Resolving plant transformation recalcitrance by Agrobacterium-mediated protein translocation

Gariboldi, I.

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.

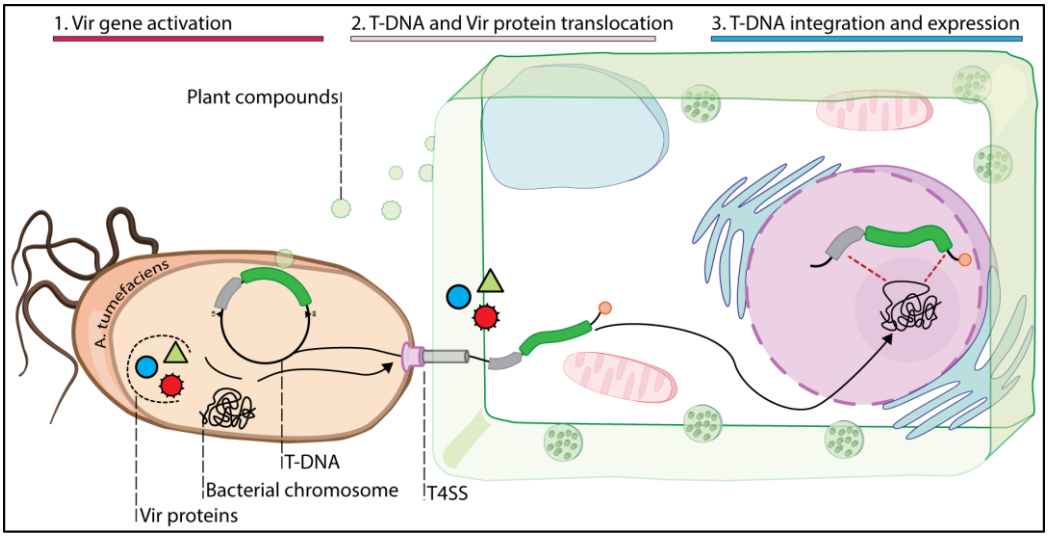

Figure 1. Schematic simplified representation of AMT and AMPT to plant cells. (1) *Vir* gene induction; The wounded plant cell secretes compounds, which induce the Agrobacterium virulence by activation of the VirA/VirG signaling cascade. Virulence proteins are produced, the T4SS is formed and the T-strand 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).

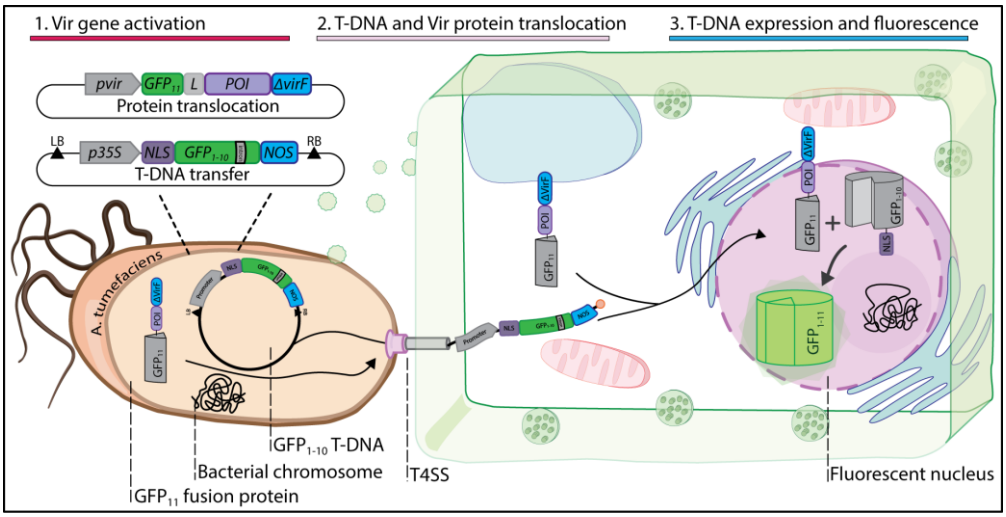

Figure 2. Schematic simplified representation of the general method for construct design and detection of AMPT to plant cells. (1) *Vir* gene induction: schematic representation of the two *Agrobacterium* constructs necessary for the split-GFP method previously developed; a protein translocation plasmid 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).

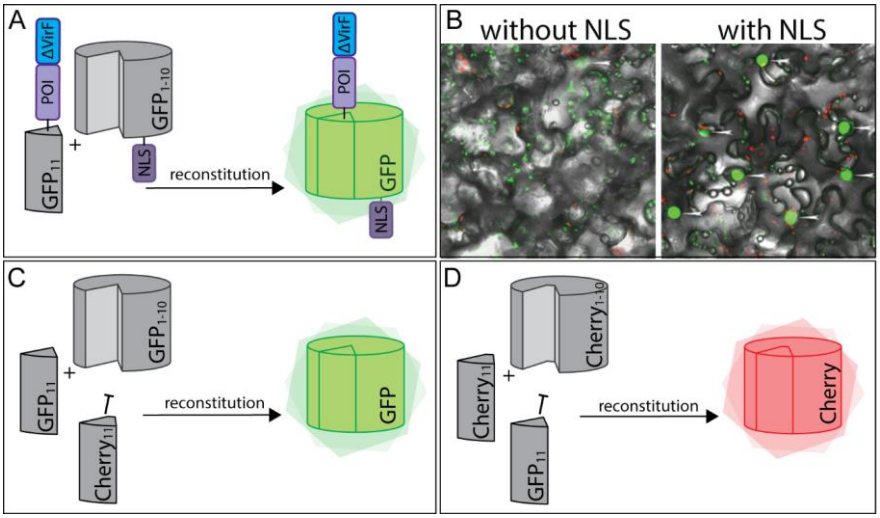

Figure 3. A schematic representation of the split-GFP and split-Cherry system and the effect of a NLS sequence. (A) The split GFP system used to detect AMPT: translocation of the fusion protein consisting of the GFP_{11}-tag, the protein of interest (POI) and the translocation signal (ΔVirF) to a plant 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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