Resolving plant transformation recalcitrance by Agrobacterium-mediated protein translocation

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Front cover: *Capsicum annuum*, Pepper, epithelial cells with nuclei showing GFP or Cherry fluorescent signal.

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Chapter 1

Introduction

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Introduction

Plants form a crucial but often undervalued component of our anthropocentric society. Essentially, they are the basis for all organic material on earth by providing all the components for life e.g. oxygen, building materials and food The crop plants used for these purposes have been optimized over centuries to meet our needs. The increasing strain on crops to deliver nutrients and materials for the world population has led to modern breeding techniques which use molecular techniques to speed up the process. Among these are genetic techniques, which however, have been restricted for many commercial markets. The common method for plant genome modifications, apart from chemical mutagenesis, is utilizing the natural gene editing capabilities of the phytopathogen *Agrobacterium tumefaciens* (Agrobacterium). For crop plants it would be useful to introduce genes that increase production or help develop new pest or stress resilient varieties. To comply with the negative public opinion against genome modification (GM) in plants, especially in the European Union, efforts have been made to find novel ways that are considered non-GM and can be used to introduce traits in crop plants to enhance the agri- and horticultural sustainability and productivity. In this chapter we will review these methods with a focus on the use of Agrobacterium and enhancing plant regeneration.

Agrobacterium: a tumor inducing plant pathogen

More than a century, the soil dwelling Agrobacterium was identified as the causative agent of the so-called crown gall tumors on host plants (Smith & Townsend, 1907). Initially, Agrobacterium was isolated from grapevine and the first recorded observation of tumor formation on plants dates back to 1679 (Malpighi, 1675). Almost 300 years later it was discovered that Agrobacterium induces tumor formation by transferring a copy of a DNA fragment (Chilton et al., 1977), termed the transfer or T-DNA and situated on the tumor inducing plasmid (Ti plasmid), to plant cells, where it integrates into the chromosomal DNA of these plant cells. The T-DNA carries genes for the biosynthesis of the plant hormones auxin and cytokinin, causing plant cells to divide and form a tumor, but also genes that cause

the tumor cells to produce amino acid-derived compounds (opines) that are used as carbon and nitrogen source by the bacterium. The plant transformation process is facilitated by Virulence (Vir) proteins encoded by the *vir* region located on the Ti plasmid. These Vir proteins help to generate the single stranded T-DNA copy (T-strand) and form the type IV secretion system (T4SS) pilus through which the T-strand together with some other Vir proteins are introduced into the plant cell. The translocated Vir proteins protect the T-strand and help to guide it towards the plant cell nucleus where it is inserted into chromosomal DNA of the plant host (Nester, 2015).

The activation of the Agrobacterium virulence machinery and of the production of Virulence proteins is energy costly. In the nutrient poor environment where Agrobacterium resides it has evolved a strategy to only activate *vir* gene expression when a suitable host plant is detected. The first step of Agrobacterium pathogenesis in a natural environment begins with the detection of wounded plant cells (Guo et al., 2017). The damaged plant cells release a variety of compounds (Fig. 1), among which phenolic compounds and sugars, that trigger the expression of the *vir* genes. The acidity, temperature and low phosphate in the plant cell environment all enhance the *vir* gene induction (Ashby et al., 1988; Baron, Domke, Beinhofer, Hapfelmeier, et al., 2001; Melchers et al., 1989; Parke et al., 1987; Subramoni et al., 2014; D. V. Thompson et al., 1988). Additionally, Agrobacterium uses quorum-sensing and quorum quenching to react on environmental parameters, such as the amount of Agrobacterium cells present on a plant cell, thereby limiting unwanted activation of the nutrient costly virulence machinery (Dessaux & Faure, 2018). The Agrobacterium *vir* genes are located in several *vir* operons, designated *virA, B, C, D, E, F, G* and *H*. Each *vir* gene encodes for a protein with a specific function related to pathogenesis in the host plant. The phenolic compound acetosyringone, originally found to be exuded by wounded tobacco cells, is generally used as the main inducer of *vir* gene expression in laboratory settings (Stachel et al., 1985). It has the strongest effect on virulence induction and it triggers the VirA/VirG bacterial two component regulatory system by activating the transmembrane sensor histidine kinase VirA (Capra & Laub, 2012). In turn VirA phosphorylates the VirG transcription factor, which promotes *vir* gene expression

by binding to the *vir* gene promoters. The induction signal is strongest not only in the presence of acetosyringone but when all inducing conditions of the plant cell environment are present (Wise & Binns, 2016). To be able to perceive signals for virulence induction, the *virA* and *virG* operons are constitutively expressed at a low level. In addition, there are chromosomally-located *vir* (*chv*) genes, that are independently regulated from the VirA/VirG regulatory system. For example, the chromosomally encoded periplasmic sugar binding VirE protein (ChvE) involved in chemotaxis and uptake of sugars (Huang et al., 1990) directly interacts with the periplasmic domain of VirA to enhance *vir* gene induction (Shimoda et al., 1990). The expression of *ChvE* is induced in response to glucose in a concentration-dependent manner (Hu et al., 2013), but glucose does not turn on *vir* expression in the absence of acetosyringone (Wise & Binns, 2016). This all is part of the bacterial strategy to limit unwanted virulence induction without a suitable plant host for infection and thus reducing the risk of resource depletion.

T-DNA transfer and Vir protein translocation via the type 4 secretion system

The generation of the T-strand and its transfer and integration into the host plant genome is facilitated by a diverse set of Vir proteins (Gelvin, 2010; McCullen & Binns, 2006; Nester, 2015). As soon as the virulence machinery is activated, DNA transfer starts with the recognition of two 25 bp imperfect direct repeats that flank the T-region and are accordingly named the left border (LB) and right border (RB) repeat. The size of the T-DNA depends on the Agrobacterium strain and can range from 10 to 30 kilobasepairs (kbp). A relaxosome consisting of the VirD1 helicase and the VirD2 endonuclease binds to the border sequence where VirD2 introduces a nick in the bottom strand. During this process it stays covalently attached to the 5’ end of the nick (Pansegrau et al., 1993; Ward & Barnes, 1988). The single stranded T-strand is subsequently released from the Ti plasmid by DNA polymerase-mediated repair of the nicks assisted by the VirD1 helicase. The covalent binding of VirD2 to the 5’end of the T-strand (T-complex) is essential for virulence, as the protein protects the DNA from nucleases and guides the transfer to the plant cell nucleus through its nuclear localization signals (Van Kregten et al., 2009). The process is enhanced by VirC1 and VirC2 by binding to the overdrive

sequence close to the RB of the T-DNA sequence (Toro et al., 1989), but it also recruits, together with three VirD2-Binding Proteins (VBP 1-3) (Guo, et al., 2007; Guo, et al., 2007), the T-DNA complex to the T4SS (Atmakuri et al., 2007). In the plant cell, the T-strand is bound by the single stranded DNA binding Virulence protein VirE2, which similar to VirD2 provides protection from nucleases and guidance to the plant cell nucleus through nuclear localization signals (Citovsky et al., 1989) (Fig. 1) (Ballas & Citovsky, 1997; Van Kregten et al., 2009). The process of T-DNA transfer and incorporation is commonly known as plant transformation with T-DNA and is termed *Agrobacterium*-mediated transformation (AMT)

The T4SS through which Agrobacterium transports the T-DNA spans the bacterial inner membrane, the periplasm and the outer membrane. It is unique among other bacterial delivery systems, as it is able to transfer DNA inter- and intra-species (Christie, 2019). It shows similarities to the bacterial conjugation system and is based on a conserved set of proteins found in most T4SS (Schröder & Lanka, 2005). Sometimes called the VirB/D4 secretion system, it is composed of twelve Vir proteins, VirB1 – 11 and VirD4, each with a specific function and expressed from the *virB* and *virD* operons located on the Ti plasmid (Christie et al., 2005). It differs from other bacterial secretion systems, such as the type three secretion system (T3SS), in its ability to transfer both DNA and Vir proteins to plant cells. The T4SS can be ordered in four subassemblies; the substrate receptor or type four coupling protein (T4CP), the inner membrane translocase (IMC), the core complex or outer membrane complex (OMC) and the extracellular pilus (Christie et al., 2014; Costa et al., 2021). The actual translocation channel is formed by the T4CP, IMC and OMC subassemblies and all four subassemblies together form the T4SS. The T4CP VirD4 situated at the base of the translocation channel recognizes the substrates, such as the T-complex, allowing them to enter the T4SS. Together with VirB4 and VirB11 from the IMC these three ATPases provide energy to transfer the substrate through the barrel like OMC, which consists of the outer membrane-associated VirB7 and VirB9 lipoproteins and the cell-envelope-spanning subunit VirB10. The extracellular pilus is used to cross the barriers of the plant cell wall and plasma membrane. It is composed of the pillin subunit VirB2 and pilus-tip adhesin VirB5 (Christie et al., 2014). It is suggested that substrates, apart from direct

transfer from the bacterial cytosol through the T4SS into the cytosol of the plant cell, enter as well from the periplasm. They could first enter the periplasm via a part of the T4SS, the IMC, and then enter the secretion chamber of the core complex (Low et al., 2014). Apart from T-DNA, Agrobacterium translocates virulence proteins VirD2, VirD5, VirE2, VirE3 and VirF to the plant cell (Lacroix et al., 2005; Vergunst et al., 2000; Vergunst et al., 2005). It was shown that Agrobacterium delivers VirE2 by presumably manipulating clathrin-mediated endocytosis (X. Li & Pan, 2017) and VirE3 is imported by the karyopherin α-dependent pathway. It mimics VirE2- interacting protein (VIP1), which is required for VirE2 nuclear import of plants (Tzfira et al., 2001; Lacroix et al., 2005; Li et al., 2020). Each translocated protein plays a different role in either DNA transfer, integration or tumor formation. VirD5 increases the transformation frequency, but it also elevates spindle instability which might allow more time for DNA repair after T-DNA integration before cytokinesis, but also causes enhanced chromosome mis-segregation (Zhang & Hooykaas, 2019) leading to DNA damage and mutation (Zhang et al., 2022). The F-box protein VirF is a subunit of a class of E3 ubiquitin ligases and part of the ubiquitin-proteasome system (Schrammeijer et al., 2001) which is often manipulated by pathogens to facilitate infection. The function of VirF is not yet fully understood, however it increases virulence in plants in a host specific way (Regensburg-Tuïnk & Hooykaas, 1993) and in Arabidopsis Agrobacterium induces expression of endogenous AtVIP1-Binding F-box protein (VBF), which substitutes VirF (Zaltsman et al., 2010). In this thesis the term AMT is used for T-DNA transfer, whereas *Agrobacterium*-mediated protein translocation (AMPT) is used to specifically indicate the transfer of proteins (of interest) by *Agrobacterium* to plant host cells.

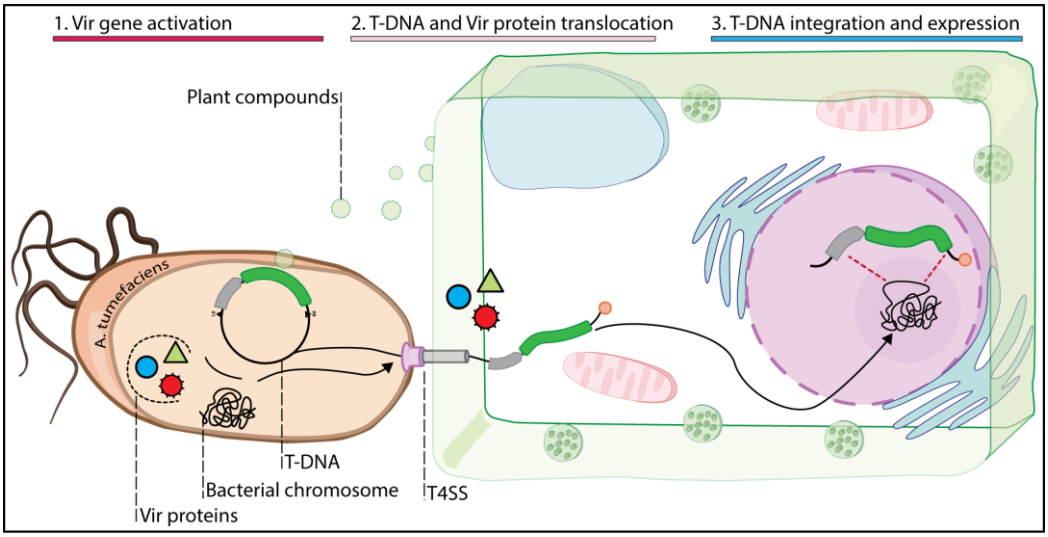

Figure 1. Schematic simplified representation of AMT and AMPT to plant cells. (1) *Vir* gene induction; The wounded plant cell secretes compounds, which induce the Agrobacterium virulence by activation of the VirA/VirG signaling cascade. Virulence proteins are produced, the T4SS is formed and the T-strand is generated. (2) T-DNA and protein translocation: The virulence proteins and the ssDNA are guided through the T4SS inside the plant cell. (3) T-DNA integration and expression: The T-DNA is protected against degradation inside the plant cell and once it reaches the plant cell nucleus it is incorporated into the plant genome from where the T-DNA genes are expression.

Application of Agrobacterium-mediated transformation (AMT) in agriculture and biotechnology

Since the discovery of the potential of Agrobacterium to introduce genes into the genome of host plants, various efforts have been made to develop it for both scientific as well as agricultural and biotechnological use. Initially, methods of direct DNA transformation were developed in parallel, such as protoplast transformation by chemical or electroshock treatment or bombardment of plant tissues with DNA-coated particles. With the increasing ease to generate desired T-DNA constructs using newly developed binary vectors (Hoekema et al., 1983) and the discoveries on the more optimal mechanism of DNA transfer compared to direct DNA transformation (Jorgensen et al., 1987), the Agrobacterium vector system has become the preferred method for both stable plant genetic modification and

transient gene expression studies in plant cells. In fact, following the discovery that not only plants but also yeast and other fungal cells are hosts for Agrobacterium-mediated transformation (Bundock et al., 1995; De Groot et al., 1998), the Agrobacterium vector system has become a common method for the genetic modification for these organisms as well.
In general, stable transformation is the most common method. The transient expression system using Agrobacterium has been used mainly for research, however it has also been used in biotechnology. A variation of techniques have been developed for transient expression (Chincinska, 2021) and the most popular is the infiltration of tobacco leaves with a syringe on the abaxial side (Yang et al., 2000). Vacuum infiltration is a popular alternative for plant species that are more difficult to infiltrate with syringe infiltration e.g. *Arabidopsis* (Leuzinger et al., 2013). The production of recombinant proteins in *N. benthamiana* via transient expression is performed on industrial scale (Spiegel et al., 2022). Although only feasible for high profit biopharmaceutical compounds, it has the potential to be scaled up via large scale leaf infiltration (Chen et al., 2014) or by using cell suspension bioreactors (O'Neill et al., 2008). The production of recombinant proteins by transient expression is generally in controlled production facilities, however also field production applications have been reported (Hahn et al., 2015). These open field production methods have raised great concern about the spread of engineered Agrobacterium strains and the resulting GM plants in the environment (Bauer-Panskus et al., 2020).

Recalcitrance to AMT: political issues

The most common use of Agrobacterium is stable genetic modification. The use is however restricted in many parts of the world, including the European Union (EU), which has many restrictions for the use of genetically modified organisms (GMOs). Since 1990 the EU Council directive 90/220/EEC is in force on the deliberate release of GMOs into the environment, amended by directive 2001/18/EC and it is focused on the introduction of heterologous genes (Eriksson, 2018). It covers established genomic techniques (EGT) which are techniques such as random mutagenesis using physical or chemical mutagens or the transfer of genetic material e.g. using AMT

(Mullins et al., 2022). In the case of all these techniques the genome is modified randomly e.g. for AMT the exogenous sequence integrates randomly into the host genome. In contrast, new genomic techniques (NGTs) that have been developed in the recent decades are designed to achieve targeted mutagenesis. A well-known example is CRISPR-Cas9, by which the plant genome can be altered at a predefined location (Doudna & Charpentier, 2014). The current directive requires an exhaustive list of assessments for a GM crop to be cultured in the field, including an environmental risk assessment and post-release monitoring (Ramsay 2022). This legislative burden has prevented the introduction and field cultivation of GM crops in the EU, where only one crop (the insect resistant maize Mon 810 expressing a *Bacillus thuringiensis* protein) has been approved for cultivation, and this approval is currently waiting its second renewal.

The European Union is discussing a draft regulation on new genomic techniques (NGTs) through which GM plants are obtained by targeted mutagenesis, cis-genesis or intra-genesis. The incorporation of genetic material from sexually incompatible organisms, transgenesis, is out of the scope of the current negotiations, even though it has been shown that horizontal gene transfer in plants is very common in nature (Aubin et al., 2021). Criteria are being developed for the risk assessment of crops generated by these NGTs (Mullins et al., 2022), and various options for NGTs in the EU are being investigated (Eriksson et al., 2018; Purnhagen et al., 2023). Recently, GM plants created by NGTs were proposed to fall in to two categories, where plants and products in category 1 would be exempt from the requirements of GMO legislation. The outcome is still insecure and the procedures are of considerable length (Garcia-Alonso et al., 2022). To circumvent the GM discussion and legislation, new methods resulting in genetically improved crops that are likely be considered non-GM are being explored, such as Agrobacterium plant genome editing using non integrating viral vectors (Gong et al., 2021).

Recalcitrance to AMT: plant pathogen interaction issues

Plant transformation is an important technique for research and industry; however, plants have developed defense strategies to repel various pathogen attacks. Agrobacterium tries to manipulate the plant defense response via its

virulence effector proteins (Tiwari et al., 2022). In turn, a plant's resistance to a pathogen is determined by its genetic traits and of the pathogen. Plants contain resistance (*R*) genes that are involved in the recognition of pathogen derived molecules. The pathogen in its turn contains matching avirulence (*avr*) genes, encoding effector proteins that overcome the effect of the plant's defense response (White et al., 2000). The plant and pathogen often reside in the same biotope and the gene-for-gene interaction can co-evolve between host-pathogen. Three scenarios can occur for a plant-pathogen interaction. In a compatible interaction the pathogen will infect the plant by successfully suppressing the host defense responses. In an incompatible interaction, the pathogen is either incapable of infecting the plant and cause disease symptoms, or its initial infection leads to a strong defense response (Yuan et al., 2021).

A plant pathogen can be recognized through its pathogen-associated molecular patterns (PAMPs) by surface pattern recognition receptors (PRRs), which induces PAMP-triggered immunity (PTI), or its effectors can be recognized by cytosolic nucleotide-binding/leucine-rich-repeat (NLR) receptors and induce effector-triggered immunity (ETI) (Bigeard et al., 2015; Cui et al., 2015). The plant hormone salicylic acid (SA) plays an important role in both PTI and ETI. Upon pathogen attack, its biosynthesis is upregulated, which in Arabidopsis leads to the activation of many SA-inducible genes through the nuclear import of the SA receptor NON-EXPRESSOR OF PR GENES 1 (*NPR1)* (Backer et al., 2019). Effectors of the pathogenic *Pseudomonas syringae* were shown to suppress defence responses by directly interacting with NPR1. Another *P. syringae* effector, AvrPto, was shown to block pattern triggered immunity (PTI) by binding PRRs, including FLS2 and EFR (Xiang et al., 2008). In non-susceptible hosts, the Pto kinase competes with PRRs for binding AvrPto and activates ETI (Chen et al., 2017). Both PTI and ETI are basal local defense mechanisms leading to diverse physiological outputs for ETI often conferring resistance by inducing a hypersensitive response (HR), which is a rapid defence response that can be induced by phytopathogenic bacteria and prevents the spread of the infection by localized cell death on the site of infection (Dixon et al., 1994; Yuan et al., 2021). The resistance upon infection spreads throughout the plant and is called systemic acquired resistance (SAR). This resistance is able to

remain active for prolonged periods of time and provides resistance to a variety of pathogens, including fungi, viruses and bacteria by the expression of pathogenesis-related (PR) genes (Ryals et al., 1996). The SAR response is triggered upon the formation of HR or any other disease symptom, and induces the accumulation of SA. Once activated it can repel pathogens that normally cause disease.

The SA response is an important factor determining recalcitrance to AMT. *Nicotiana benthamiana* plants treated with SA showed decreased susceptibility to Agrobacterium infection. (Anand et al., 2008). Moreover, exogenous application of SA to Agrobacterium cultures decreased the bacterial growth, virulence, and attachment to plant cells (Y. Peng et al., 2021; Verberne et al., 2003; Vlot et al., 2021). Ectopic expression of the bacterial *NahG* gene, encoding salicylate hydroxylase which metabolizes SA, in Arabidopsis prevented pathogen-induced accumulation of SA and prevented the subsequent SAR defense responses thereby increasing the transformation efficiency (Lawton et al., 1995). Interestingly, Agrobacterium also uses SA to regulate its own virulence. After perception of plant-derived sucrose it is able to release SA from the conjugated storage form SA-glucose (Zeier, 2021) to rapidly down-regulate *vir* gene expression and thereby preserve energy (Wang et al., 2019a). In conclusion, one has to keep in mind that Agrobacterium is a plant pathogen that triggers defense responses in plant tissues and that mitigating these defense responses might help to overcome recalcitrance to AMT or AMPT.

Plant regeneration and propagation: what can we learn from zygotic embryogenesis?

The majority of crops are flowering plants, which reproduce sexually via zygotic embryogenesis, where two haploid sexual cells, the gametes, fuse to form a diploid zygote, which then develops into an embryo. Cell division and cell differentiation change the pluripotent embryonic cells into mature somatic tissue. The gametes can be derived from the same hermaphrodite parent, or from different unisexual parents (Schmidt et al., 2015). Further development and growth of the root and shoots are maintained by stem cell zones e.g. in the shoot apical meristem (SAM) and the root apical meristem (RAM). Positioned at the tip of the

shoot, the SAM maintains pluripotent stem cells and its daughter cells differentiate into organs. The SAM and RAM remain active throughout the life span of a plant.

Early in *Arabidopsis* embryogenesis, the apical and basal patterning is formed mediated by WUSCHEL RELATED HOMEOBOX2 (WOX2) and WOX8 respectively (Breuninger et al., 2008). WOX2 is involved in the initiation of shoot stem cells by promoting the expression of HD-ZIP III transcription factors, which creates a balance of cytokinin and auxin (Zhang et al., 2017). Auxin in turn controls pattern formation during embryogenesis with the hormone minima and maxima concentrations acting as developmental signal (Friml et al., 2003; Verma et al., 2021). The stem cell inducing transcription factors WUSCHEL (WUS) and SHOOT MERISTEMLESS (STM) are required for SAM establishment and maintenance (Barton, 2010). WUS is able to move from cell to cell and part of the regulation is restricting movement by the formation of dimers (Daum et al., 2014). Stem cells express the CLAVATA3 (CLV3) peptide and its expression restricts *WUS* through signaling via the CLV1 and CLV2 receptor-like kinases (Brand et al., 2002). CLV1/2/3 are required to restrict the number of stem cells accumulating in both shoot and floral meristems and are found in the plasma membrane (CLV1 and 2) and in the apoplastic space (CLV3). During early phases of embryogenesis, the transcription factor *BABY BOOM* (*BBM*) is expressed in developing embryos and seeds (Boutilier et al., 2002). It encodes an AINTEGUMENTA-LIKE (AIL) APETALA2/ethylene-responsive element binding factor (AP2/ERF), which in *Arabidopsis* is part of an eight-member clade, which next to BBM comprises AINTEGUMENTA (ANT), AINTEGUMENTA-LIKE 1 (AIL1), PLETHORA1 (PLT1), PLT2, AIL6/PLT3, EMBRYOMAKER (EMK)/AIL5/PLT5 and PLT7. The early embryo arrest of the *bbm plt2* double mutant shows the redundant and important role of these two transcription factors in zygotic embryogenesis (Horstman et al., 2015). Interestingly, BBM transcriptionally regulates *LEAFY COTYLEDON 1* and *2* (*LEC1* and *LEC2*), as well as *FUSCA3* (*FUS3*), *ABI45 INSENSITIVE3* (*ABI3*) and *AT-HOOK MOTIF NUCLEAR LOCALIZED 15* (*AHL15*), all transcription factors playing crucial roles during zygotic embryogenesis (Horstman et al., 2017; Karami et al., 2021).

Plant reproduction via somatic embryogenesis

Apart from sexual reproduction via zygotic embryogenesis, some plants such as *Kalanchoë daigremontiana* have the ability to clonally reproduce by regenerating an entire new plant from somatic cells (Garcês et al., 2007). For other plants various laborious techniques are needed for clonal propagation by tissue culture using techniques such as stem cuttings or tissue culture. The tissue culture techniques can be divided into two methods: organogenesis or somatic embryogenesis (SE). For organogenesis plant cells or tissues are commonly cultured on media containing a specific ratio of the plant hormones cytokinin and auxin to induce shoots or roots. Generally, regeneration by organogenesis is a three-step procedure starting with the induction of cell division followed by shoot formation and rooting of these shoots. In some plants somatic cells can be induced *in vitro* to develop into to embryos using various stress treatments, plant hormones or ectopic expression of transcription factors involved in embryogenesis (Horstman et al., 2017). In *Brassica napus* and Arabidopsis, the ectopic expression of *BBM* leads to the formation of somatic embryos on the SAM and cotyledons of germinating seedings (Boutilier et al., 2002). The overexpression of *WUS* in *Arabidopsis* causes similar vegetative to embryonal conversions (Zuo et al., 2002). Apart from *WUS* and *BBM*, a number of other genes have been identified in Arabidopsis that when ectopically expressed promote somatic embryo development, among which the BBM target genes *LEC1*, *LEC2* and *AHL15* (Lotan et al., 1998; Stone et al., 2001; Karami et al., 2021).

Interestingly, these SE-inducing genes have also been used to overcome regeneration recalcitrance during transformation. The combined ectopic expression of the maize homologs of *BBM* and *WUS* resulted in enhanced regeneration of transgenic calli in a recalcitrant hybrid maize genotype. Moreover, the same method also stimulated transformation in sorghum (*Sorghum bicolor*) immature embryos, sugarcane (*Saccharum officinarum*) callus, and rice (*Oryza sativa ssp indica*) callus tissue (Lowe et al., 2016). However, regeneration proved difficult and it was shown that ectopic expression of *BBM* and *WUS* prevented further development of the transgenic calli. To circumvent constant expression, excision of a *loxP* site-flanked *WUS* and *BBM* containing fragment by Cre

recombinase has been used, where the *Cre* gene was expressed under the drought inducible promoter of the maize *rab17* gene (Lowe et al., 2016). Other gene induction systems often rely on a hormone triggered response, which uses the regulatory mechanism of steroid hormone receptors not naturally present in plants. These systems use the receptor domain of the rat glucocorticoid receptor (GR) and the ligand dexamethasone (DEX), a strong synthetic glucocorticoid (Aoyama & Chua, 1997), thus preventing constitutive expression of a heterologous gene in the host plant.

Agrobacterium-mediated translocation of heterologous proteins as solution to recalcitrance to AMT

Alternative to genetic transformation approaches the AMPT system of Agrobacterium can be used to transiently introduce proteins of interest inside the plant cell without modifying the host genome. Previously AMPT has been used to introduce proteins of interest in plant cells (Vergunst et al., 2000; Khan, 2017; Schmitz et al., 2020). The proteins of interest could be transcription factors, such as BBM or WUS, that following AMPT would promote regeneration of genetically transformed cells of regeneration recalcitrant crops (Anjanappa & Gruissem, 2021). The WUS transcription factor was shown to be required for effective regeneration of Arabidopsis mesophyll protoplasts (Xu et al., 2021) and, as presented above, the combined effect of ectopic BBM and WUS expression resulted in enhanced regeneration in recalcitrant monocot species (Lowe et al., 2016). Difficulties in approval and public opinion have halted the widespread use of Agrobacterium outside of academic settings. The use of AMPT instead of AMT, thereby circumventing genomic alteration, is currently not yet regarded as genetic modification.

Transient protein expression and visualization

In order to test the use of AMPT for improved regeneration it is important that the occurrence and efficiency of protein translocation can be monitored. Translocation of virulence proteins by Agrobacterium was demonstrated for the

first time by fusing the site-specific Cre recombinase to VirE2 and VirF, and using this in combination with a transgenic Arabidopsis line containing a *loxP*-flanked region interrupting the expression of a neomycin phosphotransferase (*nptII*) gene. Successful translocation led to excision of a disruptive region between the promoter and open reading frame, allowing to detect and monitor the efficiency of AMPT by selecting on kanamycin. It was shown that a positively charged C-terminal signal peptide on the virulence proteins is required for T4SS-mediated protein translocation. Fusing this part to the C-terminus of proteins of interest resulted in their translocation (Vergunst et al., 2000). A disadvantage of the antibiotic resistance selection system was that it did not allow for direct visualization of the process. As fluorescent proteins such as GFP appeared not be translocated by the Agrobacterium T4SS, probably due to their tight folding, the split-GFP system was adopted to visualize AMPT. For the split-GFP system, the coding region of the *GFP* gene has been split in two parts, a larger fragment coding for amino acids 1-214 comprising β-strands 1 to 10 (GFP_{1-10}, the detector) and a smaller fragment coding for amino acids 214-230 comprising β-strand 11 (GFP_{11}, the tag). Both GFP parts are non-fluorescent, however when brought together they can reassemble into a functional GFP (Ghosh et al., 2000a). In plants visualization of fluorescent molecules is more challenging because of many autofluorescent components. To increase the fluorescence intensity, the GFP molecule has been previously improved for use in plants (Pang et al., 1996). The split-GFP molecule has been optimized to prevent misfolding when the GFP_{11} tag is expressed as fusion protein. This so called superfolder GFP (sfGFP) has increased solubility which increases the fluorescence and extraction efficiency in living cells. Originally visualizing the transfer of fusion proteins tagged with GFP_{11} via the Agrobacterium T4SS using the split-GFP system relied on a host plant expressing GFP_{1-10} (Sakalis et al., 2014a), which required *a priori* transformed plants and limited the capabilities to visualize protein transfer in any genotype. However, the split-GFP system has been adapted to transfer simultaneously both GFP_{1-10} on T-DNA and GFP_{11} as fusion protein via the T4SS into the plant host cell (Khan, 2017). The general approach is an Agrobacterium strain carrying a binary vector containing a plasmid for T-DNA transfer and a second plasmid from which the fusion protein to be translocated to

the host plant cell is expressed. With this system, AMPT can be visualized in any plant species or genotype without the need for *a priori* generation of plant lines expressing the detector protein (Fig. 2).

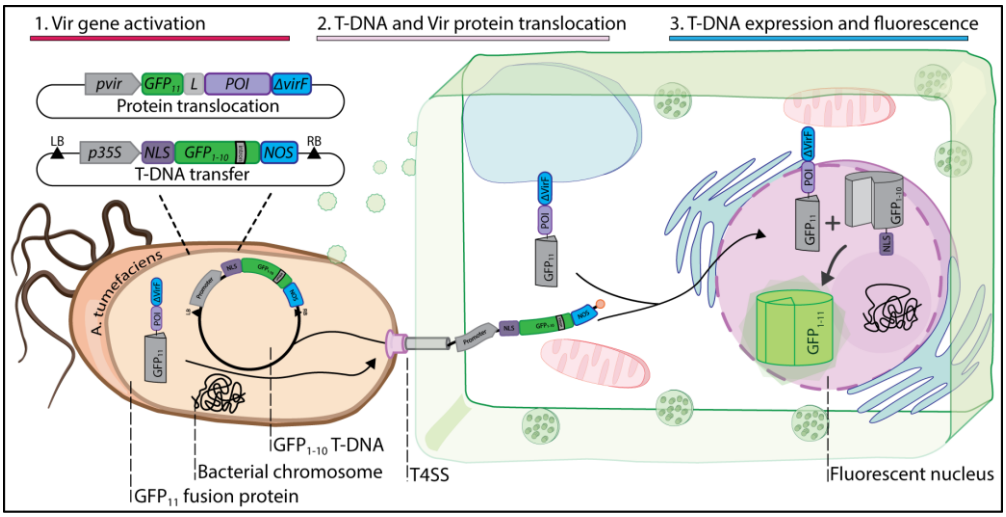

Figure 2. Schematic simplified representation of the general method for construct design and detection of AMPT to plant cells. (1) *Vir* gene induction: schematic representation of the two *Agrobacterium* constructs necessary for the split-GFP method previously developed; a protein translocation plasmid and a T-DNA transfer plasmid. Both plasmids have been engineered to be modified to suit the needs for further experiments to translocate any protein of interest. (2) T-DNA and protein translocation: both T-DNA and GFP_{11}-labelled ΔVirF fusion protein are introduced in the plant cell through the T4SS pilus and guided to the nucleus. (3) T-DNA transient expression and GFP reconstitution, T-DNA expresses GFP_{1-10}, which is targeted to the nucleus by its NLS sequence. Upon co-translocation of the GFP_{11}-labelled ΔVirF fusion protein reconstitution of GFP results in a nuclear green fluorescent signal.

Furthermore, the sensitivity of GFP fluorescence visualization was increased by addition of a NLS signal to GFP_{1-10} (Fig. 3A and B), resulting in accumulation of the fluorescent signal into the nucleus (Khan, 2017). More recently, the possibility to do multi-color imaging was added by the development of split systems for other fluorescent proteins, such as superfolder Cherry2 (sfCherry2), in animal cells. Importantly, the components of split-sfGFP and split-sfCherry2 are not interchangeable and GFP or Cherry can only be reconstituted to a

fluorescent molecule if both unique parts of the protein are present (Fig. 3C and D). This now allows to visualize the simultaneous translocation of different proteins to host cells (Kamiyama et al., 2016a; Park et al., 2017).

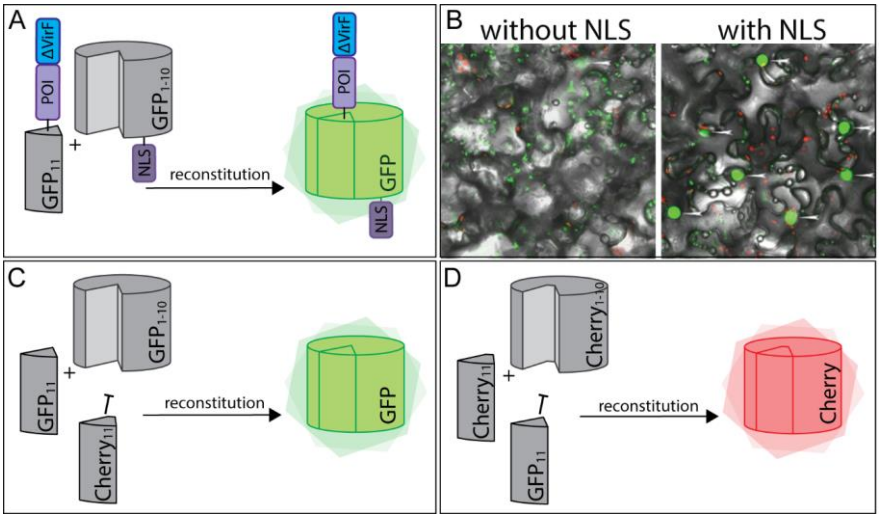

Figure 3. A schematic representation of the split-GFP and split-Cherry system and the effect of a NLS sequence. (A) The split GFP system used to detect AMPT: translocation of the fusion protein consisting of the GFP_{11}-tag, the protein of interest (POI) and the translocation signal (ΔVirF) to a plant cell expressing the nuclear localized (NLS) GFP_{1-10} reporter protein results in reconstitution of a functional green fluorescent protein (B) Comparison of detection of AMPT with a cytosolic or nuclear localized GFP_{1-10} reporter protein. (C, D) There is no cross contamination between the split-GFP and the split-Cherry system. GFP_{1-10} can only form a functional green fluorescent protein with GFP_{11} (C) and $Cherry_{1-10}$ can only reconstitute to a functional red fluorescent protein with $Cherry_{11}$ (D).

Thesis outline

The knowledge gained from AMT on plant development and physiology is tremendous. The stable and transient overexpression or inducible gene constructs gave insight in the biological function of many genetic elements in plants. The demonstration that Agrobacterium can also translocate virulence proteins and the recent advances in AMPT opened the possibilities for novel experimental insights. Moreover, growing knowledge in the interaction between pathogens and plant hosts enables finetuning of the transformation efficiency. In this thesis the

application of AMPT on wild type plants was studied to address transformation recalcitrance by AMPT of proteins which could improve regeneration or reduce the defense response against Agrobacterium.

Previous experiments using the split-GFP system to detect AMPT showed that the fluorescent signal was relatively weak compared to the GFP signal following AMT, leading to an underestimation of the AMPT frequency (Khan, 2017). In **Chapter 2** the split-GFP system was codon-optimized for expression in plants (GFP_{1-10}) or Agrobacterium (GFP_{11}-fusion protein) resulting in enhanced efficiency and fluorescence intensity. Furthermore, the use of a novel fluorophore variant, sfCherry2 (Cherry), was tested in plants and the split variant was tested for the double split-fluorophore system (ds-FP) that would allow to detect the simultaneous translocation of two proteins of interest. Whereas the Cherry protein appeared to be a suitable reporter in plant cells, the split Cherry did not work in plant cells. We therefore incorporated the Cherry fluorophore on a T-DNA alongside the split-GFP system and could successfully show that this allowed co-localization of the T-DNA derived Cherry signal with the AMPT derived split-GFP signal, termed the colocalization split-GFP (split-GFP^{col}).

In **Chapter 3** a workflow was established, combining confocal microscopy with multi-well plate reader-based quantification of fluorescent signal, to analyze GFP fluorescence reporting *vir* gene induction in Agrobacterium or to quantify simultaneous GFP and Cherry fluorescence reporting respectively AMPT and AMT in plant cells. The use of the multi-well plate reader enabled a higher throughput quantification of AMPT and AMT and time lapse analysis of *vir* gene induction and the data were verified by confocal microscopy. The plate reader method showed that the *virE* promoter resulted in much higher expression in Agrobacterium compared to the *virF* or *virD* promoter, indicating that it is the preferred promoter for expression of proteins to be translocated from Agrobacterium to plant cells. The method also allowed for optimization of the Agrobacterium induction conditions and resulted in increased AMT of Arabidopsis suspension cells.

In **Chapter 4** we used the optimized constructs and conditions from **Chapter 2** and **Chapter 3** to investigate whether AMPT of heterologous proteins could be used to modulate plant physiology and ultimately to remove bottle necks

causing transformation recalcitrance. Previously, it was shown that expression of the *P. syringae pv. Tomato DC3000* effector AvrPto or the bacterial salicylic acid hydroxylase NahG in Arabidopsis leads to higher transient expression following AMT. AMPT of AvrPto did not induce a hypersensitive response (HR) in *N. benthamiana* leaves, but instead it did enhance the efficiency of both AMT and AMPT. AMPT of NahG enhanced the efficiency of both AMT and AMPT to even a higher level. In addition, we could show that AMPT of AHL15 delayed senescence in *N. benthamiana* leaves and was able to enhance shoot regeneration on tobacco leaf discs. A slight effect on translocation was observed of N- and C-terminal tags on the fusion protein, although overall in all cases a clear physiological effect was observed in the experiments.

In conclusion, with the research described in this thesis we show that the AMPT system is capable of introducing biologically active heterologous proteins to plant cells and that this can be used to increase transformation efficiency by removing the main bottle necks of transformation recalcitrance. Moreover, the tools developed to visualize and quantify AMT and AMPT will be useful to optimize *vir* gene induction and Agrobacterium-plant cell cocultivation conditions in a high throughput manner.

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Chapter 2

A generic detection system for *Agrobacterium*-mediated DNA and protein translocation to plant cells

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Abstract

The use of *Agrobacterium tumefaciens* (Agrobacterium) for plant transformation has long focused on stable T-DNA integration or transient expression, where stable T-DNA integration was generally selected for by co-expressing an antibiotic or herbicide resistance gene. The finding that Agrobacterium also translocates Vir proteins, or heterologous proteins fused to these Vir proteins, to host plant cells has provided an interesting additional tool for the reprogramming of plant cells or the editing of their genomes. In this chapter, the split-GFP system was optimized for sensitive visualization of translocation of GFP_{11}-tagged proteins of interest to plant cells. In addition, a split-Cherry system was tested to detect the simultaneous translocation of a $Cherry_{11}$-tagged protein of interest. Unfortunately, the split-Cherry system was not suitable for the detection of protein translocation. Instead, we successfully used the *Cherry* reporter in combination with the optimized split-GFP system to visualize simultaneous T-DNA transfer and protein translocation in leaves of *Nicotiana tabacum, Nicotiana benthamiana, Solanum lycopersicum, Capsicum annuum, Brassica napus* and suspension cells of *Arabidopsis thaliana*.

Introduction

Plants are commonly genetically modified for experimental or breeding purposes using the natural DNA transfer system of the soil-borne phytopathogen *Agrobacterium tumefaciens* (Agrobacterium). It has the ability to transfer a part of its DNA (transfer DNA or T-DNA) together with Virulence proteins (Vir proteins) to the host plant cell. The T-DNA originates from the tumor-inducing plasmid (Ti plasmid) and contains all the genes necessary to cause tumor growth on the plant and to make these tumor cells produce compounds beneficial for the bacterium. The Vir proteins aid in the process of transformation by, among others, forming the type IV secretion system (T4SS) pilus and guiding the T-DNA strand towards the plant cell nucleus (Nester, 2015).

For these guiding Vir proteins, such as VirE2 and VirF, it was shown that they are translocated together with the T-DNA by the T4SS to plant cells and that a positively charged C-terminal signal sequence in these proteins is required and sufficient for translocation. Heterologous proteins C-terminally fused to this signal sequence can be introduced into plant cells by Agrobacterium-mediated protein translocation (AMPT) (Vergunst et al., 2000, 2005). To prove transfer of heterologous proteins by AMPT to plant cells, the Cre recombinase was fused to the C-terminal domain of VirE2 or VirF and translocation was tested using a transgenic *Arabidopsis thaliana (*Arabidopsis) line containing a lox-flanked DNA segment. This segment separated the coding region of the *neomycin phosphotransferase II* (*nptII*) gene from the promoter, thereby preventing its expression. Successful transfer of the Cre fusion protein led to excision of the segment and thus to restoration of *nptII* expression. This expression could be detected by the appearance of kanamycin resistant calli, thereby proving that Agrobacterium was capable of protein translocation (Vergunst et al., 2000). Although a robust system, it could only report AMPT in an indirect manner. The green fluorescent protein (GFP), often used for visualization of expression, cannot be translocated using AMPT. To directly visualize AMPT, the split-GFP system was adopted to detect the translocation of VirE2 into *Nicotiana tabacum* (Sakalis et al., 2014). The general concept of the split-GFP system is that GFP is split into two non-fluorescent parts, a larger fragment comprising

amino acids 1-214 (GFP_{1-10}, detector) and a smaller fragment comprising amino acids 214-230 ($GFP_{11,}$ tag), that are able to self-assemble into a fluorescent GFP molecule (Ghosh et al., 2000b). For this purpose, GFP has been optimized to prevent misfolding when the GFP_{11} tag is expressed as fusion with other proteins. This so called superfolder GFP, hereafter referred to as GFP, has increased solubility, which increases the fluorescence in living cells and the extraction efficiency of the protein (Pédelacq et al., 2006). In contrast to many other protein tagging techniques, the split-GFP system is highly suitable for *in vivo* work. In the first approaches to visualize AMPT using split-GFP, plants that stably express the GFP_{1-10} were co-cultivated with Agrobacterium transferring a fusion protein VirE2 N-terminally tagged with GFP_{11} (Sakalis et al., 2014). The system was further optimized by expressing the GFP_{1-10} from a T-DNA that was co-transferred with the translocated GFP_{11}-tagged protein of interest (POI), enabling direct visualization of AMPT in wild-type plants without the need for *a priori* transformation (Khan, 2017).

The above system had the disadvantage that the sensitivity of detecting AMPT was limited when compared to the transient expression of the GFP_{11}-tagged POI from a T-DNA (Khan, 2017), suggesting that many AMPT events were left undetected. Moreover, our previous AMPT data suggested that for many plant genotypes that have been reported to be recalcitrant to AMT, both T-DNA transfer and AMPT could be detected (Khan, 2017), suggesting that the main bottle neck is the regeneration of the transformed cells. Previously, it has been reported that the simultaneous expression of the transcription factors BABY BOOM (BBM) and WUSCHEL (WUS) in plant cells significantly enhances the frequency of transformation and regeneration in numerous previously difficult to transform crop species and tissues, such as maize (*Zea mays*) immature embryos and callus, sorghum (*Sorghum bicolor)* immature embryos, sugarcane (*Saccharum officinarum*) callus and rice (*Oryza sativa ssp indica*) callus (Lowe et al., 2016). A major disadvantage of this system is that it requires removal of these genes during the process of regeneration, because sustained expression of these transcription factors interfere with plant development. We therefore tested whether simultaneous introduction of the BBM and WUS proteins via AMPT would lead to enhanced regeneration without the need to remove the genes. As this required the detection of translocation of

two POIs, we adopted the split-variant of *super folder* Cherry 2 (hereafter referred to as split-Cherry), which has previously only been tested in animal cells (Feng et al., 2017; Kamiyama et al., 2016b), into our AMPT system for the detection of the second POI.

In this chapter we describe several approaches to optimize the detection of AMPT for the simultaneous translocation of two POIs. We show that the detection of AMPT by the split-GFP system can be enhanced by optimization of the *GFP$_{11}$:POI:ΔvirF* coding region for expression in Agrobacterium and by a seven-times multimerization of the GFP$_{11}$ tag. Unfortunately, it appeared impossible to detect AMPT using the split-Cherry system, either due to insufficient sensitivity of the system or because the Cherry$_{11}$ tag prohibited AMPT. Instead, we successfully used the *Cherry* reporter in combination with the improved split-GFP system, to detect simultaneous AMT and AMPT in different tissues of various plant species.

Results

Optimized detection of AMPT in tobacco leaf cells

The previously observed fluorescence by confocal microscopy after AMPT using the split-GFP system (split-GFP^{mk}) using GFP$_{11}$ protein translocation and a GFP$_{1-10}$ T-DNA transfer vector was relatively weak compared to *p35S* driven transient expression of full length GFP following DNA transfer (Khan, 2017). High laser power was required to be able to clearly visualize the signal by confocal microscopy, causing unwanted tissue damage and rapid bleaching of the fluorescent signal. One of the reasons for the weak signal could be that the coding regions in the split-GFP system were not optimized for the species-specific codon usage. We therefore optimized the *GFP$_{11}$* sequence as used in the coding region of GFP$_{11}$:POI:ΔVirF (Appendix 1a) and the sequence of the POI, BBM (Appendix 1e) for expression in Agrobacterium. At the same time, the *NLS* and *GFP* sequences forming the *NLS:GFP$_{1-10}$* coding region on the T-DNA vector were optimized for expression in plants (Appendix 1b). An Agrobacterium strain containing the resulting vectors *pvirF::GFP$_{11}$:BBM:ΔvirF* and *p35S::NLS:GFP$_{1-10}$::tNOS* was infiltrated into *Nicotiana tabacum* (tobacco) leaves. At 4 days post infiltration (dpi) nuclear fluorescent GFP signal was observed,

indicating that AMPT was successful. The fluorescence intensity of the nuclear signal was compared to that in leaves infiltrated with an Agrobacterium strain translocating a non-optimized GFP_{11}:BBM:ΔVirF fusion protein (Khan, 2017) together with the plant optimized *p35S::NLS:GFP_{1-10}::tNOS* T-DNA construct. With the Agrobacterium optimized fusion protein, significantly more positive nuclei were detected, and in those nuclei the fluorescent signal was on average 2-fold stronger compared to fluorescent nuclei obtained following AMPT of the non-optimized protein (Fig. 1A, B). These data suggest that codon optimization does lead to higher expression in Agrobacterium and thus to more AMPT events, resulting in a stronger signal in plants. Therefore, all the constructs used in subsequent experiments were codon optimized for either plant or bacterial expression. To further increase detection of the fluorescent signal, the GFP_{11} tag was multimerized seven times, resulting in the *pvirF::GFP_{11}x7:BBM:ΔvirF* construct. Multimerization of the GFP_{11} tag should provide more binding places for the abundantly overexpressed GFP_{1-10} sensor, which has been reported to lead to a significant enhancement of the signal (Kamiyama et al., 2016b; Park et al., 2017). In our experiments, more fluorescence positive nuclei were observed with the GFP_{11}x7:BBM:ΔVirF fusion proteins and the average fluorescence intensity per nucleus was 2-fold higher compared to that with the single GFP_{11}-tagged codon optimized fusion protein (Fig. 1A, B).

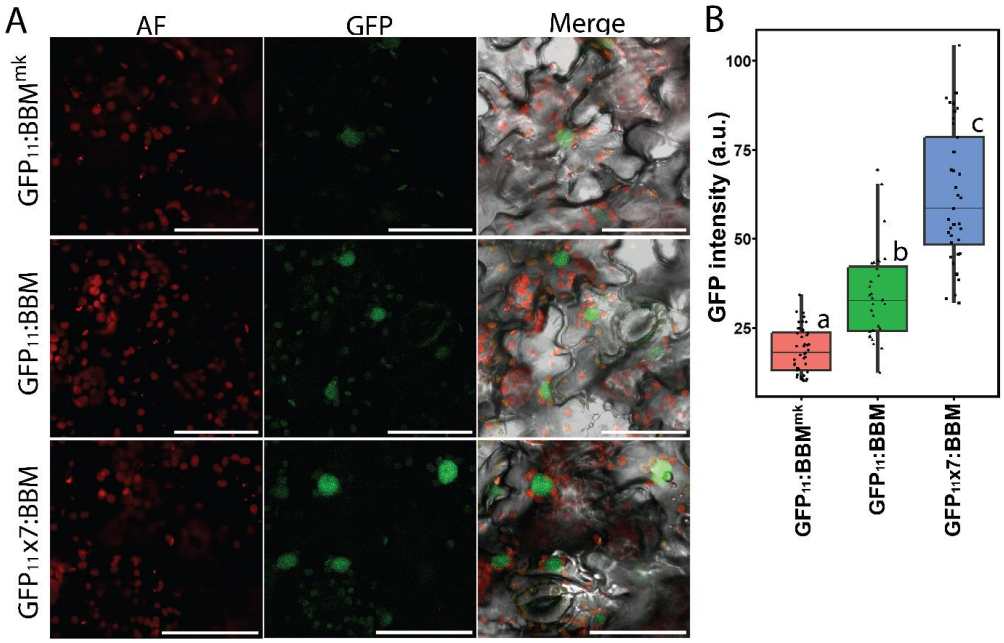


Figure 1. Enhanced detection of AMPT of BBM fusions by modifying the split GFP system. (A) Confocal microscopy images showing GFP fluorescence observed 4 dpi in 4-weeks old tobacco leaf epidermis cells after AMPT using split-GFP system variants: the non-optimized split-GFP^{mk} (*p35S::NLS:GFP$_{1-10}$^{mk}::tNOS + pvirF::GFP$_{11}$^{mk}:BBM^{mk}:ΔvirF),* the codon optimized split-GFP (*p35S::NLS:GFP$_{1-10}$::tNOS + pvirF::GFP$_{11}$:BBM:ΔvirF)* and the codon optimized split-GFP with 7 tandem GFP$_{11}$ repeats (*p35S::NLS:GFP$_{1-10}$::tNOS + pvirF::GFP$_{11}$x7:BBM:ΔvirF*). The GFP$_{11}$-BBM-ΔVirF was expressed from the protein translocation vector and NLS-GFP$_{1-10}$ was expressed from a T-DNA in the plant cell. Scale bars indicate 50 µm. AF: autofluorescence. (B) Quantification of the intensity of the nuclear GFP signal in tobacco mesophyll cells after AMPT of a non-optimized fusion protein (GFP$_{11}$:BBM^{mk}), a bacterial codon-optimized fusion protein (GFP$_{11}$:BBM) and a bacterial codon optimized fusion protein with GFP$_{11}$ multimerization (GFP$_{11}$x7:BBM). For each treatment 36 nuclei were measured in images taken from the 3th, 4th and 5th leaf of 12 tobacco plants. The dots indicate the fluorescence intensity per nucleus. Different letters indicate statistically significant differences ($p < 0.001$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test.

Testing the split-Cherry reporter for AMPT to plant cells

Our aim was to use AMPT for the simultaneous translocation of the regeneration enhancing transcription factors BBM and WUS. This required the use of a second split-fluorophore system to be able to detect AMPT of both proteins. Previously, a split-fluorophore system was reported in animal systems using Cherry (Nguyen et

al., 2013) and it was shown that it can be simultaneously used with the split-GFP system as the fluorophore fragments are not able to cross-associate (Feng et al., 2017; Kamiyama et al., 2016a). As a first approach to test the use of split-Cherry in plants, the *NLS:GFP* coding region on a positive control T-DNA construct was replaced by that of *NLS:Cherry* (Fig. 2A, Appendix 1c), codon optimized for plant expression (Puigbò et al., 2007) and containing an intron at the same relative position as in GFP (Haseloff et al., 1997) (Appendix 1b). Clear Cherry fluorescence, both nuclear and cytoplasmic, was observed in epidermis cells of 4-weeks old tobacco leaves at 4 dpi with an Agrobacterium strain containing the *p35S::NLS:Cherry::tNOS* construct, indicating that Cherry is a suitable reporter in plant cells (Fig. 2B).

The next step was to test the reconstitution of the split-Cherry parts in plant cells. Therefore, the GFP_{1-10} coding region on the T-DNA transfer construct of the split-GFP system was replaced by the $Cherry_{1-10}$ sequence. On the same T-DNA the $Cherry_{11}$*:WUS:ΔvirF* coding region was cloned behind a second *35S* promoter (Fig. 2C). Following infiltration of tobacco leaves with an Agrobacterium strain containing the resulting construct, clear nuclear and cytosolic Cherry fluorescence could be detected at 4 dpi in leaf epidermis cells (Fig. 2D). These results show that also the codon optimized split-Cherry system is functional in plants, at least when both components are expressed from a single T-DNA.

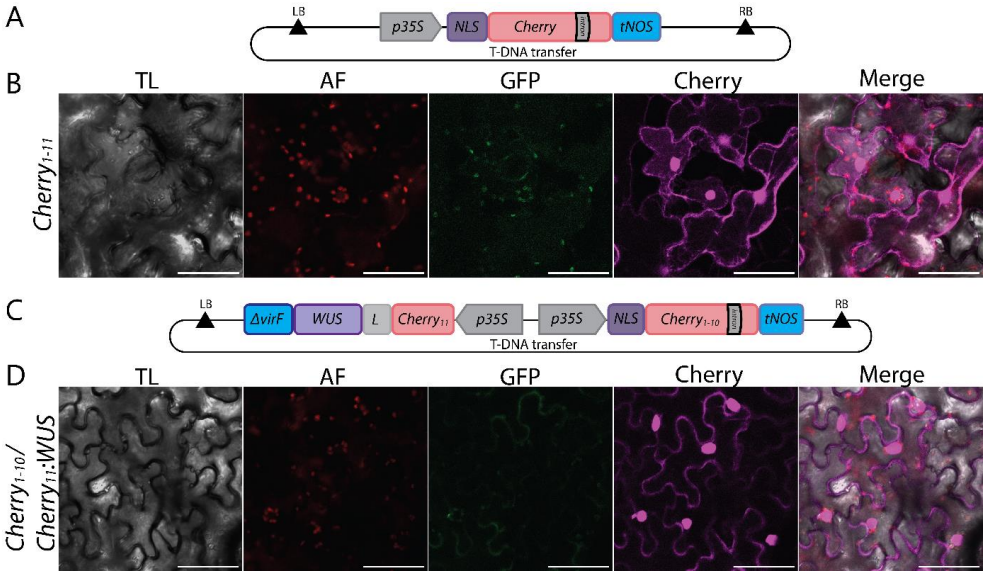


Figure 2. The Cherry fluorophore-based split system can be used as a reporter in plant cells. (A, C) T-DNA constructs *p35S::NLS:Cherry::tNOS* (A) and *p35S::NLS:Cherry$_{1-10}$::tNOS/p35S::Cherry$_{11}$:WUS:ΔvirF::tNOS* (C) to test the use of the split-Cherry system in plant cells. (B, D) Confocal microscopy images showing Cherry fluorescence at 4 dpi in leaf epidermis cells of 4-weeks old tobacco plants after AMT of a T-DNA expressing full-length Cherry (A, B) or the split-Cherry system (C,D). Scale bars indicate 50 µm. Abbreviations: TL, transmitted light; AF, autofluorescence; p35S, *Cauliflower Mosaic Virus 35S* promoter; NLS, nuclear localization signal; tNOS, nopaline synthase transcriptional terminator; L, Linker sequence coding for 9 amino acids connecting the fluorophore$_{11}$ tag and the protein of interest (POI); WUS, WUSCHEL; *Δ*VirF, 51 amino acid translocation signal of VirF; LB/RB, left/right T-DNA border.

Strategy for visualization of simultaneous AMPT of two proteins into plant cells

The detection of the simultaneous translocation of two POIs and possibly also different combinations of POIs requires a versatile cloning platform. Although the 7x multimerized GFP$_{11}$ tag significantly enhanced the sensitivity, we decided to continue with the single GFP$_{11}$ or Cherry$_{11}$ tag as it resulted in sufficient fluorescence intensity and we suspected that a 7x tag might affect the functionality of the POI fused to it. We therefore replaced the *GFP$_{11}$:POI:ΔvirF* coding region in the protein translocation plasmid by a synthetic fragment on which the individual

parts were separated by unique restriction sites, allowing easy exchange of plant promoter, POI coding region and *vir* promoter. As additional optimization, a leader (Shine and Dalgarno) sequence was placed before the ATG of the GFP_{11} for improved translation and a linker sequence coding for 9 amino acids was placed between the GFP_{11} tag and the region coding for the POI to minimize the chance that it would affect the functionality of the POI (Fig. 3A, Appendix 1a). This construct together with the previously plant optimized T-DNA construct carrying *p35S::NLS:GFP$_{1-10}$::tNOS* created the optimized split-GFP construct (split-GFP). For AMPT of a second protein, a synthetic fragment containing the same leader sequence upstream of a bacterium-optimized coding region for *Cherry$_{11}$:POI:ΔvirF* was cloned downstream of the *GFP$_{11}$:POI:ΔvirF* coding region. Also here a linker sequence was added connecting the $Cherry_{11}$ and POI coding region (Appendix 1d). This generated a polycistronic operon where transcription from a *vir* gene promoter resulted in the production of a single RNA that is subsequently translated into two fusion proteins (Fig. 3B). For modulation of the ratio of expression of the two POI fusions, the positioning of the POI coding region inside the operon can be switched. The so-called transcription distance dictates that the open reading frame closer to the transcription start will be expressed at a higher level, because there is more time for translation (Lim et al., 2011). The presence of several unique restriction enzyme sites allows easy exchange of coding regions and *vir* promoters. We named this the double split fluorophore (ds-FP) system (Fig. 3B). The unique *Xma*I and *Bam*HI restriction sites also allowed to add a second *vir* promoter depending on the experimental needs, thus creating two monocistronic operons, each with their own *vir* promoter (Fig. 3C). The *p35S::NLS:Cherry$_{1-10}$::tNOS* sequence was added to the T-DNA construct for detection of AMPT of $Cherry_{11}$-fused proteins (Fig. 3D).

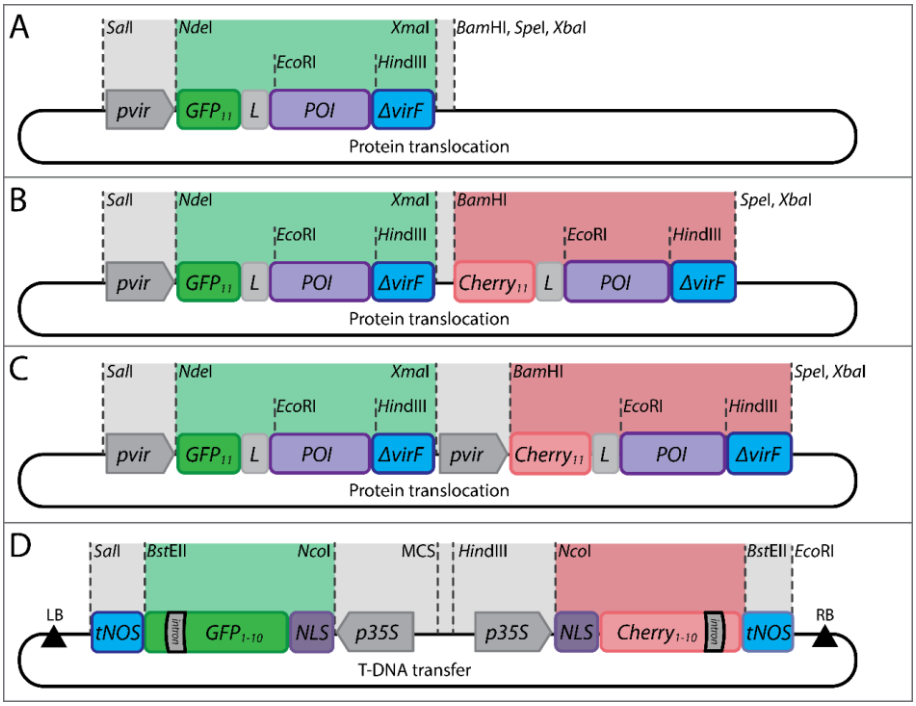


Figure 3. Vector design for the optimized split-GFP system and the ds-FP system. (A-D) All vectors were designed with unique restriction sites allowing easy exchange of individual components. (A) Protein translocation vector of the optimized split-GFP system with a coding region optimized for bacterial translation coding for GFP_{11}:POI:ΔVirF fusion protein expressed from a *vir* promoter (*pvir*). (B) Protein translocation vector for the ds-PF system with a *vir* promoter producing a polycistronic mRNA coding for the GFP_{11}:POI:ΔVirF and $Cherry_{11}$:POI:ΔVirF fusion proteins. (C) The protein translocation vector of the ds-FP system where the regions coding for the GFP_{11}:POI:ΔVirF and $Cherry_{11}$:POI:ΔVirF fusion proteins are transcribed from separate *vir* promoters. (D) T-DNA transfer vector used for both the split-GFP and ds-FP systems, containing a T-DNA carrying the *p35S::NLS:GFP$_{1-10}$::tNOS* and *p35S::NLS:Cherry$_{1-10}$::tNOS* genes to report AMPT of respectively GFP_{11}- or $Cherry_{11}$-tagged fusion proteins. The *NLS:GFP$_{1-10}$* and *NLS:Cherry$_{1-10}$* coding regions are codon-optimized for expression in plants and equipped with an intron to abolish expression in bacteria. Abbreviations: GFP, green fluorescent protein; tNOS, *Δ*VirF, 51 amino acid translocation signal of VirF; nopaline synthase transcriptional terminator; L, Linker sequence coding for 9 amino acids connecting the $fluorophore_{11}$ tag and the protein of interest (POI); p35S, *Cauliflower Mosaic Virus 35S* promoter; NLS, nuclear localization signal; LB/RB, left/right T-DNA border; MCS, multi cloning site.

Testing the ds-FP system for simultaneous AMPT of WUS and BBM to plant cells

As a first test of our newly designed ds-FP system, we infiltrated 4-weeks old tobacco leaves with an Agrobacterium strain containing the protein translocation vector *pvirF::GFP$_{11}$:WUS:ΔvirF* with bacterial codon optimized WUS (Appendix 1f) and the T-DNA construct *p35S::NLS:GFP$_{1-10}$::tNOS/p35S::NLS:Cherry$_{1-10}$::tNOS* to report AMPT of the GFP$_{11}$-tagged fusion protein (Fig. 4A). Clear nuclear GFP fluorescence was detected at 4 dpi (Fig. 4B), indicating that the split-GFP reporter of the new ds-FP system successfully detected AMPT. Next, we tested both split-GFP and split-Cherry reporters in combination with the polycistronic vector for expression in Agrobacterium. Tobacco leaves were infiltrated with an Agrobacterium strain containing the polycistronic *pvirF*::*GFP$_{11}$:WUS:ΔvirF-Cherry$_{11}$:BBM:ΔvirF* protein translocation vector and the *p35S::NLS:GFP$_{1-10}$::tNOS/p35S::NLS:Cherry$_{1-10}$::tNOS* T-DNA AMPT reporter construct (Fig. 4C). At 4 dpi again clear nuclear GFP fluorescence was detected, however, no Cherry fluorescence was observed (Fig. 4D). Introduction of the *virD* promoter in front of the *Cherry$_{11}$:BBM:ΔvirF* coding region also did not result in detectable Cherry fluorescence, whereas AMPT of the GFP$_{11}$:WUS:ΔVirF fusion protein still resulted in nuclear GFP signal (Fig. S1A, S1B). To rule out design problems with the ds-FP system, a single split-Cherry system was constructed by replacing *pvirF::GFP$_{11}$:WUS:ΔvirF* in the protein translocation vector by *pvirF::Cherry$_{11}$:WUS:ΔvirF* and replacing *p35S::NLS:GFP$_{1-10}$::tNOS* in the T-DNA transfer vector by *p35S::NLS:Cherry$_{1-10}$::tNOS* (Fig. S1C). Infiltrating tobacco leaves with an Agrobacterium strain carrying the resulting single split-Cherry system did not result in detectable Cherry fluorescence at 4 dpi (Fig. S1D). The very bright Cherry fluorescence obtained when both split-Cherry components, *Cherry$_{11}$:WUS:ΔvirF* and *NLS:Cherry$_{1-10}$*, are expressed from the *35S* promoter (Figure 2A, B) suggests that the Cherry$_{11}$ tag somehow prevents translocation of the fusion protein to the plant cell. Interestingly, the GFP fluorescence observed from the ds-FP system was significantly (1.4-fold) higher compared to the split-GFP system (Fig 4E). Somehow *GFP$_{11}$:WUS:ΔvirF* expression from the polycistronic operon is more efficient than from the monocistronic operon.

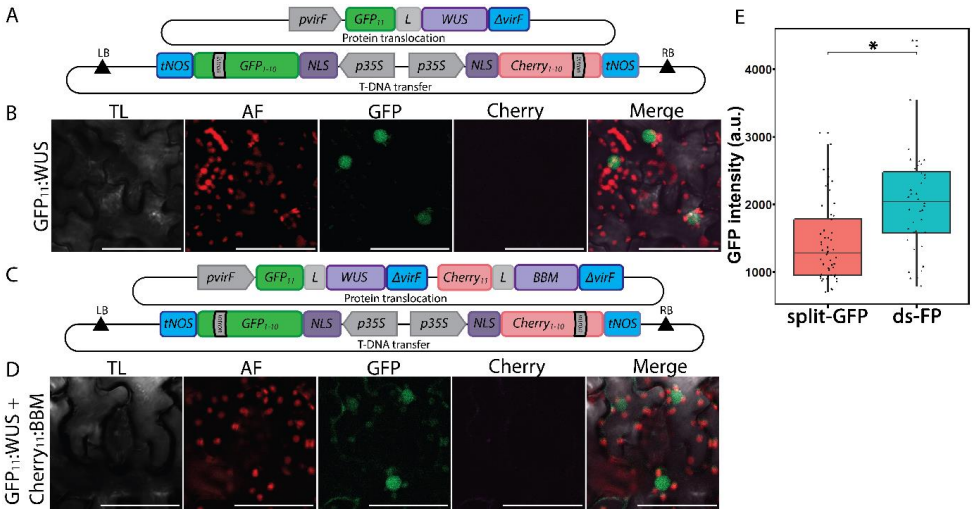


Figure 4. The ds-FP system detects AMPT of GFP_{11}-tagged but not of $Cherry_{11}$-tagged proteins. (A, C) The ds-FP system with the protein translocation construct coding for the GFP_{11}:WUS:ΔVirF fusion (split-GFP, A) or for both the GFP_{11}:WUS:ΔVirF and the $Cherry_{11}$:BBM:GFP_{11}:WUS:ΔVirF fusion (ds-FP, C). Both T-DNA transfer constructs express NLS:GFP_{1-10} and NLS:$Cherry_{1-10}$ from the *35S* promoter. See also Figure 3 for further information. (B, D) Confocal microscopy images of leaf epidermis cells of 4-weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying the split-GFP system depicted in (A) or the ds-FP system depicted in (C). Scale bars indicate 50 µm. Abbreviations: TL, transmitted light; AF, autofluorescence; WUS, WUSCHEL; BBM, BABY BOOM. (E) Quantification of the intensity of the nuclear GFP signal in tobacco mesophyll cells after AMPT using the split-GFP (B) or the ds-FP (D) system. For each treatment 50 nuclei were measured in 18 images taken from the 3th, 4th and 5th leaf of six tobacco plants. The dots indicate the fluorescence intensity per nucleus. The statistically significant difference is indicated above boxplots (*: $p < 0.05$) as determined by the Student's *t*-test with Tukey's honest significant difference post hoc test.

Use of split GFP and Cherry for detection of simultaneous AMPT and AMT to plant cells

Although the split Cherry system appeared unsuitable as reporter for AMPT, our results did show that the Cherry reporter is a good marker to detect T-DNA transfer (Fig. 2B). We therefore decided to use it in combination with the split GFP reporter for the simultaneous detection of respectively AMT and AMPT (referred to as the

split-GFP^{col} system), allowing to compare the efficiencies of the two processes, not only in tobacco, but also in plant species or genotypes that are more recalcitrant to transformation. The transient Cherry expression following T-DNA transfer can also be used as a positive control for successful leaf infiltration and activation of the Agrobacterium *vir* system by the host cells. This is important, as many economically important crop plants commonly used in various laboratory experiments show recalcitrance to AMT. In laboratory experiments, the tomato cultivar 'Moneymaker' is popular but shows low leaf transformation efficiency (Hoshikawa et al., 2019) and subsequent regeneration proves laborious (Eck et al., 2019). Plant defense responses against Agrobacterium were reported to contribute significantly to limit or completely inhibit AMT (Pitzschke, 2013).

For the simultaneous detection of AMT and AMPT, the T-DNA transfer vector was equipped with the optimized *NLS:GFP$_{1-10}$* and *NLS:Cherry* coding regions, both expressed under control of the *35S* promoter (Fig. 5A) and the protein translocation vector carrying *pvirF::GFP$_{11}$:WUS:ΔvirF* was used (Fig. 5A). An Agrobacterium strain containing this split-GFP^{col} system was used to infiltrate leaves of 4 weeks old plants of tobacco and of the crop species tomato (*Solanum lycopersicum* cv. 'Money Maker'), pepper (*Capsicum annuum* cv. 'jalapeño') and rapeseed (*Brassica napus subsp. oleifera)*. As observed previously (Fig. 2B and 2D), tobacco leaf epidermis cells showed a strong Cherry signal, marking cells transformed with the T-DNA construct. As previously observed, the GFP signal observed in the nucleus of the same cells was weaker and even absent in some cells that were marked by a clear Cherry signal. Assuming that T-DNA transfer always coincides with protein translocation, this indicates that AMPT occasionally is not detected because the number of translocated fusion proteins is too low, and that despite the improved split-GFP system this results in an underestimation of the frequency of AMPT. In tomato leaves also clear signals were observed for both AMT and AMPT, however in leaves of sweet pepper and rapeseed the Cherry and GFP signals were significantly weaker (Fig. 5B). These results show that the split-GFP^{col} system can be used in varieties of common crop plant species to report simultaneous AMPT and AMT, and thus may provide an useful tool to analyze and resolve bottle necks in transformation and regeneration.

The model plant *Arabidopsis thaliana* is also considered recalcitrant for transient transformation assays, limiting its use for rapid studies of *in planta* protein localization and interaction. Leaf infiltration protocols for *Arabidopsis* have been optimized to include prolonged induction of Agrobacterium with acetosyringone (Mangano et al., 1998), different Agrobacterium strains (Wroblewski et al., 2005) or infiltrating higher bacterial concentrations into leaves (Y. Zhang et al., 2020). However, the use of Arabidopsis cell suspension cultures has until now been limited to protoplast isolation and subsequent chemical transformation. Here we tested the split-GFP^{col} system on Arabidopsis cell suspensions and to our surprise we detected clear nuclear split-GFP signals, indicative of AMPT, co-localizing with nuclear Cherry signals indicative of AMT (Fig. S2). These results show that the split-GFP^{col} system can be used for the detection of AMPT and AMT in both leaves of different plant species (tobacco, tomato, pepper and rapeseed) and in Arabidopsis suspension cells.

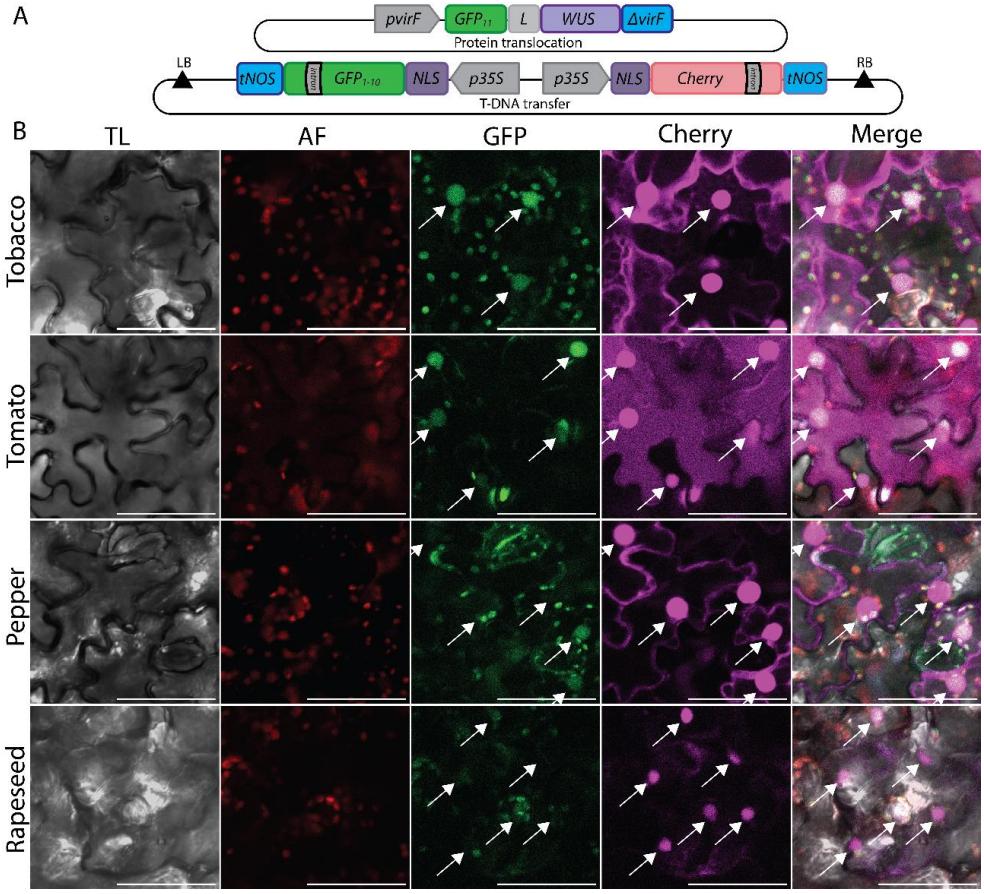


Figure 5. The use of split-GFP and Cherry (split-GFP^{col}) to detect simultaneous AMPT and AMT, respectively. (A) Schematic representation of the combined AMPT/AMT detection system split-GFP^{col}. The system comprises a T-DNA transfer vector containing the optimized *NLS:GFP$_{1-10}$* and *NLS:Cherry* coding regions, both expressed from the *35S* promoter. The protein transfer vector encodes a GFP$_{11}$-WUS-ΔVirF fusion protein expressed from the *virF* promoter. See Figure 3 for further information. (B) Confocal microscopy images of leaf epidermis cells of 4-weeks old plants of the indicated plant species. Arrows indicate co-localized GFP and Cherry fluorescence in the nucleus, indicative of simultaneous AMPT and AMT. Scale bars indicate 50 µm. Abbreviations: TL, transmitted light; AF, autofluorescence.

Discussion

In this study, the split-GFP system previously developed to visualize AMPT in plants was optimized for better translational efficiency of the individual components in

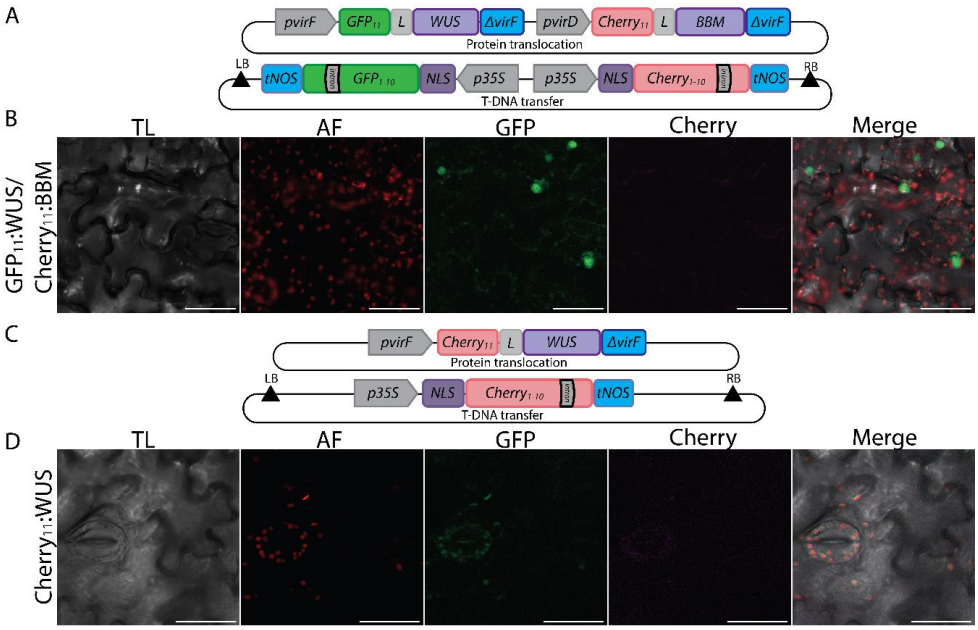
either Agrobacterium (GFP_{11} fusion protein) or plant cells (GFP_{1-10}). This resulted in significantly higher GFP fluorescence intensity and thus increased the sensitivity of AMPT detection, allowing to reduce the laser power of the confocal microscope, thus preventing photobleaching and phototoxicity (Colin et al., 2022). A further increase in the GFP intensity was achieved by multimerization of the GFP_{11} tag (GFP_{11}x7). As indicated, however, we decided not to use this, as we suspected that a repeated GFP_{11} tag might interfere with the functionality of the POI fused to it, especially when it leads to reconstitution of multiple GFPs.

To visualize AMPT of two POIs either tagged with either GFP_{11} or $Cherry_{11}$, an additional split fluorophore system (split-Cherry) was added to this optimized split-GFP system. In order to express two fluorophore-tagged proteins from a single plasmid, we either placed both coding regions in a single operon expressed from the same *vir* promoter or in two separate operons, each expressed from its own *vir* promoter. The single operon construct gave sufficient expression to detect AMPT of GFP_{11}-tagged WUS and interestingly the fluorescence was significantly higher than when the GFP_{11}-tagged WUS proteins was expressed from a monocistronic operon. This confirmed the observations in *E. coli* where increasing the operon length resulted in increased expression (Lim et al., 2011). Nonetheless, we were not able to detect AMPT of the $Cherry_{11}$-BBM fusion, also not when expressed from its own *vir* promoter. This despite the fact that our results clearly showed that the split-Cherry system works in plants when both parts are expressed from the same plasmid. The most likely reason for this is that the $Cherry_{11}$ tag prevents AMPT of the fusion protein. Possibly, the linker length or spatial arrangement of the fusion protein is limiting the transfer through the T4SS pilus. A second reason might be that the sensitivity of the split-Cherry system is insufficient to detect AMPT. Based on the experiments presented in this chapter, we cannot exclude any of these options.

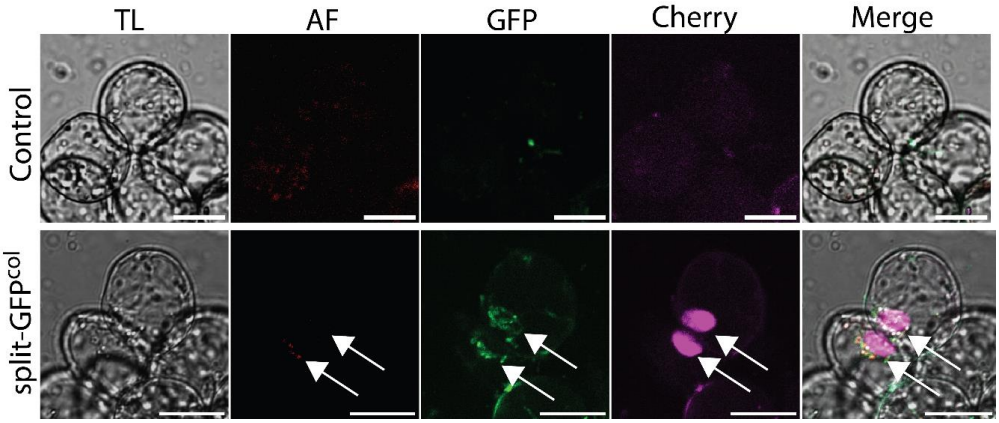
The Cherry fluorophore appeared to be a useful reporter to detect transient expression following AMT. As such, it was used in the split-GFP^{col} system to detect simultaneous AMT and AMPT in different plant species. The potential use of the split-GFP system to detect AMPT has previously been demonstrated in various plant species , such as *N. tabacum, N. benthamiana, Arabidopsis* and tulip (Khan,

2017). Here we confirmed this for tobacco, but also showed that it is possible to detect AMT and AMPT in tomato, pepper, rapeseed and for the first time in Arabidopsis suspension cells. The *Arabidopsis* cell suspension system provides a readily available and continuous supply of close to identical cells, enabling more high-throughput visualization of a variety of fusion proteins and a foundation for upscaling for fusion protein extraction or transient produced compound extraction. Both in previous work as well as in the experiments performed in this chapter, the overexpression of full length GFP or Cherry led to fluorescence observed both in the nucleus as in the cytosol. NLS activity can vary depending on flanking sequences and the target organism (Kosugi et al., 2009). The NLS sequence might be optimized to prevent signal dispersion, but in our case the cytosolic signal was also indicative for the efficiency of AMT, which was higher for tobacco and tomato and lower for pepper and rapeseed, plant species known to be more recalcitrant to AMT. As expected, the GFP fluorescence intensity marking AMPT correlated with the Cherry fluorescence intensity.

Supplementary figures



Supplementary figure 1. The split-Cherry system is not suitable for detecting AMPT. (A) Schematic representation of the ds-FP system where the regions coding for the GFP_{11}:WUS:ΔVirF and $Cherry_{11}$:BBM:ΔVirF fusion proteins are transcribed from respectively the *virF* or *virD* promoter from the protein translocation vector. The T-DNA transfer vector carries *p35S::NLS:GFP$_{1-10}$::tNOS* and *p35S::NLS:Cherry$_{1-10}$::tNOS* to report AMPT of both the GFP_{11}- and $Cherry_{11}$-tagged fusion proteins (B) Confocal microscopy images of leaf epidermis cells of 4-weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying the ds-FP system depicted in (A). (C) Schematic representation of the optimized split-Cherry system with a protein translocation vector coding for the $Cherry_{11}$:WUS:ΔVirF fusion protein expressed from the *virF* promoter and the T-DNA transfer vector carrying *p35S::NLS:Cherry$_{1-10}$::tNOS* to report AMPT of the $Cherry_{11}$-tagged fusion protein. (D) Confocal microscopy images of leaf epidermis cells of 4-weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying the split-Cherry system depicted in (C). Scale bars indicate 50 µm. Abbreviations: TL, transmitted light; AF, autofluorescence.



Supplementary figure 2. The use of split-GFP and Cherry (split-GFP^{col}) to detect simultaneous AMPT and AMT in Arabidopsis suspension cells. Confocal microscopy images of Arabidopsis suspension cells after 4 days of cocultivation with an Agrobacterium strain carrying a control vector (*p35S::NLS:GFP$_{1-10}$::tNOS/p35S::NLS:Cherry$_{1-10}$::tNOS*) or the split-GFP^{col} system (*p35S::NLS:GFP$_{1-10}$::tNOS/p35S::NLS:Cherry::tNOS + pvirF::GFP$_{11}$:WUS:ΔvirF).* Scale bars indicate 50 µm. White arrows indicate the position of GFP and Cherry positive nuclei. Abbreviations: TL, transmitted light; AF, autofluorescence.

Appendix

Appendix 1. (a-f) DNA sequences coding for: (a) empty bacterial codon optimized split-GFP construct, (b) plant codon optimized sfGFP$_{1-11}$, (c) plant codon optimized sfCherry2$_{1-11}$, (d) empty bacterial codon optimized ds-FP cloning construct (*pvir::leader*

sequence:sfGFP$_{11}$:linker:POI:ΔvirF:pvir::leader sequence:sfCherry2$_{11}$:linker:POI:ΔvirF), (e) bacterial codon optimized BBM and (f) bacterial codon optimized WUS. Highlighted are: the NLS sequence in purple, the intron sequence in yellow, the sfCherry2$_{11}$ part in red, the sfGFP$_{11}$ part in green and the linker sequence in grey. Promoter, POI, Leader (Shine & Dalgarno) sequence. start and stop sequences are in bold. Restriction enzyme recognition sites are underscored.

a) GTCGAC**|pVir|AGGAGC**GATCAT**ATG**CGCGACCACATGGTCCTGCACGAATACGTCAACGCC
GCCGGCATCACCGGCGACGGCGGCTCCGGCGGCGGCTCCGAATTC**|POI|**AAGCTTAACGTT
GCGGAACCGATTATGTTCAATGAAATCTCCGCTCTCGAGGTTATGGCAGAAGTTCGGCCCAT
AGCCCGATCCATTAAAACGGCTCACGACGATGCGCGAGCGGAATTAATGTCGGCGGACAGA
CCTCGATCAACGCGCGGTCTA**TGA**CCCGGGACTAGTTCTAGA

b) **ATG**GAGCCTCCTAAGAAGAAGAGGAAGGTTGAGCTGATGGTTTCGAAAGGCGAGGAGCTG
TTCACAGGCGTGGTGCCAATCCTGGTGGAGCTGGACGGCGACGTGAACGGCCACAAATTCA
GCGTGAGAGGCGAGGGCGAGGGCGACGCCACAATCGGCAAACTGACACTGAAATTCATCTG
CACAACAGGCAAACTGCCAGTGCCCTGGCCAACACTAGTGACAACACTGACATACGGCGTGC
AGTGCTTCAGCAGATATCCGGACCACATGAAAAGACACGACTTCTTCAAAAGCGCCATGCCA
GAGGGCTACGTGCAGGAGAGAACAATCAGCTTCAAAGACGACGGCAAATACAAAACAAGA
GCCGTGGTGAAATTCGAGGGCGACACACTGGTGAACAGAATCGAGCTGAAAGGTATGACAA
TTTACTCGAACTTCCTTTTTTAACTCGAACTATGTATATACACAACAACGTTAATAATTAAGTC
GTACTCATTTTGAATCTACTGACTCTAGATCCTGATTCACACATGTAATATAATTGCAGGGCAC
AGACTTCAAAGAGGACGGCAACATCCTGGGCCACAAACTGGAGTACAACTTCAACAGCCACA
ACGTGTACATCACAGCCAACAAACAGAAAAACGGCATCAAAGCCAACTTCACAGTGAGACAC
AACGTGGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACACCAATCGGCG
ACGGCCCAGTGCTGCTGCCAGACAACCACTACCTGAGCACACAGACAGTGCTGAGCAAAGA
CCCAAACGAGAAACGGGACCACATGGTGCTGCACGAGTACGTGAACGCCGCCGGCATCACA
TAA

c) **ATG**GAGCCTCCTAAGAAGAAGAGGAAGGTTGAGCTGGAAGAAGATAATATGGCTATTATTA
AGGAATTTATGAGATTTAAGGTTCATATGGAAGGATCTGTTAATGGACATGAATTTGAAATT
GAAGGAGAAGGAGAAGGACATCCTTATGAAGGAACTCAAACTGCTAAGTTGAAGGTTACTA
AGGGAGGACCTTTGCCTTTTGCTTGGGATATTTTGTCTCCTCAATTTATGTATGGATCTAAGG
CTTATGTTAAGCATCCTGCTGATATTCCTGATTATTTGAAGTTGTCTTTTCCTGAAGGATTTACT
TGGGAAAGAGTTATGAATTTTGAAGATGGAGGAGTTGTTACTGTTACTCAAGATTCTTCTTTG
CAAGATGGACAATTTATTTATAAGGTTAAGTTGTTGGGAATTAATTTTCCTTCTGATGGACCT
GTTATGCAAAAGGTATGACAATTTACTCGAACTTCCTTTTTTAACTCGAACTATGTATATACAC
AACAACGTTAATAATTAAGTCGTACTCATTTTGAATCTACTGACTCTAGATCCTGATTCACACA
TGTAATATAATTGCAGAAGACTATGGGATGGGAAGCTTCTACTGAAAGAATGTATCCTGAAG

ATGGAGCTTTGAAGGGAGAAATTAATCAAAGATTGAAGTTGAAGGATGGAGGACATTATGA
TGCTGAAGTTAAGACTACTTATAAGGCTAAGAAGCCTGTTCAATTGCCTGGAGCTTATAATGT
TGATATTAAGTTGGATATTACTTCTCATAATGAAGATTATACTATTGTTGAACAATATGAAAG
AGCTGAAGCTAGACATTCTACT**TAA**

d) GTCGAC**|pVir|AGGAGC**GATCAT**ATG**CGCGACCACATGGTCCTGCACGAATACGTCAACGCC
GCCGGCATCACCGGCGACGGCGGCTCCGGCGGCGGCTCCGAATTC**|GOI|**AAGCTTAACGTT
GCGGAACCGATTATGTTCAATGAAATCTCCGCTCTCGAGGTTATGGCAGAAGTTCGGCCCAT
AGCCCGATCCATTAAAACGGCTCACGACGATGCGCGAGCGGAATTAATGTCGGCGGACAGA
CCTCGATCAACGCGCGGTCTA**TGA**CCCGGG**(pVir/)AGGAGC**GATGGATCC**ATG**TACACCATC
GTCGAACAGTACGAACGCGCCGAAGCCCGCCACTCCACCGGCGACGGCGGCTCCGGCGGCG
GCTCCGAATTC**|GOI|**AAGCTTAACGTTGCGGAACCGATTATGTTCAATGAAATCTCCGCTCTC
GAGGTTATGGCAGAAGTTCGGCCCATAGCCCGATCCATTAAAACGGCTCACGACGATGCGCG
AGCGGAATTAATGTCGGCGGACAGACCTCGATCAACGCGCGGTCTA**TGA**ACTAGTTCTAGA

e) GAATTCAACAACAACTGGCTGGGCTTCTCCCTGTCCCCGTACGAACAGAACCACCACCGCAA
GGACGTCTGCTCCTCCACCACCACCACCGCCGTTGACGTCGCCGGCGAATACTGCTACGACCC
GACCGCCGCCTCCGACGAATCCTCCGCCATCCAGACCTCCTTCCCGTCCCCGTTCGGCGTCGT
CCTGGACGCCTTCACCCGCGACAACAACTCCCACTCCCGCGACTGGGACATCAACGGCTCCG
CCTGCAACAACATCCACAACGACGAACAGGACGGCCCGAAGCTGGAAAACTTCCTGGGCCG
CACCACCACCATCTACAACACCAACGAAAACGTCGGCGACATCGACGGCTCCGGCTGCTACG
GCGGCGGCGACGGCGGCGGCGGCTCCCTGGGCCTGTCCATGATCAAGACCTGGCTGCGCAA
CCAGCCGGTTGACAACGTTGACAACCAGGAAAACGGCAACGGCGCCAAGGGCCTGTCCCTG
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GGCAAGACCATCGACGACTCCGTCGAAGCCACCCCGAAGAAGACCATCGAATCCTTCGGCCA
GCGCACCTCCATCTACCGCGGCGTCACCCGCCACCGCTGGACCGGCCGCTACGAAGCCCACC
TGTGGGACAACTCCTGCAAGCGCGAAGGCCAGACCCGCAAGGGCCGCCAGGTCTACCTGGG
CGGCTACGACAAGGAAGAAAAGGCCGCCCGCGCCTACGACCTGGCCGCCCTGAAGTACTGG
GGCACCACCACCACCACCAACTTCCCGATGTCCGAATACGAAAAGGAAATCGAAGAAATGAA
GCACATGACCCGCCAGGAATACGTCGCCTCCCTGCGCCGCAAGTCCTCCGGCTTCTCCCGCG
GCGCCTCCATCTACCGCGGCGTCACCCGCCACCACCAGCACGGCCGCTGGCAGGCCCGCATC
GGCCGCGTCGCCGGCAACAAGGACCTGTACCTGGGCACCTTCGGCACCCAGGAAGAAGCCG
CCGAAGCCTACGACATCGCCGCCATCAAGTTCCGCGGCCTGACCGCCGTCACCAACTTCGAC
ATGAACCGCTACAACGTCAAGGCCATCCTGGAATCCCCGTCCCTGCCGATCGGCTCCGCCGCC
AAGCGCCTGAAGGAAGCCAACCGCCCGGTCCCGTCCATGATGATGATCTCCAACAACGTCTC
CGAATCCGAAAACAACGCCTCCGGCTGGCAGAACGCCGCCGTCCAGCACCACCAGGGCGTT
GACCTGTCCCTGCTGCAGCAGCACCAGGAACGCTACAACGGCTACTACTACAACGGCGGCAA
CCTGTCCTCCGAATCCGCCCGCGCCTGCTTCAAGCAGGAAGACGACCAGCACCACTTCCTGTC
CAACACCCAGTCCCTGATGACCAACATCGACCACCAGTCCTCCGTCTCCGACGACTCCGTCAC
CGTCTGCGGCAACGTCGTCGGCTACGGCGGCTACCAGGGCTTCGCCGCCCCGGTCAACTGCG
ACGCCTACGCCGCCTCCGAGTTCGACTACAACGCCCGCAACCACTACTACTTCGCCCAGCAGC

AGCAGACCCAGCACTCCCCAGGCGGCGACTTCCCGGCCGCCATGACCAACAACGTCGGCTCC
AACATGTACTACCACGGCGAAGGCGGCGGCGAAGTCGCCCCGACCTTCACCGTCTGGAACG
ACAACAAGCTT

f) AAGCTTGTTCAGGCGCAGTTCCAGGGAGGCGCACGGGCGGACTTCGGACTGGCCGTACTTC
CAGATGGCGCCGGAGCCGCCGTTGATGTGGTCTTCGCCGTGCATCGGGAACAGCGGCAGGG
TGCGGCGGTGTTCCAGGTAGGCGTCGCCGCCGCATTCTTCTTCTTCCTGGTGGCCTTCCAGGC
CGAACAGCGGCTTGGCGCGGTCGAAGAAGTTGTACGGGGCGGAGGAGTAGTGGTGGTCCA
TGTTGGCCCAGCCGCCGCCGACGTTGTTGTAGTTCATGGAGCAGTCCTGTTCCATGGAGCCG
TAGACGTGGGAGGACATGTAGCCGTTGGAGGCGTTGACGACGCCGCATTCGGTGCCGGAGG
AGGCGTGGTTCAGGTTGCCGTTGTTGAAGGACGGGTACGGCTTGTTGTGGTGGTACAGGTG
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TGGTGGTGCAGCAGCGGGTGGTAGTGGTCGTTGGCGGCCATCATGACGGAGTTCGGGGAG
GAGGACGGGGTGGTCATGTTGGTGCCGTTGAAGCGCTTCTTCTGGCGTTCGCGGGCCTTGTG
GTTCTGGAACCAGTAGAAGACGTTCTTGCCTTCGATCTTGCCGAACTGGCGCAGGCGGGCGG
TGATCTTCTGGATCTGGTCGGCGGTCGGGGAGCGGATGGCGTTGTTGTAGTACAGTTCCTTC
AGGATCTTGATCTGTTCGGTGGTCGGGGTCCAGCGGGTGGAGGTCTGGCGGCAGGTGTAGC
CGCCGGAGCCGGACTTGTTGTTGTTGTTGCCGGATTCCTGGTCGGCCTGGTGGTGGTGG
TGCTGGTGCTGCGGCGGTTCGAATTC

Materials and methods

Agrobacterium strains and growth conditions

The Agrobacterium strain AGL1 (C58, *RecA*, pTiBo542 disarmed, Rif, Cb) (Jin et al., 1987) used in this chapter was grown in modified LC medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.5) at 28 ºC with the appropriate antibiotics at the following concentrations: gentamicin 40 µg/ml; carbenicillin 75 µg/ml; kanamycin 100 µg/ml; rifampicin 20 µg/ml. Plasmids were introduced into Agrobacterium by electroporation, as previously described (den Dulk-Ras & Hooykaas, 1995).

Plant growth conditions

The seeds of *Nicotiana tabacum* cv. Petit Havana SR1, *Nicotiana benthamiana, Capsicum annuum cv. 'Jalapeño' (*hot pepper*) and Solanum lycopersicum* cv. 'Money Maker' (tomato) were stratified for seven days on wet soil and germinated

in high humidity under a plastic cover and seedlings were grown in growth chambers at 24 °C, 75 % relative humidity and a 16 hours photoperiod for four weeks.
The seeds of *Brassica napus* (rapeseed) were germinated in high humidity under a plastic cover and seedlings were grown in growth chambers at 21 °C, 50 % relative humidity and a 16 hours photoperiod for four weeks.
The Arabidopsis thaliana 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 subcultured at 7-day intervals. The cell culture medium consisted of a modified B5 medium (Gamborg et al., 1968) with 30 g/L sucrose and 1 µM NAA.

Agrobacterium leaf infiltration and cell suspension co-cultivation

For co-cultivation, a colony of Agrobacterium strain AGL1 with the appropriate plasmids (overview plasmids: Table 1) from a fresh one-week old plate was resuspended in 10 ml LC medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.5) supplemented with the appropriate antibiotics in a 100 ml Erlenmeyer flask and was incubated at 28 °C under continuous shaking (180 rpm) until the culture reached an OD_{600} of 1.0. The bacteria were pelleted by centrifugation in a 50 ml tube (CLS430829, Corning) at 4000 rpm for 20 minutes and resuspended in 20 ml AB minimal medium (Gelvin, 2006) with the appropriate antibiotics and grown overnight at 28 °C under continuous shaking (180 rpm) 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. The bacteria were induced overnight in induction medium in the dark on a rocking shaker at 60 rpm at room temperature. Prior to infiltration, the overnight 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 3^{th}, 4^{th} and 5^{th} leaves of four weeks old plants were infiltrated on the abaxial side using a blunt tipped 5 ml syringe with an induced Agrobacterium culture. After infiltration the plants were covered with

plastic overnight, after which the plastic was removed and the plants were incubated for three more days. Leaf discs obtained from the infiltrated parts of the leaf were placed on a microscopy slide in water, covered with a cover slip and the abaxial side of the leaf observed under the confocal microscope at 4 days post infiltration.
For cell suspension co-cultivation, five days after subculture 1.5 ml of *Arabidopsis* cell suspension was transferred to a 6-wells plate and 1.5 ml of induced Agrobacterium culture was added to a final concentration of OD_{600} = 0.4. After 36 hours under normal growth conditions the co-cultivation medium was replaced by fresh cell culture medium with 250 µg/L Timentin. The suspension cells were visualized using confocal microscopy at four days after co-cultivation.

Laser scanning confocal microscopy

Fluorescence was observed using a Zeiss Imager M1 or a Zeiss observer (Zeiss, Oberkochen, Germany) confocal laser scanning microscope, equipped with an LSM 5 Exciter using a 20x and 40x magnifying objective (numerical aperture of 0.8 and 0.65, respectively). GFP signal was detected using an argon 488 nm laser and a 505-530 nm band-pass emission filter. Chloroplast- and other auto-fluorescence was detected using an argon 488 nm laser and a 650 nm long pass emission filter. Cherry signal was detected using a 561 nm Diode laser and a 595 – 500 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). The GFP or Cherry fluorescence intensity was measured in ImageJ.

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).

For the T-DNA transfer construct, a modified version of the plasmid pSDM3764 (Khan, 2017), originating from pCambia1302, a derivative of the pPZP family of binary plasmids (Hajdukiewicz et al., 1994), was used. The pSDM3764 plasmid harbours a GFP_{1-10} sequence under control of the *Cauliflower Mosaic Virus 35S* promoter (*p35S*) and the terminator of the *nopaline synthase* gene (*tNOS*) (Sakalis et al. 2013). To engineer the optimized split-GFP construct, the NLS:GFP_{1-10} sequence of the T-DNA plasmid, pSDM3764, was replaced by restriction enzyme digestion with *Nco*I and *Bst*EII with a plant codon optimized *NLS:*GFP_{1-10}^{opt} synthetic sequence (Bio Basic inc., Canada) containing an 84 nucleotide intron IV sequence of the potato *ST-LS1* gene (Pang et al., 1996) (Appendix 1b). To engineer the double-split fluorophore (ds-FP) system, the optimized split-GFP plasmid was digested with *Bam*HI and *Eco*RI and a synthetic sequence coding for NLS:sfCherry2_{1-10}^{opt} (Appendix. 1c) driven by *p35S* and terminated by *tNOS* was inserted. To construct the AMT and AMPT co-localization construct instead of NLS:sfCherry2_{1-10}^{opt} a synthetic sequence coding for NLS:sfCherry2^{opt} was inserted into the ds-FP T-DNA transfer construct using the *Bam*HI and *Eco*RI restriction sites.

The protein translocation vector was based on pSDM6503 (Khan, 2017), a modified version of the plasmid pSDM3163 (Sakalis et al., 2014a). Plasmid pSDM6503 harbours a coding region consisting of an *AHL15* sequence N-terminally tagged via a 27 bp linker sequence to GFP_{11} and C-terminally to *Δvir*F, under control of the *virF* promoter (Khan, 2017). To engineer the optimized split-GFP construct (split-GFP^{opt}), the open reading frame and adjacent multicloning site were removed by digesting the vector with *Nde*I and *Xba*I, and inserting a compatible synthetic DNA fragment coding for bacterial codon optimized GFP_{11}^{opt}*:Linker:ΔvirF* (Appendix 1a) and with a leader sequence (L) containing a Shine-Dalgarno sequence (AGGAGC) preceding the translation initiation start site (ATG) at a previously determined optimal seven base pairs distance (Shultzaberger et al., 2001) (Fig 3A). The resulting construct (split-GFP^{opt}) was used to insert any gene of interest, bacterial codon optimized, using the restriction enzymes *Eco*RI and *Hin*dIII. To construct the double-split fluorophore system (ds-FP), the split-GFP^{opt} vector was digested with *Xma*I and *Spe*I. A synthetic

DNA sequence was inserted coding for bacterial codon optimized *sfCherry2*$_{11}^{opt}$*:Linker:ΔvirF*. The fragment contains a leader sequence on which a Shine-Dalgarno sequence (Shine & Dalgarno, 1974) had been placed, thereby creating a polycistronic construct driven by one promoter (Fig. 3B, Appendix 1d). To create a ds-FP with each fluorophore driven by a separate promoter, the construct was digested with the restriction enzymes *Xma*I and *Bam*HI to insert a PCR fragment with *Xma*I and *Bam*HI restriction sites containing the *virD* promoter in front of the *sfCherry2*$_{11}^{opt}$*:LinkerPGOI:ΔvirF* sequence (Fig. 3C).

Table 1. Plasmids and their combinations used in this study. Km^r = Kanamycin A Gm^r = Gentamycin. In the main text sfCherry2 is referred to as Cherry and the optimized superscript (^{opt}) is omitted.

Plasmid content	**Function**	**Source**
p35S::NLS:GFP$_{1\text{-}10}$*::tNOS / pNOS::Hyg*	T-DNA transfer (Km^r)	Khan, 2017
p35S::NLS:GFP$_{1\text{-}10}^{opt}$*::tNOS / pNOS::Hyg*	T-DNA transfer (Km^r)	Chapter 2
p35S::NLS:sfCherry$_{1\text{-}10}^{opt}$*::tNOS / pNOS::Hyg*	T-DNA transfer (Km^r)	Chapter 2
p35S::NLS:GFP$_{1\text{-}10}^{opt}$*::tNOS / p35S::NLS:sfCherry*$_{1\text{-}10}^{opt}$*::tNOS / pNOS::Hyg*	T-DNA transfer (Km^r)	Chapter 2
p35S::NLS:GFP$_{1\text{-}10}^{opt}$*::tNOS / p35S::NLS:sfCherry-*^{opt}*::tNOS / pNOS::Hyg*	T-DNA transfer (Km^r)	Chapter 2
pvirF::GFP$_{11}$*:BBM:ΔvirF*	Protein translocation (Gm^r)	Khan, 2017
pvirF::GFP$_{11}^{opt}$*:BBM*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2
pvirF::GFP$_{11}^{opt}$*:WUS*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2
pvirF::GFP$_{11}^{opt}$*:WUS*^{opt}*:ΔvirF:sfCherry2*$_{11}^{opt}$*:BBM*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2
pvirF::GFP$_{11}^{opt}$*:WUS*^{opt}*:ΔvirF:pVirD::sfCherry2*$_{11}^{opt}$*:BBM*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2
pvirF::sfCherry2$_{11}^{opt}$*:WUS*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2
pvirF::GFP$_{11}$*x7*^{opt}*:BBM*^{opt}*:ΔvirF*	Protein translocation (Gm^r)	Chapter 2

Table 2. Overview of primers used in this study

Primer name	Sequence
BamHI- ΔVirF -OPT Fw	**GGATCC**TCATAGACCGCGCGTTGA
NdeI-GFP11-OPT Rev	**CATATG**CGCGACCACATGGTCCTG
SalI pVirD Fw	**GTCGAC**AAACGGAGTGCATTTGTATTTTTG
SalI pVirF Fw	**GTCGAC**CCTATGATAGTCGATATTTTGGTCCG
SalI pVirE Fw	**GTCGAC**CGGCTGCTCGTCACCAACAA
NdeI pVirD Rev	**CATATG**CTTCCTCCAAAAAAAGCGGAAG
NdeI pVirE Rev	**CATATG**TTCTCTCCTGCAAAATTGCGGTTT
NdeI-pVirF Rev	**CATATG**ATCGCTCCTGTGCTTTTGAAAG
GFP11x7 Fw opt	CATATGCGCGACCACATGGTC
GFP11x7 Rev opt	GAATTC GGAGCCGCCGCC
HindIII 35S Cherry	**CCCAAGCTTCATGGAGTCAAAGATTCAAAT**
EcoRI NOS Cherry	CCG**GAATTC**CCCGATCTAGTAACATAGATGAC
NdeI SfCherry11	G**GAATTCCATATG**ATGTACACCATCGTCGAACAG
EcoRI SfCherry11	G**GAATTC**GGAGCCGCCGCCG
pSDM6500 Seq Fw	GTGATCATTTGCAGTATTCG
pSDM6500 Seq Rev	CAAGGCGATTAAGTTGGGTAA
pCambia1300 Seq Fw	CGTATGTTGTGTGGAATTGTGAGC
pCambia1300 Seq Rev	CACGGGGGACTCTTGACCATG

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Author contribution

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

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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.

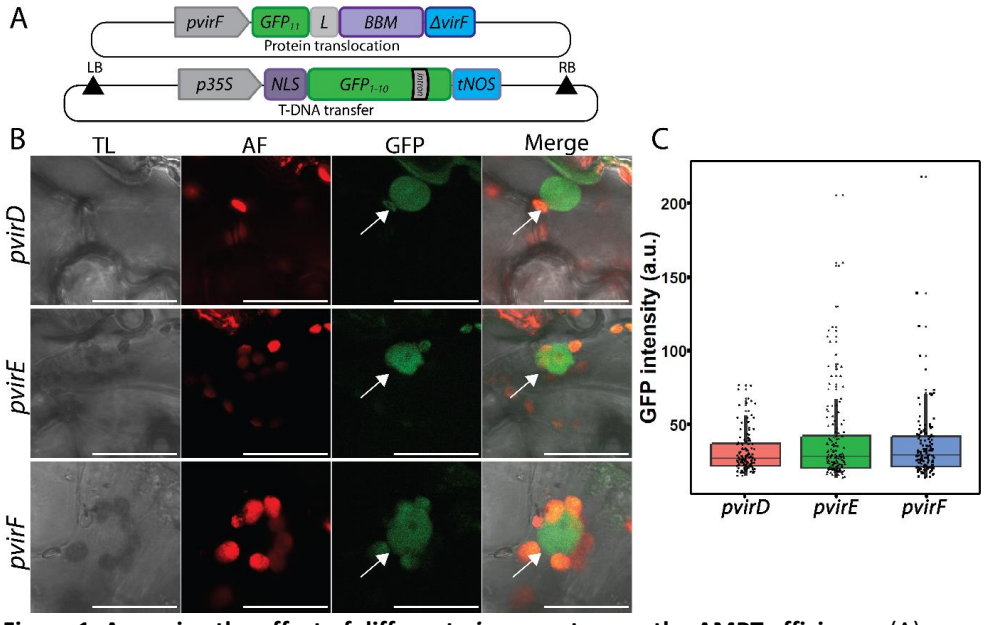

Figure 1. Assessing the effect of different *vir* promoters on the AMPT efficiency. (A) Schematic representation of the split-GFP^{mk} system with a protein translocation vector

coding for the GFP_{11}:BBM:ΔVirF fusion protein expressed from the *pvirF* promoter and the T-DNA transfer vector carrying *p35S::NLS:GFP$_{1-10}$::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$_{1-10}$::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_{1-10} 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_{1-10} 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.

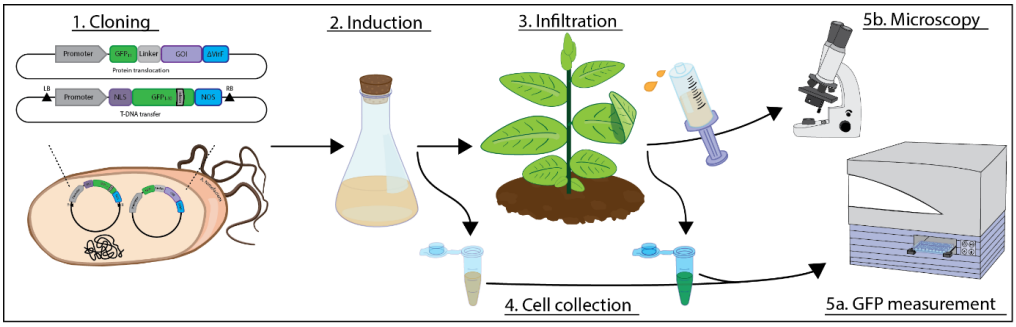

Figure 2. Schematic representation of the workflow for detection of fluorescent proteins in Agrobacterium or plant cells during leaf infiltration experiments. The appropriate constructs for fluorescent protein expression are transformed into Agrobacterium (1). The Agrobacterium cultures are induced either 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.

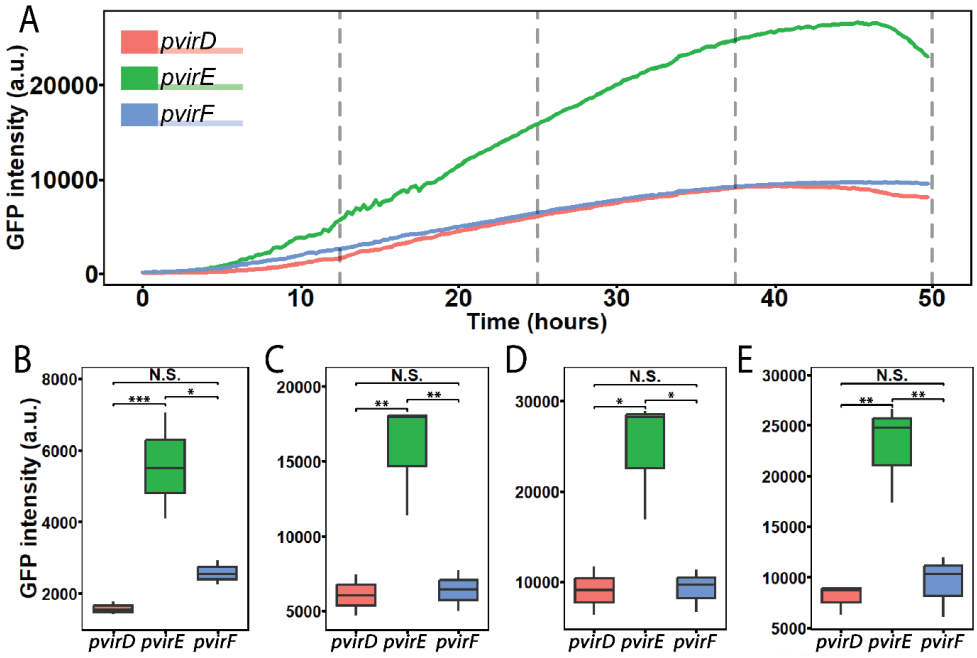

Figure 3. Relative strength of three *vir* promoters based on GFP fluorescence expressed in Agrobacterium following acetosyringone induction. (A) The GFP expression in Agrobacterium measured continuously every 5 minutes in a 96-wells plate reader at room temperature and 180 rpm agitation from start of 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).

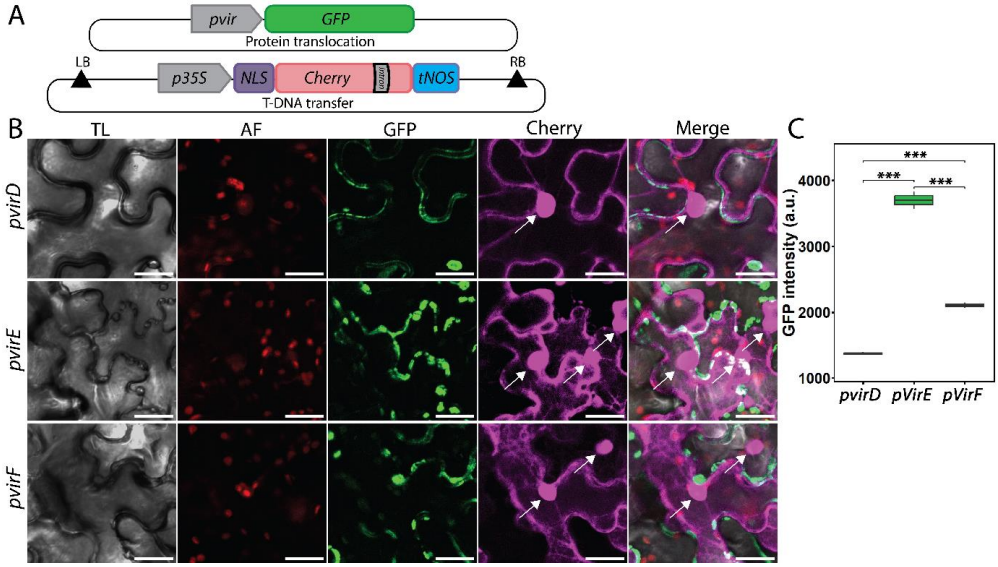

Figure 4. The combined detection of Agrobacterium *vir* gene induction and T-DNA transfer in tobacco leaf cells. (A) Schematic representation of the *vir* promoter-controlled GFP expression in Agrobacterium with a vector coding for GFP expressed from the *virD, virE or virF* promoter and the T-DNA transfer 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.

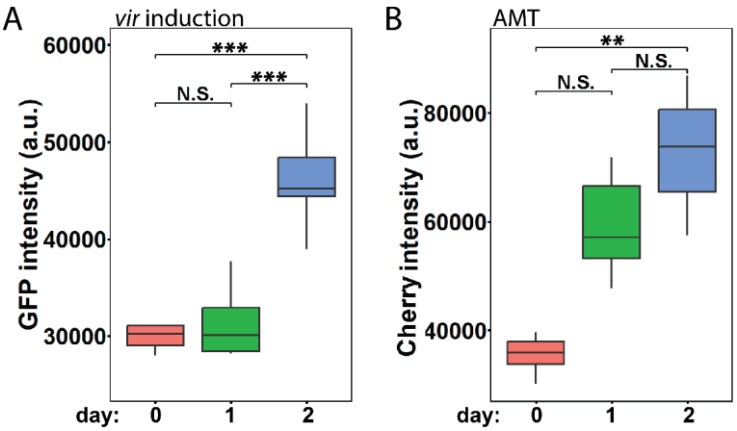

Figure 5. Agrobacterium *vir* gene expression and AMT efficiency increases by prolonged pre-induction with AS. (A, B) Agrobacterium expressing GFP under control of the *virE* promoter (*pvirE::GFP*) and carrying a T-DNA with the *p35S::NLS:Cherry::tNOS* reporter was cultured for 0, 1 or 2 days in induction 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$_{1-10}$* and plant optimized Cherry, both under control of a *35S* promoter (*p35S::NLS:GFP$_{1-10}$::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.

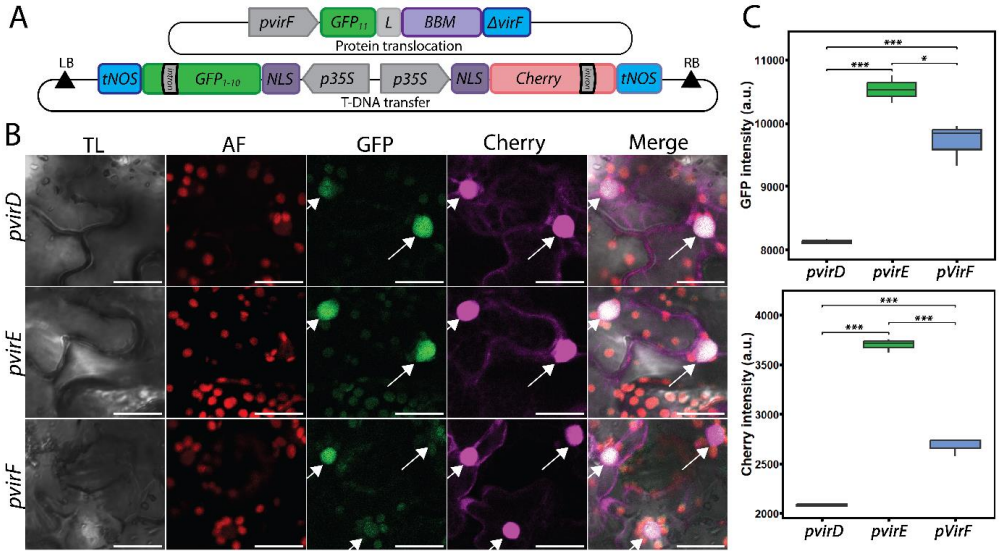

Figure 6. Visualization and quantification of GFP-reported AMPT and Cherry-reported AMT in tobacco leaf cells. (A) Schematic representation of the combined AMPT/AMT detection system split-GFP^{col}. The system comprises a T-DNA transfer vector containing the optimized *NLS:GFP$_{1-10}$* and *NLS:Cherry* 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.

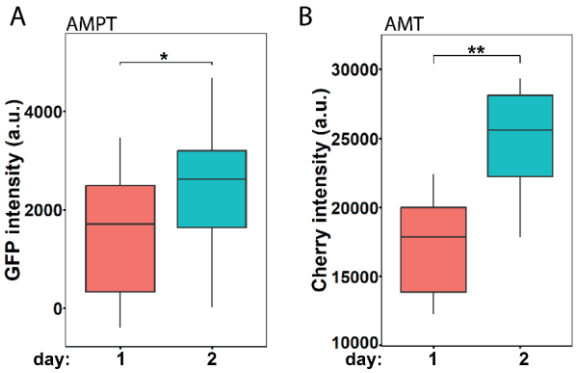

Figure 7. Improved AMT and AMPT efficiency after prolonged Agrobacterium *vir* gene induction. Quantification of GFP and Cherry fluorescence in extracts of leaves from 4 weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying the ds-FP system (*pvirE::GFP$_{11}$:BBM:ΔvirF* + *p35S::NLS:GFP$_{1-10}$::tNOS/p35S::NLS:Cherry::tNOS*) 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).

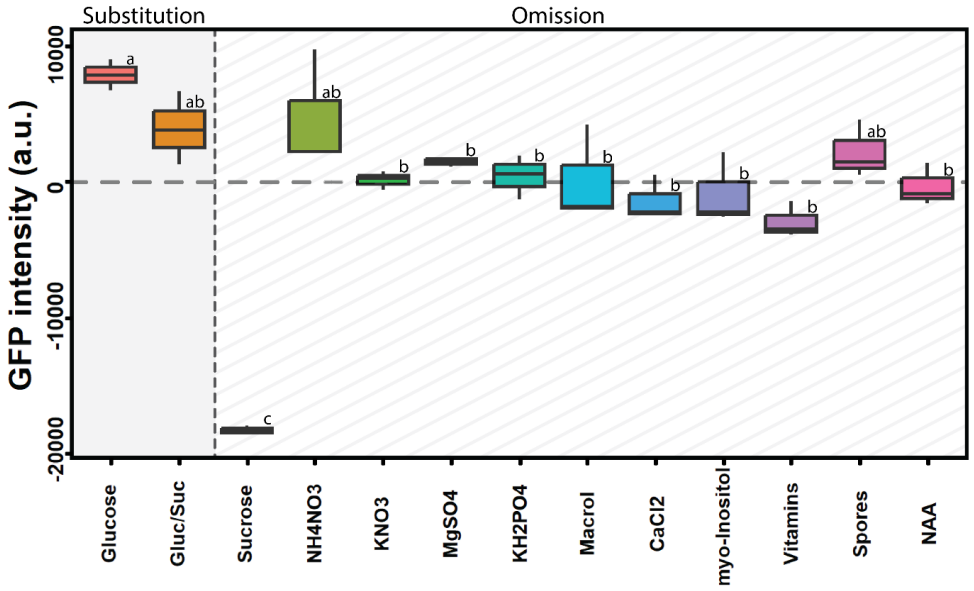

Figure 8. Co-cultivation medium optimization leads to higher Agrobacterium *vir* gene induction. The relative GFP fluorescence intensity in Agrobacterium expressing GFP under the *virE* promoter (*pvirE::GFP*) cultured in standard and different variants of Gamborg B5 medium following 2 days in *vir* 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.

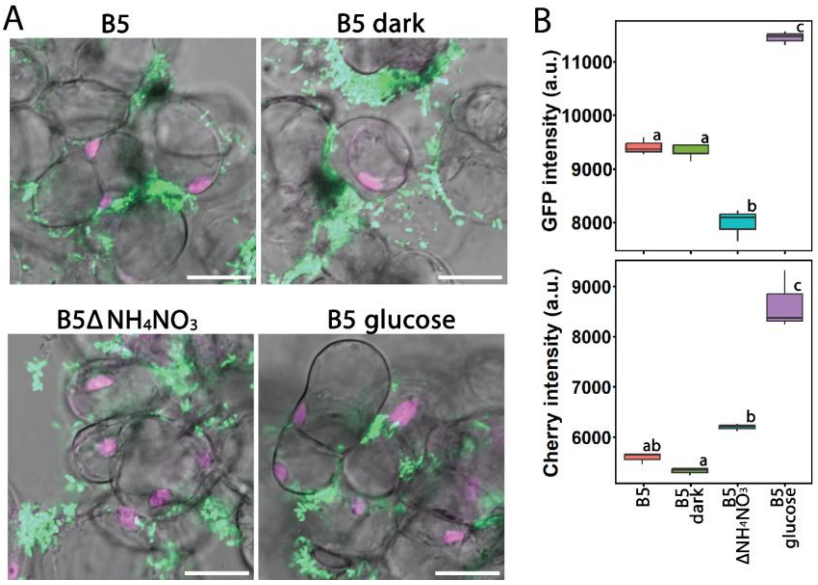

Figure 9. Increased AMT efficiency in Arabidopsis suspension cells by medium optimization. (A) Confocal microscopy images of Arabidopsis suspension cells 4 days after cocultivation with an Agrobacterium strain expressing GFP from the *virE* promoter and carrying an *p35S::NLS:Cherry::tNOS* T-DNA construct. 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

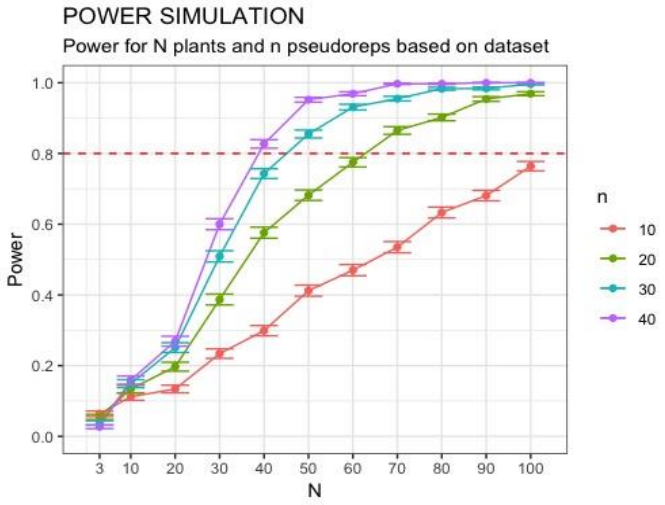

Figure S1. A large number of replicate plants are needed for GFP intensity measurements using confocal images. A power simulation based on a mixed model of collected data from GFP intensity measurements on confocal images of leaves of 4-weeks old tobacco plants at 4 dpi with an Agrobacterium strain carrying 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.

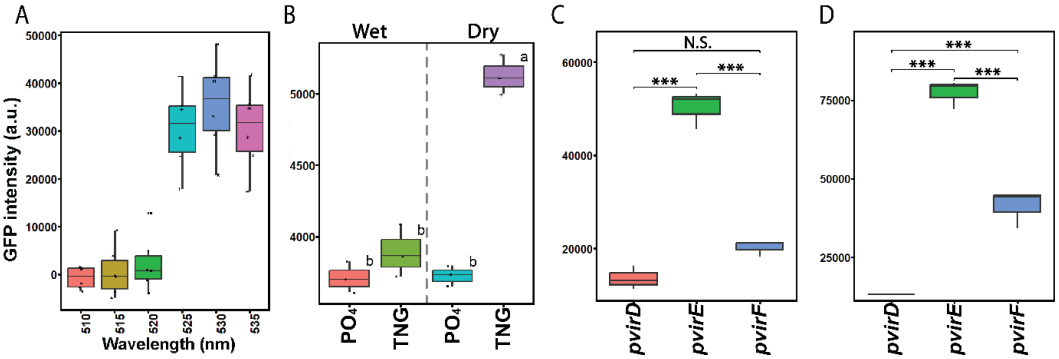

Figure S2. Optimization of the plate reader assay for measuring GFP fluorescence intensity in Agrobacterium or plant extracts. (A) GFP fluorescence measured at 6 different excitation wavelengths (nm) using a 96-wells plate reader in cultures of Agrobacterium expressing GFP under control of the *virE*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

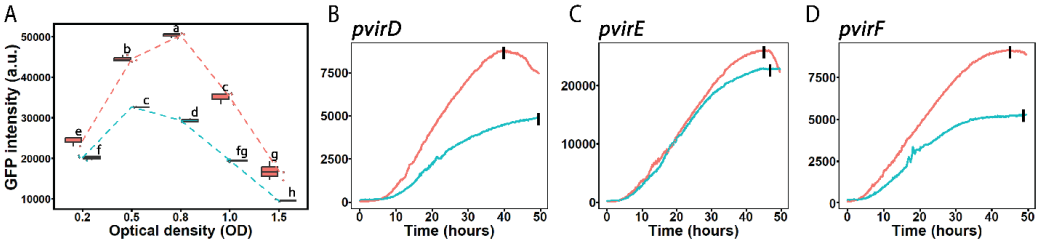

Figure S3. Increased relative GFP fluorescence intensity from Agrobacterium cultures from 1-week-old plates compared to 3-week-old. (A) Relative GFP fluorescence of induced Agrobacterium cultures at an OD_{600} of 0.2; 0.5; 0.8; 1.0 or 1.5 initiated from a 1-week-old colony (red) or a 3-week-old colony (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).

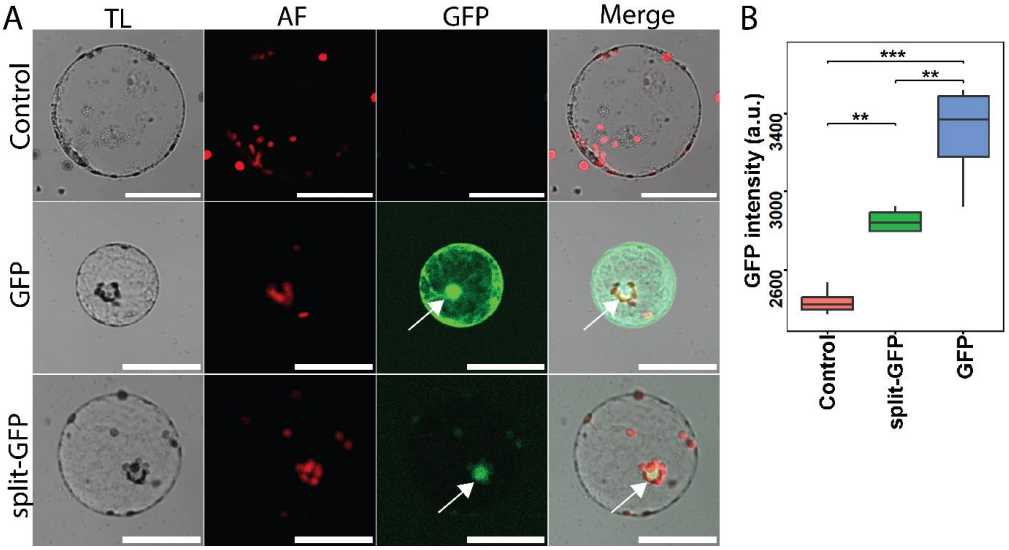

Figure S4. Detection of AMPT to tobacco protoplasts using the optimized split-GFP system. (A) Confocal microscopy images showing GFP fluorescence observed 4 dpi in tobacco protoplasts co-cultivated with Agrobacterium carrying T-DNA construct *p35S::NLS:GFP*$_{1\text{-}10}$*::tNOS* (control) or *p35S::NLS:GFP::tNOS* (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).

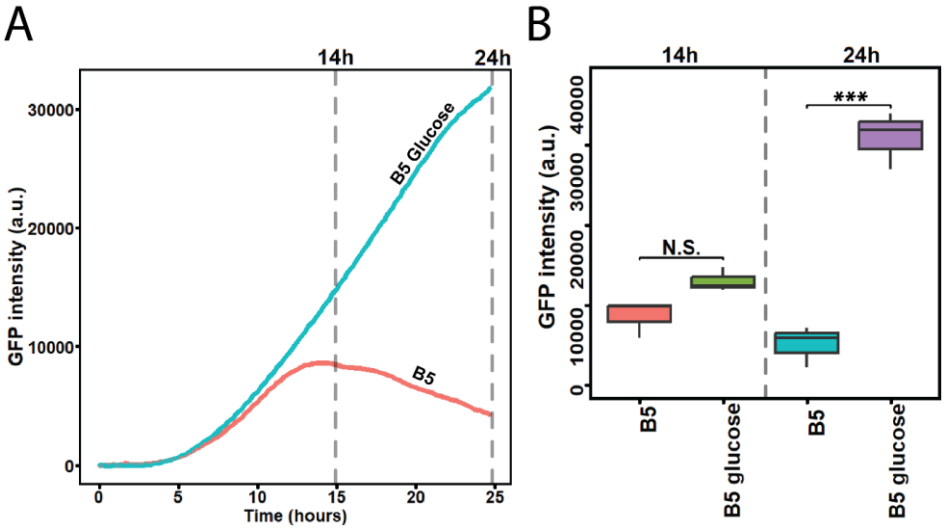

Figure S5. Comparison of Gamborg B5 media against modified B5 medium with sucrose substituted for glucose. (A) The control treatment (B5) and B5 with sucrose substituted by glucose (B5 glucose) were added to Agrobacterium expressing GFP under control of the *virE* promoter and fluorescence was measured 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$_{1\text{-}10}^{opt}$::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$_{1\text{-}10}^{opt}$::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℃ 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 °C 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 °C 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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Chapter 4

Tackling plant transformation recalcitrance by *Agrobacterium*-mediated protein translocation

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Abstract

DNA transfer by the soil bacterium *Agrobacterium tumefaciens* (Agrobacterium) is commonly used to generate transgenic plants or for CRISPR-Cas-mediated genome editing. However, Agrobacterium-mediated transformation (AMT) is only efficient in a limited number of plant species or accessions, as many are recalcitrant to this process. This recalcitrance is caused on the one hand by inefficient DNA transfer due to suppression of Agrobacterium virulence by plant cells, and on the other hand by problems with regenerating plants from the transformed cells. It has been shown that Agrobacterium also translocates Virulence (Vir) proteins to plant cells and that this system can be used to introduce heterologous proteins into plant cells. In this chapter, we investigated in *Nicotiana tabacum* (tobacco) leaves whether Agrobacterium-mediated protein translocation (AMPT) can be used to tackle some of the bottle-necks leading to recalcitrance to AMT. Interestingly, AMPT of the *Pseudomonas syringae* avirulence protein AvrPto did not induce severe effector triggered immunity (ETI) leading to leaf necrosis, which is normally observed when *AvrPto* is overexpressed under control of a *35S* promoter. Instead AMPT of AvrPto or the bacterial salicylic acid hydroxylase NahG enhanced the efficiency of both AMT and AMPT, probably by reducing recalcitrance caused by the Agrobacterium induced plant defense responses. In addition, we show that AMPT of the *Arabidopsis thaliana* AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15) to tobacco leaves reduced the senescence response induced by Agrobacterium. Furthermore, the transfer of AHL15 was able to enhance shoot regeneration on tobacco leaf discs. Based on our result we conclude that AMPT can be used to resolve bottle necks causing recalcitrance to AMT

Introduction

Plants are found all over the world and manage to thrive even in the most difficult natural habitats despite being immobile. Because of this they have evolved elaborate signalling networks for growth, reproduction and defence (Blaacutezquez et al., 2020). In order to respond to internal and external stimuli, plants have to make use of hormone signalling, so-called phytohormones, to communicate with proximal and distal parts (Anfang & Shani, 2021).

Current commercial crops have lost some of this environmental resilience by extensive breeding programs that have focussed on high production capacity. Current breeding programs are aimed at reintroducing resilience traits, but this is a laborious and time-consuming process. With the increasing knowledge on resilience genes and recently developed new techniques, introduction of these traits by directed genome editing would be preferrable. This requires efficient protocols of transformation and regeneration, for which DNA transfer by the soil bacterium *Agrobacterium tumefaciens* (Agrobacterium) is commonly used. Unfortunately, the recalcitrance of many commercial cultivars to Agrobacterium-mediated transformation (AMT) and the subsequent regeneration of genome edited plants still forms a major bottleneck.

The recalcitrance to AMT affects transient expression experiments and makes regeneration of transgenic lines difficult. It is for an important part caused by the fact that plants have developed effective defence systems that enable them to recognise phytopathogens, which in turn have co-evolved together with their host (Anderson et al., 2010). Many phytopathogenic bacteria make use of a delivery system to transfer virulence proteins e.g. to modulate the plant defence or aid in infection. Common delivery systems are the type III (T3SS) and type IV (T4SS) secretion systems, of which the T4SS also transfers DNA (Costa et al., 2021; Deng et al., 2017). Plants on the other hand have the ability to detect these effector or avirulence (Avr) proteins produced by phytopathogens by Resistance (R) proteins, which can either directly recognize the effector proteins or act via 'Guard Model' monitoring, guarding the target of the pathogen effector (Van Der Hoorn & Kamoun, 2008). This recognition induces a rapid defence response, the so-called

hypersensitive response (HR), which prevents spread of the infection by localized cell death (necrosis) on the site of infection (Klessig et al., 2018). Resistance upon infection that radiates throughout the plant is called systemic acquired resistance (SAR). The SAR response is under control of the plant defense hormone salicylic acid (SA) and N-hydroxypipecolic acid (NHP). Exogenous application of SA to Agrobacterium cultures decreased its growth, virulence, and attachment to plant cells (Verberne et al., 2003). *Nicotiana benthamiana* plants treated with SA showed decreased susceptibility to AMT (Anand et al., 2008). Compared to *N. benthamiana* and *Nicotiana tabacum* (tobacco) leaf infiltration, which is abundantly used for transient expression following AMT, Arabidopsis shows recalcitrance to AMT resulting in variable transient expression (Khan, 2017). It was shown that the transient expression efficiency in Arabidopsis leaves can be increased by expressing the *Pseudomonas syringae AvrPto* effector gene under a inducible promoter prior to infiltration (Tsuda et al., 2012b). AvrPto blocks pathogen-associated molecular pattern triggered immunity (PTI) by binding pattern-recognition receptors (PRRs) including FLS2 and EFR (Chinchilla et al., 2006; Zipfel et al., 2006). However, this only works in susceptible hosts, as in non-susceptible hosts AvrPto competes with Pto kinase for binding with PRRs (Xiang et al., 2008) and the interaction of AvrPto and Pto can activate effector-triggered immunity (ETI) (H. Chen et al., 2017). Transient expression of T-DNA is also enhanced by decreasing the endogenous SA levels by expression of *NahG*, encoding an enzyme that can metabolize SA, or by using the SA biosynthesis mutants *sid2* and *ics1* or signaling mutant *npr1* (Rosas-Díaz et al., 2017; Zhu et al., 2017). Expression of *NahG* in Arabidopsis also increased the transformation efficiency (Lawton et al., 1995).

Another bottleneck causing low efficiency in AMT is recalcitrance to regeneration. Plant somatic cells do not normally regenerate new organs or form new embryos, but can be triggered to do so by treatment with phytohormones or by overexpression of specific transcription factors with a key role in zygotic embryogenesis, such as BABY BOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL (WUS) or AT-HOOK MOTIF NUCLEAR LOCALIZED15 (AHL15) (Boutilier et al., 2002; Horstman et al., 2017; Karami et al., 2021; Zuo et al., 2002). Generally, stable Agrobacterium-mediated transformation (AMT) was used to obtain lines

overexpressing these transcription factors, leading to increased regeneration efficiencies in various plant species (Heidmann et al., 2011; Horstman et al., 2017; Lowe et al., 2016). Moreover, overexpression of *AHL15* and other *AHL* genes was found to reduce leaf senescence (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013).

Previously, it was shown that the T4SS of Agrobacterium can be used to translocate heterologous proteins to host cells (Sakalis et al., 2014; Vergunst et al., 2000, 2003). In this chapter we investigated the use of Agrobacterium-mediated protein translocation (AMPT) to resolve the two main bottle necks; the recalcitrance to Agrobacterium transformation and the recalcitrance in regeneration. First, the functionality of fusion proteins transferred to or expressed in plant cells via AMPT or after AMT, respectively, on plant physiology was established using AvrPto and AHL15. As expected, transfer or expression of AvrPto induced necrosis whereas AHL15 delayed senescence in *N. benthamiana* leaves. Next we tested AMPT of AvrPto or NahG and observed that this resulted in increased AMPT and transient AMT efficiencies. Interestingly, AMPT of AvrPto did not induce severe leaf necrosis, making it useful to enhance transient expression. Finally, we observed that shoot regeneration from tobacco leaf discs could be increased by AMPT or AMT of AHL15.

Results

AMPT of AvrPto induces necrosis in tobacco leaves

As a first approach to test whether AMPT of an heterologous protein can induce a physiological effect in plants, AvrPto from *Pseudomonas syringae pv. Tomato* (Pto) DC3000 was used, since it induces a strong hypersensitive (HR) response, resulting in programmed cell death at the site of infection in incompatible plants such as *N. benthamiana* and tobacco (Alfano & Collmer, 2004; Choi et al., 2017; Gimenez-Ibanez et al., 2014). Leaves of 4-weeks old tobacco plants were infiltrated with an Agrobacterium strain carrying either a plasmid with *pvirE::GFP$_{11}$:AvrPto:ΔvirF* for AMPT of the AvrPto fusion protein (fp), or a plasmid with *p35S::GFP$_{11}$:AvrPto:ΔvirF::tNOS* (T-DNA fp) or *p35S::AvrPto::tNOS* (T-DNA) for AMT

of a T-DNA expressing the AvrPto fusion protein or AvrPto without tags from the constitutive *35S* promoter (Fig. 1A). At 4 days after infiltration (dpi), transient overexpression of *AvrPto* induced necrosis in almost the entire infiltrated zone (98.1 %) and at 8 dpi this increased to 99.9 % (Fig. 1B). Transient expression of the *GFP$_{11}$:AvrPto:ΔvirF* fusion protein showed a milder necrosis in the leaf tissue at 4 dpi (22.7 %), but at 8 dpi this increased to 84.3 %. AMPT of the AvrPto fusion protein 4 dpi showed necrosis of 2.3 % of the leaf tissue and increased 8 dpi to 13.8 %. These results indicate that AMPT of an AvrPto fusion protein to tobacco leaf cells can induce a physiological effect in the form of necrosis. However, this effect is weaker compared to when the fusion protein or the non-fused AvrPto protein is transiently expressed following AMT. Also, it should be noted that the GFP$_{11}$:AvrPto:ΔVirF fusion protein is significantly less active compared to the AvrPto protein itself in AMT experiments.

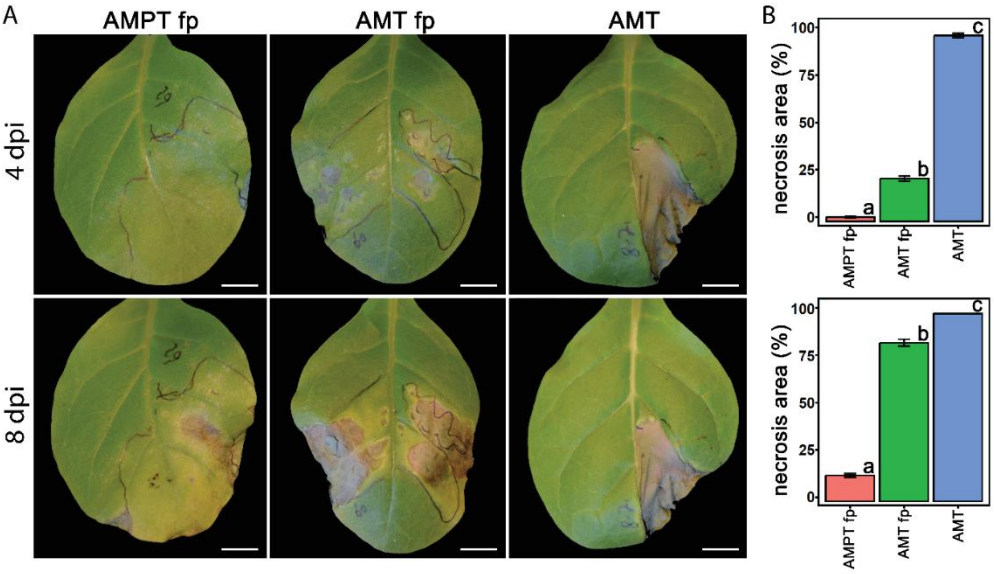

Figure 1. **AMPT and AMT of AvrPto induces necrosis in *N. tabacum* leaves.** (A) Hypersensitive response observed as necrosis in leaves of 4-weeks old tobacco 4 dpi or 8 dpi caused by AMPT of GFP$_{11}$:AvrPto:ΔVirF under control of *pvirE* (AMPT fp), or AMT of a T-DNA construct containing *p35S::GFP$_{11}$:AvrPto:ΔvirF::tNOS* (AMT fp) or *p35S::AvrPto::tNOS* (AMT). Size bars indicate 10 mm. (B) The percentage of the infiltrated leaf surface that showed necrosis at 4 dpi (upper panel for AMPT (2.3 %), AMT fp (22.7 %) and AMT (98.1 %) or at 8 dpi (lower panel) for AMPT fp (13.8 %), AMTfp (84.3 %) and AMT (99.8 %). Letters indicate statistically significant differences ($p < 0.001$) as determined by one-way analysis of variance (ANOVA)

with Tukey's honest significant difference post hoc test. Bars indicate the mean area and error bars indicate the standard error of the mean (n = 12).

AMPT of AHL15 *delays senescence in N. benthamiana leaves*

Next we tested whether AMPT of AHL15 could also induce detectable physiological changes in *N. benthamiana* leaves. Agrobacterium leaf infiltration is known to induce host defense and developmental responses in tobacco and *N. benthamiana* leaves, among which the senescence-related loss of chlorophyll (Ludwig et al., 2005; Pruss et al., 2008). Previous observations on Arabidopsis and tobacco plants overexpressing *AHL15* (Karami et al., 2020) and reports on AHL15 homologs indicated that these AT-Hook motif proteins repress leaf senescence (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013). To test whether AMPT of AHL15 could repress Agrobacterium-induced senescence in leaves of *N. benthamiana*, we infiltrated leaves of 4-weeks old plants with an Agrobacterium strain, either transferring the fusion protein GFP_{11}:AHL15:ΔVirF expressed from *pvirE* (fp), or transferring a T-DNA construct carrying *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp), *p35S::AHL15::tNOS* (T-DNA) or *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control). Plants expressing a similar fusion with the Cre recombinase were used as control, as previous work has shown that expression of the Cre recombinase does not affect Arabidopsis development (Vergunst et al., 2000). Clear yellowing could be observed in leaves infiltrated with the control strain at 7 dpi, whereas the yellowing was reduced for the other three strains (Fig. 2A). Quantification of the yellowing at 4, 5, 6 and 7 dpi using a handheld device for non-destructive relative chlorophyll content confirmed this observation (Fig 2B, 2C). These results show that AMPT of the GFP_{11}:AHL15:ΔVirF fusion protein is as effective in reducing chlorophyll breakdown as when the AHL15 fusion or the native AHL15 protein is expressed from a T-DNA following AMT.

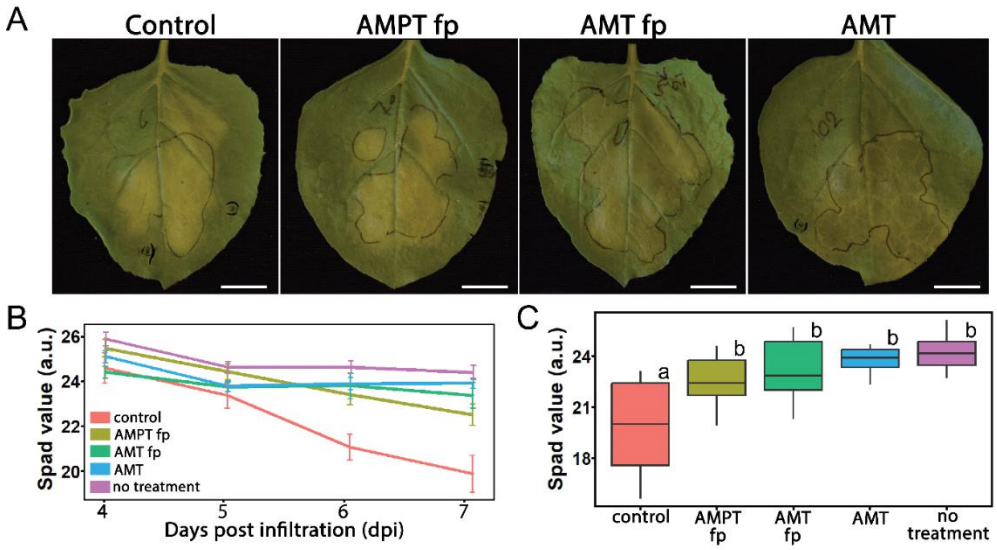

Figure 2. AMPT of AHL15 delays Agrobacterium-induced senescence in *N. benthamiana* leaves. (A) Leaves of 4-weeks old *N. benthamiana* plants at 7 dpi with an Agrobacterium strain carrying *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), ***pvirE::GFP$_{11}$:AHL15:ΔVirF*** (AMPT fp), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (AMT fp) or *p35S::AHL15::tNOS* (AMT). Size bars indicate 10 mm. (B) Quantification of the chlorophyll content in the infiltrated area of *N. benthamiana* leaves, as shown in (A) at 4, 5, 6 and 7 dpi. Error bars indicate standard deviation. (C) Quantification of the chlorophyll content of *N. benthamiana* leaves at 7 dpi. Indicated are the median, second and third quartile and whiskers extend the interquartile range by 1.5 (n = 6). Different 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.

NahG or AvrPto co-translocation increases AMPT and AMT efficiencies in tobacco

After establishing that fusion proteins following AMPT can induce the expected physiological effects in plant cells, we determined the effect of AMPT of AvrPto or NahG on the efficiency of AMPT and AMT by infiltrating 4-weeks old tobacco leaves with the split-GFP^{col} system (Fig. 3A), allowing the simultaneous detection of AMT and AMPT. Agrobacterium strains were used transferring by AMT the T-DNA construct *p35S::GFP$_{1-10}$/p35S::Cherry::tNOS* (where both fluorescent proteins carried a Nuclear Localization Signal (NLS) sequence) and by AMPT the fusion protein GFP$_{11}$:AvrPto:ΔVirF, GFP$_{11}$:NahG:ΔVirF or GFP$_{11}$:BBM:ΔVirF expressed under

control of *pvirE*. The infiltrated leaves were analyzed 4 dpi using confocal microscopy and leaf extracts were used to quantify the GFP and Cherry signals in a plate reader. Confocal analysis showed clear nuclear GFP signal from the split-GFP system, indicative of successful AMPT, co-localizing with the Cherry reporter for AMT (Fig. 3B). The GFP intensity was significantly stronger when the NahG or AvrPto fusion proteins were translocated, compared to translocation of the BBM fusion protein (Fig. 3C). The Cherry signal was enhanced by the co-translocated AvrPto fusion protein, but even stronger with a co-translocated NahG compared to the BBM control fusion protein (Fig. 3C). One has to keep in mind, however, that co-translocation of the AvrPto fusion eventually induces necrosis and can therefore only be used to enhance transient expression and not for stable transformation. The results with the NahG fusion suggested that the transformation efficiency can be increased by lowering the SA concentration in plant cells, implying that SA has a negative effect on Agrobacterium. Indeed, addition of SA to Agrobacterium cultures completely abolished *vir* gene induction (Fig. S1A) and had a severe negative effect on the growth of Agrobacterium (Fig. S1B). Our results indicate that both the AMPT and AMT can be significantly enhanced by co-translocation of NahG or AvrPto, but that concerning the efficiency and for stable transformation co-translocation of the NahG protein seems to be the best choice.

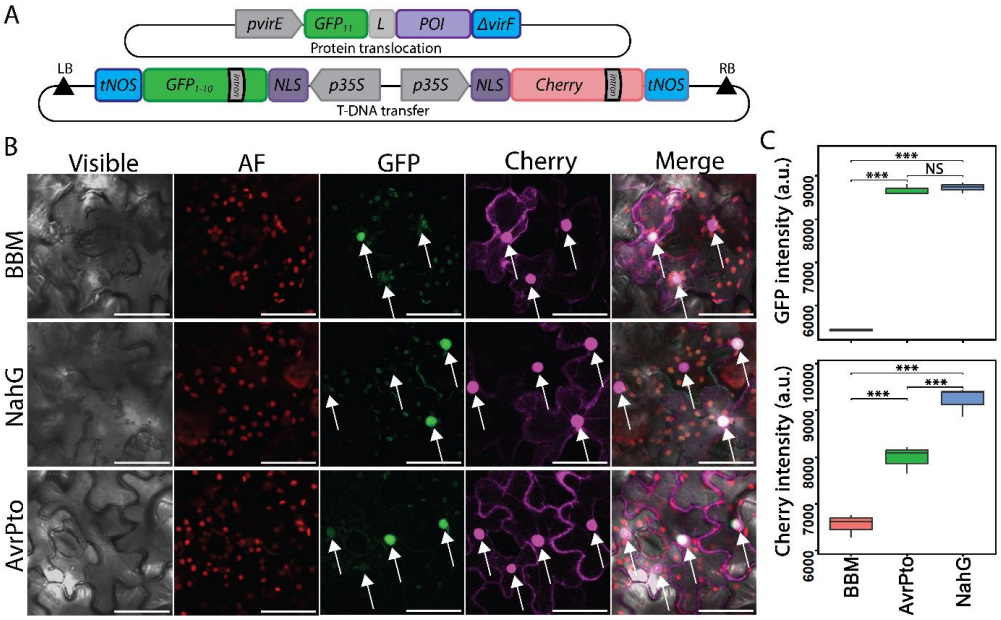

Figure 3. AMPT of AvrPto or NahG enhances protein and DNA transfer by Agrobacterium. (A) Schematic representation of the split-GFP^{col} combined AMPT and AMT detection system. The system comprises of a T-DNA transfer vector containing the *NLS:GFP$_{1-10}$* and *NLS:Cherry* coding regions, both driven by the *35S* promoter. The protein transfer vector encodes a GFP$_{11}$:POI:ΔVirF fusion protein under control of the *virE* promoter. Abbreviation: POI, protein of interest. (B) Confocal images of Cherry and GFP fluorescence observed in 4-weeks old tobacco leaf epidermis cells at 4 dpi with an Agrobacterium strain containing the split-GFP^{col} system: AMT of *p35S::NLS:sfCherry2::tNOS* and AMPT of GFP$_{11}$:BBM:ΔVirF (top), GFP$_{11}$:NahG:ΔVirF (middle) or GFP$_{11}$:AvrPto:ΔVirF (bottom). Scale bars indicate 50 µm. (C) Quantification of GFP (top) and Cherry (bottom) fluorescence in extracts of leaves imaged in (B) using a 96-wells plate reader. Measurements were adjusted to a control treatment (AMT of *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS)*. Boxplots indicate the median, second and third quartile. Whiskers extend the interquartile range by 1.5 (n = 3). Different letters above the boxplots indicate statistically significant differences ($p < 0.001$) as determined by one-way analysis of variance (ANOVA) with Tukey's honest significant difference post hoc test.

Functional analysis of AHL15 and BBM protein fusions for AMPT

Next, we tested whether we could use AMPT of AHL15 and BBM to enhance plant regeneration. For many transformation protocols regeneration forms an important rate-limiting step that can be overcome by overexpressing regeneration enhancing proteins, such as WUS and BBM, in the regenerating tissue (Lowe et al., 2016). The problem with this approach is that continuous expression of BBM significantly

alters plant development (Horstman et al., 2017), which is an undesired side effect. Co-translocation of the regeneration enhancing protein via AMPT together with the T-DNA would overcome this problem, as the protein would only be present during the transformation process. However, translocation of proteins to plant cells via AMPT requires the addition of a translocation signal to the C-terminus of the protein of interest and preferably a reporter protein to the N- or C-terminus for detection of translocation. Ideally, these additions should not interfere with the function of a protein. Tagging of proteins at the N- or C-terminus has been reported to interfere with their subcellular location or functionality (Tanz et al., 2013). In order to establish this for AHL15 or BBM, we tested overexpression of the previously generated GFP_{11}:AHL15:ΔVirF and GFP_{11}:BBM:ΔVirF protein fusions for AMPT (Chapter 2) in Arabidopsis using the *35S* promoter. As expected, control plants expressing the Cre recombinase fusion were phenotypical indistinguishable from wild-type Arabidopsis Col-0 plants. In contrast, seedlings overexpressing *GFP_{11}:BBM:ΔvirF* or *GFP_{11}:AHL15:ΔvirF* showed reduced size and abnormal leaf shape (Fig4A). This was observed in 3/60 of the *GFP_{11}:BBM:ΔvirF* and 8/60 *GFP_{11}:AHL15:ΔvirF* overexpressing seedlings. However, many positive transformants could have had too high expression preventing seedling growth and subsequently would have been counterselected. (Fig. S2A).Moreover, whereas wild-type plants showed a termination of flower production, plants overexpressing *GFP_{11}:BBM:ΔvirF* or *GFP_{11}:AHL15:ΔvirF* continued forming new flower buds (Fig 4B, top row). The inflorescence of the plants expressing *GFP_{11}:BBM:ΔvirF* showed disrupted growth and altered morphology, whereas the plants expressing *GFP_{11}:AHL15:ΔvirF* did produce flowers, although angled down slightly and with shorter stamen. Both plants overexpressing the BBM or AHL15 fusion protein did not develop siliques with seeds, not even after hand pollination. The rosette leaves of the 8-weeks old control plant showed complete senescence, however leaves of plants overexpressing the BBM or AHL15 fusion protein were still green at this moment (Fig. 4B, bottom row). The BBM fusion protein caused an abnormal rosette shape and irregular leaf shapes, whereas plants overexpressing the AHL15 fusion protein developed leaves with normal shape, although smaller in size and at a higher number. From T1 transformants with a mild AHL15 overexpression

phenotype T2 seeds could be obtained by hand pollination. The T2 seedlings showed rosette phenotypes according to the Mendelian segregation (Fig. S2B). From this analysis we concluded that the AHL15 and BBM fusions proteins are functional. We cannot exclude, however, that the fusion proteins are less active than the native proteins.

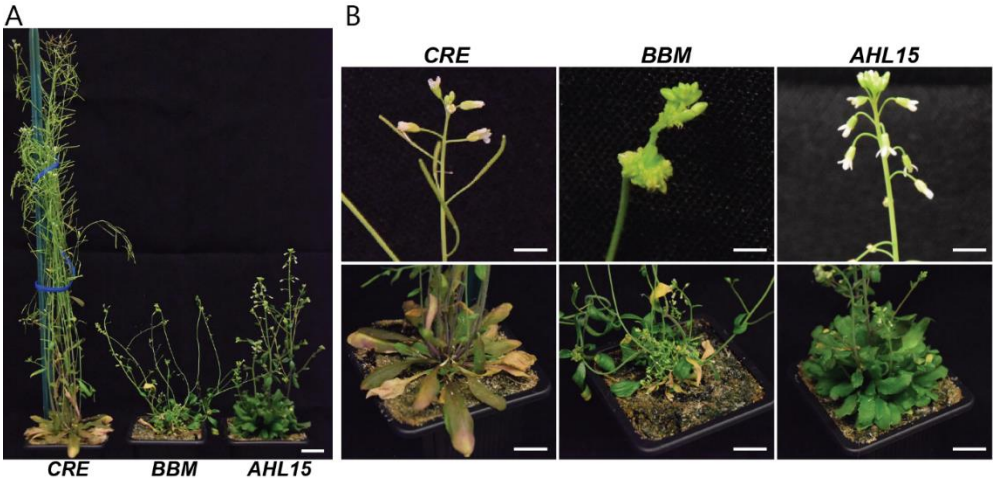

Figure 4. Altered development in Arabidopsis plants overexpressing BBM or AHL15 fusion proteins. (A) The phenotype of 8-weeks old Arabidopsis T1 plants transformed with *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (*CRE*), *p35S::GFP$_{11}$:BBM:ΔvirF::tNOS* (*BBM*) or *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (*AHL15*). (B) Close-up photos of the inflorescence (top) and rosette (bottom) of the plants in (A). Size bars indicate 20 mm.

AMPT of AHL15 increases shoot formation on tobacco leaf discs

Since the BBM and AHL15 fusion proteins for AMPT appeared functional, we selected the AHL15 fusion to see if its AMPT could enhance shoot regeneration. The fourth and fifth leaves of 4-weeks old tobacco plants were infiltrated with Agrobacterium strains transferring by AMT *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp) or *p35S::AHL15::tNOS* (T-DNA) or by AMPT GFP$_{11}$:AHL15:ΔVirF (fp). Directly after infiltration, 1.5 cm diameter leaf discs were excised and placed on shoot induction medium for two weeks. The leaf discs were subsequently transferred to medium without hormones and after two weeks this was repeated. Six weeks after infiltration, the leaf discs were photographed (Fig. 5A) and shoot formation was counted (Fig. 5B). Compared to the control

infiltration, the AMPT of the AHL15 fusion protein or its transient expression following AMT significantly enhanced the regeneration of shoots. AMPT or transient expression following AMT of the fusion protein GFP_{11}:AHL15:ΔvirF did not lead to significant differences, suggesting that the amount of protein translocated by Agrobacterium is not rate limiting for enhancing shoot regeneration (Fig. 5B), The strongest effect was observed when AHL15 without N- or C-terminal fusions was expressed from the T-DNA (Fig. 5B), suggesting that the GFP_{11}:AHL15:ΔVirF fusion has reduced activity. In conclusion, our results indicate that AMPT of regeneration enhancing proteins, such as AHL15, may be used to overcome transformation recalcitrance by enhancing plant regeneration.

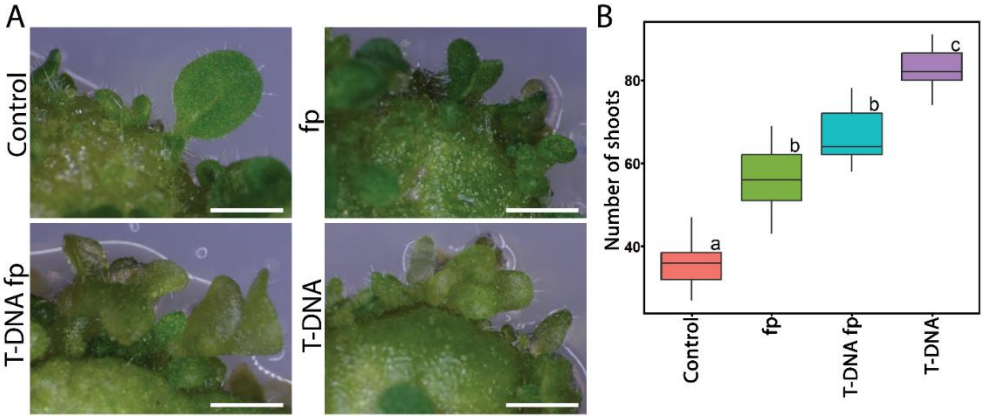

Figure 5. Increased shoot induction by expression or translocation of AHL15 (fusion) proteins in tobacco leaf cells. (A) Shoot formation observed in tobacco leaf discs 6-weeks post infiltration with an Agrobacterium strain transferring a T-DNA with *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (control), *p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS* (T-DNA fp) or *p35S::AHL15::tNOS* (T-DNA) or translocating GFP_{11}:AHL15:ΔVirF expressed under control of *pvirE* (fp). Scale bars indicate 2 mm. (B) Quantification of the number of shoots on leaf discs as shown in (A). Boxplot indicates the median, second and third quartile. Whiskers extend the interquartile range by 1.5 (n = 6). Different 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.

Discussion

The identification of key genes involved in plant resilience and the development of novel methods for directed genome editing in the last decades have provided new opportunities for the rapid introduction of beneficial traits into crop plants. Unfortunately, for many crop species or genotypes, application of this knowledge and methods is limited by their recalcitrance to AMT, which is caused by two main bottle necks; On the one hand Agrobacterium induces the production of SA as a negative feedback mechanism (Wang et al., 2019a), which inhibits Agrobacterium growth, *vir* gene induction and attachment to plant cells (Anand et al., 2008). On the other hand, some crop species or genotypes show recalcitrance to regeneration.

Previously it was shown that the induction of a plant defense response by Agrobacterium could be prevented by either overexpression in the plant cell of NahG or Pseudomonas effectors, leading to increased AMT efficiencies (Anand et al., 2008; Raman et al., 2022; Rosas-Díaz et al., 2017; Tsuda et al., 2012). Here we showed using the split-GFP system that NahG or the Pseudomonas effector AvrPto can be introduced in plants cells via AMPT, and that this enhances the efficiency of both AMT and AMPT. Introducing such proteins via AMPT has two advantages. It obviates the need for generating transgenic lines in which the proteins are continuously expressed. This expression might have a negative effect on the defense response against pathogens. Moreover, high expression of AvrPto causes a strong HR response, whereas AMPT of this protein does not in the first 4 days of infiltration, and thereby allows enhancement of transient expression. To increase the efficiency of the translocation of other proteins of interest, NahG and/or AvrPto can be simultaneously translocated. It remains to be investigated if translocation of NahG by AMPT does lower the SA levels *in planta.*

In order to check whether AMPT can also be used to solve the regeneration bottleneck, we tested translocation of the regeneration enhancing protein AHL15. First, we showed that AMPT of AHL15 reduced the senescence-inducing effect of Agrobacterium on the infiltrated leaf tissue. This is in line with the reported anti-senescence activity of AHL15 homologs (Street et al., 2008; Xiao et al., 2009; Zhao et al., 2013), suggesting that the GFP_{11}:AHL15:ΔVirF fusion protein has retained the

activity of AHL15. To confirm this, we generated Arabidopsis lines overexpressing the fusion protein and observed enlarged rosettes with bright green leaves, which was reported previously for Arabidopsis *AHL15* overexpression lines (Karami et al., 2021; Rahimi et al., 2022).

This chapter describes the potential for AMPT to increase the transformation efficiency in tobacco and induce physiological changes in Arabidopsis*, N. benthamiana* and tobacco*.* We expect that our findings will be useful for other plant species or genotypes to lower the recalcitrance to AMT and thereby open up the possibility to do transient expression experiments or even to obtain stable transgenic lines in that species or genotype. In addition, our experiments pave the way to use AMPT as a non-GM system to induce changes in plant development (e.g. flowering) or defense (e.g. SAR) through the translocation of key regulatory proteins (e.g. transcription factors) in those processes. Clear biological effects were shown after AMPT of AHL15, BBM, AvrPto or NahG fusion proteins, however whether they trigger the correct downstream processes still requires further confirmation by reporter and gene expression analysis.

Materials and methods

Agrobacterium strains and growth conditions

The Agrobacterium strain AGL1 (C58, *RecA*, pTiBo542 disarmed, Rif,Cb) (Jin et al., 1987) used in this chapter was grown in modified LC medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH = 7.5) at 28 °C with the appropriate antibiotics at the following concentrations: gentamicin 40 µg/ml; carbenicillin 75 µg/ml; kanamycin 100 µg/ml; rifampicin 20 µg/ml. Plasmids were introduced into Agrobacterium by electroporation, as previously described (den Dulk-Ras & Hooykaas, 1995).

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 T-DNA transfer vector *p35S::GFP$_{11}$:Cre:ΔvirF::tNOS* (Khan, 2017) was digested with *Nco*I and *Bst*EII to replace the *Cre* coding region with a PCR amplified *Nco*I *Bst*EII fragment containing the coding regions of *AHL15*, *NahG* or *AvrPto.* The protein translocation vector *pvirE::GFP$_{11}^{opt}$:BBM^{opt}:ΔvirF* constructed in Chapter 2 was digested with *Nde*I and *Bam*HI to replace the coding region with a codon optimized synthetic *Nde*I *Bam*HI fragment containing G*FP$_{11}^{opt}$:AHL15^{opt}:ΔvirF, GFP$_{11}^{opt}$:AvrPto^{opt}:ΔvirF or GFP$_{11}^{opt}$:NahG^{opt}:ΔvirF* (Bio Basic inc., Canada).

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::GFP$_{11}$:Cre:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:AHL15:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:BBM:ΔvirF::tNOS/ pNOS::Hyg	T-DNA transfer (Km^r)	Khan, 2017
p35S::GFP$_{11}$:AvrPto:ΔvirF/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::AHL15/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::AvrPto/ pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 4
p35S::NLS^{opt}:GFP$_{1\text{-}10}^{opt}$::tNOS / pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 2
p35S::NLS^{opt}:GFP$_{1\text{-}10}^{opt}$::tNOS / p35S::NLS^{opt}:sfCherry2^{opt}::tNOS / pNOS::Hyg	T-DNA transfer (Km^r)	Chapter 2
pvirF::GFP$_{11}^{opt}$:BBM^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 3
pVirE::GFP^{opt}	Bacterial expression (Gm^r)	Chapter 3
pVirE::GFP$_{11}^{opt}$:AHL15^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4
pVirE::GFP$_{11}^{opt}$:AvrPto^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4
pVirE::GFP$_{11}^{opt}$:NahG^{opt}:ΔvirF	Protein translocation (Gm^r)	Chapter 4

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 species and growth conditions

The seed of *Nicotiana tabacum* cv. Petit Havana SR1 (tobacco) and *Nicotiana benthamiana* 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 seeds of *Arabidopsis thaliana* Col-0 were sterilized by a pre-wash with sterile water, followed by one minute in 70% ethanol, 10 minutes in a 10% commercial bleach solution (4.5% active sodium hypochlorite) under constant agitation and five times wash with sterile water. Sterilized seeds were stratified for three days at 4 °C and germinated axenically on 1% sucrose half-strength MS medium (Murashige & Skoog, 1962) solidified with 1% Daishin agar (w/v) (Duchefa Biochemie). Seeds were germinated and seedlings axenically grown at 21 °C and a 16 hour photoperiod.

Floral dip

Arabidopsis was transformed using the floral dip method (Clough & Bent, 1998b), transgenic plants were selected by germinating sterilized seeds on medium with 50 mg/l Hygromycin B and T-DNA integration was verified by PCR analysis (List of PCR primers, table 2). Seedlings were transferred to soil and grown for five days in high humidity under a plastic cover in growth chambers at 21 °C, 50 % relative humidity and a 16 hour photoperiod for four weeks (Rivero et al., 2014).

Table 2. Overview of primers used in this study

Primer name	**Sequence**
AHL15opt Fw	ACTTCACCACCAACAACTCCGG
AHL15opt Rev	GTTGTTGCCGGATTCGTTGTCG
BBMopt Fw	CGTTGACAACCAGGAAAACGGC
BBMopt Rev	TGGTCGTCTTCCTGCTTGAAGC
WUSopt Fw	AACGTCAAGCTGAACCAGGACC
WUSopt Rev	AGTAGTGGTGGTCCATGTTGGC
NahGopt Fw	CCTTAGCACTGGAACTCT
NahGopt Rev	CAACTCGTATAACTCGCC
Cre Fw	CCGCGCGCCTGAAGATATAGAA
Cre Rev	CCATTGCCCCTGTTTCAC
SpeI AvrPto Fw	GG ACTAGT GGAAATATATGTGTCGGCG
SacI AvrPto Rev	C GAGCTC TCA TTGCCAGTTACGGTAC
EcoRI AvrPto Fw	CCG GAATTC GGAAATATATGTGTCGG
HindIII AvrPto Rev	CCCAAGCTTTTGCCAGTTACGGTAC
SpeI NahG Fw	GG ACTAGT AAAAACAATAAACTTGGCTTGCG
SacI NahG Rev	C GAGCTC TCA CCCTTGACGTAGC
EcoRI NahG Fw	CCG GAATTC AAAAACAATAAACTTGGCTTGC
HindIII NahG Rev	CCC AAGCTT CCCTTGACGTAGC

Agrobacterium leaf infiltration and fluorophore measurement

Agrobacterium induction and leaf infiltration was performed as described in Chapter 2. Fluorophore levels were measured using a plate reader as described in Chapter 3.

Senescence measurements

The third and fourth leaf of 4-weeks old *N. benthamiana* plants were infiltrated with Agrobacterium containing the appropriate plasmids (Table 1). The Agrobacterium infiltration was performed as described in Chapter 3. The senescence was measured at 3 dpi using a handheld SPAD-502plus meter (Konica Minolta, Langenhagen, Germany) at three spots of the infiltrated area per leaf using six plants per treatment. The measurements in one leaf were averaged and the statistical analysis was performed per leaf number. The measurements were repeated at 4, 5, 6 and 7 dpi on the same spots on the leaves.

Phytohormone treatment

Agrobacterium virulence induction and growth in response to SA was measured in a 96-wells plate reader. The Agrobacterium cultures were induced as described in Chapter 2 and measurements performed as described in Chapter 3. Each well of the 96-wells plate was loaded with 150 µl induced *Agrobacterium*. The Agrobacterium cultures were treated with SA dissolved in 10% DMSO to a final concentration of 0.425; 2.125; 4.25; 8.50 or 12.75 mM.

Organogenesis quantification

The leaves of soil grown 4-weeks old *N. tabacum* were infiltrated with Agrobacterium containing the appropriate plasmid(s) (Table 2). The position on and the number of the leaf for infiltration and for the subsequent leaf disc was described in Chapter 2. After infiltration, excess Agrobacterium infiltration medium on the leaf disc was removed by a sterile water wash and the leaf was subsequently dried by placing it shortly on sterile filter paper. The round leaf discs (1.5 cm) were

cut using a cork borer, dried on sterile filter paper and immediately placed on solid shoot induction medium containing 1x MS, 3% sucrose, 1% Daishin agar, 200 µM AS, 2 mg/l BAP and 0.2 mg/l NAA. After two weeks, leaf discs were transferred to 3% sucrose MS plates without hormones and AS but containing 100 µg/ml Timentin or 500 mg/l cefotaxime. Six leaf discs were observed per treatment and shoot formation was counted using a Zeiss Axiozoom v16 (Zeiss, Oberkochen, Germany) stereomicroscope.

Laser scanning confocal microscopy

Fluorescence was observed using a Zeiss Imager M1 or a Zeiss Observer (Zeiss, Oberkochen, Germany) 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 595 – 500 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). The GFP or Cherry fluorescence intensity was measured in ImageJ.

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Author contribution

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

Supplemental figures

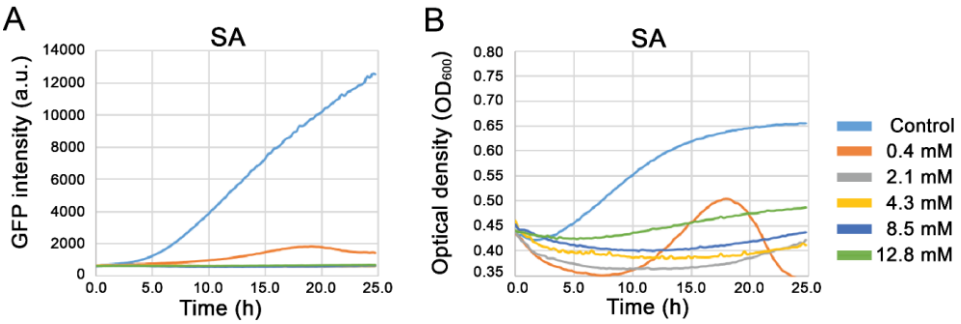

Figure S1. Agrobacterium *vir* gene induction and growth is severely inhibited by SA. (A) Time lapse measurements of GFP fluorescence from and Agrobacterium strain expressing *GFP* under control *pvirE* treated with 200 uM acetosyringone in the absence or presence of SA. (B) Timelapse measurement of the optical density (OD_{600}) of the Agrobacterium cultures in (A). Data represent the mean of three replicates.

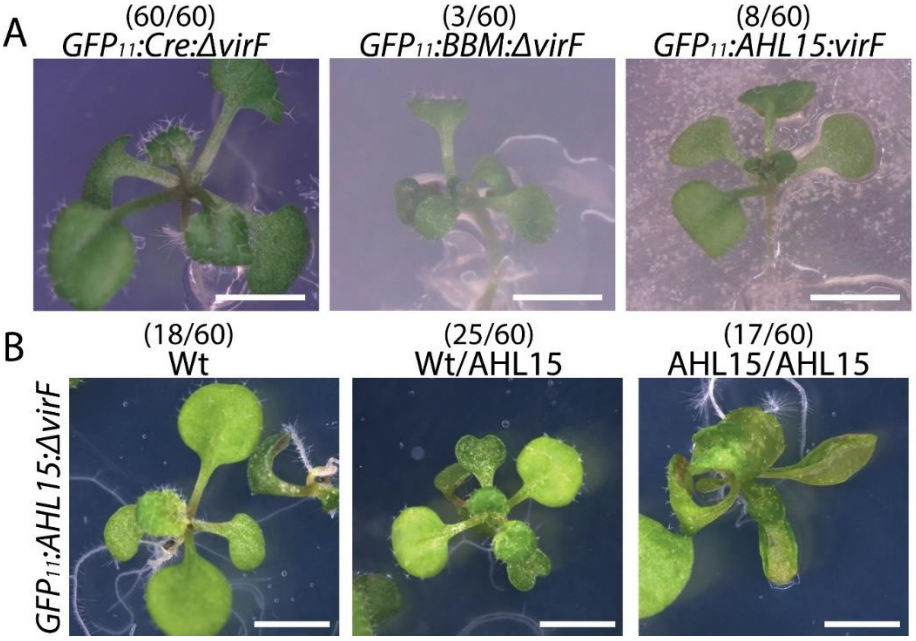

Figure S2. Phenotypes of seedlings overexpressing BBM or AHL15 fusion proteins (A) Phenotype of T1 seedlings overexpressing the indicated fusion protein under the *35S* promoter. The numbers above the pictures indicate in how many of 60 seedlings the phenotype was observed. (B) The phenotypes of T2 seedlings of an Arabidopsis line follow a typical Mendelian ratio and show either wild-type phenotype (Wt) or are heterozygous (Wt/AHL15) or homozygous (AHL15/AHL15) AHL15 phenotypes by overexpressing *GFP11:AHL15:ΔvirF* under control of the *35S* promoter. The number of observations is indicated above the figure. Size bars indicate 10 mm.

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Summary

Plants play a crucial role in human life, providing not only essential components such as oxygen, food and building materials, but all organic material on earth. Over many centuries, crop plants have been optimized by classical breeding for yield, pest resistance and product quality. With the current global climate change and restrictions in the use of pesticides it has become increasingly important to generate new crop varieties that are able to grow well under adverse conditions through their resilience to biotic and abiotic stresses. The discovery that the phytopathogenic bacterium *Agrobacterium tumefaciens* (Agrobacterium) causes tumorous growth on plants by transferring a DNA copy (transfer or T-DNA) of part of the tumour inducing (Ti) plasmid to cells of its host plant has led to the application of this natural DNA transfer system to boost crop improvement by introducing beneficial genes in crop plants. Although the number of genetically modified (GM) crops and the area cultivated with them are increasing worldwide, in the EU only one GM maize is currently cultivated and the use of other GM crops for food and feed use is strictly controlled. Nonetheless, Agrobacterium-mediated transformation (AMT) has abundantly been used for scientific research, where it has been instrumental in identifying valuable traits. The finding that Agrobacterium not only transfers DNA but also translocates various virulence proteins encoded by the *vir* region on the Ti plasmid, opened up the possibility to use this Agrobacterium-mediated protein translocation (AMPT) to trigger changes in plant cells without the need for introducing DNA. The aim of the research described in this thesis was to further develop the AMPT system to trigger changes in plant cells that may resolve recalcitrance to plant regeneration or AMT, resulting in improved propagation of plants or in more efficient generation of GM plants.

Chapter 1 reviews the natural mechanism of genetic modification by Agrobacterium, through which the T-DNA and virulence proteins are transferred into plant cells via the type IV secretion system to generate transgenic plants. The virulence proteins protect the T-DNA during transfer and facilitate its integration into the host genome. Initially, AMPT was detected by activation of an antibiotic resistance gene through excision of an insert by the translocated CRE recombinase. These experiments showed that a signal sequence in the C-terminal part of Vir proteins is required for translocation, and that AMPT can be used to introduce heterologous proteins into

plant cells. More recently, the split-GFP system was adopted, where the protein fused to the small GFP_{11} fragment was translocated into plant host cells expressing the larger GFP_{1-10} fragment, leading to GFP fluorescence upon successful AMPT. **Chapter 1** discusses the challenges in applying AMT and AMPT for crop improvement, which are not limited to the political ones described above. A major practical bottleneck is that efficient protocols for AMT are limited to specific plant species or genotypes. This recalcitrance to transformation lies in difficulties in regenerating whole plants from the transformed cells and also in the plant defense response triggered by Agrobacterium itself. For the recalcitrance to regeneration several strategies are reviewed, among which the overexpression of transcription factors with an important role in zygotic embryogenesis, such as BABY BOOM (BBM), LEAFY COTYLEDON 1 and 2 (LEC1 and LEC2), and AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15), leading to the plant hormone-independent formation of organs or even embryos on somatic plant tissues. The latter process is referred to as somatic embryogenesis. At the end of **Chapter 1**, the potential applications of AMPT in agriculture and biotechnology are discussed. AMPT of aforementioned transcription factors could be used as a non-GM approach to alleviate regeneration recalcitrance. Similarly, AMPT of proteins that interfere with plant defense responses could be used to enhance AMT.

In **Chapter 2**, we investigated the optimization of the previously developed split-GFP system for more sensitive visualization of AMPT of GFP_{11}-labeled proteins of interest in plant cells. GFP_{1-10} is transcribed from a T-DNA that is co-transferred with a GFP_{11}-labeled protein of interest (POI), enabling direct visualization of AMPT in wild-type plants. For this optimization, the codon usage was adjusted for expression in bacteria and plants, for AMPT and AMT, respectively. The sensitivity of the split-GFP system was further enhanced by multimerizing the GFP_{11} label seven times (GFP_{11}x7). To increase versatility and simultaneously visualize two proteins of interest via AMPT, the split-Cherry system was tested. However, we were unable to detect the translocation of $Cherry_{11}$-labeled fusion proteins. In contrast, the Cherry system, combined with the optimized split-GFP system, successfully visualized both AMT and AMPT in the leaves of *Nicotiana benthamiana*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Capsicum annuum*, *Brassica napus*, and suspension cell lines of *Arabidopsis thaliana*.

In **Chapter 3**, we developed a sensitive assay based on a 96-well plate reader to measure fluorescence in Agrobacterium cultures or plant extracts after AMPT or

AMT. This method allowed us to enhance AMT in *Arabidopsis thaliana* suspension cell lines by optimizing the plant medium composition, Agrobacterium culture age, and optical density. We demonstrated that the *virE* promoter results in higher GFP expression in Agrobacterium than the *virF* or *virD* promoter and that *virE* promoter-driven expression of the protein to be translocated results in higher AMPT efficiencies.

In **Chapter 4**, we investigated whether AMPT could reduce AMT recalcitrance by introducing heterologous proteins into plant cells. We found that AMPT of the avirulence protein AvrPto from *Pseudomonas syringae* did not trigger severe defense responses, such as effector-mediated immunity. This typically occurs when AvrPto is overexpressed under the constitutive *35S* promoter and leads to leaf necrosis. The efficiency of both AMT and AMPT increased with AMPT of bacterial salicylate hydroxylase NahG or AvrPto, likely due to a reduction in the defense response usually induced by Agrobacterium. To explore whether AMPT could alleviate regeneration recalcitrance in plants, we introduced AHL15 via AMPT into tobacco leaves and showed that this reduced the senescence response induced by Agrobacterium. We also discovered that the transfer of AHL15 increased shoot regeneration on tobacco leaf discs, despite the fact that the GFP_{11} N-terminal tag and VirF C-terminal tag required for AMPT of AHL15 reduced its regeneration enhancing capacity.

In conclusion, we propose that the two main two bottlenecks of AMT of plants, i) the recalcitrance of plants to AMT and ii) the difficulties in regenerating whole plants from transformed cells**,** can be overcome by AMPT of heterologous proteins involved in the modulation of plant defense responses or the activation of embryogenesis. For this, a sensitive AMPT system was developed to both visualize and measure Cherry and GFP fluorescence from respectively AMT and AMPT using microscopy and a plate reader. The visualization of the simultaneous AMPT of two heterologous proteins, however, needs additional work to optimize the system.

Samenvatting

Planten spelen een cruciale rol in onze samenleving doordat ze essentiële componenten, zoals zuurstof, voedsel en bouwmaterialen, produceren die het leven van mensen mogelijk maken. Gedurende vele eeuwen zijn landbouwgewassen geoptimaliseerd door klassieke veredeling met het oog op opbrengst, resistentie tegen plagen en productkwaliteit. Door de huidige wereldwijde klimaatverandering en beperkingen in het gebruik van pesticiden is het steeds belangrijker geworden om nieuwe gewasvariëteiten te ontwikkelen, die goed kunnen groeien onder ongunstige omstandigheden door hun veerkracht tegen biotische en abiotische stressfactoren. De ontdekking dat de fytopathogene bacterie *Agrobacterium tumefaciens* (Agrobacterium) tumorachtige groei bij planten veroorzaakt door een DNA-kopie (transfer of T-DNA) van een deel van het tumor-inducerende (Ti) plasmide over te brengen naar gastheercellen, heeft geleid tot de toepassing van dit natuurlijke DNA-overdrachtsysteem voor gewasverbetering door gunstige genen in gewassen in te brengen. Hoewel het aantal genetisch gemodificeerde (GM) gewassen en het daarmee bebouwde areaal wereldwijd toeneemt, wordt in de EU momenteel slechts één GM-maïs geteeld en is het gebruik van andere GM-gewassen voor voedsel- en voederdoeleinden streng gereguleerd. Desondanks is Agrobacterium-gemedieerde transformatie (AMT) veelvuldig toegepast in wetenschappelijk onderzoek, waar het van essentieel belang is gebleken bij het identificeren van waardevolle eigenschappen. De bevinding dat Agrobacterium niet alleen DNA overdraagt, maar ook verschillende virulentie-eiwitten gecodeerd door de *vir*-regio op het Ti-plasmide, heeft de mogelijkheid geopend om deze Agrobacterium-gemedieerde eiwittranslocatie (AMPT) te gebruiken om veranderingen in plantencellen teweeg te brengen zonder DNA in te brengen. Het doel van het onderzoek beschreven in dit proefschrift was om het AMPT-systeem verder te ontwikkelen om veranderingen in plantencellen teweeg te brengen die de weerbarstigheid tegen plantregeneratie of AMT kunnen verminderen om daarmee de efficiëntie van plantenvermeerdering of de generatie van GM-planten te verhogen.

Hoofdstuk 1 behandelt het natuurlijke mechanisme van genetische modificatie door Agrobacterium, waarbij het T-DNA en de virulentie-eiwitten via het type IV-

secretiesysteem naar plantencellen worden overgebracht om plantencellen genetisch te modificeren. De virulentie-eiwitten beschermen het T-DNA tijdens de overdracht en vergemakkelijken de integratie ervan in het genoom van de gastheer. In eerste instantie werd AMPT gedetecteerd door translocatie van het CRE recombinase dat vervolgens in de plantencel door excisie van een DNA insertie een antibioticumresistentiegen activeert. Deze experimenten toonden aan dat een signaalsequentie in het C-terminale deel van Vir-eiwitten nodig is voor translocatie, en dat AMPT kan worden gebruikt om heterologe eiwitten in plantencellen te brengen. Meer recentelijk is het split-GFP-systeem toegepast, waarbij het eiwit dat is gefuseerd met het kleine GFP_{11}-fragment wordt getransloceerd naar gastheercellen die het grotere GFP_{1-10}-fragment tot expressie brengen, wat leidt tot GFP-fluorescentie bij succesvolle AMPT. **Hoofdstuk 1** bespreekt verder de uitdagingen bij de toepassing van AMT en AMPT voor gewasverbetering, die niet beperkt blijven tot de hierboven genoemde politieke kwesties. Een belangrijke praktische beperking is dat efficiënte protocollen voor AMT beperkt zijn tot specifieke plantensoorten of genotypen. Deze weerbarstigheid tegen transformatie hangt samen met moeilijkheden bij het regenereren van volledige planten uit de getransformeerde cellen en met de afweerreactie van de plant tegen Agrobacterium zelf. Voor de weerbarstigheid tegen regeneratie worden verschillende strategieën besproken, waaronder de overexpressie van transcriptiefactoren die een belangrijke rol spelen bij zygotische embryogenese, zoals BABY BOOM (BBM), LEAFY COTYLEDON 1 en 2 (LEC1 en LEC2), en AT-HOOK MOTIF NUCLEAR LOCALIZED 15 (AHL15), wat leidt tot de plantenhormoon-onafhankelijke vorming van organen of zelfs embryo's op plantenweefsels. Dit laatste proces wordt somatische embryogenese genoemd. Aan het einde van **Hoofdstuk 1** worden de potentiële toepassingen van AMPT in de landbouw en biotechnologie besproken. AMPT van eerder genoemde transcriptiefactoren zou kunnen worden gebruikt als een niet-GM-benadering om regeneratieproblemen te verminderen. Evenzo zou AMPT van eiwitten die de afweerreacties van planten tegen Agrobacterium verstoren kunnen worden gebruikt om AMT te verbeteren.

Hoofdstuk 2 beschrijft de optimalisatie van het eerder ontwikkelde split-GFP-systeem voor een gevoeligere visualisatie van AMPT van GFP_{11}-gelabelde eiwitten in plantencellen. Het reportereiwit GFP_{1-10} wordt daarbij geproduceerd vanaf een T-

DNA dat samen met een GFP_{11}-gelabeld eiwit van interesse (POI) wordt overgedragen, wat directe visualisatie van AMPT in niet-transgene planten mogelijk maakt. Voor deze optimalisatie werd het codongebruik aangepast voor expressie in bacteriën en planten voor respectievelijk AMPT en AMT. De gevoeligheid van het split-GFP-systeem werd verder verhoogd door het GFP_{11}-label zeven keer te multimeriseren (GFP_{11x7}). Om de veelzijdigheid te vergroten en gelijktijdig twee eiwitten van interesse via AMPT te visualiseren, werd het split-Cherry-systeem getest. Dit systeem bleek echter niet in staat om de translocatie van $Cherry_{11}$-gelabelde fusie-eiwitten te detecteren. Daarentegen werd met het Cherry-systeem, gecombineerd met het geoptimaliseerde split-GFP-systeem, zowel AMT als AMPT succesvol gevisualiseerd in de bladeren van *Nicotiana benthamiana*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Capsicum annuum*, *Brassica napus*, en suspensiecellijnen van *Arabidopsis thaliana*.

In **Hoofdstuk 3** wordt de ontwikkeling van een gevoelige assay gebaseerd op een 96-wells microplaatlezer beschreven waarmee fluorescentie in Agrobacterium-culturen of in plantenextracten na AMPT of AMT gemeten kan worden. Deze methode stelde ons in staat AMT te verbeteren in suspensiecellijnen van *Arabidopsis thaliana* door de samenstelling van het plantmedium, de leeftijd van de Agrobacterium-cultuur en de optische dichtheid te optimaliseren. Daarbij bleek de *virE* promoter een hogere GFP-expressie in Agrobacterium te geven dan de *virF* of *virD* promoter en daarmee tot een hogere AMPT-efficiëntie te leiden.

In **Hoofdstuk 4** is onderzocht of met behulp van AMPT de weerbarstigheid voor AMT verminderd kan worden door heterologe eiwitten in plantencellen te brengen. Daarbij is gevonden dat AMPT van het *Pseudomonas syringae* avirulentie-eiwit AvrPto geen sterke afweerreactie zoals effector-gemedieerde immuniteit opwekt, die typisch optreedt bij overexpressie van AvrPto onder de constitutieve *35S* promoter en tot het afsterven van het hele blad kan leiden. Zowel AMT- als AMPT-efficiënties namen toe bij AMPT van bacteriële salicylaat-hydroxylase NahG of AvrPto, waarschijnlijk door een vermindering van de door Agrobacterium geïnduceerde afweerreactie. Om te onderzoeken of AMPT regeneratieweerbarstigheid in planten kan verminderen, introduceerden we AHL15 via AMPT in tabaksbladeren en toonden aan dat dit de door Agrobacterium geïnduceerde verouderingsrespons verminderde. Ook ontdekten we dat AMPT van

AHL15 de scheutregeneratie op tabaksbladschijfjes kan verhogen. Dit ondanks het feit dat de aanwezigheid van de voor AMPT van AHL15 benodigde N-terminale GFP_{11} tag en de C-terminale VirF-tag het regeneratie stimulerende effect van dit eiwit verminderde.

Concluderend kunnen we op basis van het in dit proefschrift beschreven onderzoek stellen dat de twee belangrijkste knelpunten van AMT bij planten, namelijk i) de weerbarstigheid van planten tegen Agrobacterium-transformatie en ii) de moeilijkheden bij regeneratie van planten uit getransformeerde cellen, kunnen worden overwonnen door AMPT van heterologe eiwitten die betrokken zijn bij respectievelijk de modulatie van afweerreacties of de activering van het embryogenese programma. Hiervoor werd een gevoelig AMPT-systeem ontwikkeld om zowel GFP- als Cherry-fluorescentie na respectievelijk AMPT en AMT te visualiseren en te meten met behulp van microscopie en een plaatlezer. De visualisatie van de gelijktijdige AMPT van twee heterologe eiwitten gelabeld met GFP en Cherry vereist echter nog verdere optimalisatie.

Curriculum vitae

Ivo Gariboldi was born on December 3th 1990 in Leiden, the Netherlands. Here education started at the basisschool 'De Tweemaster' and later moved on to the Stedelijk Gymnasium Leiden, with a profile in biology and natural science oriented 'natuur en gezondheid'. Again, he decided to pursue education in Leiden and obtained his BSc and MSc degrees in Biology with the specialization Plant Sciences and Natural Products in 2016. During his Bachelor studies, he followed a minor at the University of Bangor, United Kingdom and back in Leiden concluded an internship looking into the role of recently identified transcription factors in alkaloid biosynthesis in *Catharanthus roseus* at the Plant Cell Physiology group under the supervision of Prof. Dr. Johan Memelink at the Institute of Biology Leiden (IBL). Later on, in his Master studies during an internship he investigated the design of an optimized generic split-GFP-based reporter system to establish *Agrobacterium* mediated protein translocation in *wild-type* plants at the IBL under supervision of Prof. Dr. Remko Offringa. He was briefly at Wageningen University where he studied ecological aspects of bio-interactions and physiology and development of plants in horticulture. After this he subsequently moved to Sweden to investigate the auxin conjugating enzyme GH3.5 in its role in restricting fruit growth in *Arabidopsis thaliana* under supervision of Dr. Emma Larsson in the Plant Biology research group of Prof. Dr. Eva Sundberg at the Swedish University of Agricultural Sciences in Uppsala. In 2017, he returned to the group of Prof. Dr. Remko Offringa at the IBL and started his PhD project, partially financed by BASF SE and co-supervised by Dr. Maarten Stuiver, with the central theme to adopt Agrobacterium mediated protein translocation for modulation of plant development and resolve recalcitrance to *Agrobacterium*-mediated transformation. In 2022, he started at the Netherlands Organisation for Scientific Research (NWO) as program secretary in the department of NWA/KIC/NGF in The Hague and since 2024 works at Plantum in Gouda, a Dutch trade organization for companies in the breeding, propagation and cultivation of seeds and young plants, where he focusses as policy specialist on biodiversity and genetic resources. Currently he lives in Oegstgeest with his sons Gianluca 'Luca' (* Leiden, 2022), Matteo (* Leiden, 2025) and his partner Marissa Cherelle Vacher.