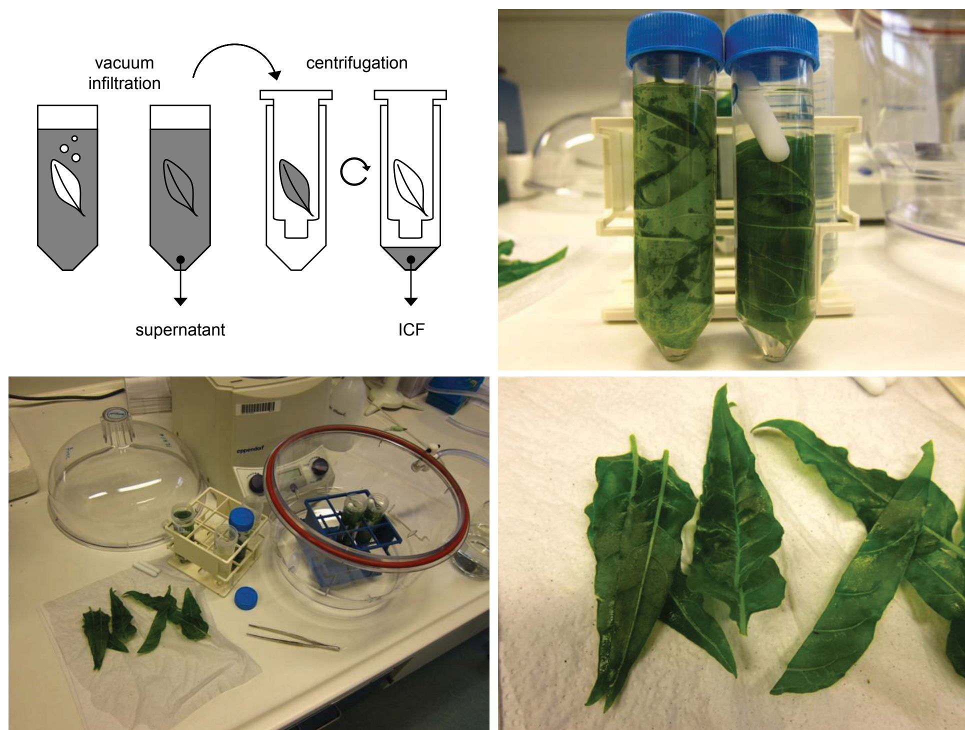
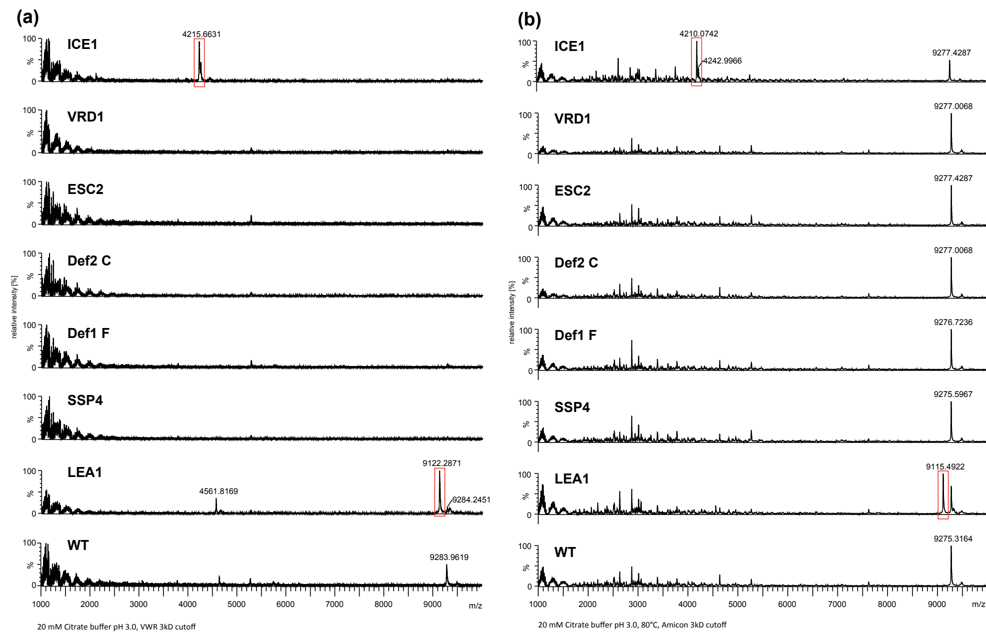
### 5.1.4. Supplemental material




**Supplementary Figure 5.1: Illustration of the vacuum infiltration procedure.** *N. attenuata* leaves were submerged in infiltration buffer and exposed to a vacuum inside a desiccator. A complete infiltration was indicated by the darkening of the leaves and a more translucent appearance. Infiltrated leaves were removed from the buffer, blotted on a paper towel and placed inside the barrel of a syringe. The remaining infiltration buffer was collected as "supernatant". The infiltrated leaves were centrifuged and the extracted liquid was collected as intercellular fluid (ICF).

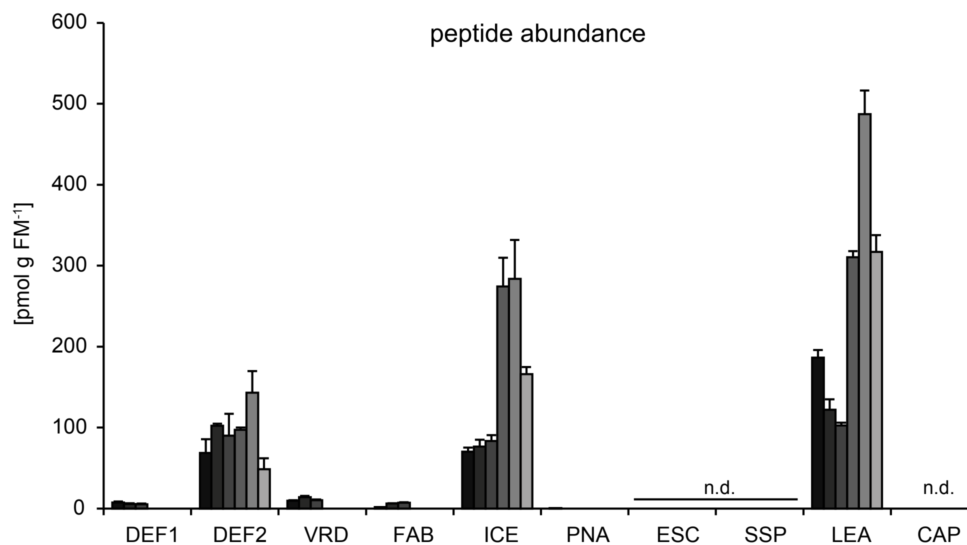



**Supplementary Figure 5.2: Comparison of the MALDI-TOF mass spectra from different lines.** Spectra were acquired from the intercellular fluid (ICF) of WT and transgenic plants in the mass range 1–10 kDa. Peaks within the expected mass ranges from the ICE and LEA lines are indicated. (a) ICF was extracted with citrate buffer (pH 3.0) and desalted by ultrafiltration (VWR 3K columns). (b) ICF was extracted with citrate buffer (pH 3.0), heat treated (80°C) and desalted by ultrafiltration (Amicon 3K columns). MALDI-TOF instrument was operated in linear ion mode.

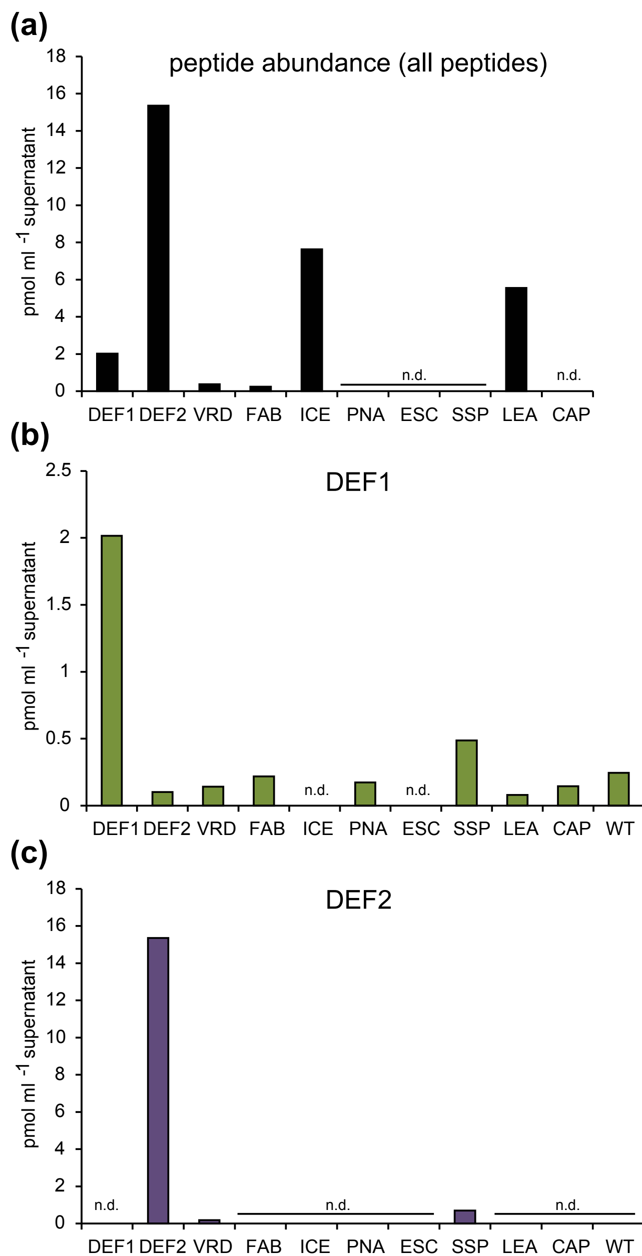
**Supplementary Table 5.1:** Expected masses of AMP peptides after tryptic digest. The peptide masses (minimum 300 Da) were computed using the Expasy server (`http://web.expasy.org/peptide_mass/`). Tryptic peptides confirmed by MS$^E$ are underlined.

| line | monoisotopic mass | position | peptide sequence |
|---|---|---|---|
| DEF1 | 1885.8571 | 6-22 | AESNTFEGFCVTKPPCR |
| DEF1 | 829.3405 | 42-48 | CICYKPC |
| DEF1 | 567.2773 | 30-34 | FTDGK |
| DEF1 | 438.2017 | 2-5 | STCK |
| DEF1 | 434.2431 | 24-27 | ACLK |
| DEF1 | 401.2871 | 38-40 | ILR |
| DEF1 | 337.1540 | 35-37 | CSK |
| DEF2 | 1863.9091 | 5-21 | TESNTFPGICITKPPCR |
| DEF2 | 894.3774 | 29-36 | FTDGHCSK |
| DEF2 | 767.3249 | 41-47 | CLCTKPC |
| DEF2 | 650.3178 | 23-28 | ACISEK |
| DEF2 | 401.2871 | 37-39 | ILR |
| DEF2 | 379.1646 | 2-4 | ECK |
| VRD | 1294.5588 | 13-24 | CLIDTTCAHSCK |
| VRD | 918.3518 | 39-46 | TCYCLVNC |
| VRD | 811.3767 | 27-34 | GYIGGNCK |
| VRD | 595.2942 | 2-6 | TCMIK |
| VRD | 576.2776 | 8-12 | EGWGK |
| VRD | 464.2286 | 35-38 | GMTR |
| FAB | 1753.7455 | 12-27 | FNGPCLTDTHCSTVCR |
| FAB | 848.3468 | 33-40 | GGDCHGFR |
| FAB | 572.1699 | 43-47 | CMCLC |
| FAB | 553.2616 | 28-32 | GEGYK |
| FAB | 458.3085 | 1-4 | LLGR |
| FAB | 376.1939 | 9-11 | SNR |
| ICE | 1708.6552 | 11-25 | EDQGPPFCCSGFCYR |
| ICE | 716.3838 | 26-31 | QVGWAR |
| ICE | 470.2068 | 32-35 | GYCK |
| ICE | 363.2060 | 2-4 | CIK |
| ICE | 335.1496 | 8-10 | GCR |
| ICE | 318.1772 | 5-7 | NGK |
| PNA | 3022.1537 | 11-40 | LCGNGLCCSQWGYCGSTAAYCGAGCQSQCK |
| PNA | 591.2667 | 1-5 | QQCGR |
| PNA | 518.2681 | 6-10 | QASGR |
| ESC | 886.4992 | 25-32 | EVGLDVVR |
| ESC | 877.4447 | 33-41 | TGIDIAGCK |
| ESC | 857.5454 | 13-20 | NLLISGLK |
| ESC | 551.3187 | 1-5 | GIFSK |
| ESC | 417.2456 | 21-24 | NVGK |
| ESC | 388.2554 | 6-9 | LAGK |

| | | | |
|---|---|---|---|
| ESC | 308.0911 | 44-46 | GEC |
| SSP | 886.5145 | 19-26 | FPSIPIGR |
| SSP | 755.3327 | 30-35 | FVQCCR |
| SSP | 682.3341 | 1-6 | SFGLCR |
| SSP | 553.2551 | 10-14 | GFCAR |
| SSP | 365.1602 | 27-29 | CSR |
| SSP | 304.1655 | 37-38 | VW |
| LEA | 1811.8302 | 73-89 | CGVSIPYQISPNTDCSK |
| LEA | 1518.7910 | 57-72 | SYSGINLGNAAGLPGK |
| LEA | 1179.5900 | 11-21 | MAPCLPYVTGK |
| LEA | 963.4928 | 1-10 | AIGCNTVASK |
| LEA | 947.4437 | 22-32 | GPLGGCCGGVK |
| LEA | 878.4223 | 45-52 | QAVCNCLK |
| LEA | 715.4097 | 33-39 | GLIDAAR |
| LEA | 589.2940 | 40-44 | TTPDR |
| LEA | 432.2816 | 53-56 | TLAK |
| CAP | 2361.9602 | 1-28 | GYGGHGGHGGHGGHGGHGGHGHGGGGHG |
| CAP | 3258.3015 | 1-38 | GYHGGHGGHGGGYNGGGGHGGHGGGYNGGGHHGGGGHG |




**Supplementary Figure 5.3: Biological and analytical variability of AMPs quantified using nanoUPLC-MS$^E$.** The AMP amounts were calculated from three to six individual biological replicates, each derived from the intercellular fluid extraction of a single *N. attenuata* plant. Error bars indicate the standard error of 3 – 5 technical replicates, n.d. = not detected.




**Supplementary Figure 5.4: Determination of AMP abundance in the supernatant.** After vacuum infiltration 15 mL of infiltration buffer (MES, pH 5.5) were desalted using reversed phase cartridges, spiked with BSA and analyzed using nanoUPLC-MS$^E$, n.d. = not detected; (a) Comparison of all peptides from the supernatant (b) Comparison of DEF1 abundance in the supernatant (c) Comparison of DEF2 abundance in the supernatant.

# 6 | Manuscript IV

## 6.1. *In planta* manipulation of native endophytic *Bacillus* spp. in *Nicotiana attenuata*: Engineering an aposymbiotic plant

Plant associated bacteria were inoculated into *N. attenuata* leaves by pressure infiltration and re-isolated for colony forming unit counting.

# *In planta* manipulation of native endophytic *Bacillus* spp. in *Nicotiana attenuata*: Engineering an aposymbiotic plant

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## Summary


- Plant-inhabiting endophytic bacteria are believed to help plants resist biotic or abiotic stresses, but the importance of normal plant colonization is unknown, due to the inability to grow endophyte free plants in nature.
- We wanted to create near-aposymbiotic plants and transformed *Nicotiana attenuata* to ectopically express different antimicrobial peptides (AMPs). Transgenic plants were tested for *in vivo* activity using several native endophytic bacteria.
- The expression of the knottin-like antimicrobial peptide (Mc-AMP1) from the common ice plant (*Mesembryanthemum crystallinum* L.) showed strong *in vivo* activity against different beneficial *Bacillus* strains. All tested native isolates of *B. pumilus* showed consistently reduced colony forming units, whereas *B. megaterium* isolates were highly variable in their susceptibility. Native fungal isolates (*Alternaria* and *Fusarium*) as well as the leaf pathogenic bacterium *Pseudomonas syringae* were not affected.
- AMP expressing plants can manipulate plant beneficial bacteria, and the *N. attenuata* ICE lines are excellent tools to further investigate the impact of non-culturable bacteria on the fitness of plants grown in nature. The heterogeneity of resistance found among individual endophytic isolates suggests that bacterial communities have a capacity to tolerate antimicrobial activity, a trait readily missed in metagenomic studies.

### 6.1.1. Introduction

Plants are surrounded by a vast and diverse community of soil bacteria, some of which are able to form close associations and important mutualistic relationships with plants (Gaiero *et al.* 2013). Plant-microbe interactions play an important role in plant health and productivity and have received increasingly attention for their roles in agricultural as well as natural ecosystems (Berendsen *et al.* 2012; Pavlo *et al.* 2011; Zamioudis and Pieterse 2012). Yet little is known about the relevance of asymptomatic endophytic bacteria naturally present in many host plants (Bulgarelli *et al.* 2012; Reinhold-Hurek and Hurek 2011; Rosenblueth and Martínez-Romero 2006).

Endophytes are bacteria which live within the plant and can be isolated from surface-sterilized plant tissues (Hallmann *et al.* 1997). Most reside as free-living cells within the intercellular space of the apoplast and are generally considered to be either harmless, or to benefit plants under certain conditions (Compant *et al.* 2010; Gaiero *et al.* 2013; Turner *et al.* 2013a). It is also speculated that plant endophytes might play important roles in aboveground defenses against herbivores (Badri *et al.* 2013; Pineda *et al.* 2013). Classical culture-dependent approaches have been used for the analysis of endophytes, as they provide single strains, essential for plant re-inoculation experiments (Long *et al.* 2010). But plant growth promoting abilities or other effects of single strains are commonly overestimated, as the use of high bacterial concentrations leads to an 'over-inoculation' of the plant, which does not resemble native conditions or realistic concentrations. The reconstruction and establishment of artificial communities refines this approach, but is likewise restricted to the availability of culturable bacteria.

Modern molecular techniques as 454 pyrosequencing enable a more comprehensive and culture-independent survey of endophytic bacteria and allow the *in situ* identification of previously overlooked communities (Bulgarelli *et al.* 2012; Lundberg *et al.* 2012; Peiffer *et al.* 2013). High-throughput sequencing technologies can reveal rare taxa and whole community compositions, but only provide observational data, without the ability to reveal the contribution of particular taxa to the extended phenotype of a host plant. The challenge is the development of experimental procedures that reveal the overall contribution of endophytic bacteria to the fitness of a plant, grown under natural conditions (Gaiero *et al.* 2013).

Our goal was to meet this challenge by creating a transgenic native plant which itself manipulates its endophytic bacteria. This aposymbiotic host plant could be compared to plants with natural bacterial communities, to reveal otherwise hidden phenotypes (Partida-Martínez and Heil 2011). To do so, we ectopically expressed cysteine-rich antimicrobial peptides (AMPs) in the wild tobacco *Nicotiana attenuata* to create an

'antimicrobial' plant. AMPs are small, cationic peptides, rich in disulfide bridges, which have been shown to inhibit the growth of a broad range of microbes. AMPs usually contain signal peptides, that target them for secretion into the apoplast, where endophytic bacteria usually reside (Bednarek *et al.* 2010).

The antimicrobial peptide Mc-AMP1 from the common ice plant (*Mesembryanthemum crystallinum* L.) consists of an N-terminal signal peptide and a short 37 amino acid long mature peptide. It has an average molecular mass of 4.2 kDa and belongs, due to the distinct 'knot' connection motif of the disulfide bridges, to the knottin sub-family of antimicrobial peptides (Pelegrini *et al.* 2011). These types of peptides show activity solely against gram-positive bacteria and fungi under *in vitro* conditions (Farrokhi *et al.* 2008; Odintsova and Egorov 2012; Stotz *et al.* 2013). Gram-positive bacteria are common soil bacteria and frequently found in close association with plants. The plant beneficial bacteria *Bacillus pumilus* and *B. megaterium* have been found as plant endophytes and within the rhizosphere, exerting disease suppressive or plant growth promoting abilities (Gutierrez-Manero *et al.* 2001; Lima *et al.* 2005; López-Bucio *et al.* 2007; Sari *et al.* 2007; Zou *et al.* 2010). These bacteria were also commonly associated with *N. attenuata* (Long *et al.* 2010), and in particular colonization by *B. megaterium* restored normal growth in field-grown ethylene-insensitive plants, in part by supplying plants with reduced sulfur (Meldau *et al.* 2012, 2013).

*N. attenuata* is a post-fire annual plant inhabiting the Great Basin Desert, and has been intensively studied as an ecological model for determining fitness effects of single genes within the native habitat (Schuman *et al.* 2012; Steppuhn *et al.* 2004). Targeted genetic manipulation allows reverse genetic studies for analyzing the consequences of different defense strategies against biological or environmental stresses. For the *in planta* manipulation of *N. attenuata´s* microbiome, we made 11 different constructs for the ectopic overexpression of different AMPs. The selection of epigenetically stable plant lines reduced the availability to 10 constructs with trans-generational stable gene expression (Weinhold *et al.* 2013), which were further analyzed for a stable peptide localization within the apoplast [manuscript in review]. Here we report the *in vivo* activity and direct effects on native endophytic bacteria in transgenic AMP expressing *N. attenuata* plants.

The aim of this study was to use AMPs to engineer truly 'antibacterial' plants which have *in planta* activity against native bacteria. For this purpose the transgenic plant should exhibit no developmental or growth effects, show stable gene expression levels, and AMPs in the apoplast. Conditions within the plant apoplast are tremendously different than controlled laboratory conditions, and antimicrobial activity *in planta* can vanish due to increased salt concentrations, protease-based degradation, or inhibition by phenolic compounds (Zeitler *et al.* 2013). Effects on endophytic bacteria cannot be evaluated

using classical 'resistance' assays, and we developed a leaf infiltration method suitable for testing the activity against non-pathogenic bacteria. We demonstrated that transgenic *N. attenuata* ICE plants showed taxa-specific *in vivo* activity against native endophytic *Bacillus* isolates, without affecting Proteobacteria or phytopathogenic fungi.

### 6.1.2. Material and Methods

**Plant cultivation**

*Nicotiana attenuata* Torr. ex S. Watson seeds were originally collected in 1988 from a natural population at the DI Ranch in Southwestern Utah. Wild-type seeds from the $30^{th}$ inbreed generation were used for the construction of transgenic plants. Seeds of the same generation were always used as controls (e.g. WT $33^{rd}$ inbreed generation propagated together with $T_3$ transgenic plants). Seeds were germinated on Gamborg's B5 Medium (Duchefa) as described in Krügel *et al.* (2002) and incubated in a growth chamber (Percival, day 16 h 26°C, night 8 h 24°C). Ten-days-old seedlings were transferred to Teku pots for ten days, and later into 1 L pots and cultivated in the glasshouse under constant temperature and light conditions (day 16 h 26-28°C, night 8 h 22-24°C).

**Plant transformation and line screening**

For the construction of the plant transformation vector pSOL9ICE the antimicrobial peptide Mc-AMP1 of the common ice plant (*Mesembryanthemum crystallinum* L.) was selected from the PhytAMP database (`http://phytamp.pfba-lab-tun.org/main.php`) (ID:PHYT00272) and the cDNA sequence retrieved from NCBI (GenBank:AF069321). Other AMPs (DEF1, DEF2, VRD, FAB, LEA, PNA SSP) are further described in (Weinhold *et al.* in review). The genes were synthesized in sequential PCR reactions and cloned in pSOL9 binary plant transformation vectors under a constitutive cauliflower mosaic virus promoter (35S). Plants were transformed by *Agrobacterium tumefaciens*-mediated gene transfer as previously described (ibid.). Per construct at least 10 independently transformed transgenic plant lines were screened and selected following the workflow described in Gase *et al.* (2011), including flow cytometry, segregation analysis and diagnostic PCRs for testing completeness of T-DNA insertion using the primers PROM FOR and TER REV. If not otherwise stated, homozygous $T_4$ plants from independent regeneration events with single T-DNA insertions were used for all experiments: ICE 6.4.2.1 (A-09-748), ICE 8.4.1.1 (A-09-804), ICE 1.1.1.1 (A-09-653). The continuous numbering identifies the plant from which seeds were collected and the digits indicate the seed generation.

**Gene expression and southern blot analysis**

RNA was isolated by a modified salt precipitation method described in Weinhold *et al.* (2013). Quantitative Real Time-PCR (qRT-PCR) was performed on the Mx3005P QPCR System (Stratagene) from four biological replicates described in Weinhold *et al.* (ibid.) using the following primers: ICE-94F (5'-AATGGAAAAGGATGTCGAGAGG-3'), ICE-167R (5'-CATCCAACCTGACGGTAACAGAA-3'). For southern blot analysis, genomic DNA was isolated from seedlings and the DNA blotting performed as described in Weinhold *et al.* (ibid.) using a radiolabeled PCR fragment of the *hygromycin phosphotransferase* II gene (*hpt*II) as probe.

**Bacterial strains**

Endophytic *Bacillus* spp. strains were previously isolated from *N. attenuata* plants grown in native Utah soils described in Long *et al.* (2010). The *Arthrobacter* sp. and *Rhodococcus* sp. isolates were retrieved from *N. attenuata* seedlings germinated from seeds, which had been buried in a seedbank in their native habitat in Utah, USA. After a year in the soil, seeds were dug up, surface-sterilized and germinated on GB5 medium. Endophytic bacteria were isolated from 15-day-old seedlings similar as described in Long *et al.* (ibid.). Related bacterial type strains were retrieved from the German culture collection DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen): *Bacillus pumilus* DSM 1794, *Bacillus megaterium* DSM 32, *Kocuria rhizophila* DSM 11926, *Micrococcus luteus* DSM 20030, *Arthrobacter aurescens* DSM 20116, *Rhodococcus erythropolis* DSM 43066. All bacterial strains were stored at -80°C in 20% (v/v) glycerol stocks and sub-cultured on LB-Lennox agar plates (28°C). Only culture media for *Pseudomonas syringae* pv *tomato* DC3000 were supplemented with the antibiotics Rifampicin (25 $\mu$g mL$^{-1}$) and Tetracyclin (5 $\mu$g mL$^{-1}$).

**Bacterial DNA isolation and 16S rDNA sequencing**

Genomic DNA was isolated from bacterial isolates using a modified CTAB method. Cell pellets from overnight cultures were pre-digested for 30 min in 450$\mu$L Lysozyme buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 5 mg mL$^{-1}$ Lysozyme (Fluka). Cells were lysed for 30 min at 65°C in 800 $\mu$L CTAB buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl). After addition of 500 $\mu$L chloroform, tubes were centrifuged (1 min at 16.100 g) in a tabletop centrifuge. The supernatant was again phase-separated with 700 $\mu$L chloroform after addition of 70 $\mu$L 10% CTAB solution. The aqueous phase was precipitated with 1 volume of isopropanol and the pellet washed

twice in 400 $\mu$L 70% ethanol, air dried and dissolved in 50 $\mu$L nuclease-free water (Ambion). The amplification of the 16S rDNA was performed with 100 ng template DNA in a final volume of 20 $\mu$L containing 0.05 U $\mu L^{-1}$ JumpStart Taq DNA Polymerase using the provided reaction buffer (Sigma-Aldrich), 200 $\mu$M dNTP Mix (Fermentas) and 0.5 $\mu$M of the following primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). The amplification was performed with following program: 94°C for 1 min, followed by 29 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s and a final chain elongation step of 72°C for 5 min. The PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and sequenced from both sides with primers 27F and 1492R using the BigDye Terminator mix v3.1 (Applied Biosystems). The sequences were manually trimmed using EditSeq (DNAStar Lasergene 8) and deposited in GenBank. Alignment and phylogenetic tree construction were performed with MEGA5 (Tamura *et al.* 2011) using the CLUSTALW algorithm, neighbor-joining and bootstrapping. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances computed using the Maximum Composite Likelihood method.

**Nucleotide Sequence Accession Numbers**

Obtained sequences were deposited in GenBank under the accession numbers: KJ476709 - KJ476726.

**Clone library of root colonizing bacteria**

Plant roots were harvested from *N. attenuata* plants, growing in a wild population in the Great Basin Desert, southwestern Utah (USA). Roots from the two depicted plants (Fig. 6.1a) were surface washed with tap water, and all visible soil particles removed. Frozen roots were transported to the MPI-CE in Jena, Germany and ground over liquid nitrogen. The gDNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals) including the rhizoplane and endosphere fraction. The 16S rDNA region was amplified as indicated above using the primers 1492R and 799F (5'-AACAGGATTAGATACCCTG-3'), which avoids amplification of plastid DNA. PCR products were gel separated on a 1% agarose gel and the plant mitochondrial sequences separated from bacterial sequences, which were gel excised and purified using the NucleoSpin Extract II kit (Macherey-Nagel). DNA fragments were cloned into the pGEM-T Easy vector system (Promega) and 112 individually picked clones were Sanger sequenced using the BigDye Terminator mix v3.1 (Applied Biosystems). Sequences were manually evaluated for chimeras by using Bellerophon (Huber *et al.* 2004). The 89 remaining non-chimeric OTUs were aligned

online using the Ribosomal Database Project pipeline and illustrated in a phylogenetic tree, constructed with the RDP tree builder using a weighted neighbor-joining method.

**Bacteria leaf inoculation assay**

For all inoculation assays, the bacterial strains were grown as liquid overnight culture in LB-Lennox medium (28°C, 320 rpm). Bacterial cells were harvested by centrifugation in a table top centrifuge (1500 $\times$ g, 2 min), the supernatant was discarded and cell pellet washed, resuspended and diluted in sterile infiltration buffer (10 mM Sodium Phosphate buffer pH 7.0) to a final $OD_{600}$ of 0.001 to 1.0, depending on the taxon: OD 0.001 for *Pseudomonas syringae* pv *tomato* DC3000, OD 0.02 for *Bacillus pumilus*, OD 0.04 for *Arthrobacter* sp., OD 0.2 for *Rhodococcus* sp. / *Kocuria rhizophila* / *Bacillus endophyticus*, OD 0.4 for *Micrococcus luteus* and OD 1.0 for *Bacillus megaterium*, which has the biggest cell size of all bacteria and needed to be infiltrated within a much higher optical density than most other bacteria. Tested, but for this method unsuitable strains were: *Bacillus pichinotyi*, *Paenibacillus* sp., *Lactobacillus plantarum* and *Lysinibacillus sphaericus*. For the leaf inoculation assay, fully expanded rosette leaves were used when plants started elongation (approx. 35 days old). Per leaf ca. 300 – 400 $\mu$L bacterial solution was injected by pressure infiltration on both sides of the midrib using a 1 mL syringe without needle. Leaves were blotted dry on paper towels and the infiltrated area was marked with a pen (edding AG, Ahrensburg, Germany). For re-isolation, two leaf-discs were punched out per leaf using a cork-borer with 8 mm diameter. Samples were usually taken after 0, 2, 4 and 6 days-post-inoculation (dpi) from two leaves per plant (technical replicate) and 4 plants per genotype (biological replicate). The two leaf discs (together 1 $cm^2$) were squeezed with a sterile pistil in 400 $\mu$L dilution buffer (10 mM $MgCl_2$) in a 1.5 mL reaction tube and serial diluted ($10^{-1}$ to $10^{-5}$, depending on bacterial taxon). From the three highest dilutions, 40 $\mu$L were spotted on a square LB-Lennox agar plate (Fig. 6.2) and incubated at 28°C. Colonies were counted for most taxa after overnight incubation, for *P. syringae* after 2 days of incubation and for *M. luteus* after 3 days of incubation. A standard kinetic consists of 4 biological replicates $\times$ 2 technical replicates $\times$ 3 dilutions $\times$ 3 genotypes $\times$ 4 sampling days = 288 spots for CFU counting, which were plotted as log CFU $cm^{-2}$ leaf area.

**Fungi inoculation assay**

Fungal strains were isolated from the leaves of diseased *N. attenuata* plants, collected from a natural population in the Great Basin Desert in Southwestern Utah (USA) (S.

Schuck *et al.* in review). Isolates were maintained on potato dextrose agar (PDA) (Sigma-Aldrich) in the dark at 26°C. For detached leaf assay (Vleeshouwers *et al.* 1999), fully expanded leaves of elongating greenhouse-grown *N. attenuata* plants were detached and placed in a square plate on a moist paper towel. From 10-day-old fungal cultures of *Fusarium brachygibbosum* [U4] 6 mm diameter agar-plugs were pushed out of the mycelium using a flame-sterilized cork borer and placed on the adaxial side of a detached leaf. Plates were sealed twice using parafilm and incubated in a Percival growth chamber at constant temperature and light conditions (day 16 h 26°C, night 8 h 24°C). The lesions below the agar plug became visible after 3 days and the diameter was observed until 7 dpi, when leaves started senescing (n = 12 plates). This experiment was repeated twice with similar outcomes. For seedling resistant assay, sterilized seeds of WT and transgenic plants were germinated on GB5 media in a concentric pattern. WT seeds had been collected together with the transgenic lines to synchronize seed dormancy and time of germination. After 7 days a 12 mm agar-plugs containing fungal mycelium from 8-day-old fungal cultures (*Alternaria* sp. [U10], *Alternaria* sp. [U11], *Fusarium oxysporum*) were placed in the center and incubated in a Percival growth chamber at constant temperature and light conditions (day 16 h 26°C, night 8 h 24°C). The seedling mortality was observed every third day (n = 7 plates).

### 6.1.3. Results

**Antimicrobial peptide expression in *Nicotiana attenuata***

The heterologous expression of AMPs in plants is a common practice for the engineering of disease resistant crops, but little is known about the potential impact on beneficial endophytic bacteria. We designed transgenic plants not for increased pathogen resistance, but rather to manipulate potentially beneficial endophytic bacteria within the intact plant. From the previously analyzed culturable endophytic community of *N. attenuata*, we knew that many isolates belong to the genus *Bacillus* (Long *et al.* 2010), of which some showed growth promoting effects on the plant (Meldau *et al.* 2012). For a culture-independent overview about the root inhabiting community, we harvested wild *N. attenuata* plants from a native population in the Great Basin Desert Utah (USA) and sequenced the root associated bacterial community (Fig. 6.1a). The obtained clone library showed operational taxonomic units (OTUs) from typical endophytic taxa like *Streptomyces*, *Nocardioides*, *Micrococinae*, *Bacilliales*, *Proteobacteria* and *Bacteriodetes* (Hallmann and Berg 2006) and was dominated by gram-positive bacteria (62%) with the most abundant genera of *Streptomyces* (21%) and *Bacillus* (10%) (Fig. 6.1b). *N. attenuata* plants were transformed with constructs for the ectopic expression of various AMPs belonging to

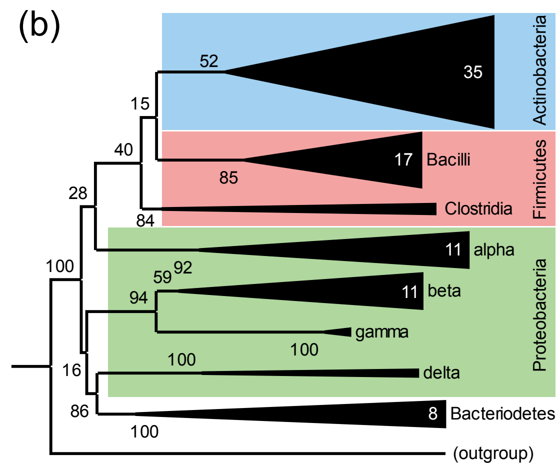


**Figure 6.1: Overview of the culture-independent endophytic bacterial community of *N. attenuata* grown in its native environment.** (**a**) Plants were harvested from a natural wash population in southwestern Utah and root associated bacteria were amplified using the primers 799F and 1492R. (**b**) 89 operational taxonomic units (OTUs) were aligned using the pipeline of the Ribosomal Database Project (RDP) and the phylogenetic tree constructed with the RDP tree builder using a weighted neighbor-joining method and rooted to *Aquifex aeolicus* as an outgroup. Identified phylotypes were tabulated and the numbers of OTUs (if >3) per phylum shown.

different peptide families (Table S6.1), and several AMPs (*e.g.* FAB, LEA, PNA, SSP and ICE) are reported to have activity against gram-positive bacteria (Pelegrini *et al.* 2011; Thouzeau *et al.* 2003; Yang *et al.* 2006). All transgenic plants were screened according to the workflow described in Gase *et al.* (2011), and in parallel, we developed a leaf infiltration method to test the transgenic plants for *in vivo* activity against the gram-positive bacterium *Bacillus pumilus*. The pre-screening of 8 different genotypes in the $T_2$ generation by *Bacillus pumilus* inoculation and re-isolation, showed a reduction in colony forming units (CFU) only for the ICE lines compared to WT plants (Fig. 6.2). This was the first indication of *in vivo* activity for any of the ectopically expressed AMPs. However, the two independent plant lines used at this stage (ICE 1.2 and ICE 6.8) contained multiple insertions of the transgene.

### Creation of lines with single transgene insertion

The ICE lines were transformed to express *Mesembryanthemum crystallinum* antimicrobial peptide 1 (Mc-AMP1), cloned behind a constitutive 35S promoter (Fig. 6.3a). This small AMP consists of a 37 amino acid long mature peptide sequence and has a knottin-like folding motif (Fig. 6.3b,c). For use in ecological experiments, transgenic plants should

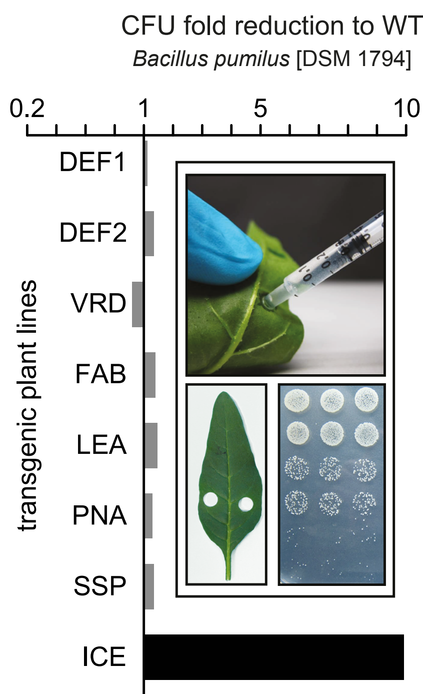


**Figure 6.2: *In planta* activity assays with different antimicrobial peptide expressing *N. attenuata* plant lines ($T_2$ stage).** The colony forming units (CFU) of re-isolated *B. pumilus* were compared with those of WT plants. The transgenic plants expressed different antimicrobial peptides: DEF1 (NaDefensin1 from *N. attenuata*), DEF2 (NaDefensin2 from *N. attenuata*), VRD (VrD1 from *Vigna radiata*), FAB (Fabatin-1 from *Vicia faba*), LEA (LJAMP2 from *Leonurus japonicus*), PNA (Pn-AMP2 from *Ipomoea nil*), SSP (Spheniscin-2 from *Aptenodytes patagonicus*), ICE (Mc-AMP1 from *Mesembryanthemum crystallinum*) see Table 6.1. *Bacillus pumilus* DSM 1794 was pressure-infiltrated into leaves and re-isolated 2 days post inoculation (dpi). The bars represent the fold reduction to WT as a mean from 2 independent plant lines per transgenic construct. The inset illustrates the standardized experimental procedure used for the *in planta* activity measurements. About 300 - 400$\mu$L bacterial solution were injected by pressure infiltration into fully expanded leaves of WT and transgenic *N. attenuata* plants, followed by re-isolation of leaf disks, serial dilutions ($10^{-1}$ – $10^{-3}$) and the spotting of the last three dilutions on LB plates for CFU counting.

be diploid, homozygous for a single transgene and retain gene expression over multiple generations. As previously reported, many of the independently transformed AMP expressing plant lines indicated the occurrence of unwanted epigenetic gene silencing, and were excluded from further propagation (Weinhold *et al.* 2013). Likewise, ICE lines with unstable gene expression were excluded in each generation, leaving, in the end, only 3 of 10 independent transformed plant lines (Fig. 6.4a) However, these 3 remaining lines (ICE 1, ICE 6 and ICE 8) indicated the presence of multiple T-DNA insertions by showing an unusual segregation ratios (offspring with approximate 6.25% antibiotic sensitivity) (Supplementary Fig. 6.1a). Southern blot analysis performed on the first homozygous versions ($T_2$) of each line confirmed the presence of multiple T-DNA insertions (Supplementary Fig. 6.1b). As the T-DNA loci segregated independently, it was possible to separate them by the selection of hemizygous $T_2$ plants, followed by the selection of homozygous $T_3$ plants, to create single insertion transgenic plants, which were further used in this study (Supplementary Fig. 6.1). From line ICE 1 two homozygous versions were created by segregation (ICE 1.1.1 and ICE 1.5.2), both from the same callus regeneration event, but each harboring a different copy of the T-DNA. This became important since the two versions of line ICE 1 differed in their *in vivo* activity against *B. pumilus*, performed on the first homozygous generation ($T_3$) (Supplementary Fig. 6.2a,b). Line ICE 1.5.2 showed a lack of *in vivo* activity and a similar CFU count

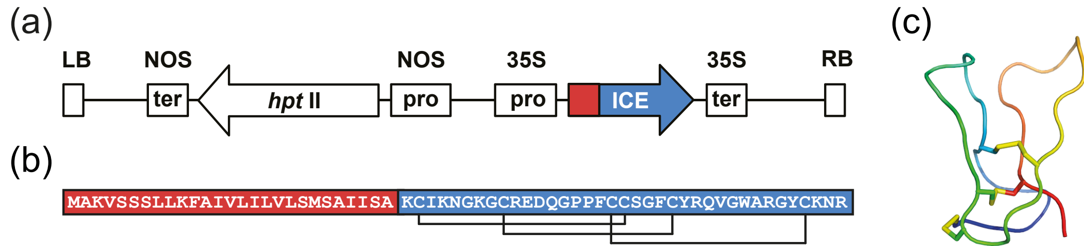


**Figure 6.3.: Schematic representation of the transgenic cassette and peptide sequence used in the overexpression lines.** (**a**) AMPs were constitutively expressed by a 35S promoter of the cauliflower mosaic virus and *hygromycin phosphotransferase* II (*hpt*II) served as a resistance marker. (**b**) Amino acid sequence of the antimicrobial peptide Mc-AMP1 from the common ice plant *M. crystallinum* that was expressed in the transgenic *N. attenuata* ICE lines. The signal peptide is indicated in red, the mature peptide domain in blue, and the expected connection pattern of cysteine residues are indicate by black lines. (**c**) The 3D structure was simulated using SWISS-MODEL (`http://swissmodel.expasy.org/`) and drawn with the PYMOL softwarepackage 0.99rc6 (2006 DeLano Scientific).

as re-isolated from WT plants. A diagnostic PCR for the amplification of the flanking regions of the transgene indicated an incomplete expression cassette for line ICE 1.5.2 (Supplementary Fig. 6.2c). However in comparison with WT plants, both ICE 1 lines had a similar reduction in growth and showed about 8% (±2%) smaller rosette diameters compared to WT plants at 43 days post germination (t-test; $P < 0.0001$) (Supplementary Fig. 6.2d). As the growth reduction in ICE 1 was independent of the insertion position, and even independent of antimicrobial activity, we concluded that this must be caused by a side effect of the callus regeneration process during the transformation of this line. From all ICE lines with antibacterial activity, the second homozygous seed generation ($T_4$) was collected to obtain seeds for further experiments.

### Obtaining side-effect free plants with stable gene expression

The common appearance and aggravation of epigenetic gene silencing within each generation of the transgenic plants (Weinhold *et al.* 2013), required that we confirm expression stability within the $T_4$ generation of the single insertion plant lines (ICE 1.1.1.1, ICE 6.4.2.1 and ICE 8.4.1.1). Quantitative RT-PCR confirmed consistent high gene expression levels of the ectopically expressed transgene in all three independent lines (Fig. 6.4b). RNA was isolated from 4 biological replicates each and the mean overexpression of the three lines was 23.1 fold (± 6.0) compared to actin as the reference gene with a CV of 2% between the three independent lines. A previously conducted promoter methylation analysis performed with line ICE 1.1.1.1 (ibid.) indicated low levels of cytosine

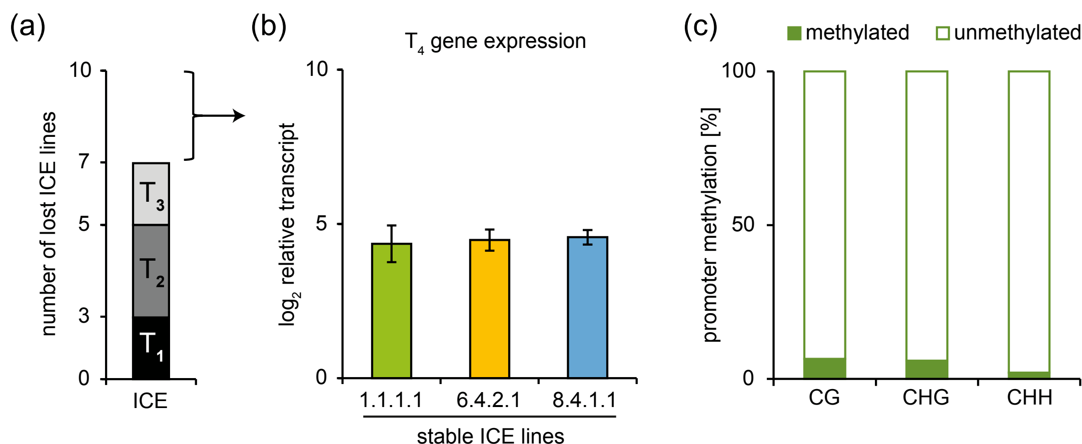


**Figure 6.4.: Transgene expression stability in *N. attenuata* ICE lines ($T_4$ stage).** (**a**) During the screening process, 7 of 10 independently transformed ICE lines lost gene expression by epigenetic transgene silencing (Gase *et al.* 2011). (**b**) Gene expression strength in $T_4$ stage of the remaining 3 ICE lines with single insertions (ICE 1.1.1.1, ICE 6.4.2.1 and ICE 8.4.1.1). Transcript abundance was determined by qRT-PCR of cDNA obtained from rosette-stage leaves of homozygous plants. Bars indicate the $\log_2$ fold expression relative to actin as the reference gene ($\pm$SD, n = 4 plants). (**c**) Methylation analysis of the 35S promoter from line ICE 1.1.1.1 (rosette stage leaves of 30 day old $T_4$ plants) showing low methylation levels within all possible methylation sites (CG, CHG, CHH), data from Weinhold *et al.* (2013).

methylation within the 35S promoter of the transgenic cassette (Fig. 6.4c). Altogether these results indicated that the $T_4$ generations of the three lines were epigenetically stable, regarding transgene expression. The lines ICE 6.4.2.1 and ICE 8.4.1.1 were finally selected for experiments, as they showed no growth reduction in the glasshouse compared to WT plants (Fig. 6.5a,b). All ICE lines flowered and produced fertile seeds and showed no indication of any developmental changes due to accumulation of the antimicrobial peptide (Fig. 6.5c). Line ICE 6 showed rather a marginal increase in stalk height compared to WT (Fig. 6.5b) (40-46 dpg, t-test $P < 0.09$).

### *In vivo* activity against *Bacillus* spp.

Since endophytic bacteria are likely be localized in the apoplast, secretion of the AMP outside of the cell and deposition within the apoplast is necessary in order to affect potentially beneficial endophytic bacteria. We used a standardized pressure infiltration method, depicted in the insert of Fig. 6.2, to infiltrate different bacteria into the leaf apoplast and test for *in vivo* activity of the transgenic plants. The bacteria were re-isolated in leaf discs every second day using a cork borer and for each time point 4 plants were used as biological replicates. Infiltration of *B. pumilus* DSM 1794 confirmed the phenotype

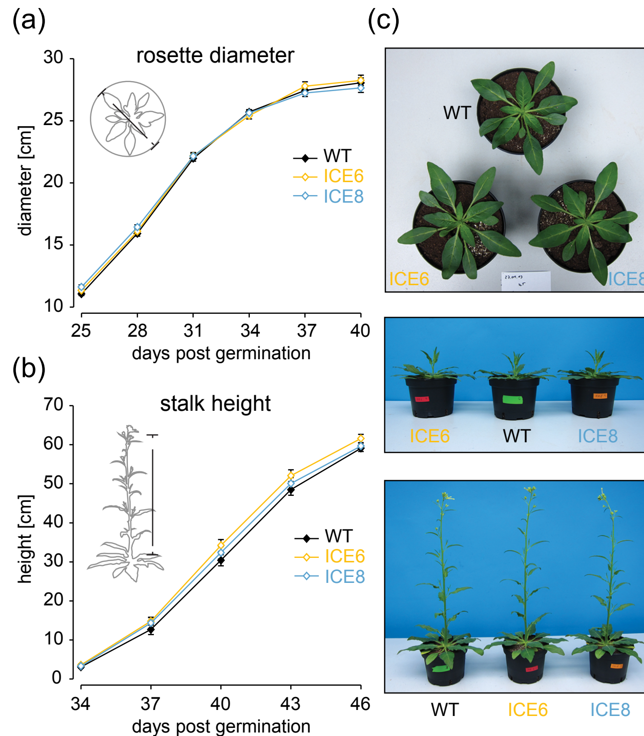


**Figure 6.5: Growth of transgenic *N. attenuata* ICE overexpression lines compared to WT.** (**a**) Rosette diameter and (**b**) stalk height of $T_4$ plants were quantified in the glasshouse until 46 days post germination. Plants were distributed in a randomized design (±SEM, n = 28 plants). (**c**) Pictures of WT and transgenic ICE overexpression plants at 30, 34 and 40 days post germination.

in the finally selected $T_4$ plants and showed consistent CFU reduction after 2 days post inoculation (dpi) (Fig. 6.6a). After 6 dpi, a 9 to 39 fold CFU decrease was observed in comparison to WT (all lines t-test; $P < 0.0003$). The outcome of the infiltration / re-isolation method was completely different when the leaf pathogen *Pseudomonas syringae* pv *tomato* DC3000 was used in the same setup, as it showed no differences in CFU counts between ICE lines and WT (Fig. 6.6b). This was consistent with the expectations that the expressed knottin has a selective activity solely against gram-positive bacteria.

### Activity against native endophytic bacteria from *N. attenuata*

The activity of antibiotics against laboratory strains of bacteria does not imply effectiveness against wild strains (Wright 2007). Native soil bacterial isolates from different ecosystems have shown non-anthropogenic resistance to various antibiotics (Cytryn 2013; Shade *et al.* 2013). A plant within its natural habitat is exposed to a tremendous diversity of soil bacteria, and we used native isolates for activity measurement and testing the limits of these transgenic plants.

The used endophytic bacteria were previously isolated from *N. attenuata* grown in native soils (Long *et al.* 2010) and most were closely related to *B. pumilis* and *B. megaterium*. The native bacterial isolates #5, #77 and #45 showed a similar reduction

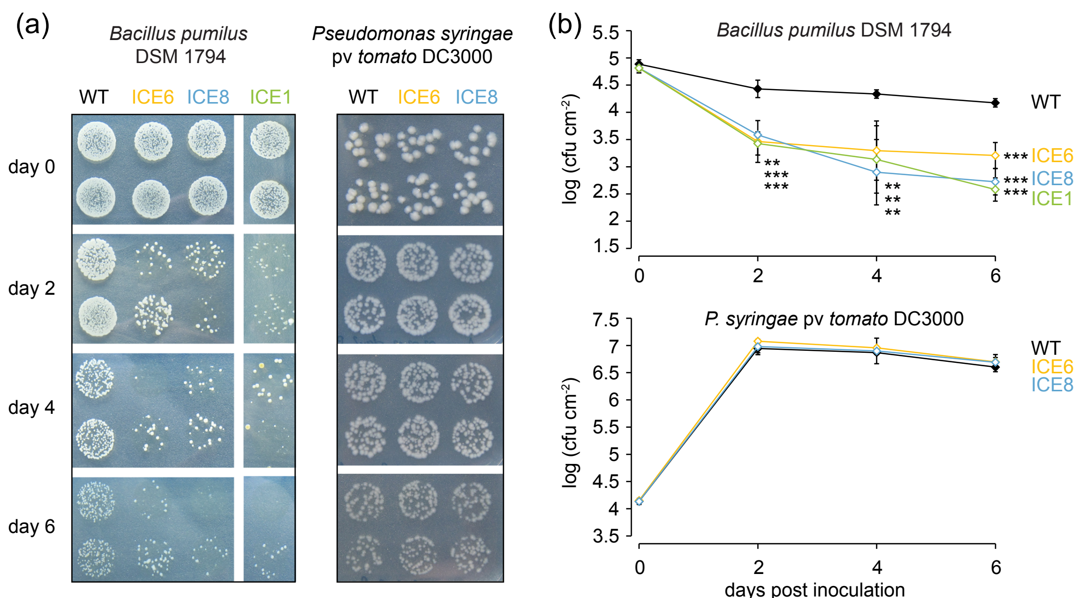


**Figure 6.6.: *In planta* activity of $T_4$ stage transgenic ICE overexpression *N. attenuata* lines against *Bacillus pumilus* DSM 1794 and *Pseudomonas syringae* pv *tomato* DC3000.** (**a**) Bacteria were injected by pressure infiltration into fully expanded leaves, re-isolated at 0, 2, 4 and 6 days post inoculation, serially diluted and spotted on LB plates for colony counting. The pictures show two technical replicates per genotype and time point ($10^{-1}$ dilution for *B. pumilis* and $10^{-2}$ and $10^{-4}$ dilutions for *P. syringae*). (**b**) The mean colony forming units (CFU) were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 4 plants). Asterisks indicate statistically significant differences between WT and transgenic plants (t-test;* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

in CFU counts as observed for the lab strain *B. pumilus* DSM 1794 when infiltrated in the ICE lines (Fig. 6.7, Supplementary Fig. 6.3a). Other isolates [#58; #88] showed a weaker, but overall significant reduction in the transgenic plants compared to WT (Fig. 6.7). Notably, in all experiments with different *B. pumilus* strains the two transgenic lines seem to differ in their effects to reduce CFUs: Line ICE 8 showed a significantly stronger reduction in CFUs than line ICE 6, when all fold changes to WT at 2-6 dpi were compared (paired t-test; P = 0.0018) (Fig. 6.7).

In contrast, *B. megaterium* could not be easily assayed in a 6-day infiltration kinetic. The *B. megaterium* type strain DSM 32 showed a strong decrease in CFU counts in the WT plants already at 2 dpi, and there was no significant reduction in the transgenic plants (Supplementary Fig. 6.3b,c). The native *B. megaterium* isolates [#131, #38] behaved similarly, showing a strong CFU decrease at 2 dpi in WT plants. However, the native isolates showed very distinct susceptibilities (Supplementary Fig. 6.3b,c). Isolate #38, in contrast to isolate #131, showed reductions in CFU in the transgenic plants at every time point, and the strongest reductions already at the day of infiltration (0 dpi): A full 6 day

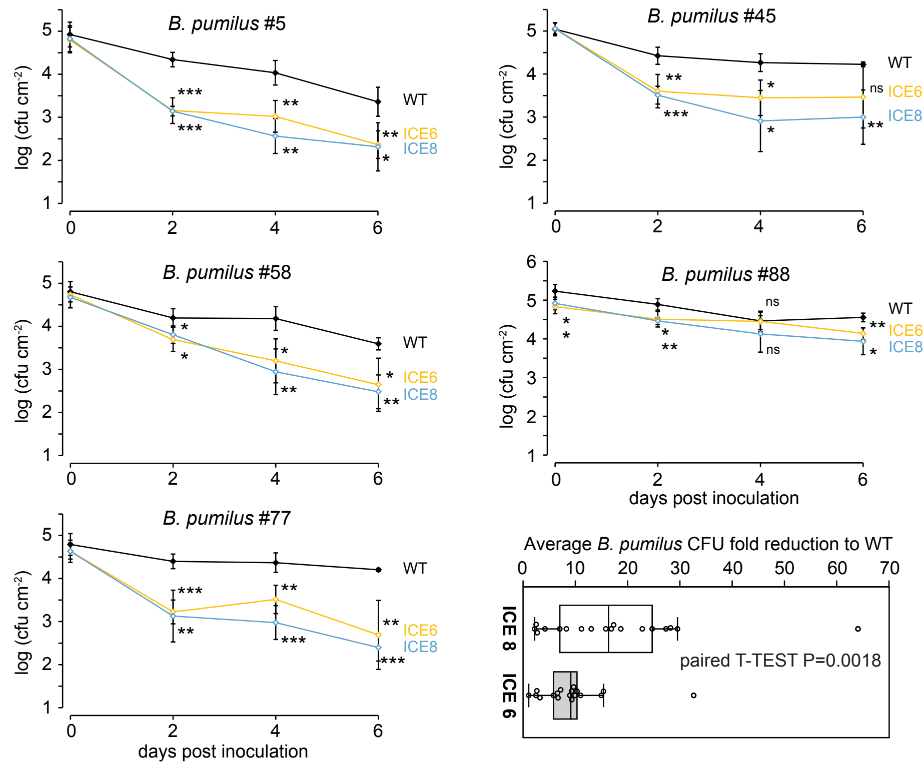


**Figure 6.7.: *In planta* activity of $T_4$ stage transgenic ICE overexpression *N. attenuata* lines against native, endophytic bacteria.** *Bacillus pumilus* isolates #5, #58, #77, #45 and #88 were injected by pressure infiltration into fully expanded leaves and the mean colony forming units (CFU) were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 4 plants). Asterisks indicate statistically significant differences between WT and the transgenic plants (t-test;* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant). The average *B. pumilus* CFU fold reduction compared to WT indicates differences in antimicrobial activity strength between line ICE 6 and line ICE 8. Dots represent average fold reduction at 2-6 dpi and the medians are shown as the centered lines limited by 25th and 75th percentiles and 1.5 times extended whiskers after Tukey (n = 18 sample points; paired t-test; $P < 0.01$).

kinetic required the infiltration of 96 leaves and the 0 dpi samples were usually taken 3 to 6 hours after the actual infiltration process. Therefore we refined the experiments for a higher temporal resolution and *B. megaterium* isolates were re-isolated henceforth at 6 h post inoculation. The comparison of all four native *B. megaterium* isolates revealed very distinct levels of susceptibility (Fig. 6.8). Isolates #7 and #38 showed strong CFU reductions in the transgenic plants, in contrast to isolates #131 and #126, which showed no significant reduction (t-test; $P > 0.1$). Although line ICE 8 continued to indicate a tendency for stronger antibacterial activity than line ICE 6, this was not significant for *B. megaterium* (Supplementary Fig. 6.3d).

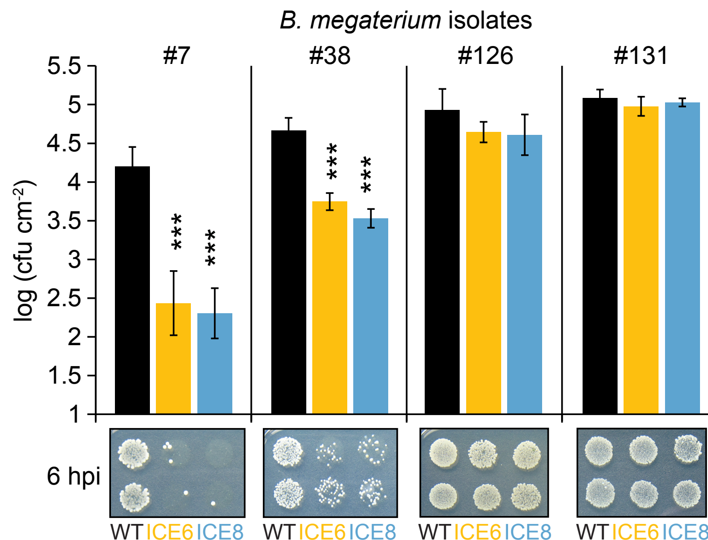


**Figure 6.8.: *In planta* activity of transgenic ICE overexpression *N. attenuata* lines against native endophytic *Bacillus megaterium* isolates (#7, #38, #126, #131).** Bacteria were injected by pressure infiltration into fully expanded leaves, re-isolated at 6 hours post inoculation (hpi) and the mean colony forming units (CFU) were plotted as log CFU $cm^{-2}$ leaf area ($\pm$SD, n = 4 plants). Asterisks indicate statistically significant differences between WT and the transgenic plants (t-test;* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant).

### Activity against native and laboratory strains of Actinobacteria

To test for antibacterial effects on Actinobacteria, we used the native isolates *Arthrobacter* sp. [S02] and *Rhodococcus* sp. [S05] together with closely related type strains from the culture collection. These bacteria were well suited for the leaf infiltration method, but showed no difference in CFU from ICE lines and WT plants (Fig. 6.9, Supplementary Fig. 6.4). A commonly used gram-positive indicator organism for antimicrobial activity is *Micrococcus luteus* DSM 20030, because of its exquisite sensitivity to many antibiotics, including lysozyme (Fleming and Allison 1922) but also insect defensins (Cociancich *et al.* 1993). But *M. luteus* proved to be inappropriate for the leaf infiltration assays and colonies were commonly overgrown by other bacteria, due to its slow growth. However, the samples at 2 dpi indicated – surprisingly – an increase in CFU number of *M. luteus* in the transgenic plants (Supplementary Fig. 6.5a,b). This unexpected outcome was confirmed in an independent experiment (Supplementary Fig. 6.5c). In comparison, *Kocuria rhizophila* DSM 11926 showed no de- or increase in CFU (Supplementary Fig. 6.5). Overall, the transgenic plants indicated no activity against most Actinobacteria and Proteobacteria tested, but variable activity against different *Bacillus* strains (Fig. 6.9). Although all *B. megaterium* isolates were phylogenetically closely related with more than 99.8% similarity in 16S rDNA sequences (1443/1446 bp), they showed the most

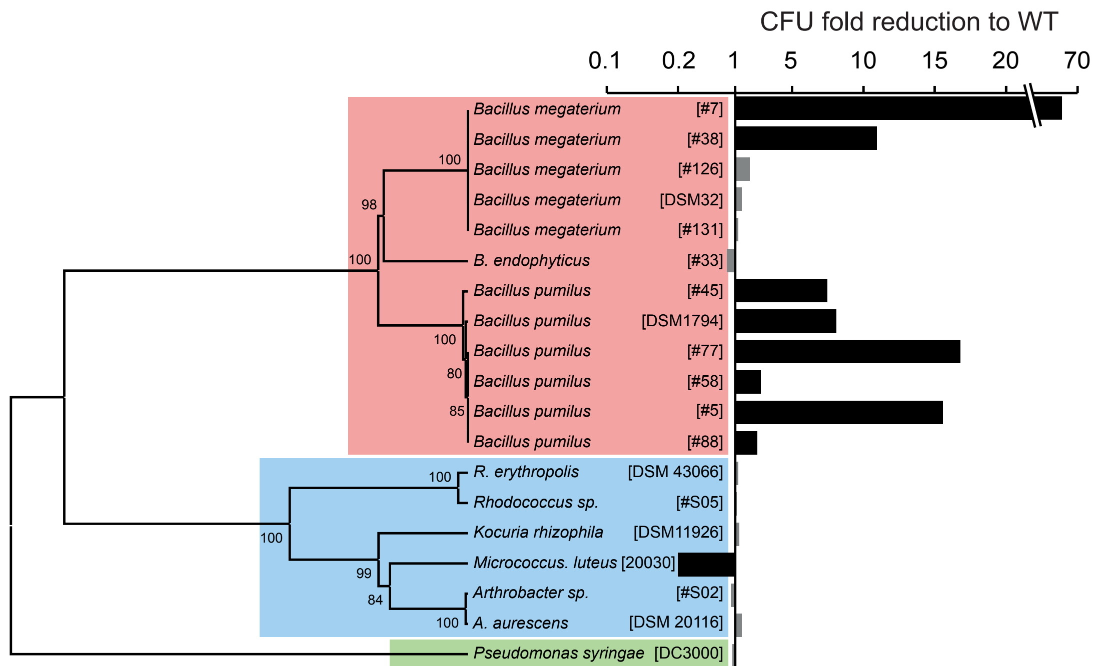


**Figure 6.9.: Neighbor-joining tree showing the relative phylogenetic distribution of all bacterial strains used for *in planta* inoculations.** The 16S rDNA was amplified using primers 27F and 1492R and aligned in MEGA5 using the CLUSTALW algorithm. Bootstrap values are shown adjacent to the branches, representing the percentage support for the clusters (1000 replicates). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances computed using the Maximum Composite Likelihood method. The bars next to the isolates show the CFU fold reduction to WT averaged from both transgenic ICE lines at 2 days post inoculation (6 h post inoculation for *B. megaterium*). Bars in black indicate that the changes to WT were significant in both transgenic lines.

distinct susceptibilities among all bacteria used (Fig. 6.9). The isolates also responded very differently, compared to the culture collection strain DSM 32, emphasizing the importance of including native isolates in such experiments.

### Activity against phytopathogenic fungi *Fusarium* and *Alternaria*

Antimicrobial peptide expressing plants are usually only challenged with phytopathogenic fungi, since increased antifungal resistance is the most desired trait for genetically engineered crop plants. We used native fungal isolates of the genus *Fusarium* and *Alternaria*, which had been isolated from diseased *N. attenuata* plants in native populations (S. Schuck *et al.* in review). The inoculation of detached leaves with agar plugs containing the fungal isolate, *Fusarium brachygibbosum* U4, resulted in no observable difference in lesion diameter between the transgenic and WT plants (Supplementary Fig. 6.6a,b). The

*Alternaria* isolates showed less defined and quantifiable lesions and were instead used for seedling resistance assays. To compensate for irregular fungal growth, the seedlings were placed in a concentric pattern with the same phenotypes in opposing positions (Supplementary Fig. 6.6c). The average seedling mortality of the transgenic plants showed no reduction compared to WT, when challenged with *Fusarium oxysporum,* or two differentially aggressive *Alternaria sp*. isolates, indicating no increased resistance of the transgenic plants against phytopathogenic fungi (Supplementary Fig. 6.6d-f).

### 6.1.4. Discussion

**Endophyte manipulation for ecological research**

The overexpression of AMPs is a common approach in crop disease resistance engineering, and has been employed in various plants, including tobacco (Koo *et al.* 2002; Yang *et al.* 2007), potato (Osusky *et al.* 2004; Portieles *et al.* 2010), banana trees (Ghag *et al.* 2012) and Arabidopsis (Wu *et al.* 2013). Transgenic plants were commonly challenged with fungal or oomycete plant pathogens and often claimed to be suitable for field protection (López-García *et al.* 2012). But little is known about the potential impact of AMP expression on beneficial endophytic bacteria, as such effects were rarely considered or investigated.

We used the ecological model plant *N. attenuata*, transformed for ectopic expression of different AMPs, and showed that transgenic plants can have strong antibacterial activity against native endophytic *Bacillus spp.* when expressing the knottin Mc-AMP1 from the common ice plant *M. crystallinum*. This is, to our knowledge, the first time that the activity of AMP-expressing plants against native endophytic bacteria has been analyzed. As the natural history of *N. attenuata* is well known and has been intensively studied, these transgenic lines are excellent candidates for further in-depth field studies in the native environment, to estimate potential effects of endophytic bacteria on plant fitness. Although the clone library obtained from the wild *N. attenuata* plants was relative small and could show just a glimpse of the diversity of the root associated bacteria, it indicated that spore forming, gram-positive bacteria were highly abundant, expectable for a dry and semiarid environment. Using a transgenic plant, which targets a dominant bacterial group, enhances the probability to reveal effects, important for plant survival in nature.

To be able to use transgenic plants for comparative studies, they should be free of side effects, which can be caused either by the callus-regeneration process during plant transformation or by the accumulation of the peptide within the plant. Some AMPs are reported to have phytotoxic effects on plant protoplasts (Zeitler *et al.* 2013) or cause

reduced root growth, if applied to seedlings at high concentrations (Allen *et al.* 2008). For other peptides, the wrong subcellular localization could lead to altered leaf morphology in transgenic plants (Lay *et al.* 2014). Peptides which might also be involved in physiological processes (Okuda *et al.* 2009) should generally be avoided, as this could lead to direct phenotypic changes. The same is true for plants impaired in phytohormone production or perception, which are altered in diverse plant processes and could have misleading secondary effects on endophyte composition (Kniskern *et al.* 2007). We observe no side effects of peptide overproduction in the ICE lines. The selective targeting on bacteria, without changing the physiology of the plant, is a requirement that allows attributing phenotypes to endophyte presence. Although plant line ICE 1.1.1.1 contained only a single T-DNA insertion and showed stable gene expression and antibacterial activity, it will be excluded from further experiments, due to the reduced growth performance. The two finally selected lines had no growth deficiencies and no indication of epigenetic gene silencing and showed stable transgene expression and antibacterial activity in the $T_4$ generation.

### ***In vivo* activity against native endophytic *Bacillus* spp.**

Newly discovered AMPs are roughly grouped in their activity against gram-positive or gram-negative bacteria, usually drawn from *in vitro* tests with lab strains (Slavokhotova *et al.* 2011; Yang *et al.* 2006). Similarly, the activity of heterologously expressed AMPs is sometimes tested under controlled conditions after peptide extraction (De Bolle *et al.* 1996). Although these experiments can confirm correct folding and activity *ex vivo*, they do not indicate whenever bacterial growth inhibition can be expected within the plant. Experiments showed that antimicrobial activity *in vitro* can be reduced by adding leaf extracts to the pure peptides (Güell *et al.* 2011). It turned out that AMP activities can be inhibited by divalent cations, which could abrogate their activity in the apoplast.

We used a leaf infiltration method to test the *in vivo* activity of the expressed AMPs against different bacteria and most of the tested strains grew sufficiently to yield quantifiable CFU results. Only *B. megaterium* needed to be infiltrated with bacterial suspension of very high optical density, due to their considerable large cell size, and the best results could be observed 6 h after infiltration. AMPs can act very quickly on bacteria and it was reported that a reduction in *P syringae* could be seen already 30 min after AMPs were sprayed on the leaf surface Zeitler *et al.* 2013.

*P. syringae* DC3000 is commonly used as a standard pathogen in numerous studies (reviewed in Xin and He 2013), but the ecological relevance of this pathogen for *N. attenuata* is unknown and we focused on plant-associated bacteria which had been isolated

directly from *N. attenuata*. The genera *Arthrobacter* and *Rhodococcus* are, interestingly, known for their ability to degrade and metabolize nicotine (Brandsch 2006; Gong *et al.* 2009; Yao *et al.* 2012). However, they seemed to be not affected by the ICE lines. In contrast, all *B. pumilus* strains, from the culture collection as well as endophytic isolates, were strongly affected in the ICE lines. As these bacteria are known for plant beneficial abilities, a field assay might reveal if the natural occurrence of these bacteria play any essential role for plant fitness in nature. The big question is, if the transgenic plants show an altered community, when grown under natural conditions. When endophytic bacteria are of high importance, the transgenic plants might suffer under certain environmental conditions. On the other hand, they might also benefit from peptide expression, by increased resistance against yet unknown pathogens.

In a previous study, transgenic potato plants were challenged with *Streptomyces scabies* (Rivero *et al.* 2012), the causative agent of tuber scab disease and a rare example of a gram-positive plant pathogen. It is remarkable that many plant AMPs are preferentially active against gram-positive bacteria (Cândido *et al.* 2011), although not many gram-positive plant pathogens are known. Having a plant that shows effects on *Bacillus* strains is a great opportunity to target preferable beneficial bacteria. However, manipulating the large group of Actinobacteria would be exciting but very ambitious, as this group is in particularly well known for antibiotic production. The test with *M. luteus* showed an unexpected increase in CFU number when re-isolated from the ICE lines. A possible explanation could be that the cocci of this bacterium usually form tetrads and if AMP activity weakens these cell aggregations, they could fall apart and would lead to a 4 fold increase in CFU counts. We observed an approximate 5.2 ($\pm$ 1.2) fold increase in CFUs from transgenic plants compared to WT. But *M. luteus* CFUs were generally difficult to count because they were commonly overgrown on the media, due to their slow growth. As a replacement and (regarding plants) more relevant strain we used the close relative *Kocuria rhizophila* (Tang 2003). This strain grows faster, gave better quantifiable results and showed clearly no CFU reduction in the ICE lines. Overall most Actinobacteria seem not to be affected by the ICE lines, whereas most *Bacillus* isolates showed reductions in CFUs.

### *In planta* inhibition of AMP activity

However, in contrast to quantitative assays using bacteria, only few published studies show quantitative data on antifungal activity; instead it is still common to present mere simple photographs or qualitative disease indices Jung *et al.* 2012; Koch *et al.* 2012; Rivero *et al.* 2012; Verma *et al.* 2012. Detached leaf assays, or other plant infection assays can vary tremendously in their results, depending on the fungus used. We observed

that the native fungal isolate *Fusarium brachygibbosum* U4 produced defined lesions on detached *N. attenuata* leaves, which could be precisely measured. Three replicated detached leaf experiments indicated no differences between WT and transgenic plants, although knottins have been reported to have also antifungal activities. *In vitro* tests with a similar knottin (Mj-AMP2) from *Mirabilis jalapa* (68 % similarity to Mc-AMP1) indicated a high peptide resistance against different proteases, strong heat stability (100°C for up to 10 min) and a constant activity retained over the pH range of 2-11 (Cammue *et al.* 1992). Nevertheless, AMPs are notoriously sensitive to divalent cations, and low concentrations of $Ca^{2+}$ or $Mg^{2+}$ can drastically reduce activity under *in vivo* conditions. Tobacco plants expressing Mj-AMP2 did not show altered resistance against the fungi *Botritis cinerea* or *Alternaria longipes* (De Bolle *et al.* 1996). However if extracts from the transgenic plants were cleaned up using reversed phase columns, they showed an enhanced antifungal activity compared to extracts from WT plants (ibid.). This is a strong indication that antifungal activity of knottins is abolished when tested *in planta*. Likewise, the Mc-AMP1 expressing *N. attenuata* ICE plants did not display enhanced resistance against *Fusarium* and *Alternaria* isolates.

**Conclusions**

Before the application of »antimicrobial« plants in the field or the release of GM crop plants, endophytic isolates can be used to estimate effects on native bacteria. Although this depends on the availability of culturable bacteria, it can reveal differences among single strains, which would be readily missed in the 'rough' phylogenetic grouping used in current high throughput sequencing approaches. Recent studies looking only at the community level have revealed no or low differences between transgenic and control plants, as the relative short amplicon sizes does not allow very deep phylogenetic comparisons. The tremendous diversity of soil bacteria is a rich reservoir of genetic variability and single isolates can show distinct susceptibilities even in phylogenetically nearly indistinguishable strains. The replacement of sensitive strains by resistant ones might allow bacteria to outcompete antimicrobial activity by diversity, leading to species specific shifts in the community, which are beyond the resolution of current 454 pyrosequencing analysis.

## Acknowledgements

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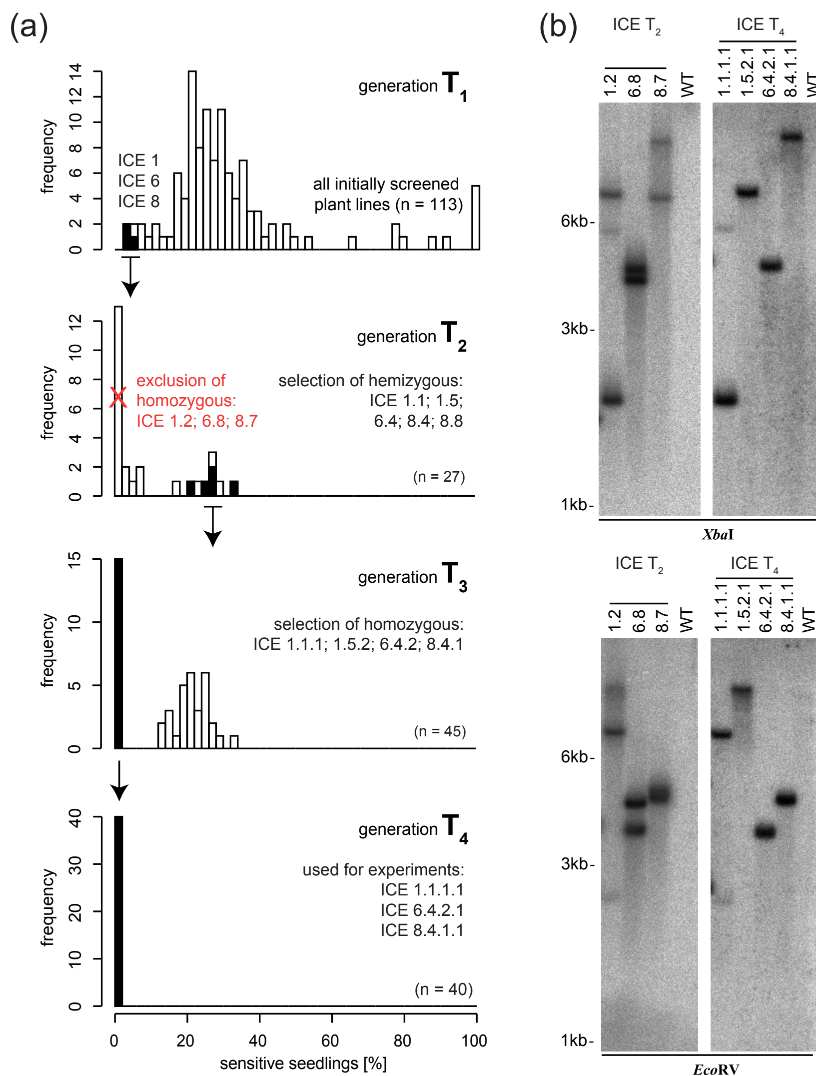
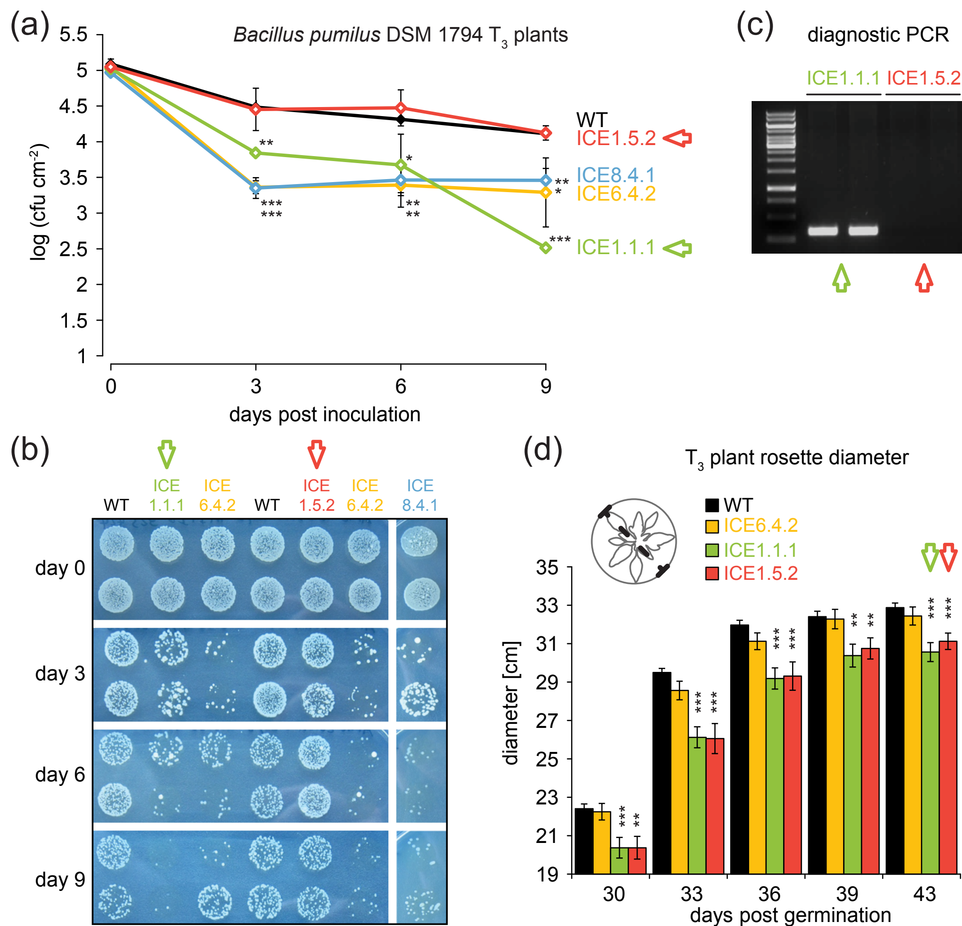
### 6.1.5. Supplementary data

**Supplementary Table 6.1:** List of the transgenic *Nicotiana attenuata* lines, used in this study. The names and origin of the ectopically expressed antimicrobial peptides are shown.

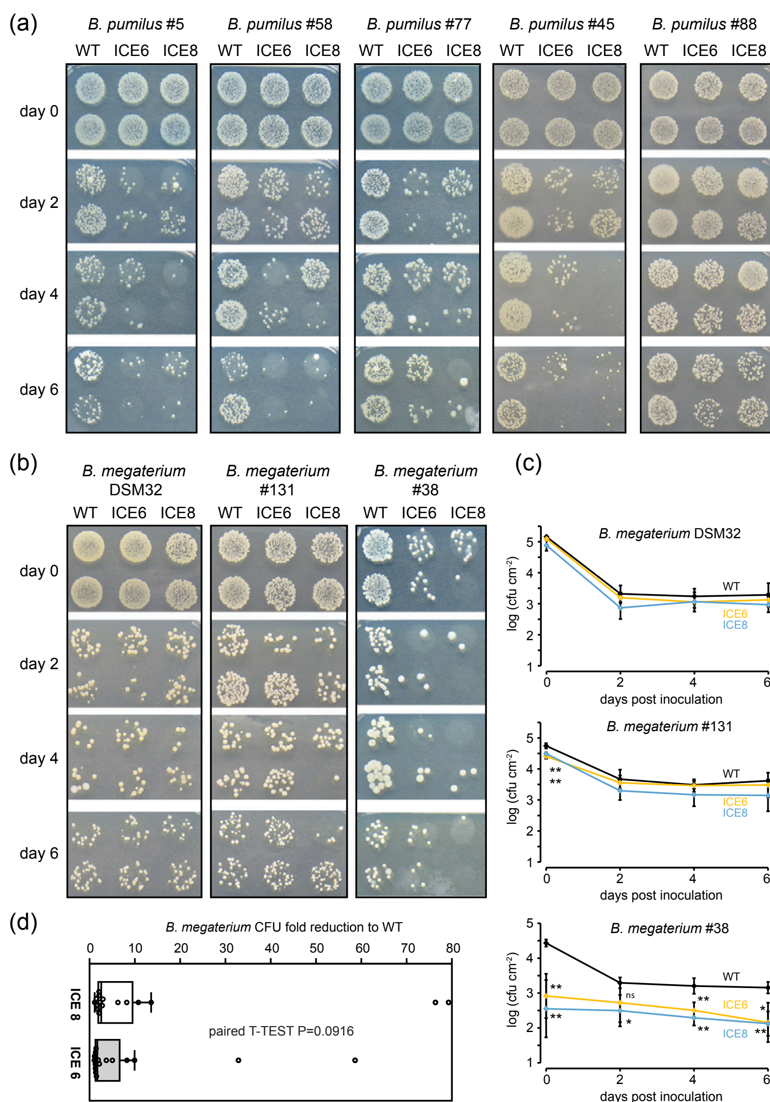
| Plant line | Construct | Trans formation | Expressed peptide | Peptide family | Organism of origin |
|---|---|---|---|---|---|
| DEF1 C.3 | pSOL9DEF1 | A-09-167 | NaDefensin1 | defensin | *Nicotiana attenuata* |
| DEF1 G.1 | pSOL9DEF1 | A-09-168 | | | |
| DEF2 C.7 | pSOL9DEF2 | A-09-230 | NaDefensin2 | defensin | *Nicotiana attenuata* |
| DEF2 F.5 | pSOL9DEF2 | A-09-278 | | | |
| VRD 1.9 | pSOL9VRD | A-09-652 | VrD1 | defensin | *Vigna radiata* |
| VRD 4.7 | pSOL9VRD | A-09-668 | | | |
| FAB 1.8 | pSOL9FAB | A-09-662 | Fabatin-1 | defensin | *Vicia faba* |
| FAB 9.3 | pSOL9FAB | A-09-865 | | | |
| LEA 1.7 | pSOL9LEA | A-09-721 | LJAMP2 | lipid transfer protein | *Leonurus japonicus* |
| LEA 5.6 | pSOL9LEA | A-09-761 | | | |
| PNA 8.6 | pSOL9PNA | A-09-823 | Pn-AFP2 | hevein | *Ipomoea nil* |
| PNA 9.9 | pSOL9PNA | A-09-825 | | | |
| SSP 4.6 | pSOL9SSP | A-09-775 | Spheniscin-2 | avian defensin | *Aptenodytes patagonicus* |
| SSP 6.5 | pSOL9SSP | A-09-671 | | | |
| ICE 1.1.1.1 | pSOL9ICE | A-09-653 | Mc-AMP1 | knottin | *Mesembryanthemum crystallinum* |
| ICE 6.4.2.1 | pSOL9ICE | A-09-748 | | | |
| ICE 8.4.1.1 | pSOL9ICE | A-09-804 | | | |



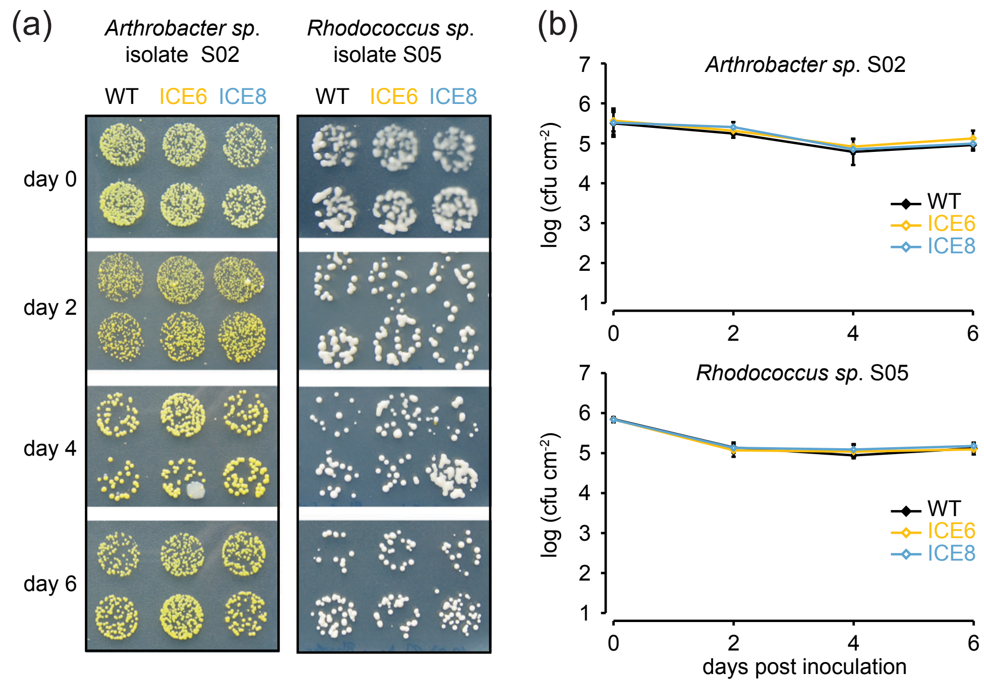

**Supplementary Figure 6.1: Selection scheme for the creation of single insertion, transgenic ICE lines.** (**a**) Segregation analysis of the resistance marker loci as shown in Weinhold *et al.* (2013) was determined by germinating transgenic *N. attenuata* seedlings on antibiotic containing medium. The independent regenerated plant lines ICE 1, ICE 6 and ICE 8 indicated the presence of two independent T-DNA loci by showing a segregation rate aproximating 6.25% in the $T_1$ generation. The initially selected homozygous plants ($T_2$) indicated multiple insertions in southern blots and were excluded from further analysis. Alternative selected $T_2$ plants with hemizygous segregation patterns (around 25% sensitive seedlings) were propagated and resulted finally in $T_4$ plants with single insertions that were used for experiments. (**b**) Southern blot analysis for the determination of T-DNA copy number. Genomic DNA was isolated from homozygous seedlings and digested in separate reactions with *Xba*I or *Eco*RV. A radiolabeled fragment of the hygromycin resistance gene (*hpt*II) served as probe. The two versions created from line ICE 1 (ICE 1.1.1 and ICE 1.5.2) harbored T-DNA insertions at different positions.



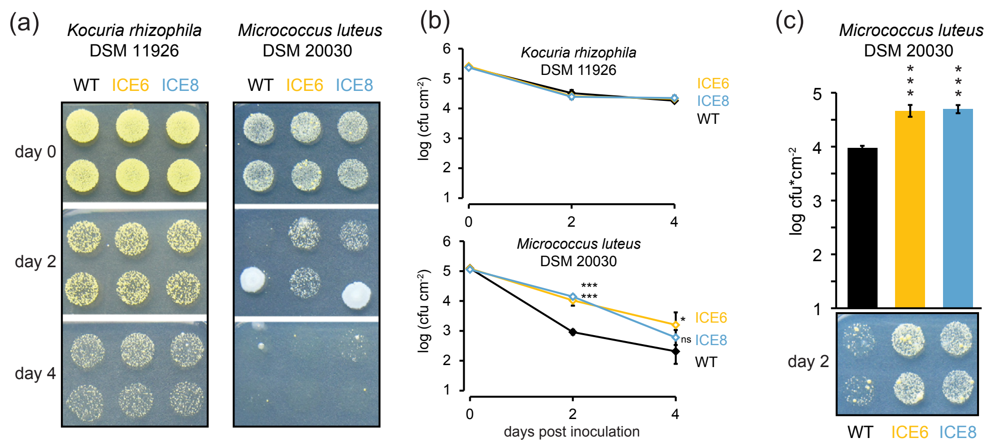

**Supplementary Figure 6.2: Screening for *in planta* activity of the first homozygous generation ($T_3$) from the single insertion ICE lines (ICE 1.1.1, ICE 1.5.2, ICE 6.4.2 and ICE 8.4.1).** (**a**) The mean colony forming units (CFU) after inoculation of *Bacillus pumilus* DSM 1794 were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 2; ICE 6 and WT n = 4). Asterisks indicate statistically significant differences between WT and the transgenic plants revealing a lack of antibacterial activity in line ICE 1.5.2 (t-test;* P $\leq$ 0.05, ** P $\leq$ 0.01, *** P $\leq$ 0.001). (**b**) Serial dilutions of the re-isolated bacteria showing two technical replicates ($10^{-1}$ dilution) per genotype and time point. (**c**) A diagnostic PCR testing for the completeness of the T-DNA insert (Gase *et al.*, 2011) was performed on two plants per line and indicated an incomplete expression cassette for line ICE 1.5.2. (**d**) Rosette diameter of glasshouse grown plants was smaller in ICE 1 plants (±SEM, n = 8; ICE 6 and WT n = 16).



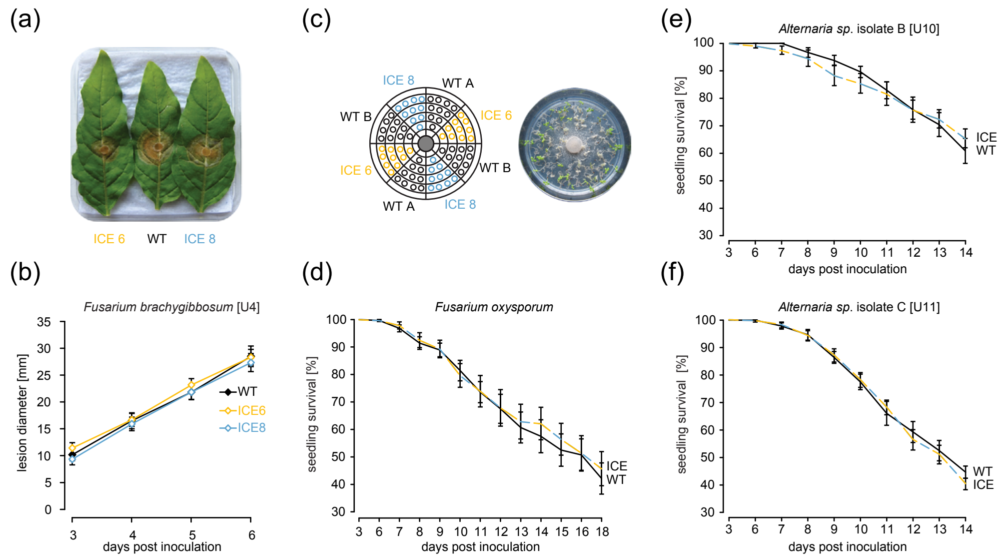

**Supplementary Figure 6.3: *In planta* inoculation of native endophytes.** (**a**) Different endophytic *Bacillus pumilus* isolates #5, #58, #77, #45 and #88 were infiltrated into leaves of WT and transgenic ICE overexpression *N. attenuata* lines. Serial dilutions of the re-isolated bacteria were spotted on LB plates and colonies counted after overnight incubation at 28°C summarized in **Fig. 7**. (**b**) *In planta* inoculation of *Bacillus megaterium* (DSM32 type strain and isolates #131 and #38). Colonies were counted after overnight incubation at 28°C. (**c**) *B. megaterium* CFUs were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 4 plants). Asterisks indicate statistically significant differences between WT and the transgenic plants (t-test;* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant). (**d**) The average *B. megaterium* CFU fold reduction to WT between line ICE 6 and line ICE 8. Dots represent average fold reduction at 0 to 6 dpi and the medians are shown as the centered lines limited by 25th and 75th percentiles and 1.5 times extend whiskers after Tukey. (n = 16 sample points).




**Supplementary Figure 6.4: *In planta* inoculation of endophytic *Arthrobacter sp.* #S02 and *Rhodococcus sp.* #S05 isolates into the leaves of WT and transgenic ICE overexpression *N. attenuata* lines.** (**a**) Serial dilutions of the re-isolated bacteria showing two technical replicates ($10^{-2}$ dilution) per genotype and time point. (**b**) The mean colony forming units (CFU) were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 4 plants). No statistical significant difference between WT and transgenic ICE lines were observed.




**Supplementary Figure 6.5: *In planta* inoculation of *Kocuria rhizophila* DSM 11926 and *Micrococcus luteus* DSM 20030 into leaves of WT and transgenic ICE overexpression *N. attenuata* lines.** (**a**) Bacterial solutions ($OD_{600}$ OD 0.2) were injected into fully expanded rosette leaves by pressure infiltration. Serial dilutions of the re-isolated bacteria were spotted on LB plates and colonies counted after overnight incubation (for *K. rhizophila*) or after incubation for three nights (for *M. luteus*) at 28°C. (**b**) The mean colony forming units (CFU) were plotted as log CFU $cm^{-2}$ leaf area (±SD, n = 4 plants). Asterisks indicate statistically significant differences between WT and the transgenic plants (t-test;* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns = not significant). (**c**) Repetition of the *M. luteus* infiltration using a higher cell number ($OD_{600}$ OD 0.4) resulting in a similar outcome at 2 dpi showing increased CFU number in the transgenic lines.




**Supplementary Figure 6.6: Fungal resistance assays with WT and transgenic ICE overexpression *N. attenuata* lines.** (**a**) Detached leaf assay with *Fusarium brachygibbosum* U4 isolate. Agar-plugs were placed on the adaxial side of detached leaves, incubated in a petri dish on moist filter paper. (**b**) The increase in lesion diameter was observed for 7 days post inoculation (dpi) (mean ±SE, n = 12 plates). (**c**) Seedling resistance assays against *Fusarium* and *Alternaria*. Seeds from WT and transgenic ICE overexpression *N. attenuata* lines were germinated on GB5 media in a concentric pattern, placing seeds from the same lines at opposing positions to compensate for unequal growth of the fungus. After 7 days post germination, a 12 mm diameter agar-plug containing fungal mycelium was placed in the center. Seedlings were observed every third day and mean seedling mortality is shown for each genotype (±SEM, n = 7 plates). Seedling resistant assay against (**d**) *Fusarium oxysporum,* (**e**) *Alternaria sp*. isolate B [U10], (**f**) *Alternaria sp*. isolate C [U11].

# 7 | General Discussion

> *"If I could do it all over again, and relive my vision in the twenty-first century, I would be a microbial ecologist. Ten billion bacteria live in a gram of ordinary soil, a mere pinch held between thumb and forefinger. They represent thousands of species, almost none of which are known to science. Into that world I would go with the aid of modern microscopy and molecular analysis. I would cut my way through clonal forests sprawled across grains of sand, travel in an imagined submarine through drops of water proportionately the size of lakes, and track predators and prey in order to discover new life ways and alien food webs."*
>
> — Edward O. Wilson (Entomologist) (Wilson 1994)

## 7.1. Plant-Microbe Mutualism

THE field of plant microbe interactions is skewed by the common perspective of seeing most »microbes« in *sensu latu* mainly as pathogens. With the primary focus on the host, the overall diversity of bacteria in and around a plant is reduced to only a few players. Plant pathogens have been intensively studied for the exploration of *Pathogen-Associated Molecular Pattern* (PAMP) and the elicitation of plant defenses (Dodds and Rathjen 2010). Certainly, understanding the infection patterns of plant pathogens is of immense agricultural importance (Dangl *et al.* 2013), but in microbial ecology the huge disguised diversity of non-pathogenic bacteria, with enigmatic functions, is much more intriguing. Endophytic bacteria are commonly overlooked as they have no direct or observable effect on the plant (Hardoim *et al.* 2008), and revealing merely the abundance is still today a difficult task, even with modern molecular sequencing techniques. Commensalistic bacteria can colonize certain plant niches in high cell numbers, without being recognized.

Studies about plant-microbe mutualism are dominated by only two major systems, arbuscular mycorrhizal fungi (AMF), the »*mother of plant root endosymbioses*« (Parniske

2008), and nodulating *Rhizobia*, well known for their role in nitrogen fixation in legumes. Whereas diazotrophic *Rhizobia* represent only a specific form of symbiosis with a single plant family, AM fungi are able to interact with nearly 80 % of all land plants (Parniske 2008). Therefore research focused for decades mainly on the AMF *Rhizophagus irregularis*[1], as the best studied mutualistic plant-fungus system. Until just two decades ago *Piriformospora indica* was discovered (Verma *et al.* 1998), a plant colonizing heterobasidiomycetous fungus belonging to the *Sebacinaceae* family, and until then completely unknown. Further studies showed that *Sebacinales* could be found in almost all examined plants around the world, and have been recognized as ubiquitous and important players. *Sebacinales* form mutualistic associations with a wide host range and have been found in mosses, ferns and most angiosperms, including the non-mycorrhizal *Brassicaceae* (Weiss *et al.* 2011). Thereby they represent an excellent example of a previously *overlooked* or *ignored* fungal endophyte of global ecological importance for many different plant species.

The perspective on plant associated bacteria might also change. Culture-independent techniques are more commonly used and allow the detection of unknown or rare bacterial taxa (Pedrós-Alió 2012). The exploration of endophytes by using high-throughput sequencing approaches has gained increasing interest in recent years and revolutionized the field of plant-microbe interactions. But despite progression in sequencing technologies, only minor innovations were made regarding common obstacles of bacterial DNA amplification from plant material, as the co-amplification of plastidal and mitochondrial DNA (Chelius and Triplett 2001; Hanshew *et al.* 2013; Lundberg *et al.* 2013). As with all PCR-based methods, the (phylogenetic) detection range is restricted solely by the used primers[2], and modern sequencing technologies renders such investigations certainly more affordable, but not more profound. The same primers have been used more than 20 years for the amplification of conserved 16S rDNA sequences (Lane 1991), and it is not surprising that the representation in databases is biased towards sequences matching to those primers (Klindworth *et al.* 2013). Yet, bacterial sequences from soil habitats are underrepresented in databases, leading to insufficient classifications (Gans *et al.* 2005), and the percentage of »*microbial dark matter*« can only be estimated (Rinke *et al.* 2013).

Recently, first attempts were made for the systematic analysis of endophytes from different *A. thaliana* accessions and close related plants, to investigate if a »core« bacterial community can also be defined in plants (Lundberg *et al.* 2012; Schlaeppi *et al.* 2014). Besides observed stochastic effects, plant genotypes and soil types seem to be the main

[1] Formely known as *Glomus intraradices*, but according to recent molecular results reclassified to the genus *Rhizophagus* (Krüger *et al.* 2012).

[2] Or as Rudolf Amann stated, *a PCR-based method is a PCR-biased method*.

factors driving the composition of the endophytic community in plants (Berg and Smalla 2009; Bulgarelli *et al.* 2012; Hardoim *et al.* 2008; Schlaeppi *et al.* 2014; Weinert *et al.* 2011). Microbial ecology will benefit from technical advances in sequencing technology, but as this is restricted to the observation of abundances only, it cannot be used for determining functionality of certain taxa.

This thesis aimed to manipulate the plant microbiome in transgenic plants which are suitable for field releases in the native environment. For the creation of »antimicrobial« plants, a set of antimicrobial peptides from different plants and animals were selected and constitutively expressed in *N. attenuata*. Transgenic plants with stable gene expression and peptide accumulation were selected and confirmed for antimicrobial activity against native endophytic bacteria *in vivo*. In MANUSCRIPT I the usual screening protocol for transgenic plants was extended and optimized, to include the use of flow cytometry and diagnostic PCRs as a standard procedure. This allowed the early exclusion of unsuitable plant lines and a faster and more efficient screening process. MANUSCRIPT II details advice for easy and early detection of epigenetically silenced plant lines and showed ways for avoidance and recovery. All finally selected AMP overexpression lines showed trans-generational stable levels of gene expression. In MANUSCRIPT III a method was established for the quantification of AMPs in the transgenic plants. A work flow for intercellular fluid extraction and processing was developed to quantify the absolute amounts of peptides within the apoplast of the transgenic plants. The plants showed huge differences in the accumulation of various AMPs. Finally, in MANUSCRIPT IV the *in vivo* antibacterial activity for a particular peptide was confirmed, which reduces different endophytic bacteria, previously isolated from *N. attenuata*.

## 7.2. Transgenic plants fight back

### 7.2.1. Heterologous protein expression after more than two decades

Starting with 11 different constructs for the expression of antimicrobial peptides resulting in more than 113 independently transformed plant lines, set a solid basis for the selection of suitable transgenic plants. The selection of at least two independently transformed plant lines from a single construct is a time-consuming process and requires several steps which can take up to 12 months. Therefore the optimization of the plant screening process, as presented in MANUSCRIPT I, was necessary. The exclusion of polyploid plants (AMP overexpression lines showed unusual high occurrence of polyploidy with up to 57 %, MANUSCRIPT I, Fig. 3) allowed the reduction of line numbers at an early stage and allowed for the creation of more than 20 stable plant lines from 10 different constructs

within only 18 month. The generation of such an unusual high number of different constructs and independently transformed plants was based on initial concerns about the uncertainty in peptide stability and *in planta* activity. During the screening process for the selection of homozygous plants, each generation indicated a newly occurring loss of transgene expression (Fig. 7.1A) following a typical pattern of epigenetic gene silencing (MANUSCRIPT II). After only three generations of inbreeding 49 of 113 independent

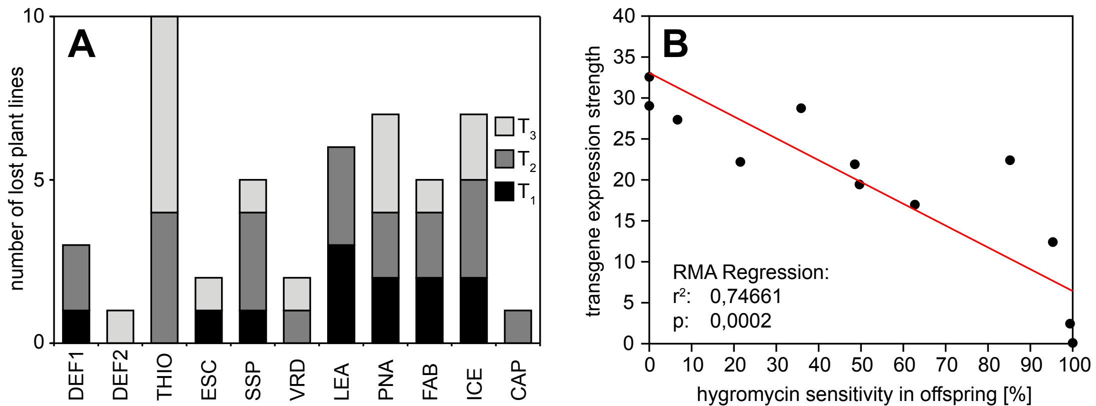



**Figure 7.1.: Impact of transgene silencing in *N. attenuata*.** (A) Numbers of lost plant lines due to transgene silencing. Within three inbred generations ($T_1$, $T_2$ and $T_3$) several lines from the eleven antimicrobial peptide expressing genotypes were excluded during screening, due to loss of transgene expression. Modified and extended from MANUSCRIPT I, Fig. 4 (Gase *et al.* 2011). (B) Correlation of gene expression strength and loss of hygromycin resistance. The mean transgene expressions (relative to actin) from secondary regenerated PNA 1.2 plants ($T_3$) were plotted to the mean hygromycin resistance in their offspring ($T_4$). Modified and extended from MANUSCRIPT II, Fig. 8 (Weinhold *et al.* 2013).

sense expression *N. attenuata* lines (43 %) were discarded, due to indications of gene silencing. Among all lines transformed with the THIO construct, numerous plants showed loss of gene expression (Fig. 7.1A) and none of the independently transformed lines was suitable for experiments. Although the promoter methylation of transgenes had been described in other plant species (in particular well studied in *N. benthamiana*) it has never before been analyzed in *N. attenuata*. The analysis of the methylation status of the 35S promoter revealed strong methylation levels among plant lines, which also showed transcriptional gene silencing. Changes in methylation were usually considered to happen after a generational change, while it is considered being more static during plant development (Feng *et al.* 2010; Law and Jacobsen 2010). The analysis of different developmental stages revealed an augmented methylation during the vegetative phase of *N. attenuata* development (MANUSCRIPT II, Fig. 7). The speed of the increasing methylation demonstrated that transgenic plants can lose expression of the transgene within a single generation leading to a phenotype loss in older plant stages. One pitfall

about promoter methylation is that it stays covert until it is too late. A slight methylation increase seems to not affect gene expression until a certain threshold, but as soon as differences can be detected on transcriptional level, the downregulation is already severe. Since transgenic plants showed on average a methylation increase of more than 3 % per day, this process happens very fast, resulting in huge expression differences among biological replicates. To be able to predict the probability of the occurrence of gene silencing, the observation of any variegation in the phenotypes becomes important. If no visual marker (*e.g. gfp*) is available, as in my case, the accurate selection based on the resistance marker can be a simple but powerful indicator. In MANUSCRIPT II the following criteria were proposed to detect unstable expressing plant lines: (A) unusual segregation rates with >50 % of sensitive seedlings, (B) intermediate phenotypes of seedlings with unclear levels of resistance and (C) large differences in gene expression among isogenic plants. These criteria are sufficient for the fast detection and exclusion of unsuitable plant lines, but a robust method to test epigenetic stability is the determination of gene expression in multiple generations and the analysis of the promoter methylation status. For example the $T_4$ generation of the ICE lines, used in MANUSCRIPT IV, showed uniform expression strength of the transgene and the offspring remained resistant in the $T_5$ generation.

### 7.2.2. Transgenic plants for ecological research. A critical view

A transgenic plant can be affected from various side effects, which are not all evident. The most obvious is a direct insertion effect. The integration of the T-DNA into the plant genome at a random position can simply hit an un-targeted gene or promoter, resulting in the unintended creation of a knockout plant. The produced phenotype would only become visible in a homozygous plant and debunks this as insertion effect. More common, but less explicit, are regeneration effects. The callus regeneration process itself is a source of somaclonal variations, from which some are of epigenetic nature. Frederick Meins reported 1983 about heritable variations in plant cell cultures (Meins 1983), and DNA methylation patterns were shown to be highly variable among regenerated plants suggesting an altered DNA methylation machinery during cell culture conditions (Miguel and Marum 2011). Cell culture conditions can lead to genome wide decrease in DNA methylation (hypomethylation), as recently shown for rice (*Oryza sativa*), where a simple tissue culture step caused stable and heritable epigenome changes (Stroud *et al.* 2013). De-methylation of promoter regions can lead to transcriptional activation, and a genome wide hypomethylation can result in undesired increase in transposon activity. This can introduce random mutations and would create unpredictable phenotypes in each independently regenerated plant line (Lisch 2013). Merely the callus regeneration process

can be a source of phenotypic changes, without having a transformation process even being involved. Depending on the plant species, these somaclonal variations can result in severe phenotypes as for the oil palm (*Elaeis guineensis*) making clonal propagation of this plant nearly impossible (Jaligot *et al.* 2000). If the transgene expression If a plant phenotype should be accounted for being caused by transgene expression, it has to be demonstrated in two or more independent plant lines, generated in separate *in vitro* regeneration events. The callus regeneration step has the potential to reduce the undesired developmental increase in promoter methylation and could be used for a temporal reactivation of epigenetically silenced transgenes (MANUSCRIPT II). From the regenerated plants, several showed re-occurring loss of gene-expression, which correlated with the subsequent loss of hygromycin resistance in the offspring (Fig. 7.1B). The fastest and easiest way to test for gene expression stability in a particular plant line, is the analyzes of the marker gene expression in the offspring, simply by germinating seeds on hygromycin containing media. Testing only the same generation will be insufficient, as the DNA methylation process of *N. attenuata* is highly dynamic and could show an increase of up to 50 % in absolute CG methylation within only 15 days of normal plant growth (MANUSCRIPT II Fig. 7).

On the other hand does this dynamic methylation processes imply that epigenetic adaption might play a much greater role in driving plant evolution, as suggested by Fedoroff (2012). Unlike in animals in which the germline is sequestered, plants develop germ cells relative late in their life cycle and any vegetatively acquired epigenetic change can be inherited to the offspring, giving plants an enormous potential to flexibly adapt to a rapid changing environment (Lang-Mladek *et al.* 2010; Verhoeven *et al.* 2010). Possible scenarios of epigenetic inherited stress resistance in plants is a topic which is often discussed in reviews, but rather rarely supported by experiments (Bräutigam *et al.* 2013; Gutzat and Mittelsten Scheid 2012; Holeski *et al.* 2012; Sahu *et al.* 2013).

## 7.3. The multiple roles of antimicrobial peptides

> "*In the era of genomics, if a gene is not annotated, it is not investigated.*"
>
> — Lease and Walker 2006

The numbers of cysteine-rich peptides (CRPs) resembling antimicrobial peptides were highly underestimated in the first genome assemblies of *A. thaliana* and about 80 % were un-annotated leading to a general under-prediction of these peptides in plant genomes (Silverstein *et al.* 2007). The genome of *A. thaliana* contains more than 300 defensin-like genes (estimated), which are preferentially expressed in reproductive organs

(Silverstein and Graham 2005). This highly diverse number of peptides do not play a sole role in plant immunity, but also in many other plant developmental processes (Takeuchi and Higashiyama 2012). Plant defensins and defensin-like peptides have evolved specific functions during plant evolution and exhibit multiple functions in cell-to-cell communication and pollen-pistil interactions. They can inhibit the activity of different enzymes ($\alpha$-amylases or proteases), confer heavy metal tolerance (Mirouze *et al.* 2006) and act as ion channel blockers or pollen tube attractants (reviewed in Kim *et al.* (2009) and Carvalho and Gomes (2009)). They play a role in the regulation of the fertilization process and many of these peptides were reported to be expressed specifically in the synergid cells of the female gametophyte (De Coninck *et al.* 2013). In the Wishbone flower (*Torenia fournieri*) defensin-like peptides act as pollen tube attractants and were aptly named »LUREs«. In *Brassicaceae*, the small (~6 kDa) defensin-like peptide (S-locus cysteine-rich protein) is involved in the self-incompatibility system, coating the male pollen and recognized by the S-locus receptor kinase (SRK) to prevent self pollination (Higashiyama 2010). In *Zea mays* a defensin-like peptide (ZmES4) is expressed in the female synergid cells and induces the pollen tube burst by membrane depolarization via potassium channel opening. This is an astonishing similar mechanism which these peptides also use to inhibit the growth of fungal hyphae (De Coninck *et al.* 2013).

All these peptides are structurally related, show a common folding motive and have highly conserved cysteine residues. Nonetheless, they are extremely versatile and structural similarity does not allow the prediction of any function (Marshall *et al.* 2011; Weerden and Anderson 2013). Most plant peptides which showed antimicrobial activity were isolated from seeds, where they are highly accumulated in the endosperm to protect the germinating seedling from pathogen infections (Cândido *et al.* 2011). Taking seed-derived AMPs for ectopic expression (as the case for FAB, VRD, PNA, LEA and ICE) decreases the chances for the accidental selection of peptides with regulatory functions, which could lead to developmental side-effects in transgenic plants (Lay *et al.* 2014; Stotz *et al.* 2009). Only one of the lines (CAP) expressed a root-derived AMP, which encodes a glycine-rich pro-peptide, that is further cleaved in two smaller peptides with slightly different activity. Cysteine-free AMPs are not very common in plants, but this glycine-rich peptide from the roots of *Capsella bursa-pastoris* have been reported for antimicrobial activity (Odintsova *et al.* 2013; Park *et al.* 2000).

## 7.4. Peptide stability in the leaf apoplast

The deposition of proteins and peptides into the apoplast is a natural process in plants to reinforce the cell-wall against microbial invasions. Fungal or bacterial pathogens try to

enter a plant and degrade the cell-wall using lytic enzymes such as polygalacturonases, which the plant tries to circumvent by inhibiting them. But plants can also counterattack and weaken the fungal cell-walls in return with chitinases or 1,3-$\beta$-glucanases. The fraction of defense related proteins that were found in the plant cell-wall proteome add up to 21 % of all proteins (Boudart *et al.* 2005; Zhu *et al.* 2006). Among these also many members of peptidase and protease families contribute to the protection of the plant and different serine-, aspartyl- and cysteine-proteases could be found in intercellular fluid extracts of plants (Boudart *et al.* 2005; Delannoy *et al.* 2008). The intercellular space is a peptidase rich environment which impedes many approaches of using the secretory pathway in plants for heterologous protein production (Doran 2006).

Although cysteine-rich AMPs are (compared to most other proteins) usually relatively resistant against proteolytic cleavage and in general quite sturdy (high tolerance against heat and acids), they are not fully immune against proteases and can be digested by trypsin (otherwise the UPLC-MS$^E$ measurements in MANUSCRIPT III would not have worked out). Therefore non-plant AMPs need to be evaluated for *in planta* stability when heterologously expressed in plants (Hamamoto *et al.* 2002; Marcos *et al.* 2008; Mills *et al.* 1994). Of course, this depends mainly on the peptide, but also on the respective plant system, and stability in one plant species does not infer the same for another species.

The expression of the hemipteran »Thanatin« and the crustacean »Penaeidin4-1« was reported to work in plants and increased the disease resistance in Arabidopsis and creeping bentgrass, respectively (Wu *et al.* 2013; Zhou *et al.* 2011), although the latter authors admit that they were unsuccessful in detecting the expressed peptide in immunoblots. Earlier reports using the lepidopteran »Cecropin B« showed instability in *N. tabacum* (Allefs *et al.* 1995; Florack *et al.* 1995). This type of linear $\alpha$-helical AMP was always degraded when secreted to the apoplast, and showed only stability in *N. tabacum* when transiently expressed and localized in the vacuole or in vesicles (Company *et al.* 2014). The amphibian AMP »esculentin-1« (which I used for the ESC lines) showed hints of exopeptidase degradation when expressed in *N. tabacum* (Ponti *et al.* 2003).

Although AMPs have been expressed in many plant species, no universal detection method was available and only a very limited number of AMPs have been shown to be detectable on immunoblots. The development of a modern, gel-free, LC-MS$^E$ proteomic method (MANUSCRIPT III), which allows the simultaneous label-free quantification of various AMPs, was a great leap forward and essential for the evaluation of the different transgenic plant lines. The analysis of the ESC lines *e.g.* showed no evidence for the presence of the peptide (including MALDI-TOF/MS and nanoUPLC-MS$^E$ analysis) and tests with fungal resistance assays and bacteria infiltration were likewise negative (unpublished), assuming peptide instability in *N. attenuata*. Other peptides as PNA (Koo

*et al.* 2002) and LEA (Yang *et al.* 2007) were selected precisely for this reason, since previous publications report stable expression in transgenic *N. tabacum* plants. Peptide stability in *N. attenuata* could be clearly approved for the LEA line, which was positive in all MALDI-TOF/MS and nanoUPLC-MS$^E$ analysis. The LEA line was used as a »positive control« during the development of the peptide extraction step (using different buffers) and the optimization of the peptide desalting process (using micro-dialysis, ultra-filtration and solid phase extraction systems). The expressed lipid-transfer protein showed high peptide amounts in the apoplast of *N. attenuata*. In comparison, the PNA peptide was barely detectable. The quantitative comparison of all constructs showed the highest peptide amounts for the DEF2, LEA and ICE lines (MANUSCRIPT III Fig. 5.4), confirming that the peptides of these lines show the desired apoplastic stability.

It has been reported that the occurrence of undesired developmental abnormalities in the leaf shape of transgenic plants is due to cytotoxic effects of high AMP amounts (Lay *et al.* 2014). However, such phenomena were not observed in the transgenic plants described here, and all high peptide accumulating lines were free of pleiotropic growth effects. The detection of the peptides in the intercellular fluid (ICF) does not only confirm peptide stability but also the correct extracellular localization. A signal peptide is no warrant for the extracellular secretion, and some defensins show accumulation in intracellular compartments (Oomen *et al.* 2011; Reimann-philipps *et al.* 1989). In particular C-terminal domains (as present in DEF1, DEF2 and PNA) are discussed as vacuolar signaling domains (Lay *et al.* 2014).

### 7.4.1. Summary of AMP quantification

Plausible explanation for the detectability of each peptide are given below: DEF1 and DEF2 are endogenous AMPs of *N. attenuata* and peptide stability seems to be obligatory. These were well detectable and the differences in peptide amount can be easily explained by differences in gene expression strength. Nevertheless they are expected to be vacuolar localized and their appearance in the ICF is surprising, but could be a result of a secretion via glandular trichomes. The CAP peptide was not found since it has, in comparison to all others, different characteristics (cysteine-free and a nearly neutral p*I*) and may simply evade the detection (discussed in MANUSCRIPT III). The SSP and ESC peptides are both either proteolytically degraded or not secreted due to a non-functional signal peptide, which was only for these two cases fused to the AMP sequences. The PNA peptide might be vacuolar localized and was therefore barely detectable in the apoplast. The remaining VRD, FAB, ICE and LEA peptides were all found, but only ICE and LEA showed very high accumulation levels. Coincidently these were the only peptides from which the sequences were not adapted in codon usage. For the other AMPs (ESC, SSP, PNA, FAB,

VRD) minor modifications were made before gene synthesis according to the codon usage table of *N. tabacum*, to increase probability of high expression efficiency in *N. attenuata*. If these minor sequence modifications resulted in an inefficient translation process is just speculation, but it would fit to the observed pattern in peptide detectability. Noteworthy is also the fact that the ICE and LEA lines showed the lowest numbers of tetraploidy occurrence (MANUSCRIPT I Fig. 3), whereas they showed a very strong appearance of gene silencing (Fig. 7.1A)

## 7.5. Activity spectrum of antimicrobial peptides

Data on the activity of newly isolated AMPs derives in most cases from *in vitro* tests using the purified peptide in agar diffusion assays, estimating the spectrum of activity (*e.g.* antifungal or antibacterial activity against gram-positive or gram-negative bacteria). These pre-tests are often the only available information, against which groups of bacteria a peptide has a possible antimicrobial activity. The controlled supplementation with different salt concentrations showed that the activity depends strongly on the ionic composition of the media and that AMPs are relatively easy inhibited by divalent cations as $Ca^{2+}$ and $Mg^{2+}$ (Cammue *et al.* 1992; Carvalho and Gomes 2009; Terras *et al.* 1993). There is a huge discrepancy in peptide activity regarding *in vitro* assays and *in vivo* assays, which is one of the reasons why these peptides were after all not applicable for most clinical purposes. A possible *in vivo* inhibition within a plant is a likely scenario, and AMPs need to be tested experimentally to confirm their activity under normal physiological conditions of a transgenic plant (MANUSCRIPT IV). This is also true for AMPs from plants, as their native sub-localization (floral tissue, endosperm or secretion via trichomes) differs from their artificial localization within all tissue due to the ectopic expression.

Most plant defensins isolated so far exhibit only a broad antifungal activity, and have no or minor antibacterial activity (Carvalho and Gomes 2009). This data might be biased, since most plant AMPs are anyhow preferentially tested against fungal plant pathogens. But this strongly limits the usefulness of plant AMPs when bacteria are targeted. All plant AMPs for heterologous expression, were selected if antibacterial activity has been reported (Chen *et al.* 2002; Koo *et al.* 1998; Park *et al.* 2000; Pelegrini *et al.* 2011; Yang *et al.* 2006; Zhang and Lewis 1997). Most of these peptides seem to be mainly active against gram-positive bacteria, and this information was used to narrow down potential microbial targets.

A leaf infiltration method was developed with *Bacillus pumilus*, to screen for *in planta* activity in the transgenic lines. This method was a reliable test for plants minimizing false

positives (which was however common in antifungal screens). The colony forming unit (CFU) counts of this bacterium were fully reproducible in diverse glasshouse chambers and not influenced from plant age, temperature or season (which is *e.g.* not the case for *P. syringae*). The tests with *Bacillus pumilus* showed only for the ICE lines *in vivo* activity which was proven satisfactory (MANUSCRIPT IV). Although the LEA peptide was highly accumulated (and maybe the best expressable peptide of them all) this line lacked *in planta* activity against *B. pumilus*. Similarly, detached leaf and seedling resistant screens did not indicate increased resistance against *Phytophtora nicotianae*, *Botrytis cinerea*, *Fusarium oxysporum* or *Alternaria brassicicola* (unpublished). The CAP and ESC peptides were the only AMPs with putative activity against gram-negative bacteria and the plant lines were hence infiltrated with *P. fluorescens* and *P. syringae*, but showed also no reduction of these bacteria (unpublished). Besides that, both peptides were not detectable in the nanoUPLC-MS$^E$ method (MANUSCRIPT III). The ICE lines, as the only positive candidates, were further characterized within their activity range and tested with native bacteria, which have been isolated as endophytes from *N. attenuata*. Importantly, this experiment excluded the possibility that the shown susceptibilities were artifact of the repeated culturing of the used bacterial strains. Infiltrations of these »wild« isolates were, for comparability reason, always performed together with closely related strains from the culture collection. However, the direct comparison of native isolates and culture collection strain showed no indications for a higher resistance in wild isolates. Interestingly, infiltration with isolates of *B. megaterium* showed the most distinct results (MANUSCRIPT IV, Fig. 6.8, 6.9). These four strains were highly diverse in their susceptibilities, but basically indistinguishable on 16S rDNA[3] It would not have been possible to distinguish them via 16S rDNA sequencing, if they would have been mixed together. Using them from separate culture stocks allowed the estimation of the individual discrepancy in susceptibility. The differences in closely related strains suggest that the diversity of the bacterial soil community might be able to countervail for antimicrobial activity. Since such intra-species differences are beyond the resolution of current sequencing approaches, they would be missed in a bare community analysis. However, pyrosequencing analysis of field grown plant samples are currently under process and will be part of future publications. The plant growth of the ICE lines did not indicate obvious differences to control plants when grown under field conditions (unpublished). But further experiments are necessary addressing current shortcomings of the planting procedures for more refined and realistic conditions regarding plant-microbe relationship.

[3]As *B. megaterium* has 3 ambiguous base pairs in the 16S rDNA, they matched 99.8 % and not 100 %.

## 7.6. Plant-microbe interaction - a plant's perspective

Concerning the whole life span of *N. attenuata*, this annual plant does not only touch the soil with the roots, but spends most of the time buried in the ground (Fig. 7.2). Due to the focus on plant-herbivore interactions most of the research on *N. attenuata* devotes to the late stages, and visible upperground plant parts. The germination of *N. attenuata* is

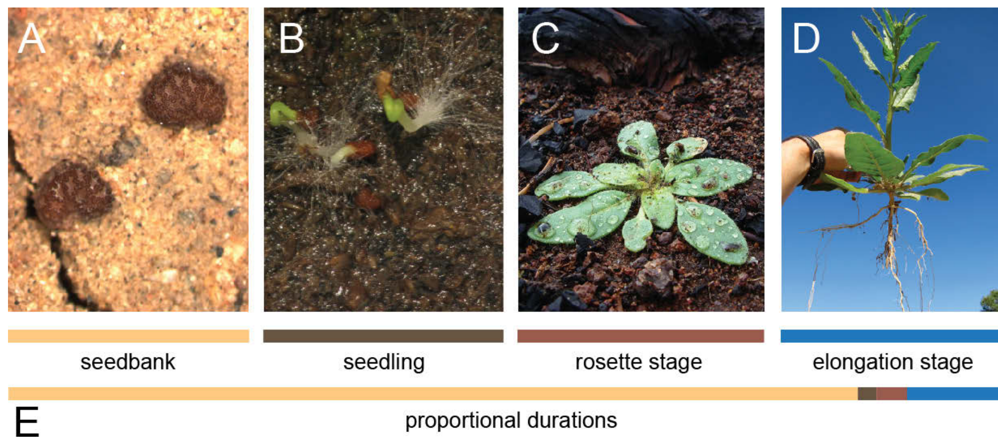



**Figure 7.2.: Life stages of *N. attenuata*.** (A) Seeds stay dormant for several years in long lasting seed banks (B) until smoke-derived cues combined by sufficient hydration brings eligible conditions for germination. (C) A rosette stage plant growing between charred debris on the »New Harmony burn« shortly after a rain shower. (D) Elongating plant excavated from a natural wash population, showing parts of the root system structure. (E) Illustration of the proportional duration for each life cycle stage of *N. attenuata*.

done in a standardized procedure placing surface sterilized seeds on sterile media, on which seedlings develop for ten days until they were placed in potting soil, comprised of an artificial peat mixture. This procedure is equal for field releases and plants are bedded out to make first contact with the soil community when they already reached early rosette stage.

In nature, plants are constantly surrounded and embedded in soil, and the longest episode of *N. attenuata´s* life is probably the seed stage, where they rest several decades coping with seasonal moisture fluctuations and the increasing possibility to decay. The microbial impact on a plant has probably its summit during the early germination stage, when the seed coat breaks open and the root system emerges. A young seedling has to find its way to protrude the moist soil layer and reach the light. Similar as »imprinting« describes a phase-sensitive learning period for animals where they can only perceive things at a particular age, plants might be more amendable for endophytic colonization at early developmental stages. The field experimental setup for *N. attenuata* was developed

to release transgenic plants for plant-herbivore studies, and deprives plants at early stages to interact with the soil community.

Even the complexity of plant-herbivore interactions with all the multi-trophic levels is not yet understood, although herbivores are visually observable, show much lower genetic diversity and their damage to the plant is conspicuous and quantifiable. Observing microbes from field grown plant is much more sophisticated, and little is known about the abundance and importance of associates or pathogens since they can only be »visualized« using molecular techniques (Gans *et al.* 2005). People are just beginning to understand the significance of microbial interactions around the root system regarding plant health and fitness (East 2013) and the value of endophytic bacteria for agricultural application (Senthilkumar and Anandham 2011). In the past, most work on plant stress response was performed on plants devoid of a natural microbiome and the inclusion of the bacteriosphere would bring a holistic view into plant physiological studies. Endophytes can remarkably increase the stress tolerance of plants (Kim *et al.* 2012; Torres *et al.* 2012), and acknowledging that plants live in harmony with these little friends (Lelie *et al.* 2009) will help future research to understand the importance of this micro-environment in a more natural context.

# 8 Summary

PLANTS are surrounded by diverse microbial communities inhabiting the plants rhizosphere, the leaf surface area but also inner parts of a plant. These endophytic bacteria are tolerated by the plant immune system and seem to form a tight mutualistic relationship with its host. Endophytic bacteria are believed being important for plant growth, plant health and plant resistance, estimated from inoculation experiments with single strains. In order to unravel the effect of their natural abundance, the colonization needs to be influenced within a plant. The wild tobacco *Nicotiana attenuata* is a non-domesticated, ecological model plant that can be genetically manipulated and allow heterologous gene expression. For this thesis I transformed *N. attenuata* for the expression of antimicrobial peptides with the goal to establish a toolbox for plant microbe interaction studies. Antimicrobial peptides (AMPs) are small cysteine-rich peptides with a broad spectrum activity against different bacteria or fungi, and part of the innate immunity of plants and animals. Constitutive expression within a transgenic plant could be used to manipulate the natural abundance of endophytic bacteria, if a transgenic plant shows high AMP accumulation and *in planta* activity under the physiological conditions of the plant apoplast.

I selected a comprehensive set of 11 different AMPs from various AMP families for ectopic expression in *N. attenuata* and developed an optimized screening method for the selection of suitable plant lines. Several transgenic plants showed gene expression loss and epigenetic gene silencing, and the analysis of the promoter methylation status allowed the selection of plant lines with trans-generational stable gene expression. I further showed that the augmentation of the methylation levels occurred independently from a generational change. Plants developed epigenetic changes solely during normal plant development and showed rapid methylation increase in somatic cells (more than 3% per day!). A secondary callus regeneration step could avoid somatic transgene methylation and was used to recover gene expression in the affected lines.

To assess the localization and stability of the expressed AMPs direct within the plants, a universal nanoUPLC-MS$^E$ method was developed allowing an absolute peptide

quantification and comparison of various AMPs from the intercellular fluid (ICF) of different transgenic plant lines. The results revealed desired high peptide accumulation in the apoplast of the LEA lines (expressing a lipid-transfer protein from motherwort) and the ICE lines (expressing a knottin from the common ice plant). Both AMPs have reported *in vitro* activity against gram-positive bacteria.

The analysis of the non-culturable bacterial community from *N. attenuata* plants from a wild population in the Great Basin Desert in Utah, showed that most bacteria belong to gram-positive groups, and *Streptomyces* and *Bacillus* dominated the root associated community. I tested the transgenic plants using a leaf infiltration method for *in planta* antibacterial activity against various bacterial strains. Only the ICE lines showed the ability to reduce infiltrated *Bacillus pumilus*, and were further characterized in their activity spectrum using culture collection strains (DSMZ) and native endophytic bacteria, previously isolated from *N. attenuata*. They showed consistent reductions of most native endophytic *Bacillus* spp. isolates, but no effects on Proteobacteria and most Actinobacteria. *B. megaterium* isolates showed strong heterogeneity among the isolates with highly distinct susceptibilities.

AMPs were in the past only used to increase the resistance of crop plants against phytopathogens, and potential effects on beneficial endophytic bacteria were usually ignored or not examined. The relevance of endophytic bacteria for natural plant growth is still largely unknown, since it was not possible to grow an aposymbiotic plant under natural conditions. The ICE lines showed *in planta* activity against endophytic bacteria and stable and high peptide expression which makes them valuable tools for the exploration of fitness effects of endophytic bacteria on *N. attanuata*.

# 9 | Zusammenfassung

Pflanzen sind von vielfältigen mikrobiellen Lebensgemeinschaften umgeben, welche die Rhizosphäre, die Blattoberfläche und sogar die inneren Pflanzenteile besiedeln. Endophytische Bakterien werden vom Immunsystem der Pflanze toleriert und scheinen in einer engen mutualistischen Beziehung zu ihrem Wirt zu stehen. Endophyten sind vermutlich wichtig für das Wachstum, aber auch für die Gesundheit und Resistenz einer Pflanze. Um die Bedeutung in der natürlichen Umgebung zu entschlüsseln, müsste eine bakterienfreie Pflanze geschaffen werden, welche von sich aus endophytische Bakterien beeinflussen kann.

Der wilde Tabak *Nicotiana attenuata* ist eine nicht-domestizierte Pflanze, die sich gentechnisch manipulieren lässt und wird daher als ökologisches Modellsystem genutzt. In meiner Thesis habe ich *N. attenuata*-Pflanzen für die Überexpression von antimikrobiellen Peptiden transformiert, um sie als Instrument für Pflanzen-Mikroben-Interaktionsstudien zu etablieren. Antimikrobielle Peptide (AMPs) sind kleine Cystein-reiche Peptide mit einem breiten Wirkungsspektrum gegen verschiedene Bakterien und Pilze und Teil der angeborenen Immunität von Tieren und Pflanzen. Eine konstitutive Expression in transgenen Pflanzen kann endophytische Bakterien nur dann manipulieren, wenn die transgenen Pflanzen hohe Mengen von AMPs produzieren und die Peptidaktivität unter den physiologischen Bedingungen der Pflanze (*in planta*) erhalten bleibt.

Für diese Arbeit wurden 11 verschiedenen AMPs aus verschiedenen Peptidfamilien selektiert, in *N. attenuata* überexprimiert und geeignete Pflanzenlinien durch ein optimiertes Selektionsverfahren ausgewählt. Viele der transgenen Pflanzen zeigten einen Verlust der Genexpression durch epigenetische Gen-Inaktivierung und die Analyse der Promoter-Methylierung erlaubte die Selektion von Pflanzenlinien mit stabiler Genexpression über mehrere Generationen. Ich konnte weiterhin zeigen, dass die Etablierung der Promoter-Methylierung unabhängig von einem Generationswechsel ist. Pflanzen entwickelten epigenetische Veränderungen nur während des normalen Wachstums und zeigten einen rasanten Anstieg der Promoter-Methylierung in somatischen Zellen (mehr als 3 % pro

Tag!). Eine sekundäre Kallus-Regeneration konnte die somatische Promoter-Methylierung verhindern und die Genexpression in den betroffenen Linien wiederherstellen.

Um die Lokalisierung und die Stabilität der exprimierten AMPs innerhalb der Pflanzen direkt zu untersuchen, wurde eine universelle nanoUPLC-$MS^E$ Methode entwickelt, wodurch die absolute Quantifizierung der Peptide und der Vergleich von verschiedenen Pflanzenlinien möglich wurden. Die Ergebnisse zeigten die gewünschte hohe Peptidakkumulation im Apoplasten der LEA-Linie (exprimiert ein Lipid-Transfer-Protein aus dem japanischen Löwenschwanz, *Leonurus japonicus* ) und der ICE Linie (exprimiert ein Knottin aus dem Eiskraut, *Mesembryanthemum crystallinum*). Für beide AMPs wurde *in vitro*-Aktivität gegen gram-positive Bakterien beschrieben.

Die kultur-unabhängige Analyse der Bakteriengemeinschaft von *N. attenuata*-Wurzeln aus einer natürlichen Population im Südwesten von Utah (USA) zeigte eine Dominanz von gram-positive Bakterien. Die transgenen Pflanzen wurden *in planta* mittels Blattinfiltrationsverfahren auf antibakterielle Aktivität gegen verschiedene Bakterienstämmen getestet. Nur die ICE-Linien wiesen eine Aktivität gegen *Bacillus pumilus* auf und wurde weiter in ihrem Wirkungsspektrum charakterisiert und sowohl gegen Bakterienstämme aus der Kultursammlung als auch gegen native endophytische Bakterien getestet, welche zuvor von *N. attenuata* isoliert wurden. Die ICE-Linie zeigte eine konsistente Reduktion der meisten nativen *Bacillus*-Arten, aber keine Wirkung auf Proteobakterien und die meisten Actinobakterien. Isolate von *Bacillus megaterium* zeigten eine besonders starke Heterogenität und sehr unterschiedliche Sensibilität gegen die AMP-Expression der ICE-Linie.

In der Vergangenheit wurden AMPs meist nur exprimiert, um die Resistenz von Nutzpflanzen gegenüber Pflanzenpathogenen zu erhöhen, aber mögliche Auswirkungen auf nützliche endophytische Bakterien blieben weitgehend unbeachtet. Die Bedeutung von endophytischen Bakterien für Pflanzen in ihrer natürlichen Umgebung ist immer noch weitgehend unerforscht, da es bisher nicht möglich war eine Endophyten-freie Pflanze wachsen zu lassen. Die ICE-Linien zeigten Aktivität *in planta* gegen endophytische Bakterien, stabile Genexpression und hohe Peptid-Akkumulation und sind daher ein wertvolles Instrument für die Erforschung des Einflusses endophytischer Bakterien auf die Fitness von *N. attanuata*.

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# 11 | Acknowledgment

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— Dr. Sheldon L. Cooper B.S M.S M.A Ph.D Sc.D (Physicist)

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# 12 Curriculum Vitae

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2012 **Weinhold A, Baldwin IT** »*Bringing the field to the lab - Why antimicrobial plants should be tested with native bacterial isolates*« Poster, 28th New Phytologist Symposium "Functions and ecology of the plant Microbiome", New Phytologist Trust, Rhodes, Greek, May 2012

2011 **Weinhold A, Baldwin IT** »*Friendly fire - Why antimicrobial plants should be tested with beneficial bacteria and in field experiments*« Poster, Institute Symposium 2011, MPI for Chemical Ecology, Jena

2010 **Long H., Sonntag D., Weinhold A, Meldau S., Groten K., Wissgott A., Baldwin IT** »*Plant Microbe interactions - From Koch´s postulates to Microbial Communities*« Poster, SAB Meeting 2010, MPI for Chemical Ecology, Jena

2009 **Weinhold A, Gase K, Baldwin IT** »*Analyzing the bacterial root community of the desert plant Nicotiana attenuata*« Poster, Symposium of the Plant Science Center Zürich: Plant-Microbe Interactions, Basel 2009

**List of Publications**

in prep. **Santhanam R., Luu VT, Weinhold A, Goldberg J, Oh Y. and Baldwin IT:** »*Native endophytic bacteria protect their natural host N. attenuata from a sudden wilt disease in nature*« in preparation

2014 **Luu VT, Schuck S, Kim SG, Weinhold A and Baldwin IT:** »*Jasmonic acid signaling mediates resistance of the wild tobacco Nicotiana attenuata to its native Fusarium but not Alternaria fungal pathogens*« Plant, Cell and Environment doi: 10. 1111/pce.12416

2014 **Schuck S, Weinhold A , Luu VT and Baldwin IT:** »*Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant Nicotiana attenuata*« PLoS ONE 9(7): e102915

in review **Weinhold A, Baldwin IT:** »*In planta manipulation of endophytic Bacillus spp in Nicotiana attenuata by the expression of antimicrobial peptides* « submitted to New Phytologist, (2014)

in review **Weinhold A, Wielsch N, Svatoš A, Baldwin IT:** »*Label-free nanoUPLC-MS$^{E}$ based quantification of antimicrobial peptides from the leaf apoplast of Nicotiana attenuata*« submitted to BMC Plant Biology, (2014)

2013 **Weinhold A, Kallenbach M, Baldwin IT:** »*Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic Nicotiana attenuata plants*« BMC Plant Biology, 13(99)

2011 **Gase K, Weinhold A, Bozorov TA, Schuck S, Baldwin IT:** »*Efficient screening of transgenic plant lines for ecological research*« Molecular Ecology Resources, 11(5), 890-902

Jena, March 20, 2014

Arne Weinhold

# 13 | Eigenständigkeitserklärung

Entsprechend § 5 Abs. 4 der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, dass mir die geltende Promotionsordnung der Fakultät bekannt ist und dass ich die vorliegende Promotion eigenständig angefertigt und alle von mir benutzen Quellen angegeben habe. Personen, die mich bei der Erhebung und Auswahl des Materials sowie bei der Erstellung der Manuskripte unterstützt haben, sind in der Auflistung der Manuskripte (MANUSCRIPT OVERVIEW Chapter 2) genannt oder werden, im Falle von Beiträgen geringeren Ausmaßes, in den Danksagungen am Ende der entsprechenden Manuskripte genannt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen noch haben Dritte für Arbeiten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Dienstleistungen erhalten. Die vorgelegte Dissertation wurde weder als Prüfungsarbeit für eine Staatliche oder andere Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, March 20, 2014

Arne Weinhold

Universität Jena
2014

# A | Apendix

**Table A.1.:** Primers used for synthesis of antimicrobial peptide genes

| Name | Sequence 5’-3’ |
|---|---|
| ICE7F-39 | GCGGCGCTCGAGATGGCCAAGGTTTCATCTTCCTTGCTG |
| ICE5F-46 | GTTTCATCTTCCTTGCTGAAATTTGCTATTGTGTTGATTCTGGTAC |
| ICE3F-46 | CTATTGTGTTGATTCTGGTACTGAGCATGTCAGCCATTATATCTGC |
| ICE1F-47 | ATGTCAGCCATTATATCTGCAAAATGCATCAAAAATGGAAAAGGATG |
| ICE2R-45 | AATGGAGGACCTTGATCCTCTCGACATCCTTTTCCATTTTTGATG |
| ICE4R-45 | ACGGTAACAGAAGCCAGAGCAACAGAATGGAGGACCTTGATCCTC |
| ICE6R-45 | AATAGCCACGAGCCCATCCAACCTGACGGTAACAGAAGCCAGAGC |
| ICE8R-43 | GCGGCGAAGCTTAGCGGTTTTTGCAATAGCCACGAGCCCATCC |
| LEA13F-41 | GCGGCGCTCGAGATGGCTGCCTTGATCAAGTTGATGTGCAC |
| LEA11F-45 | TGATCAAGTTGATGTGCACAATGCTGATCGTGGCGGCGGTGGTTG |
| LEA9F-45 | ATCGTGGCGGCGGTGGTTGCTCCGCTGGCTGAGGCGGCGATAGGG |
| LEA7F-45 | TGGCTGAGGCGGCGATAGGGTGCAACACGGTGGCTTCCAAGATGG |
| LEA5F-45 | CACGGTGGCTTCCAAGATGGCCCCATGTCTACCGTACGTCACCGG |
| LEA3F-45 | TGTCTACCGTACGTCACCGGAAAAGGGCCGCTCGGCGGGTGCTGC |
| LEA1F-45 | GGCCGCTCGGCGGGTGCTGCGGTGGCGTAAAGGGTCTCATCGACG |
| LEA2R-45 | TGCCTATCCGGCGTGGTCCGTGCGGCGTCGATGAGACCCTTTACG |
| LEA4R-44 | GTTTTCAGGCAGTTGCAAACCGCCTGCCTATCCGGCGTGGTCCG |
| LEA6R-45 | GATGCCGGAGTACGACTTGGCAAGCGTTTTCAGGCAGTTGCAAAC |
| LEA8R-45 | GGGAGTCCGGCGGCGTTGCCGAGGTTGATGCCGGAGTACGACTTG |
| LEA10R-46 | TAAGGAATGCTGACACCACATTTGCCGGGGAGTCCGGCGGCGTTGC |
| LEA12R-45 | AGCAATCAGTATTAGGGCTGATCTGGTAAGGAATGCTGACACCAC |
| LEA14R-44 | GCGGCGAAGCTTAGTGCACCTTTGAGCAATCAGTATTAGGGCTG |
| SSP9F-32 | GCGGCGCTCGAGATGACTCAATTCAATATCCC |
| SSP7F-35 | ATGACTCAATTCAATATCCCAGTAACCATGTCATC |
| SSP5F-45 | TCCCAGTAACCATGTCATCTAGCTTAAGCATAATTTTGGTCATTC |
| SSP3F-45 | AAGCATAATTTTGGTCATTCTTGTATCTTTGAGAACTGCACTCTC |
| SSP1F-46 | CTTTGAGAACTGCACTCTCATCTTTTGGACTTTGCAGGCTTAGAAG |
| SSP2R-42 | CCTACCTCTAGCACAAAATCCTCTTCTAAGCCTGCAAAGTCC |
| SSP4R-45 | CAATTGGAATAGAAGGGAATCTACACCTACCTCTAGCACAAAATC |
| SSP6R-45 | ATTGAACAAACCTAGAACATCTGCCAATTGGAATAGAAGGGAATC |
| SSP8R-41 | TTACCAAACTCTTCTGCAACATTGAACAAACCTAGAACATC |

| | |
|---|---|
| SSP10R-30 | GCGGCGAAGCTTACCAAACTCTTCTGCAAC |
| FAB9F-32 | GCGGCGCTCGAGATGGAGAGGAAAACACTCAG |
| FAB7F-43 | ATGGAGAGGAAAACACTCAGCTTTACGTTCATGCTCTTCCTTC |
| FAB5F-45 | ACGTTCATGCTCTTCCTTCTCTTAGTAGCTGATGTTTCGGTGAAG |
| FAB3F-45 | AGCTGATGTTTCGGTGAAGACATCAGAGGCGTTATTAGGCCGCTG |
| FAB1F-45 | AGGCGTTATTAGGCCGCTGTAAGGTTAAGAGCAACAGGTTTAATG |
| FAB2R-45 | GTGTGTGTCCGTCAAGCATGGTCCATTAAACCTGTTGCTCTTAAC |
| FAB4R-45 | CTTCTCCCCTGCATACCGTTGAACAGTGTGTGTCCGTCAAGCATG |
| FAB6R-45 | CGTGACAATCACCACCTTTGTAACCTTCTCCCCTGCATACCGTTG |
| FAB8R-45 | GACACATACAGCGGCGACGGAAGCCGTGACAATCACCACCTTTGT |
| FAB10R-38 | GCGGCGAAGCTTAACAAAGACACATACAGCGGCGACGG |
| ESC9F-32 | GCGGCGCTCGAGATGACTCAATTCAATATCCC |
| ESC7F-45 | ATGACTCAATTCAATATCCCAGTAACCATGTCATCTAGCTTAAGC |
| ESC5F-46 | CATGTCATCTAGCTTAAGCATAATTTTGGTCATTCTTGTATCTTTG |
| ESC3F-45 | GGTCATTCTTGTATCTTTGAGAACTGCACTCTCAGGTATTTTCTC |
| ESC1F-45 | GCACTCTCAGGTATTTTCTCTAAATTGGCTGGGAAAAAGATTAAG |
| ESC2F-45 | TTCTTGAGTCCGCTTATGAGCAGGTTCTTAATCTTTTTCCCAGCC |
| ESC4F-45 | ATCAAGGCCAACTTCCTTGCCTACGTTCTTGAGTCCGCTTATGAG |
| ESC6F-45 | CAATGTCTATCCCAGTTCTGACCACATCAAGGCCAACTTCCTTGC |
| ESC8F-45 | CATTCACCTTTAATTTTACAACCGGCAATGTCTATCCCAGTTCTG |
| ESC10F-33 | GCGGCGAAGCTTAACATTCACCTTTAATTTTAC |
| VRD9F-32 | GCGGCGCTCGAGATGGAGAGAAAAACTTTCAG |
| VRD7F-43 | ATGGAGAGAAAAACTTTCAGCTTCTTGTTCTTGCTCCTTCTTG |
| VRD5F-45 | TTGTTCTTGCTCCTTCTTGTCTTAGCCTCTGATGTGGCTGTAGAG |
| VRD3F-44 | CCTCTGATGTGGCTGTAGAGAGAGGGGAGGCTAGAACTTGTATG |
| VRD1F-45 | GGAGGCTAGAACTTGTATGATTAAGAAAGAAGGGTGGGGAAAATG |
| VRD2R-41 | CACAAGTTGTGTCAATTAAGCATTTTCCCCACCCTTCTTTC |
| VRD4R-45 | TAACCACGGTTCTTGCATGAATGTGCACAAGTTGTGTCAATTAAG |
| VRD6R-45 | TCATGCCTTTGCAATTTCCACCTATGTAACCACGGTTCTTGCATG |
| VRD8R-45 | ATTAACGAGGCAATAGCAGGTGCGCGTCATGCCTTTGCAATTTCC |
| VRD10R-36 | GCGGCGAAGCTTAACAATTAACGAGGCAATAGCAGG |
| PNA11F-37 | GCGGCGCTCGAGATGAAATACTGTACTATGTTTATTG |
| PNA9F-43 | ATACTGTACTATGTTTATTGTTCTCTTGGGTTTAGGCAGCTTG |
| PNA7F-44 | TCTTGGGTTTAGGCAGCTTGTTGCTTACACCAACAACAATAATG |
| PNA5F-43 | TTACACCAACAACAATAATGGCACAACAGTGCGGGAGACAAGC |
| PNA3F-45 | AACAGTGCGGGAGACAAGCTAGTGGGAGGCTGTGCGGTAACGGAC |
| PNA1F-45 | GAGGCTGTGCGGTAACGGACTTTGCTGTAGCCAGTGGGGCTACTG |
| PNA2R-45 | CTCCACAGTATGCGGCAGTGGAGCCACAGTAGCCCCACTGGCTAC |
| PNA4R-45 | GATTTGCATTGGCTCTGGCAACCAGCTCCACAGTATGCGGCAGTG |
| PNA6R-45 | TAGTGGTGGTGGAAGAAGCAGCAGTAGATTTGCATTGGCTCTGGC |
| PNA8R-45 | CTTAGCGGTTGATTGGTTTGCAGTGGTAGTGGTGGTGGAAGAAGC |
| PNA10R-45 | AGTTGGCACCGCCGGCAGGATCTGACTTAGCGGTTGATTGGTTTG |
| PNA12R-31 | GCGGCGAAGCTTAGTTGGCACCGCCGGCAGG |