Resolving plant transformation recalcitrance by Agrobacterium-mediated protein translocation

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Chapter 3

The dynamics of *Agrobacterium*-mediated protein translocation to plant cells

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Abstract

Since the first discovery that the soil borne phytopathogen *Agrobacterium tumefaciens* (Agrobacterium) induces tumors on host plants by transferring DNA, a diverse repertoire of protocols using Agrobacterium-mediated DNA transfer for plant transformation has been developed. A routinely performed method is the generation of stable transformants by the floral dip method in the model plant *Arabidopsis thaliana* (Arabidopsis). In contrast, transient transformation allows a more rapid analysis of gene expression, protein localization or protein-protein interaction, often performed by infiltrating leaves of *Nicotiana tabacum* (tobacco) or *Nicotiana benthamiana* with Agrobacterium. Although Agrobacterium transformation is a popular method in a wide range of plant species, some plants among which Arabidopsis remain recalcitrant to stable and transient transformation.

In this chapter, we developed a sensitive 96-well plate reader-based assay to measure fluorophore levels indicative of Agrobacterium virulence induction or Agrobacterium-mediated protein translocation (AMPT) or transformation (AMT). By using this method we could show that the *virE* promoter gives considerably higher expression in Agrobacterium compared to the *virF* or *virD* promoter, and that the increased production of the protein to be translocated leads to higher AMPT efficiencies. Moreover, the plate reader method allowed us to optimize Agrobacterium culture age and optical density and plant medium composition, leading to increased AMT to Arabidopsis suspension cells.

Introduction

The soil bacterium *Agrobacterium tumefaciens* (Agrobacterium) is able to transfer DNA, the so-called transfer- or T-DNA, and virulence proteins to cells of host plants (Vergunst et al., 2005). However, a few crucial steps are required before it can efficiently do so and the first step is the detection of the host plant cell. In its natural environment, the Agrobacterium *virulence* (*vir*) genes are activated by wounded plant cells by chemical signaling (Guo et al., 2017). These inducing signals include a variety of phenolic compounds, sugars, acidity, temperature and low phosphate (Ashby et al., 1988; Baron, Domke, Beinhofer, & Hapfelmeier, 2001; Melchers et al., 1989; Parke et al., 1987). In laboratory settings, the phenolic compound acetosyringone, found to be exuded by wounded tobacco cells, is generally used as the inducer (Stachel et al., 1985) and phenolics are the main signals for induction (Hwang et al., 2017). The inducing signals activate the typical bacterial two component regulatory system VirA/VirG, where the transmembrane receptor VirA (Melchers et al., 1989) phosphorylates the VirG transcription factor leading to binding of VirG to the promoters and activation of *vir* genes.

Since the discovery of T-DNA transfer to plant cells and the development of the binary vector system, a diverse repertoire of transformation protocols has been developed. Protocols are often optimized for a specific experimental set-up, plant species and target tissue. An efficient and routinely performed method to generate stable transformant is the floral dip method, which is generally used to generate stable transformants in the model plant *Arabidopsis thaliana* (Arabidopsis) (Clough & Bent, 1998a). However, the analysis of these stable transformants with promoter-reporter construct or expressing heterologous genes is time-consuming. The Arabidopsis mesophyll protoplast transformation by chemical PEG-calcium transfection of plasmid DNA overcomes this drawback for part of the applications (Yoo et al., 2007). Another approach for the rapid analysis of transient expression and a popular method for *in vivo* characterization is the infiltration of *Nicotiana tabacum* (tobacco) and *Nicotiana benthamiana* leaves with Agrobacterium carrying a construct to be transferred on a T-DNA (Yang et al., 2000). The technique uses a syringe to infiltrate the Agrobacterium suspension via the abaxial side into the spongy mesophyll of a tobacco leaf. The method is adapted for various other plant species (Chincinska, 2021), however tobacco leaf infiltration remains most popular because of its ease and efficiency for transient expression analysis in laboratory and industrial settings (Spiegel et al., 2022). The expression of leaf infiltrated T-DNA constructs was first determined using the β-*glucuronidase* (*GUS*) reporter gene by histochemical staining or measuring GUS activity and protein translocation independently of T-DNA transport was reported using the indirect genetic approach Cre/Lox system (Vergunst et al., 2000). More recently, methods have been developed to directly visualize Agrobacterium-mediated protein translocation (AMPT) in tobacco using the split-GFP system (Khan, 2017). In the previous chapter, the visualization of AMPT by the split-GFP system was further developed and optimized for increased sensitivity and accuracy.

Although Agrobacterium-mediated transformation (AMT) is a popular method for gene transfer to a wide range of plant species, some plants remain recalcitrant to transformation, making (transient) transformation experiments difficult to perform. These are mainly monocotyledonous plant species, although varieties of dicotyledonous species normally considered susceptible to AMT can also be recalcitrant (Benoit Lacroix & Citovsky, 2022). Generally, it is assumed that in a laboratory setting the co-cultivation conditions have to be optimized for each plant species, variety and tissue type. Careful consideration has to be given to the culture conditions favoring both the plant growth and bacterial virulence (De Saeger et al., 2021). Agrobacterium must be successfully primed in a virulent state and the plant tissue must allow regeneration of the transformed cells. The most common medium to induce the Agrobacterium *vir* genes has a low pH, similar to plant media, but is lacking valuable nutrients for plant growth. Another important component of plant and induction media are sugars. A chromosomally encoded periplasmic sugar-binding protein, ChvE, mediates sugar-induced virulence in Agrobacterium synergistically through the VirA/VirG two-component system (Cangelosi et al., 1990). ChvE binds aldose monosaccharides, specifically to D-glucose, and has the ability to increase induction of *vir* genes when glucose is added (He et al., 2009; W. T. Peng et al., 1998). However, sucrose and not glucose is typically is added to plant and induction media, which reduces virulence by binding to SghR resulting in the expression of SghA. This hydrolase frees salicylic acid (SA) from the storage form SA β-glucoside (SAG), which in turn inhibits VirA (Wang et al., 2019b). Since SghA does not have a typical translocation signal, it is assumed that hydrolysis of SAG occurs in the bacterium itself. This mechanism probably allows Agrobacterium to down-regulate its virulence following successful infection, thereby saving energy. However, in a (transient) transformation experiment, this down-regulation of virulence is likely to have unwanted effects on the efficiency.

Although transient AMT is a popular method, in Arabidopsis leaves it does not seem to reach the high levels of transient expression seen in tobacco leaves. Some research has reported modifying the culture conditions has greatly improved the Agrobacterium transformation efficiency (J. F. Li et al., 2009; Wu et al., 2014), while others report no significant increase (Wroblewski et al., 2005).

In this chapter we describe the development of a sensitive 96-well plate reader-based detection method to measure fluorescence in a high-throughput manner. This method was used on the one hand for the detection of *vir* gene induction and to evaluate *vir* promoter strength in Agrobacterium, and on the other hand for the detection of fluorophores transferred to plant cells by AMPT or AMT. We show that the *virE* promoter (*pvirE*) is stronger compared to *pvirD* or *pvirF* and thus the better choice for driving the bacterial expression of proteins that are target for AMPT to plant cells. In addition, the plate reader method allowed to identify optimal medium conditions for Agrobacterium co-cultivation with Arabidopsis cell suspension cultures.

Results

Quantification of AMPT to plant cells using split-GFP fluorescence

In the previous chapter, the split-GFP system for AMPT visualization in plants was optimized for brighter fluorescence. Using this optimized system, the effect of different *vir* promoters, *pvirD*, *pvirE* and *pvirF*, on the protein translocation efficiency was tested in tobacco (Fig. 1A). Per Agrobacterium strain the third, fourth and fifth leaf of four tobacco plants were infiltrated and four days post infiltration (dpi) six GFP positive nuclei were imaged per leaf and the fluorescence was quantified. Although some variation was observed, and higher fluorescence signals were obtained with the *virE* and *virF* promoter constructs, no significant difference was observed for the average fluorescence obtained after AMPT using the different promoter constructs (Fig. 1B). It has been reported that GFP measurements from leaves suffer mostly from within leaf variation more than between plant variation. The position on the leaf and the leaf number selected were the greatest source of variation in GFP intensity measurements (Bashandy et al., 2015; Kim et al., 2021). The current experimental set-up used a defined number of leaves and infiltration positions were consistent overall. Simulations using the same statistical test as applied above (Arnold et al., 2011) indicated that approximately 40 plants need to be infiltrated and that fluorescence of 40 nuclei has to be measured per promoter construct to reach a power of at least 80% (Fig. S1). As this is practically impossible, we decided to develop a different assay to quantify *vir* gene induction and monitor AMPT and AMT.

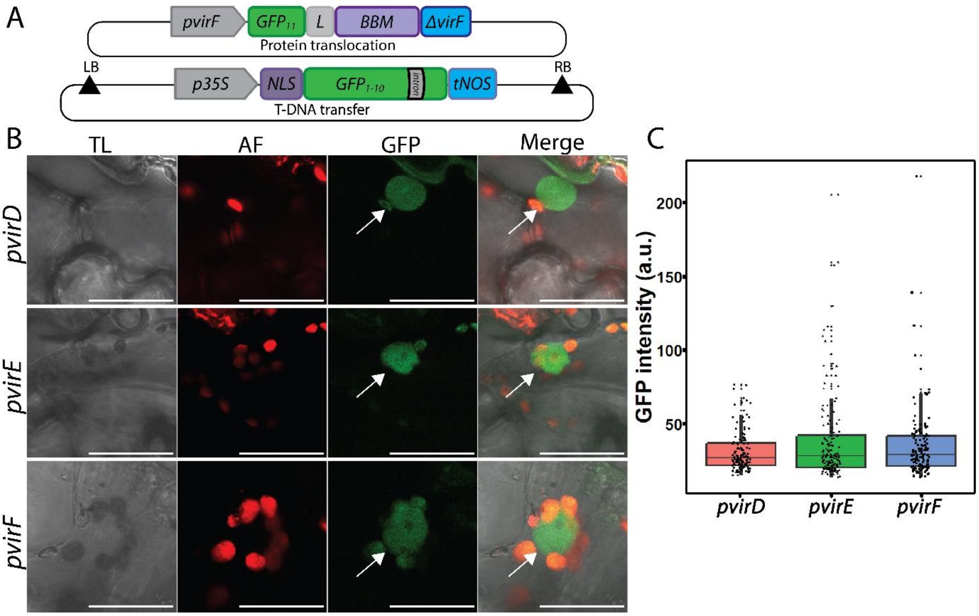

Figure 1. Assessing the effect of different *vir* promoters on the AMPT efficiency. (A) Schematic representation of the split-GFP^{mk} system with a protein translocation vector

coding for the GFP_{11}:BBM:ΔVirF fusion protein expressed from the *pvirF* promoter and the T-DNA transfer vector carrying *p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS* to report AMPT of the GFP_{11}-containing fusion protein. (B) Confocal microscopy images showing GFP fluorescence from the split-GFP system observed 4 dpi in leaf epidermis cells of 4-weeks old tobacco plants. The GFP fluorescence is indicative of AMPT of GFP_{11}:BBM:ΔVirF expressed in Agrobacterium under control of either *pvirD*, *pvirE* or *pvirF* and of the AMT with T-DNA containing *p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS*. Scale bars indicate 50 µm. TL: transmitted light; AF; autofluorescence. (C) Quantification of the intensity of nuclear GFP signal from confocal images as shown in (B). Statistical significance was determined by one-way analysis of variance (ANOVA with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 138).

A sensitive plate reader-based assay to detect and quantify vir *gene induction, AMPT and AMT*

The GFP intensity measurements using a confocal microscope showed larger variation than expected. Previously, it was found that between leaf and within leaf sampling was a major component to cause variation in measurements (Bashandy et al., 2015) and that the leaf number selected for infiltration proved important for optimal expression (Kim et al., 2021). To reduce this variation, the infiltration and sampling in the subsequent experiments followed a standardized protocol. Per tobacco plant the 3th, 4th and 5th leaf were infiltrated at three positions, starting from the base of the leaf closest to the main vein and moving towards the tip of the leaf. Leaf discs were taken from the infiltrated areas of the leaf and extracts of these leaf discs were measured for GFP fluorescence in a plate reader. Previously, a plate reader assay-based system was developed using purified $GFP_{1\text{-}10}$ and GFP_{11} tagged fusion proteins isolated from the transformed host (Cabantous & Waldo, 2006). In our plate reader-based assay, we directly measured reconstituted GFP in the extracts following simultaneous AMPT of a GFP_{11}-fusion protein and AMT of a $GFP_{1\text{-}10}$ expressing gene, and we used expression of the co-transferred Cherry reporter gene of the split-GFP^{col} system described in Chapter 2 as a measure for AMT (Fig. 2A). A variant of this system expressing a full length GFP in Agrobacterium under a *vir* promoter allowed to monitor *vir* gene induction and to compare this to the Cherry-reported AMT efficiency (Fig. 2). Depending on the experimental requirements, constructs for AMT, AMPT or Agrobacterium expression containing a fluorescent marker were inserted in the desired Agrobacterium strain (Fig. 2, Step 1). Agrobacterium cultures were initiated and bacteria were induced with AS (Fig. 2, step 2). The induced bacteria were used to syringe infiltrate the abaxial side of tobacco leaves (Fig. 2, step 3) and samples were taken from the bacterial culture and measured in the plate reader (Fig. 2, Steps 4 and 5a) to detect fluorescence in Agrobacterium from GFP under control of a *vir* promoter and simultaneously measure the optical density (OD) of the Agrobacterium culture. The infiltrated plant material was either visualized using a confocal microscope (Fig. 2, Step 5b) or extracts from leaf discs (Figure 2, Step 4) were measured in a plate reader (Fig. 2, Step 5a). This allowed to measure extracts from infiltrated plant material in a reproducible and high throughput manner. The methods also allowed the addition of more technical replications by a simple pipetting step and because the variation within samples was lower it eliminated the need for many biological repeats, which are difficult to compare between experiments.

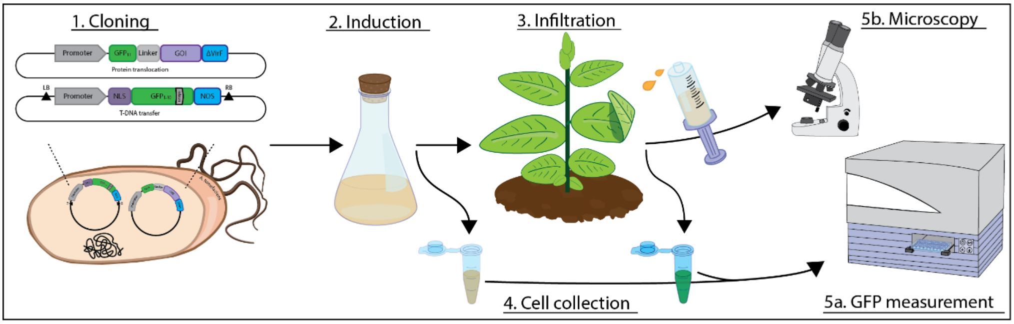

Figure 2. Schematic representation of the workflow for detection of fluorescent proteins in Agrobacterium or plant cells during leaf infiltration experiments. The appropriate constructs for fluorescent protein expression are transformed into Agrobacterium (1). The Agrobacterium cultures are induced either in the presence or absence of factors to be investigated (2). The induced Agrobacterium cultures are infiltrated into the abaxial side of host plant leaves (3). The Agrobacterium cultures and the infiltrated plant material are harvested at the end point or in a timelapse manner (4). The fluorescent proteins in bacteria or plant cells are measured after extraction using a multi-well plate reader (5a) or visualized using a confocal microscope (5b).

Quantification of vir promoter induction in Agrobacterium using the plate reader assay

To analyze whether the expression level of protein fusions designed for AMPT can determine the efficiency of AMPT, the Agrobacterium *virD*, *virE* and *virF* promoters were selected to drive the expression of the *GFP_{11}:BBM:ΔvirF* fusion. Each of these promoters has previously been successfully used to express proteins for AMPT (Khan, 2017; Sakalis et al., 2014a). However, the strength of these VirG responsive promoters has never been determined (Qian et al., 2021).
In a first approach to compare the promoter strength, the three promoters (*pvirD*, *pvirE* and *pvirF*) were cloned upstream of full length GFP that was optimized for bacterial translation (Chapter 2). The highest signal to noise ratio with the plate reader was obtained with the 530 nm (+- 5 nm) emission wavelength bandpass filter (Fig. S2A) and by fluorophore extraction from flash frozen leaf discs with TNG buffer added after (dry) instead of before (wet) homogenization (Fig. S2B). To exclude, when measuring GFP fluorescence in bacteria, that the small volume of the bacterial culture in the 96-wells plate affected the promoter induction, results were compared to those obtained with 50 ml cultures in test tubes sampled after 24 hours. Both methods showed a similar pattern in promoter strength, with *pvirE* giving the highest expression followed by *pvirF* and lowest by *pvirD* (Fig. S2C – D). For the *virE* promoter, the strongest GFP fluorescence was recorded from Agrobacterium cultures at an OD of 0.8 initiated from 1-week-old colonies grown on plates (Fig. S3A). Using 3-week-old colonies to start the culture resulted in significantly lower fluorescence values (Fig. S3B – D). For each Agrobacterium strain containing a promoter-reporter, induction cultures were measured every 5 minutes for a 48 hours period at constant 180 rpm agitation at room temperature in a plate reader (Fig. 3A). Based on the GFP fluorescence, the expression driven by each of the three promoters significantly differed at 16 hours (Fig. 3B), 24 hours (Fig. 3C), 36 hours (Fig. 3D) and 48 hours (Fig 3E). The *virE* promoter resulted in the strongest induction of GFP expression, whereas *pvirD* and *pvirF* were much less active, with *pvirD* resulting in the lowest expression.

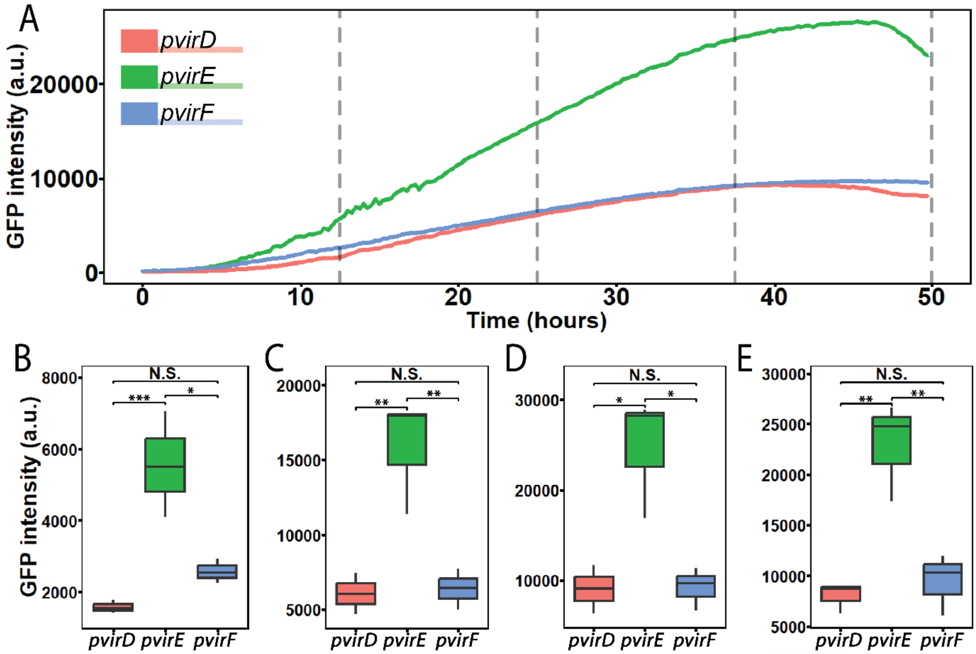

Figure 3. Relative strength of three *vir* promoters based on GFP fluorescence expressed in Agrobacterium following acetosyringone induction. (A) The GFP expression in Agrobacterium measured continuously every 5 minutes in a 96-wells plate reader at room temperature and 180 rpm agitation from start of induction (t = 0) to 48 hours. Vertical dashed lines indicate timepoints of statistical analysis (t = 16, t = 24, t = 36 and t = 48). (B-E) Timepoint measurements of GFP expression in Agrobacterium control of *pvirD*, *pvirE*, *pvirF* at 16 hours (B), 24 hours (C), 36 hours (D) and 48 hours (E). Statistically significant differences are indicated above the boxplots (for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) and not significant (N.S.)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

Combined detection of Agrobacterium vir *gene induction and T-DNA transfer in tobacco leaf cells*

In the previous experiment, the three selected *vir* promoters *pvirD*, *pvirE* and *pvirF* were expressed in Agrobacterium and showed a significant difference in promoter strength. The virulence of Agrobacterium is regulated by an inducible system, which senses external stimuli originating from wounded plant cells. Compounds produced by the host plant interact with bacteria and affect their virulence (Venturi & Fuqua, 2013). For example, Agrobacterium has several mechanisms for *quorum* *sensing*, a type chemical communication between bacteria that ensures a coordinated control of the population and effects the expression of genes involved in pathogenesis. Following tumor induction by a wild-type Agrobacterium strain, the tumor cells release opines. These opines are used by the bacteria as carbon and nitrogen source, but at the same time they activate the transcription of TraR, a transcriptional regulator involved in the synthesis of N-acyl-homoserine lactones (AHLs), known for their function in quorum sensing (Baltenneck et al., 2021; Christie & Gordon, 2015; Lang & Faure, 2014). It is to be expected that the presence of plant cells, in the absence of opines produced by tumor cells, may affect the induction of *vir* genes.

To investigate if the previously observed promoter strength in Agrobacterium would be affected by the presence of plant cells, Agrobacterium expressing full length GFP either under control of the *virD*, *virE* or *virF* promoter was infiltrated in 4-weeks old tobacco leaves. Simultaneously, a T-DNA was transferred to the host plant carrying a *35S* promoter-controlled plant optimized Cherry reporter gene to visualize transformation. Confocal imaging of the leaves at 4 dpi showed clear GFP fluorescence from *vir* promoter driven GFP expression in Agrobacterium in the plant apoplastic space and both nuclear and cytosolic Cherry fluorescence in plant cells from the T-DNA expressed Cherry reporter (Fig. 4A). Extracts from infiltrated leaves were measured in the plate reader. Similar to the *in vitro* measurements of the promoter strength, the GFP fluorescence intensity was highest under control of *pvirE* and lowest under *pvirD* (Fig. 4B).

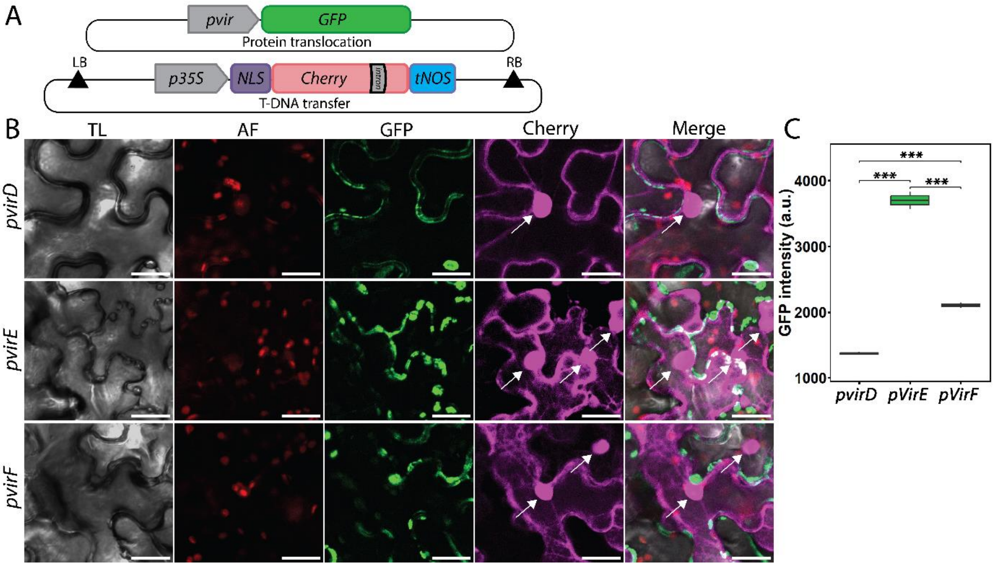

Figure 4. The combined detection of Agrobacterium *vir* gene induction and T-DNA transfer in tobacco leaf cells. (A) Schematic representation of the *vir* promoter-controlled GFP expression in Agrobacterium with a vector coding for GFP expressed from the *virD, virE or virF* promoter and the T-DNA transfer vector carrying *p35S::NLS:Cherry::tNOS* to report AMT. (B) Confocal microscopy images showing GFP fluorescence in Agrobacterium cells expressing GFP under control of three different *vir* promoters (*pvirD*, *pvirE* or *pvirF*) and Cherry fluorescence in tobacco cells after AMT of *p35::NLS:Cherry::tNOS* at 4 dpi of leaves of 4 weeks old tobacco plants. Scale bars indicate 50 µm and arrows indicate Cherry positive plant cell nuclei. TL: transmitted light; AF; autofluorescence. (C) GFP fluorescence measured using a plate reader in extracts of tobacco leaves at 4 dpi with Agrobacterium expressing GFP under control of *pvirD*, *pvirE* or *pvirF.* Statistically significant differences are indicated above the plots ($p < 0.001$ (***)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

The *vir* promoter-controlled GFP expression in Agrobacterium together with the transient expression of Cherry from the T-DNA enables to compare *vir* gene induction with the resulting transformation efficiency while Agrobacterium is in contact with the plant host cells. The induction time in commonly used Agrobacterium co-cultivation protocols ranges from 12 to 24 hours (Gelvin, 2006; Wu et al., 2014). However, many protocols limit the induction time to less than 8 hours or omit the induction phase completely (Clough & Bent, 1998b; J. F. Li et al., 2009). Previously we showed that *vir* gene-controlled GFP fluorescence increased in prolonged induction cultures up to 48 hours (Fig. 3E). This suggests that for many protocols prolonged induction before cocultivation may enhance Agrobacterium virulence and the resulting efficiency of AMT or AMPT.

To investigate this, based on the Cherry fluorescence we monitored the effect of induction time of Agrobacterium cultures grown at the previously established OD of 0.8 for 0, 1 or 2 days on the AMT efficiency. The leaves of 4-weeks old tobacco plants were infiltrated by Agrobacterium expressing GFP under the control of the *virE* promoter (*pvirE::GFP*) and carrying a T-DNA construct with the cherry reporter (*p35S::NLS:Cherry::tNOS*). The fluorescence measured in 4 dpi leaf extracts of 4-weeks old tobacco was strongest after 2 days of induction for both the GFP expressed in Agrobacterium (Fig. 5A) as for the Cherry expressed in plant cells (Fig. 5B). The longer induction time of Agrobacterium had a positive effect on virulence induction and transient Cherry expression from T-DNA.

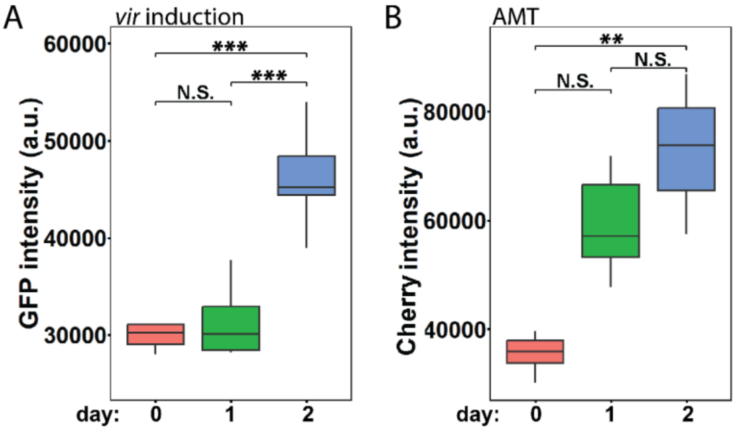

Figure 5. Agrobacterium *vir* gene expression and AMT efficiency increases by prolonged pre-induction with AS. (A, B) Agrobacterium expressing GFP under control of the *virE* promoter (*pvirE::GFP*) and carrying a T-DNA with the *p35S::NLS:Cherry::tNOS* reporter was cultured for 0, 1 or 2 days in induction medium with AS. Bacterial cultures we subsequently used to infiltrate leaves of 4 weeks old tobacco plants. At 4 dpi the GFP (A, *vir* induction) or Cherry (B, AMT) fluorescence was measured in extracts from leaf discs of the infiltrated part in a 96-wells plate reader. Statistically significant differences are indicated above the plots ($p < 0.01$ (**), $p < 0.001$ (***), not significant (N.S.)) as determined by one-way analysis of

variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

Quantification of GFP-reported AMPT using Cherry-reported AMT as reference

The three *vir* promoters *pvirD*, *pvirE* and *pvirF* showing significant difference in promoter strength in Agrobacterium were subsequently used to test if higher protein production in Agrobacterium would lead to higher AMPT. For this the previously described split-GFP^{col} system was used. Leaves of 4-weeks old tobacco plants were infiltrated with Agrobacterium expressing plant optimized *GFP*$_{11}$*:BBM:ΔvirF* under control of *pvirD*, *pvirE* or *pvirF*. Simultaneously, a T-DNA was transferred to the host plant encoding *GFP*$_{1\text{-}10}$ and plant optimized Cherry, both under control of a *35S* promoter (*p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS* and *p35S::NLS:Cherry::tNOS*, respectively), to quantify transient expression *in planta.* The leaves were imaged at 4 dpi and showed clear GFP fluorescence from split-GFP in the plant nucleus and co-localization of the GFP signal with the T-DNA expressed Cherry signal (Fig. 6A). Quantification of the GFP signal relative to the Cherry signal in leaf extracts showed that AMPT of the GFP$_{11}$:BBM:ΔVirF fusion was most efficient when expressed from the stronger *virE* promoter and lowest when expressed from the weaker *virD* or *virF* promoters (Fig. 6B). These results indicate that expression of the target protein for AMPT can be rate limiting, and that the use of a strong promoter is important for efficient AMPT. To extend the capabilities of the split-GFP^{col} system we investigated if it could be extended to protoplasts, which are often used for flowcytometry experiments. Leaves of 4-weeks old tobacco *plants* were first enzymatically digested at 4 dpi to remove the cell walls (Fig. S4A) and GFP fluorescence was measured in protoplast extracts after AMT and AMPT (Fig. S4B). The GFP fluorescence from AMPT using the split-GFP^{opt} system showed a lower signal to noise ratio in protoplasts (1.17) compared to leaf extracts (1.94). However, the average GFP intensity was 3.7 times stronger in leaf extracts. Although the split-GFP^{opt} system in combination with the plate reader was successfully used to detected GFP signal from AMPT, the generation of protoplasts is time-consuming, adds complexity to the experiment and the GFP intensity is lower.

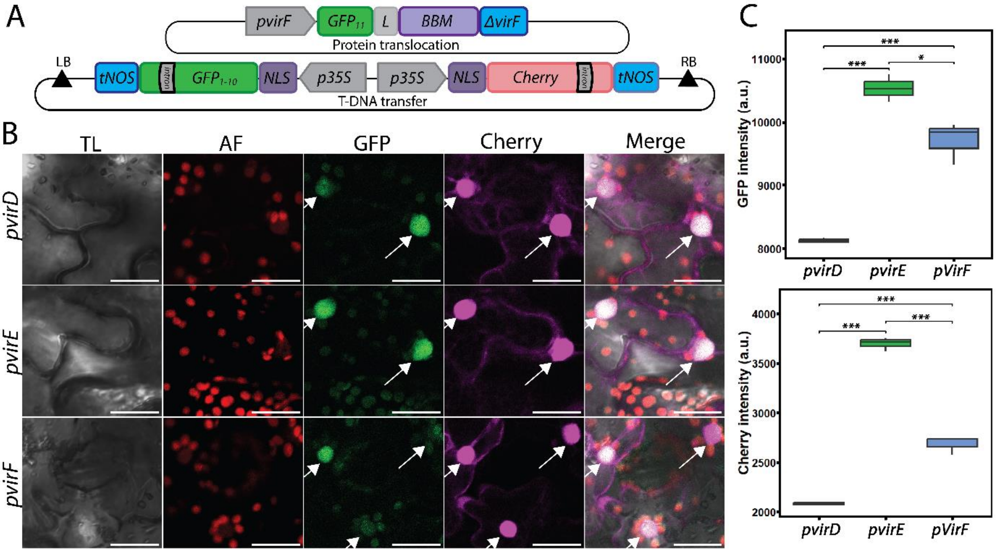

Figure 6. Visualization and quantification of GFP-reported AMPT and Cherry-reported AMT in tobacco leaf cells. (A) Schematic representation of the combined AMPT/AMT detection system split-GFP^{col}. The system comprises a T-DNA transfer vector containing the optimized *NLS:GFP$_{1-10}$* and *NLS:Cherry* coding regions, both expressed from the *35S* promoter. The protein transfer vector encodes a GFP$_{11}$:BBM:ΔVirF fusion protein expressed from the *virF* promoter. (B) Confocal microscopy images showing GFP and Cherry fluorescence 4 dpi in 4-weeks old tobacco leaf epidermis cells transformed by Agrobacterium utilizing the ds-FP system to transfer *p35S::NLS:Cherry::tNOS* on T-DNA and a fusion protein GFP$_{11}$:BBM:ΔVirF under either control of *pvirD*, *pvirE* or *pvirF*. Scale bars indicate 50 µm and arrows indicate plant cell nuclei. TL: transmitted light; AF; autofluorescence. (C) Quantification of GFP and Cherry fluorescence measured using a plate reader in extracts of tobacco leaves at 4 dpi as shown in (B). Statistically significant differences are indicated above the plots ($p < 0.05$ (*), $p = 0.001$ (***) and not significant (N.S.)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

In the previous experiments a timeseries was performed on the efficiency of Agrobacterium *vir* gene induction and AMT. Here we investigate the effect of Agrobacterium induction time on both the AMT and AMPT efficiency by measuring fluorescence of Cherry and GFP 4 dpi from infiltrated 4-weeks old tobacco leaves using the split-GFP^{col} system. The AMPT efficiency, as measured by the GFP fluorescence, was significantly higher after 2-days induction compared to 1-day induction (Fig. 7A). The same observation was made for the Cherry fluorescence measured from the same leaf disc extracts (Fig. 7B). In conclusion, increasing the induction time of Agrobacterium has a positive effect on both the AMPT and AMT efficiency.

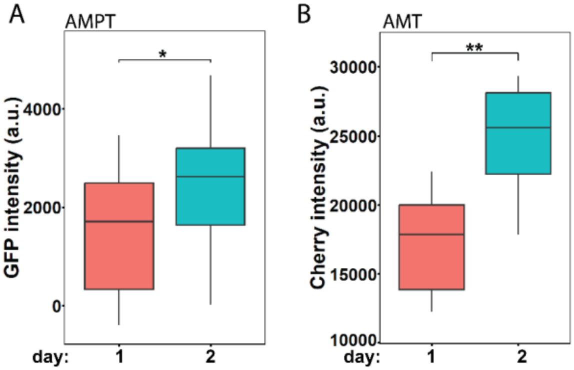

Figure 7. Improved AMT and AMPT efficiency after prolonged Agrobacterium *vir* gene induction. Quantification of GFP and Cherry fluorescence in extracts of leaves from 4 weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying the ds-FP system (*pvirE::GFP_{11}:BBM:ΔvirF* + *p35S::NLS:GFP_{1-10}::tNOS/p35S::NLS:Cherry::tNOS*) after 1 or 2-days *vir* gene induction. Statistically significant differences are indicated above the plots ($p < 0.05$ (*), $p < 0.01$ (**)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

Optimization of co-cultivation conditions to enhance Agrobacterium virulence

Previously, various experiments have been performed to investigate the optimal induction conditions for Agrobacterium virulence, among which varying the pH, temperature and sugar composition. (Melchers et al., 1989). Here we used our high-throughput plate reader assay to pinpoint elements in the composition of the cocultivation medium critical for AMPT and AMT. Arabidopsis suspension cells were used as target, as they would be handy cell system for transient expression, provided that their relative recalcitrance to AMT could be overcome. First, we investigated the effect of the individual medium components on Agrobacterium virulence induction by measuring *pvirE::GFP* expression in Agrobacterium for 48 hours. To test this Agrobacterium cells were resuspended to an OD_{600} of 0.8 in 100 µl induction medium and 50 µl plant medium was added. As Arabidopsis cell suspension cultures are grown in Gamborg B5 medium (Gamborg et al., 1968), we made variants this medium where various components were omitted or substituted one at a time and compared these against standard B5 medium (Fig. 8, horizontal dotted line). The pH for all B5 variants was corrected to 5.7, as this was optimal for Agrobacterium virulence (Melchers et al., 1989; Ohyama et al., 1979). The substitution of 3% sucrose by 3% glucose showed the only significant increase of virulence in Agrobacterium (Fig. 8). The effect was reduced in medium containing 1.5% sucrose and 1.5% glucose, confirming that the glucose concentration is important. These results are in line with previous publications (Boyko et al., 2009; Wise & Binns, 2016). To investigate the effect of glucose on Agrobacterium virulence induction in more detail, a timelapse measurement was performed (Fig. S5A). The virulence induction of Agrobacterium did not show significant difference in the first 14 hours between B5 glucose and normal B5 medium, but was significantly stronger after 24 hours in B5 glucose medium, whereas GFP fluorescence decreased in B5 medium (Fig. S5B). The omission of sucrose and thereby a complete absence of sugars dramatically reduced Agrobacterium virulence, indicating the basal necessity of sugar in the medium and confirming that sucrose per se does not inhibit the virulence induction process. The omission of ammonium nitrate (NH_4NO_3) or spores (H_3BO_3, $MnSO_4$, $ZnSO_4$, KI, Na_2MoO_4, $CuSO_4$, $CoCl_2$) did not significantly affect virulence induction. This is in contrast to previous observations where increased ammonium nitrate enhanced the Agrobacterium transformation efficiency in tobacco using MS-0 medium (Boyko et al., 2009; Maheshwari & Kovalchuk, 2013).

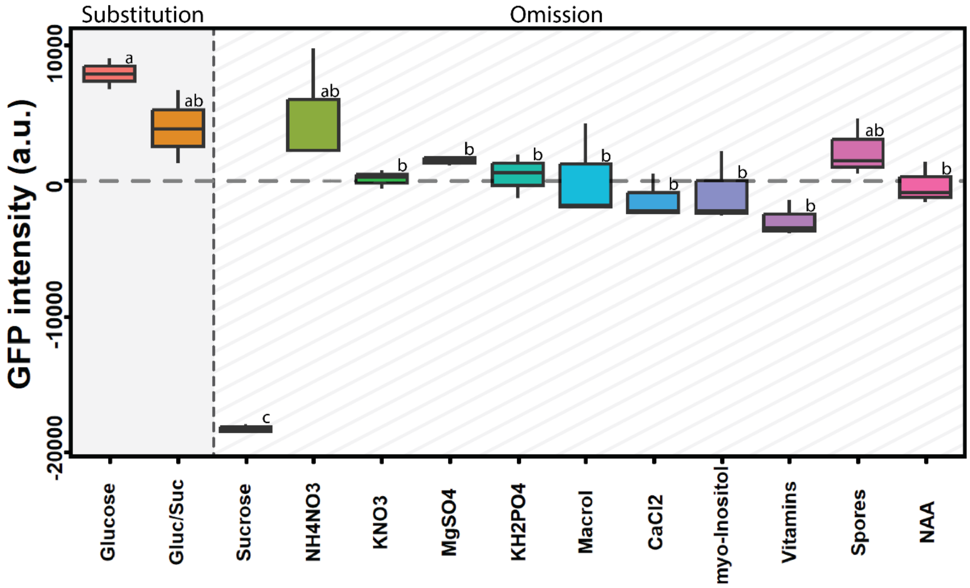

Figure 8. Co-cultivation medium optimization leads to higher Agrobacterium *vir* gene induction. The relative GFP fluorescence intensity in Agrobacterium expressing GFP under the *virE* promoter (*pvirE::GFP*) cultured in standard and different variants of Gamborg B5 medium following 2 days in *vir* inducing medium. Modified Gamborg B5 media are compared against standard Gamborg B5 medium (horizontal dotted line put at 0) and letters indicate statistically significant differences ($p < 0.05$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted ($n = 3$). Abbreviations for Gamborg B5 medium: MacroI (NH_4NO_3, KNO_3, $MgSO_4*7H_2O$, KH_2PO_4), Vitamins (Thiamine*HCL, Pyridoxine HCL, Nicotinic acid) Spores (H_3BO_3, $MnSO_4*H2O$, $ZnSO_4*7H_2O$, KI, $Na_2MoO_4*2H_2O$, $CuSO_4*5H_2O$, $CoCl_2*6H_2O$).

Enhanced AMT to Arabidopsis suspension cells using optimized culture conditions

Arabidopsis is a well-studied model plant with an extensively annotated genome. However, transient transformation experiments have been hampered and Arabidopsis is generally accepted to be a recalcitrant plant species for transient expression by Agrobacterium infiltration, either by syringe or submersion under vacuum (Wu et al., 2014). Various protocols and optimization steps have been proposed to increase the transient AMT efficiency in Arabidopsis (Kim et al., 2009). Here we investigated the effect of our culture medium optimizations on

Agrobacterium transformation of Arabidopsis cell suspension cultures. In addition, we tested co-cultivation of Agrobacterium with plant cells in the dark, as it has been shown that light-grown Agrobacterium showed reduced motility, reduced attachment in tomato roots and smaller tumors in infected cucumber plants (Oberpichler et al., 2008).

The Arabidopsis cell suspension cultures were co-cultivated in normal or modified (NH_4NO_3 omitted or glucose instead of sucrose) B5 medium with Agrobacterium and washed after two days to remove the excess of bacteria to prevent overgrowth and imaged with a confocal microscope (Fig. 9A). The GFP and Cherry fluorescence was measured 4 dpi in the co-cultivation cultures. Based on the Cherry fluorescence measurements, the transient AMT efficiency was significantly higher when B5 medium with glucose was used (B5 glucose). The dark treatment or omission of ammonium nitrate (B5-NH_4NO_3) lead to slightly reduced or increased efficiencies, respectively, but results were not statistically significant (Fig. 9B). The AMPT efficiency was significantly higher with B5-glucose medium, similar to AMT. However, the efficiency was reduced with B5-NH_4NO_3 medium compared to B5 medium with or without dark treatment. Based on the images, the attachment of Agrobacterium to the plant cells increased in the dark, as previously reported, but not in other treatments (NH_4NO_3 and glucose). However, the increased attachment did not lead to a higher transient AMPT or AMT efficiency (Fig. 9B), indicating that in the Arabidopsis cell suspension system attachment is not rate limiting.

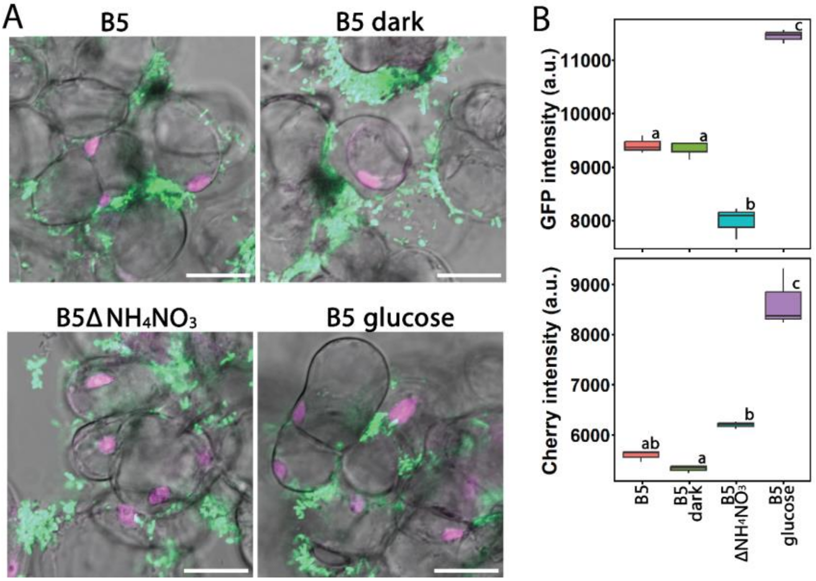

Figure 9. Increased AMT efficiency in Arabidopsis suspension cells by medium optimization. (A) Confocal microscopy images of Arabidopsis suspension cells 4 days after cocultivation with an Agrobacterium strain expressing GFP from the *virE* promoter and carrying an *p35S::NLS:Cherry::tNOS* T-DNA construct. Cocultivation was performed in B5 medium in light (B5) or dark (B5 dark), in B5 medium with glucose instead of sucrose (B5-glucose), or in B5 medium without NH_4NO_3 (B5-NH_4NO_3). Scale bars indicate 50 µm. (B) Quantification of the intensity of GFP and Cherry fluorescence in extracts of Arabidopsis suspension cells shown in (A) in a 96-wells plate reader. Letters indicate the statistically significant different classes (for GFP $p < 0.001$ and for Cherry $p < 0.05$), as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted ($n = 3$).

Discussion

In this chapter the split-GFP^{col} system was used to establish a multi-well plate reader assay for rapid screening of AMPT and AMT efficiencies in wild-type plants. The system allowed to quantify GFP and Cherry fluorescence in both extracts of infiltrated tobacco leaves and cocultivated Arabidopsis cell suspensions. The Agrobacterium syringe infiltration into tobacco leaves proves a robust system for rapid transient expression experiments. However, microscopy measurements of fluorescence are laborious and the variation in the results of within and between experiments can be considerable. The described plate reader method uses a simple extraction of the fluorophore from the infiltrated plant tissue or a direct measurement in cell suspension cultures, enabling high-throughput scalability of plant numbers. To reduce the variation in the system, the harvesting of leaf discs of infiltrated leaves was standardized, as it has been shown that in GFP and GUS quantification experiments the GFP fluorescence intensity was dependent on the position on the leaf, the leaf number and the days post infiltration (dpi) (Bashandy et al., 2015; Kim et al., 2021; Sheludko et al., 2007; Wroblewski et al., 2005). The source of variation was reported to be the highest within leaf samples (53 %), the variation in leaf number, also called leaf position, was reported 17 % and the variation between plants was 19 % (Bashandy et al., 2015).

Alternatives for high throughput Agrobacterium transient expression analysis have made use of *in vitro* complementation of split-GFP components or a fluorescence-activated cell sorter (FACS) (DeBlasio et al., 2010; Kaddoum et al., 2010). FACS enables single cell measurements but, although high efficiency numbers have been reported, this has the drawback that it relies on generating protoplasts (Pasternak et al., 2021; Yoo et al., 2007). Protoplasts require careful handling and the method is very dependent on the generation of reproducible protoplasts. To reproduce *in planta* conditions with the least effect on expression, the protoplasts have to be harvested from the correct tissue (Faraco et al., 2011). Determining the quantity of translocated proteins into plant cells by Agrobacterium has been challenging, because of the attachment of the bacterium to the plant cell and is therefore present in protein isolates from plant tissue. (Hwang & Gelvin, 2004). The split-GFP used in the ds-FP system reassociates only *in planta* and makes complete removal of Agrobacterium unnecessary for AMPT efficiency determination.

In this chapter the promoter strength in Agrobacterium expression and AMPT efficiency was shown for the *virD*, *virE* and *virF* promoters. The difference in Agrobacterium promoter strength can be used for tuneable expression and subsequent translocation to plant cells. Previously the detection *in planta* of the relative low level fluorescent signal using a confocal laser scanning microscope was hampered by autofluorescence of endogenous cellular or media components in plant tissue. The autofluorescence spectrum of the plant cell components is overlapping with the emission wavelength of GFP and Cherry (Billinton & Knight, 2001). The low detection sensitivity was restricting the detection sensitivity and lead to low signal-to-noise ratios, hampering visualization of weaker signal. The optimized protocol described in this chapter increases the sensitivity for fluorescence signal detection.

The improvement of Agrobacterium *vir* gene induction has been investigated extensively (Costa et al., 2021). For higher transformation efficiency, research has focused on modifying the binary plasmid system (Anand et al., 2018; De Saeger et al., 2021), alternate inducible promoter systems or optimized strain selection (Brewster et al., 2012). Further optimization of Agrobacterium could be achieved by engineering the chromosomal background (M. G. Thompson et al., 2020). Here it is shown that the medium composition can be rapidly optimized using the plate reader assay leading to increased expression in Agrobacterium and AMPT efficiency. The replacement of glucose in plant media for co-cultivation with Agrobacterium led to significantly higher AMPT efficiencies. It has been described that Agrobacterium has two modes to attach to the plant cell: lateral and polar attachment. The medium composition during co-cultivation can affect which attachment mode is preferred and polar attachment increases the number of bacteria able to bind the plant cell (Matthysse, 2014).

In summary, the high-throughput method developed here for GFP and Cherry fluorescence intensity measurements in Agrobacterium or *in planta* allows for both visualization and quantification of the fluorescent signal in various plant systems e.g., leaves, cell suspension or protoplast. The plant cell suspension system provides a continuous supply of close to identical cells in each experiment and coupled with the described method in this chapter allows for high-throughput analysis of AMPT. The method was used to optimize expression in Agrobacterium of recombinant proteins and for subsequent AMPT. Furthermore, the method allows rapid optimization of co-cultivation conditions for diverse experimental set-ups.

Supplemental figures

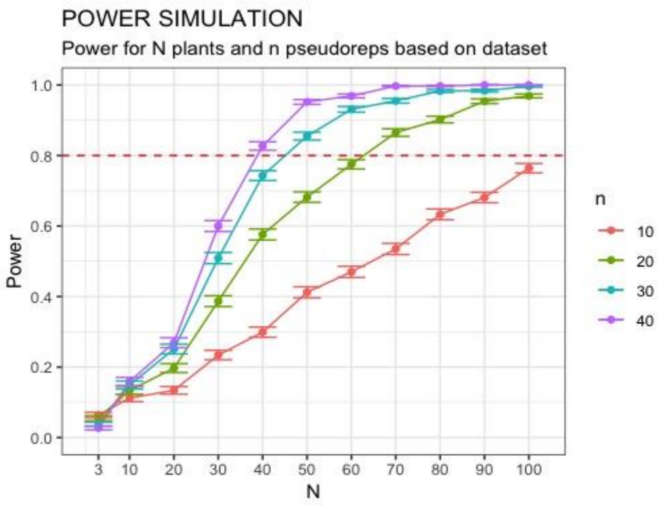

Figure S1. A large number of replicate plants are needed for GFP intensity measurements using confocal images. A power simulation based on a mixed model of collected data from GFP intensity measurements on confocal images of leaves of 4-weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying a T-DNA with *p35S::GFP*$_{1\text{-}10}$*::tNOS* and a vector expressing the GFP$_{11}$:BBM:ΔVirF fusion protein from either the *virD*, *virE* or *virF* promoter. The model rendered 1000 simulated datasets for the number of replications needed per promoter and the number of pseudo-replications needed per plant. N = number of replications (i.e. the number of plants), n = number of pseudo-replications (i.e. the number of nuclei observed) per plant. Error bars = 95% confidence interval.

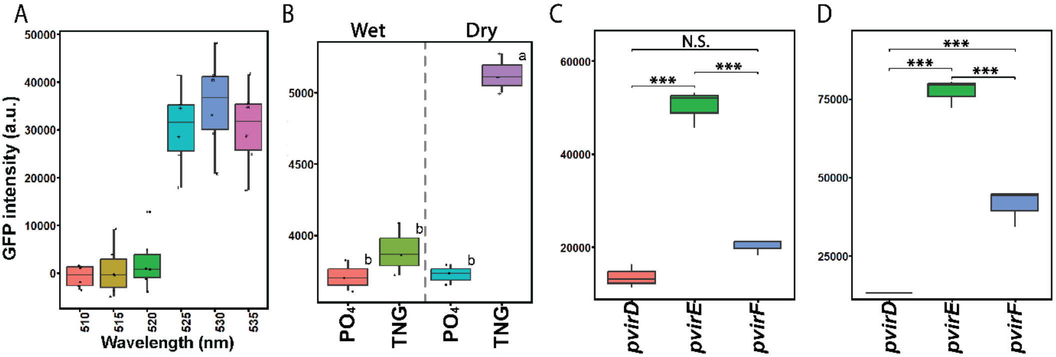

Figure S2. Optimization of the plate reader assay for measuring GFP fluorescence intensity in Agrobacterium or plant extracts. (A) GFP fluorescence measured at 6 different excitation wavelengths (nm) using a 96-wells plate reader in cultures of Agrobacterium expressing GFP under control of the *virE* promoter. (B) GFP fluorescence measured using a

96-wells plate reader in extracts of tobacco leaves at 4 dpi with an Agrobacterium strain carrying an *p35S::NLS:Cherry::tNOS* T-DNA construct. The GFP was extracted from leaves with extraction buffer (PO_4 or TNG) added during homogenization (wet) or after homogenization (dry). (ANOVA). (C) The GFP fluorescence measured in a 96-wells plate reader from Agrobacterium cultures expressing GFP under the *virD*, *virE* or *virF* promoter pre-induced in 50 ml Falcon tubes (C) or in 96-wells plates (D). (B – D) Statistically significant differences are indicated above the plots ($p < 0.001$ (***) and not significant (N.S.)) or as letters ($p < 0.05$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

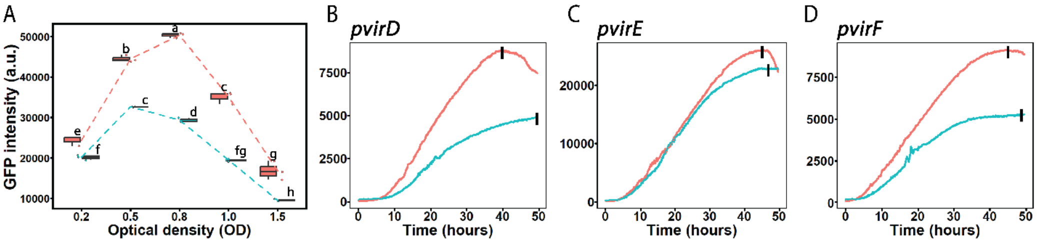

Figure S3. Increased relative GFP fluorescence intensity from Agrobacterium cultures from 1-week-old plates compared to 3-week-old. (A) Relative GFP fluorescence of induced Agrobacterium cultures at an OD_{600} of 0.2; 0.5; 0.8; 1.0 or 1.5 initiated from a 1-week-old colony (red) or a 3-week-old colony (blue) expressing GFP without an intron under control of a *virE* promoter. Letters indicate the statistically significant different classes ($p < 0.01$) as was determined by one-way analysis of covariance (ANCOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3). (B-D) The relative GFP fluorescence of induced Agrobacterium cultures at OD_{600} of 0.8 initiated from a 1-week-old colony (red) or a 3-week-old colony (blue) expressing GFP under control of either *pvirD* (B) , *pvirE* (C) or *pvirF* (D). The peak of GFP fluorescence measurements did not significantly differ between the two cultures, as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

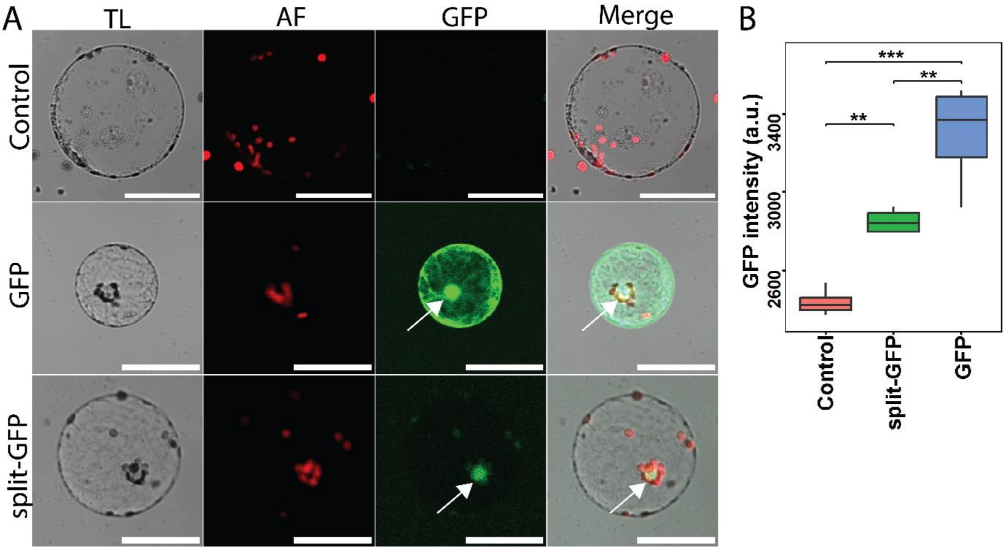

Figure S4. Detection of AMPT to tobacco protoplasts using the optimized split-GFP system. (A) Confocal microscopy images showing GFP fluorescence observed 4 dpi in tobacco protoplasts co-cultivated with Agrobacterium carrying T-DNA construct *p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS* (control) or *p35S::NLS:GFP::tNOS* (GFP), or the split-GFP system (split-GFP; *p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS* + *pvirF::GFP*$_{11}$*:BBM:ΔvirF*). Scale bars indicate 50 µm. TL: transmitted light; AF: autofluorescence. (B) Quantification of the intensity of GFP fluorescence in a 96-wells plate reader in tobacco protoplasts 4 dpi as shown in (A). Statistically significant differences are indicated above the plots ($p < 0.01$ (**) and $p < 0.001$ (***)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted (n = 3).

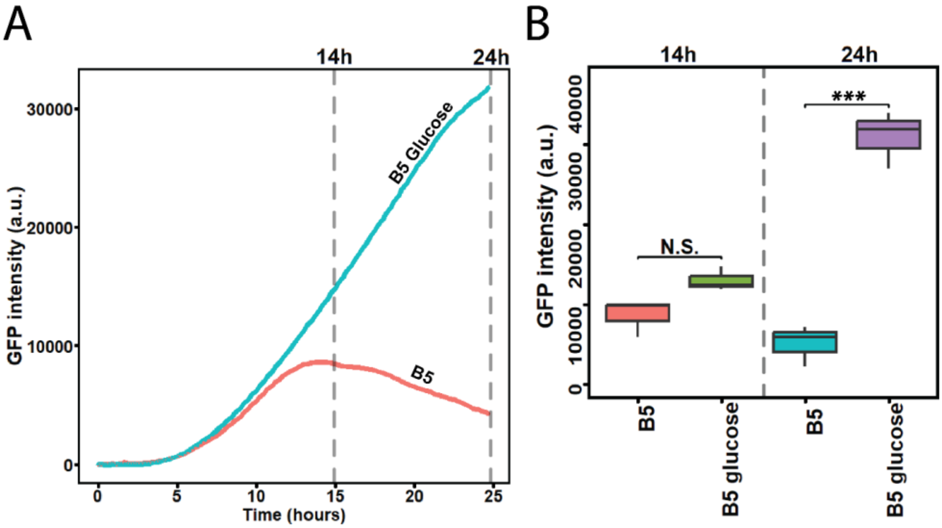

Figure S5. Comparison of Gamborg B5 media against modified B5 medium with sucrose substituted for glucose. (A) The control treatment (B5) and B5 with sucrose substituted by glucose (B5 glucose) were added to Agrobacterium expressing GFP under control of the *virE* promoter and fluorescence was measured in a 96-wells plate reader for 24 hours. (B) Quantification of the intensity of GFP fluorescence of the 14 hour and 24 hour timepoints in (A). Statistical significance is indicated above the plots plots ($p < 0.001$ (***), not significant (N.S.)) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 and individual values are plotted ($n = 3$).

Materials and Methods

Agrobacterium strains and growth conditions

Agrobacterium strain AGL1 (C58, *RecA*, Rif^r, pTiBo542 disarmed, Cb^r) (Jin et al., 1987) was used in all experiments and was grown in LC medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.5) at 28 °C. Plasmid combinations listed in Table 1 were introduced into AGL1 as previously described (den Dulk-Ras & Hooykaas, 1995) and transformed bacteria were selected with the appropriate antibiotics at the following concentrations: 40 µg/ml gentamicin; 100 µg/ml kanamycin; 75 µg/ml carbenicillin; 20 µg/ml rifampicin.

Plasmid construction

The plasmids described in this chapter are listed in Table 1. All cloning steps were performed in *E. coli* strain DH5α (CGSC#: 14231) (Laboratories, 1986). PCR amplifications were done with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Landsmeer, the Netherlands) and resulting plasmids were verified by sequencing. Primers used to construct the plasmids are listed in Table 2. Sequences were codon optimized using the web base tool OPTIMIZER (Puigbò et al., 2007).

The protein translocation vector *pvirF::*GFP_{11}^{opt}*:*BBM^{opt}*:ΔvirF* constructed in Chapter 2 was used here to replace the *virF* promoter with *pvirD* or *pvirE*. The plasmid was digested with *Sal*I and *Nde*I and ligated either with a compatible synthetic DNA fragment (Bio Basic inc., Canada) containing *pvirD* or with a compatible PCR fragment containing *pvirE*. For the Agrobacterium expression of GFP under control of the *virD, virE* or *virF* promoter, the GFP_{11}^{opt}*:*BBM^{opt}*:ΔvirF* open reading frame in the above vectors was removed by digesting with *Nde*I and *Bam*HI and replaced by a compatible synthetic DNA fragment coding for bacterial codon optimized GFP^{opt}.

Table 1. Plasmids and their function used in this study. In the main text sfCherry2 is referred to as Cherry and the optimized superscript (^{opt}) is omitted.

Plasmid content	**Function**	**Source**
p35S::NLS^{opt}:GFP_{1-10}^{opt}::tNOS / pNOS::Hyg	T-DNA transfer (Kmr)	Chapter 2
p35S::NLS^{opt}:$sfCherry2^{opt}$::tNOS / pNOS::Hyg	T-DNA transfer (Kmr)	Chapter 2
p35S::NLS^{opt}:GFP_{1-10}^{opt}::tNOS / p35S::NLS^{opt}:$sfCherry2^{opt}$::tNOS / pNOS::Hyg	T-DNA transfer (Kmr)	Chapter 2
pvirD::GFP_{11}^{opt}:BBM^{opt}:ΔvirF	Protein translocation (Gmr)	Chapter 3
pvirE::GFP_{11}^{opt}:BBM^{opt}:ΔvirF	Protein translocation (Gmr)	Chapter 3
pvirF::GFP_{11}^{opt}:BBM^{opt}:ΔvirF	Protein translocation (Gmr)	Chapter 3
pvirD::GFP^{opt}	Agrobacterium expression (Gmr)	Chapter 3
pvirE::GFP^{opt}	Agrobacterium expression (Gmr)	Chapter 3
pvirF::GFP^{opt}	Agrobacterium expression (Gmr)	Chapter 3

Table 2. Overview of primers used in this study.

Primer name	**Sequence**
*Sal*I pvirE Fw	GTCGACCGGCTGCTCGTCACCAACAA
NdeI pvirE Rev	CATATGTTCTCTCCTGCAAAATTGCGGTTT
pSDM6503 Seq Fw	GTGATCATTTGCAGTATTCG
pSDM6503 Seq Rev	CAAGGCGATTAAGTTGGGTAA

Agrobacterium induction

For leaf infiltration or co-cultivation of suspension cells, a colony of Agrobacterium strain AGL1 containing the appropriate plasmids (Table 1) from a one-week old plate was resuspended in 10 ml LC medium supplemented with the appropriate antibiotics in a 100 ml Erlenmeyer flask and was incubated at 28 °C under 180 rpm shaking until the culture reached an OD_{600} of 1.0. The bacteria were pelleted by centrifugation in a 50 ml Falcon tube at 4000 rpm for 20 minutes and resuspended in a 20 ml AB minimal medium (Gelvin, 2006) with the appropriate antibiotics and grown overnight at 28 °C under 180 rpm shaking until an OD_{600} of 0.8. The bacteria were pelleted as described above and resuspended in 20 ml induction medium (Gelvin, 2006) containing 200 µM acetosyringone (CAS# 2478-38-8, Sigma-Aldrich, Saint Louis, USA) and cultures were incubated on a rocking shaker at 60 rpm at room temperature.

Plant growth conditions

Nicotiana tabacum cv. Petit Havana SR1 (tobacco) seeds were stratified for seven days on wet soil and germinated in high humidity under a plastic cover at 24 °C and 16 hours photoperiod. Seedlings were grown in growth chambers at 24 °C, 75 % relative humidity and 16-hours photoperiod for four weeks.
The *Arabidopsis thaliana* (Arabidopsis) T87 cell suspension was derived from seedlings of *Arabidopsis thaliana (L.) Heynh. accession Columbia* (Axelos et al., 1992). The cell suspension was maintained as previously described (Ostergaard et al., 1996) under continuous light at 22°C with rotary shaking at 120 rpm and sub

cultured at seven-day intervals in cell culture medium consisting of B5 medium (Gamborg et al., 1968) with 30 g/L sucrose and 1 µM NAA.

Leaf infiltration

Prior to tobacco leaf infiltration, the induced Agrobacterium cultures were pelleted as described above and resuspended in half-strength MS medium (Murashige & Skoog, 1962) to an OD_{600} of 0.8. For the detection of AMPT or AMT, the third, fourth and fifth leaves of four weeks old plants were infiltrated on the abaxial side at three positions, starting from the base of the leaf closest to the main vein and moving towards the tip of the leaf using a blunt tipped 5 ml syringe with the induced Agrobacterium cultures. Following infiltration, the plants were covered with plastic overnight, after which the plastic was removed and the co-cultivation continued for three days under growth conditions as described above for tobacco.

Cell suspension co-cultivation

For co-cultivation of Agrobacterium with Arabidopsis suspension cells, five days after subculture 1.5 ml of cell suspension was transferred to a 6-wells plate. The induced Agrobacterium cultures were diluted in induction medium to an OD_{600} of 0.8 and 1.5 ml of the diluted culture was added to the 1.5 ml cell suspension. After 16 hours under normal growth conditions, most of the medium was removed and replaced by fresh cell culture medium, which after 48 hours was supplemented with 250 µg/L Timentin. This washing step prevented overgrowth of unbound Agrobacterium, which enabled a higher number of Agrobacterium cells to be added at the start of the cocultivation, resulting in higher numbers attached to the plant cells (Matthysse et al., 1978). The suspension cells were visualized four days after co-cultivation using a Zeiss Imager M1 or a Zeiss Observer (Zeiss, Oberkochen, Germany) confocal microscope or GFP or Cherry fluorescence was measured in a 96-wells plate reader as described below.

GFP and Cherry extraction from plant material

At 4 dpi, 1 cm leaf discs were collected using a cork borer (Catalog number: HECH41593006, VWR, Amsterdam, The Netherlands) from each of the infiltrated parts of the third, fourth and fifth leaf of each plant, starting from the first vein and between the veins as close as possible to the main rib. Nine leaf discs infiltrated with the same Agrobacterium strain were pooled in 2 ml Eppendorf microcentrifuge tubes with two 3 mm tungsten carbide beads. The tubes with harvested leaf discs were flash frozen in liquid nitrogen and, when needed, stored at -80 ºC for later isolation. The frozen leaf discs were homogenized in a TissueLyser II (Qiagen Benelux b.v., Venlo, The Netherlands). Depending on the experiment, before (wet) or after (dry) the homogenization 600 µl of TNG buffer (50 mM Tris-HCl, 0.1 M NaCl, 10 % Glycerol pH = 7.4) or a Na phosphate buffer (pH = 7) was added. Plant cells were disrupted for one minute at 1800 rpm. Plant cell debris was pelleted in a cooled tabletop centrifuge (5415 R, Eppendorf Nederland b.v., Nijmegen, The Netherlands) at maximum speed at 4 °C for 30 minutes and the supernatant was collected. For analysis 150 µl of the supernatant was either directly loaded in a 96-wells plate for analysis or stored at -80 ºC for later analysis.

Laser scanning confocal microscopy

Fluorescence was observed using a Zeiss Imager M1 or a Zeiss Observer (Zeiss, Oberkochen, Germany) confocal microscope equipped with the LSM 5 Exciter confocal laser unit using a 20x and 40x magnifying objective (numerical aperture of 0.8 and 0.65, respectively). GFP signal was detected using a 488 nm argon laser and a 505-530 nm band-pass emission filter. Chloroplast- and other auto-fluorescence was detected using a 488 nm argon laser and a 650 nm long pass emission filter. The Cherry signal was detected using a 561 nm diode laser and a 580 – 610 nm band-pass filter. Visible light was detected using the transmitted light detector. Images were collected using ZEN black edition (Zeiss, Oberkochen, Germany) imaging software and processed in ImageJ (Schneider et al., 2012).

96-wells plate reader assay

For detection of GFP fluorescence in Agrobacterium, two methods were used. Cultures were either induced in 50 ml test tubes and transferred to a 96-wells plate (96 well plate Nunc optical bottom black #165305, Fisher Scientific GmbH, Schwerte, Deutschland) for measurement, or induced directly in 96-wells plates, allowing for continuous measurements. In both cases, a 100 ml Erlenmeyer flask with 10 ml LB medium in was inoculated with an Agrobacterium colony and the bacterial culture was grown to an OD_{600} of 0.8 as described above. The bacteria were pelleted by 20 minutes centrifugation in a 50 ml Falcon tubes at 4000 rpm and resuspended in 20 ml induction medium (IM) (Gelvin, 2006) with or without 200 µM acetosyringone (AS). The bacteria were transferred to either 50 ml test tubes (5 ml) or a 96-wells plate (150 µl). The plastic test tubes were incubated on a rocking shaker at 50 rpm at room temperature in the dark. After incubation, 5 ml each tube sample was concentrated by centrifuging, and re-suspended in 5 ml TNG-buffer. From each sample 150 µl was transferred to a 96-wells plate. The GFP and Cherry fluorescence intensity from Agrobacterium and plant tissue was measured in a Tecan Spark 10M (Tecan Life Sciences, Männedorf, Switzerland) plate reader with an excitation wavelength of 488 nm (20 nm bandwidth) and emission wavelength of 530 nm (20 nm bandwidth). The growth of Agrobacterium was measured at OD_{600} in 96-wells plate with clear glass bottoms (96 well plate Nunc optical cover glass-base bottom black #164588, Fisher Scientific GmbH, Schwerte, Deutschland). Measurements were taken every five minutes at constant 180 rpm agitation at room temperature. Three biological repeats were used per treatment, in which Agrobacterium in IM or IM + AS are regarded as separate treatments.

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Author contribution

Ivo Gariboldi and Remko Offringa conceived and designed the experiments. Ivo Gariboldi, Jaap Tromp and Koen van Oostrom constructed plasmids, performed the experiments and performed statistical analysis. Ivo Gariboldi and Jaap Tromp performed the microscopic analysis. Ivo Gariboldi and Koen van Oostrom performed the plate reader experiments. Ivo Gariboldi and Remko Offringa analyzed the results and wrote the manuscript.

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