Molecular engineering of plant development using Agrobacterium-mediated protein translocation

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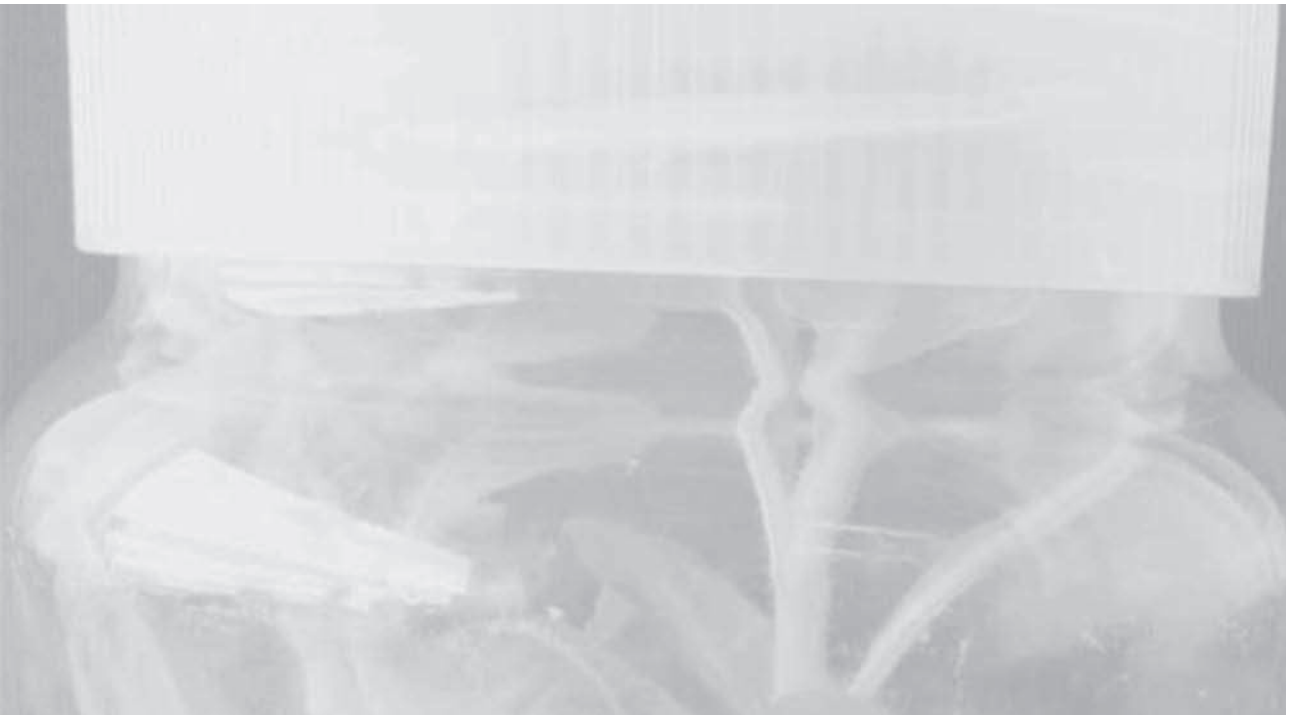

AGROBACTERIUM-MEDIATED TRANSLOCATION OF PLANT DEVELOPMENTAL REGULATORS TO PLANT CELLS

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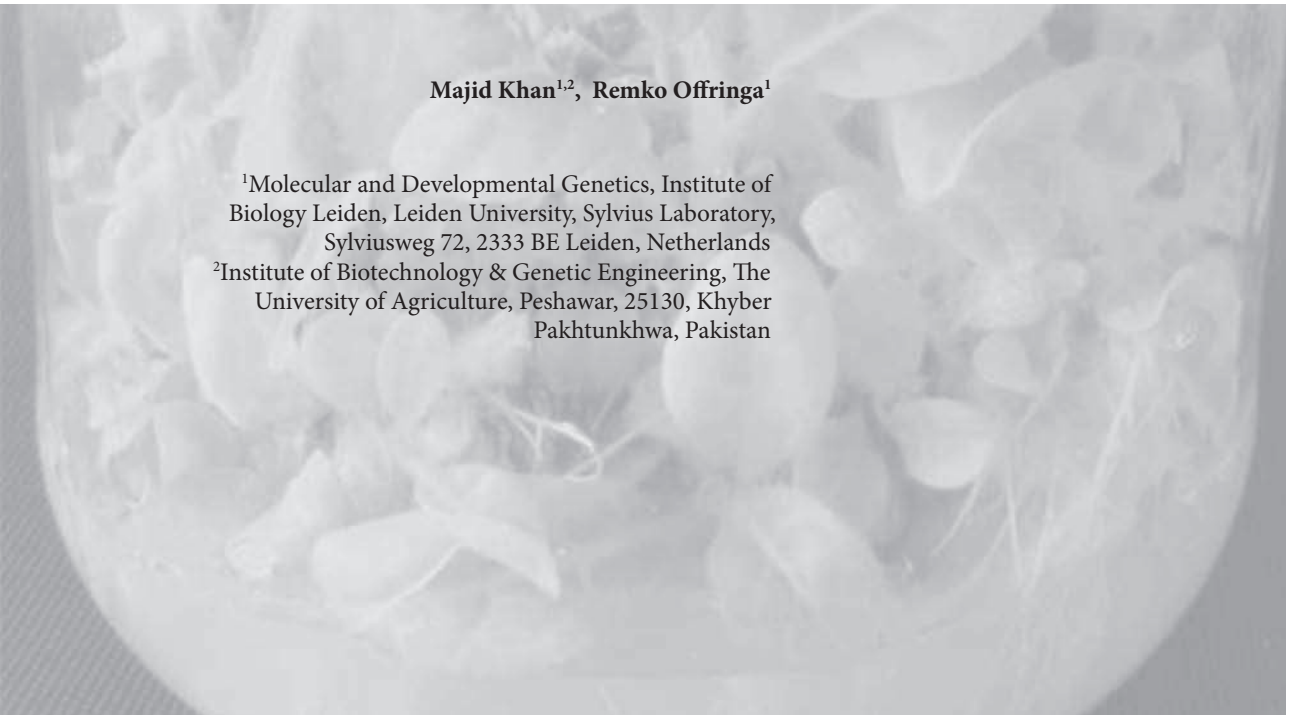

Majid Khan[1,2], Remko Offringa[1]

[1]Molecular and Developmental Genetics, Institute of Biology Leiden, Leiden University, Sylvius Laboratory, Sylviusweg 72, 2333 BE Leiden, Netherlands
[2]Institute of Biotechnology & Genetic Engineering, The University of Agriculture, Peshawar, 25130, Khyber Pakhtunkhwa, Pakistan

ABSTRACT

Agrobacterium tumefaciens is generally used for DNA transfer to plants and filamentous fungi. During *Agrobacterium*-mediated transformation (AMT), a transfer DNA (T-DNA) is produced from the tumor inducing (Ti) plasmid of *A. tumefaciens* with the help of virulence (Vir) proteins encoded by *vir*-genes. The VirB and VirD4 proteins assemble into a type 4 secretion system (T4SS) through which T-DNA is transferred to the host cell. Several Vir proteins (such as VirE2 and VirF) have been shown to be transferred to the host cell independent of the T-DNA. Previously, it has been shown that Vir protein translocation can be used to introduce heterologous DNA modifying proteins such as Cre recombinase to plant cells. Here, we show that *A. tumefaciens* can also be used to translocate plant developmental key regulators such as BABYBOOM (BBM) and REJUVENATOR/AT-HOOK CONTAINING NUCLEAR PROTEIN-LIKE 15 (AHL15/RJV) to cells of *Arabidopsis thaliana* and *Nicotiana tabacum*, when fused to the 50 amino acid C-terminal part of VirF (dVirF). Our results show that *Agrobacterium*-mediated translocation of the BBM-dVirF and AHL15-dVirF fusion proteins slow down the senescence process of the infiltrated leaf discs, and also significantly enhances tobacco shoot regeneration. In conclusion, *Agrobacterium*-mediated protein translocation (AMPT) can be used as a non-GMO approach to induce developmental changes in plant cells.

Keywords: *Agrobacterium*. VirF. Protein translocation. Developmental regulators. Non-GMO. *Nicotiana tabacum*

INTRODUCTION

In 2015, the Global Harvest Initiative reported that "accelerating productivity growth is a necessary component to achieve food and nutrition security". In the time of the green revolution from the 1930s until the late 1960s this was mainly achieved through improved agricultural practices and by enhancing crop productivity through classical breeding (da Silva et al., 2015; Zeigler, 2015).The development of technologies to genetically modify crops has provided new possibilities to introduce traits such as disease or pest resistance in a relatively short time frame, opening up to a more efficient and sustainable production of crops without the use of pesticides or other chemicals (Toenniessen et al., 2003). Although the global use of GM technology is limited by the European market, it is an invaluable tool for scientific research purposes that is applied all over the world for crop improvement (Khush, 2012). Of all the methods developed to generate GM plants, *Agrobacterium tumefaciens* is the most commonly used gene transfer machine (Ziemienowicz, 2014).

A. tumefaciens is a gram-negative soil born tumor-inducing plant pathogen first named as *Bacterium tumefaciens* by Erwin Smith and Charles Townsend in 1907. *A. tumefaciens* is a natural trans-kingdom DNA and protein transfer organism, and because of this characteristic it is harmful to plants and useful to scientists (Nester, 2014). *A. tumefaciens* contains a specific plasmid called the tumor inducing (Ti) plasmid, which is responsible for the virulence trait of the bacterium. Because of this virulence effect, *Agrobacterium* induce tumor formation or crown gall disease in plants (Pitzschke and Hirt, 2010). The injured plant cells exude phenolic compounds which stimulate the activation of *Agrobacterium* virulence by a two component regulatory system consisting of VirA and VirG. VirA is a transmembrane receptor that perceives the phenolic compounds in a pH- and temperature-sensitive manner, after which the VirA histidine kinase domain activates the VirG transcription factor through phosphorylation of the aspartic acid in its receiver domain (McCullen and Binns, 2006). The phosphorylated VirG in turn activate the transcription of *vir*-operons at the *vir*-region of Ti plasmid which result in the expression of about 25 Vir proteins. Among these induced Vir proteins is the VirD2 relaxase assisted by VirD1, nicks the bottom strand at the T-region which is flanked by imperfect 25bp right and left border repeats (RB and LB) at the Ti plasmid, resulting in the release of a single stranded transfer DNA (T-DNA) (Nester, 2014; Bourras et al., 2015). VirD2 remains attached to the 5′end of the T-strand protecting it from 5' to 3' exonucleolytic attack. VirD2 guides the T-strand to the type 4 secretion system (T4SS)-like pilus structure formed by the VirD4 coupling protein and 11 VirB proteins (van Kregten et al., 2009) and subsequently to the plant cell nucleus by virtue of a nuclear localization sequence in its C-terminus (Howard et al., 1992). Other virulence proteins, such as VirE2 and VirF, are translocated to the plant cell independently from the T-DNA, where they help in protection and integration of T-DNA into the plant genome and assist the process of tumor formation (Vergunst et al., 2000).

An aspect that limits the use of GM technology in both research and application is that several important crop species are still recalcitrant to DNA transformation and regeneration, while in other species the transformation efficiency is highly dependent on the genotype (Sharma et al., 2005). Even if a specific cultivar is transformable, the yield of this procedure is often very low. The most problematic steps in making transgenic plants are the selection and regeneration of

a plant from a transformed plant cell (Crouzet and Hohn, 2002). For selection usually antibiotic and herbicide resistance genes such as *nptII* (Horsch et al., 1985) and *bar* (D'Halluin et al, 1992) are used, which are not only considered as bio-ethically unacceptable, but also cause pleiotropic effects in transgenic plants (Miki et al., 2009). Moreover, addition of antibiotics or herbicides to the selection medium can have a serious impact on the plant regeneration efficiency (Humara and Ordas, 1999; Tran and Sanan-Mishra, 2015). As an alternative, therefore in several cases Green Fluorescent Protein (GFP) has been used as fluorescent reporter to select transgenic cells and allow regeneration of transgenic plants in the absence of antibiotics or herbicide selection (Elliott et al., 1998; Ghorbel et al., 1999; Stewart and C., 2001).

In a similar way, genes that enhance the regeneration process could be used to enrich transgenic cells during regeneration. Such genes would not only assist in the selection of transgenic plants but also allow the production of marker free transgenic plants (Khan et al., 2011). Several genes are available that induce shoot formation or somatic embryogenesis upon overexpression. For examples *Brassica napus BABY BOOM (BnBBM)* gene (Boutilier, 2002), *Arabidopsis* AT-hook motif nuclear-localized 15 or *REJUVENATOR* (*RJV/AHL15*) (Karami et al., 2017) or the *Agrobacterium* isopentenyl transferase (*ipt)* gene that mediates a rate limiting step in cytokinin biosynthesis (Kunkel et al., 1999). The *ipt* gene has been used many times as morphological marker to generate transgenic plants; however, plants that contain this gene show unwanted phenotypic changes, such as sterility and dwarf stature (Guivarc'h et al., 2002; Kant et al., 2015; Zubko et al., 2002). Obtaining phenotypically normal transgenic plant lines requires deletion of the marker gene after transformation, for example by site-specific recombinase-mediated excision, or by using an inducible expression system (Yau and Stewart, 2013). The latter system has successfully been used in combination with the *BBM* gene to enhance the regeneration of transgenic lines in tobacco and sweet pepper (Heidmann et al., 2011; Srinivasan et al., 2007).

An alternative method to improve the selection and regeneration of transgenic lines would be to co-introduce a regeneration-enhancing protein together with the gene of interest. Previously it has been shown that *A. tumefaciens* translocate Vir proteins independent of T-DNA to plant cells, and that the *Agrobacterium* protein translocation system can be used to translocate heterologous proteins (fused with VirE2 or VirF) to its host cell (Vergunst et al., 2000; Li et al., 2014; Sakalis et al., 2014). *Agrobacterium*-mediated protein translocation (AMPT) seems to work for different proteins, including several recombinases (CRE, I-SceI) and virulence proteins that vary in size considerably (van Kregten et al., 2011; Vergunst et al., 2005). This direct transfer of protein using the *Agrobacterium* translocation machinery can not only be used as a promising tool for research, but also for the production of marker free transgenic lines. Here, we demonstrated translocation of plant developmental regulators such as BBM or AHL15/RJV protein fused to the 50 C-terminal amino acids of VirF (dVirF) to *Arabidopsis* and tobacco cells. Using the AMPT system we were unable to induce hormone-independent tissue regeneration. However, we showed that BBM-dVirF and AHL15-dVirF fusion proteins translocation delayed leaf explants senescence and significantly enhanced hormone-induced shoot regeneration in *N. tabacum*.

RESULTS

Agrobacterium-mediated protein translocation (AMPT) occurs at low efficiency compared to T-DNA transfer

Previous research has shown that *A. tumefaciens* can translocate the Cre recombinase to plant cells using the Cre recombinase Reporter Assay For Translocation (CRAFT) (Vergunst et al., 2005). In this system, introduction or expression of the Cre-recombinase in cells of *Arabidopsis* line *pcb1* containing a floxed marker disrupting a *35S::GFP* gene can be sensitively monitored, since removal of the floxed marker leads to restoration of the *35S::GFP* reporter gene, and thus to abundant GFP expression (Fig. 1a) which can be easily monitored by fluorescence microscopy. Unlike the previous experiments carried out with root explants, we tested whether transfer could be detected in the whole plants and leaves would allow a more quantitative assessment of the numbers of cells with protein translocation.

We suspected, however, that it would require more proteins per cell to induce a developmental change than to achieve site directed recombination, as the latter can theoretically be performed by a single recombinase protein (Alberts et al., 2002). Instead, nuclear factors such as BBM require binding to promoters of multiple target genes to induce developmental changes (Passarinho et al., 2008). To optimize AMPT in our hands and compare its efficiency to AMT, we used the same *Arabidopsis* CRAFT system as described above (Vergunst et al., 2005; Hodges et al., 2006; Dulk-Ras et al., 2014). As part of this optimization, various methods of Agro-infiltration were tested, such as seedling vacuum infiltration, or syringe infiltration of leaf discs or of leaves on an intact plant. Seedlings of *Arabidopsis* line *pcb1* were vacuum infiltrated with *Agrobacterium* strain AGL1 carrying either binary vector with a T-DNA carrying *p35S::Cre* (Fig. 1b), or *pvirF::GFP*$_{11}$*-Cre-dvirF* (Fig. 1c) without T-DNA to compare T-DNA transfer or protein translocation, respectively. AGL1 strain containing *pvirF::GFP*$_{11}$*-dvirF* (Fig 1d) was used as a negative control.

Following 3-4 days of co-cultivation, the seedlings were analyzed for GFP signals. A global analysis of the seedlings using fluorescence stereomicroscopy showed that only 3 of the 20 seedlings co-cultivated with the AGL1 strain containing the *35S::Cre* T-DNA showed brightly fluorescent leaves (Fig. 2a), whereas the leaves of the remaining 17 seedlings showed randomly dispersed single, double, or multiple fluorescent cells (Fig. 2b). In contrast, of the 20 seedlings infiltrated with strain AGL1 (*pvirF-GFP*$_{11}$*-Cre-dvirF*), only 3 seedlings showed single, double and multiple fluorescent cells (Fig. 2c and 2d), whereas the remaining 17 seedlings did not show any GFP signal. Also the 20 seedlings infiltrated with the control strain AGL1 *pvirF::GFP*$_{11}$*-dvirF* did not show GFP signals except for some auto-fluorescence (Fig. 2e). These results show that a functional GFP$_{11}$-Cre-dVirF fusion protein is translocated from AGL1 to *Arabidopsis* leaf cells, and that the vacuum infiltration of *Arabidopsis* seedlings leads to quite variable AMT and AMPT efficiencies. More detailed confocal microscopy showed that following AMT of *35S::Cre* 90-95% of cells were GFP positive in 3 of the 20 seedlings (Fig. 2f), whereas in the majority of the seedlings 20-30% of the cells expressed GFP. After AMPT of the GFP$_{11}$-Cre-dVirF fusion 6-8% of the cells were GFP positive in three of the 20 seedlings (Fig. 2g), whereas the majority of the seedlings did not show GFP signals. This led us to conclude that the Cre recombination

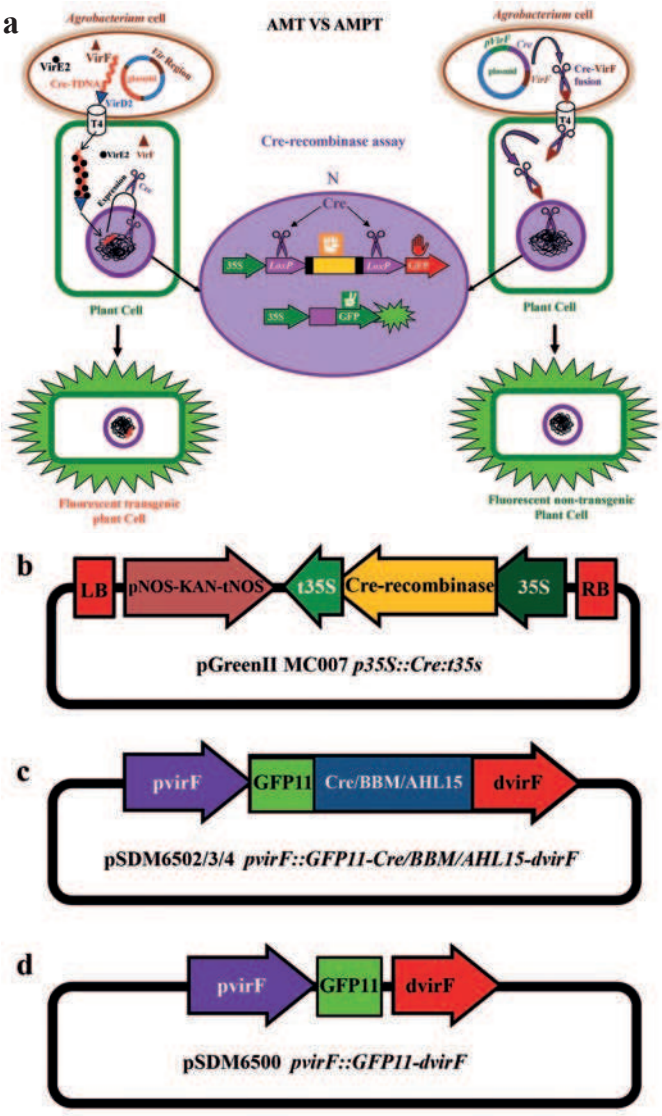

Figure 1. General strategy and DNA constructs for detection of *Agrobacterium*-mediated T-DNA transfer (AMT) or protein translocation (AMPT) to plant cells. (a) Schematic representation of the Cre-recombinase assay for detection of *Agrobacterium*–mediated T-DNA transfer or protein translocation. The *Cre* gene or Cre-VirF fusion protein are transferred by *Agrobacterium* to recipient plant cells containing a *35S::GFP* reporter gene disrupted by a *floxed* insert. Removal of the *floxed* insert by Cre-recombinase leads to restoration of the *35S::GFP* reporter gene, resulting in fluorescent plant cells. (b) T-DNA construct for AMT of the *Cre* gene under 35S promoter and terminator, with kanamycin as selection marker. LB and RB are left and right T-DNA border repeats. (c) Constructs used for translocation of GFP_{11}-Cre-dVirF, GFP_{11}-BBM-dVirF, or GFP_{11}-AHL15-dVirF fusion proteins by *Agrobacterium*. GFP_{11} comprises the C-terminal part of GFP that can complement GFP_{1-10} in a split-GFP assay. dVirF comprises the last 50 amino acids of VirF containing the translocation signal peptide. The fusion proteins are expressed under the *virF* promoter. (d) Control construct for translocation of the GFP_{11}-dVirF fusion expressed under the *virF* promoter from *Agrobacterium* to plant cells.

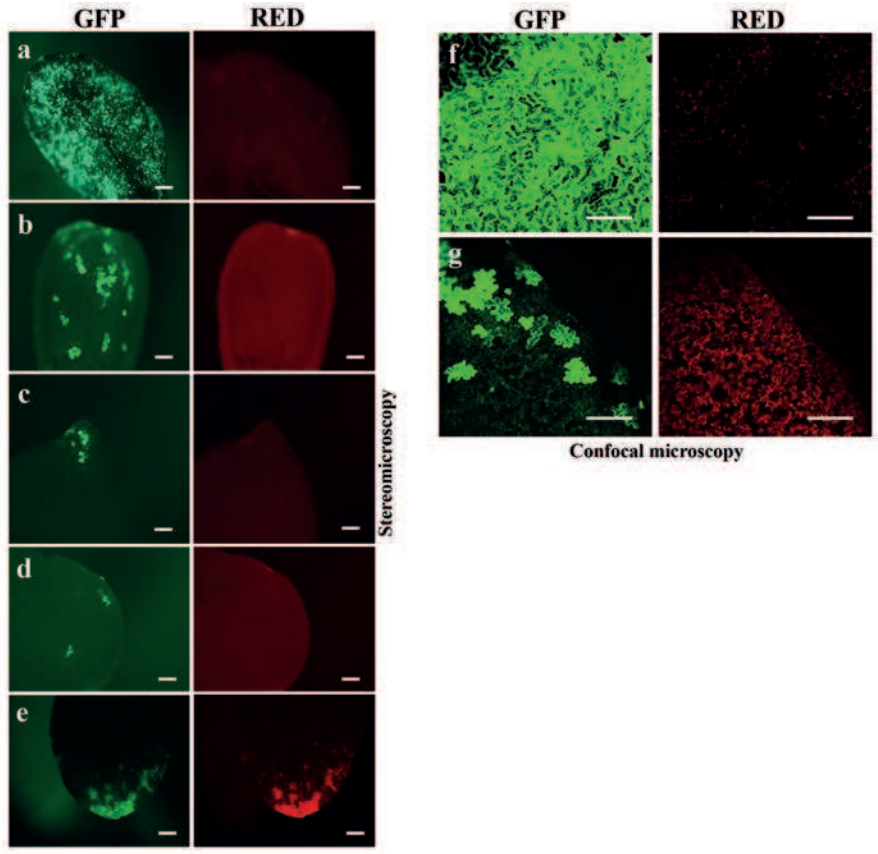

Figure 2. GFP based Cre-recombinase assay via AMT or AMPT in leaf tissues of *Arabidopsis thaliana pcb1* seedlings. (a-e) Detection of GFP expression by fluorescent stereomicroscopy following Cre-mediated restoration of the *GFP* reporter gene in *Arabidopsis pcb1* seedlings after vacuum infiltration and 3-4 days cocultivation with *Agrobacterium* AGL1 strain transferring the *p35S::Cre::t35S* T-DNA (AMT, a, b) or with AGL1 strain harboring *pvir::GFP11-Cre-dVirF* and translocating the GFP11-Cre-dVirF protein (AMPT, c, d). No GFP fluorescence was observed after translocation of the GFP11-dVirF control protein (e). (f, g) Confocal microscopy of GFP expressing cells as a result of AMT (f) or AMPT (g). The left panel shows the GFP signal in the green channel, and the right panel shows autofluorescence in the red channel. Scale bar is 0.1 mm.

in *Arabidopsis* seedlings carried out by AMPT is at least one order of magnitude less efficient than by AMT. AMT of the *p35S::GFP*$_{11}$*-Cre-dvirF* construct to different *Arabidopsis pcb1* lines showed (Fig. S1) that the variability could be explained by the *pcb1* line used in these experiments. Other lines showed much higher efficiencies. Still, however, the low percentage of GFP positive cells after AMPT is striking, and might be related to the amount of protein that is translocated or to the fact that the Cre fusion protein is not able to efficiently reach the nucleus following translocation.

AMPT of plant developmental regulators to tobacco and *Arabidopsis* cells

To use AMPT for the developmental studies of plants we used the split GFP system for the visualization of fused protein translocation in plant cells. In split GFP system the GFP gene is split into two non-fluorescent fragments GFP1-10 and GFP11 (Van Engelenburg and Palmer, 2010). GFP1-10 is overexpressed in the marker line/recipient cell while GFP 11 is fused with the protein of interest to be visualized in the marker plant cells (Li et al., 2014; Sakalis et al., 2014).

The presence of the GFP_{11} part in the *pvirF::GFP$_{11}$-Cre/BBM/AHL15-dvirF* constructs allowed us to use the split-GFP system to detect protein translocation and their localization using tobacco and *Arabidopsis* reporter lines constitutively expressing the GFP_{1-10} part under control of the *35S* promoter. For tobacco, sterile leaf discs were syringe infiltrated with *Agrobacterium* suspension, while for *Arabidopsis* sterile root explants were co-cultivated with the appropriate *Agrobacterium* strains. Two *A. tumefaciens* strains were used as controls: LBA1100 containing construct *pvirF::GFP$_{11}$-dvirF* was used as positive control and LBA2587 (*virD4* deletion mutant) containing construct *pvirF::GFP$_{11}$-BBM-dvirF* was used as a negative control. After 3 days of co-cultivation, the samples were observed by confocal microscopy. GFP signals were observed in 20 to 30 percent of the tobacco leaf disc epithelial cells (Figure 3a, b and c). In case of the co-cultivated *Arabidopsis* root segments even 60 to 70 percent of the root cells were GFP positive (Figure 3d). In both cases, most of the GFP signals were found in the cytosol, but in some cells also nuclear GFP signals were found (data not shown), which confirms previous observations that VirF is a nuclear localized effector protein (Tzfira et al., 2004) and BBM and AHL15 are also transcription factors. From these results we concluded that the developmental regulators BBM and AHL15 can be translocated by *Agrobacterium* to plants cells with similar efficiencies as Cre, which prompted us to test the use of these proteins to enhance plant regeneration.

AMPT of plant developmental regulators decreases leaf senescence and enhances shoot regeneration in tobacco

In-vitro selection and regeneration of stable transgenic plants from tissue explants is an important and difficult step in AMT, especially for some important but regeneration recalcitrant crop plants, such as sweet pepper. One way to facilitate regeneration during AMT would be to translocate regeneration enhancing proteins together with the T-DNA construct. The developmental regulators AHL15 and BBM are good candidates proteins which have already been shown to induce somatic embryos when ectopically expressed (Boutilier, 2002; Karami et al., 2017) and we showed above that they can be translocated by AMPT to plant cells.

To test the effect of developmental regulator translocation on tobacco regeneration, sterilized leaf discs were infiltrated with strain AGL1 containing *pvirF::GFP$_{11}$-BBM-dvirF*, or *pvirF::GFP$_{11}$-AHL15-dvirF*. Strain AGL1 containing *pvirF::GFP$_{11}$-dvirF* was used as a negative control. To investigate whether a co-transferred T-DNA would enhance the efficiency of protein translocation, we introduced a binary vector carrying the *35S::GFP$_{1-10}$* T-DNA construct into these strains. Leaf discs were cultured on shoot induction medium for one week (including the co-cultivation period of 3 days) and were subsequently transferred to hormone free medium (only containing antibiotics to suppress Agrobacteria) to observe the effect of BBM-VirF and

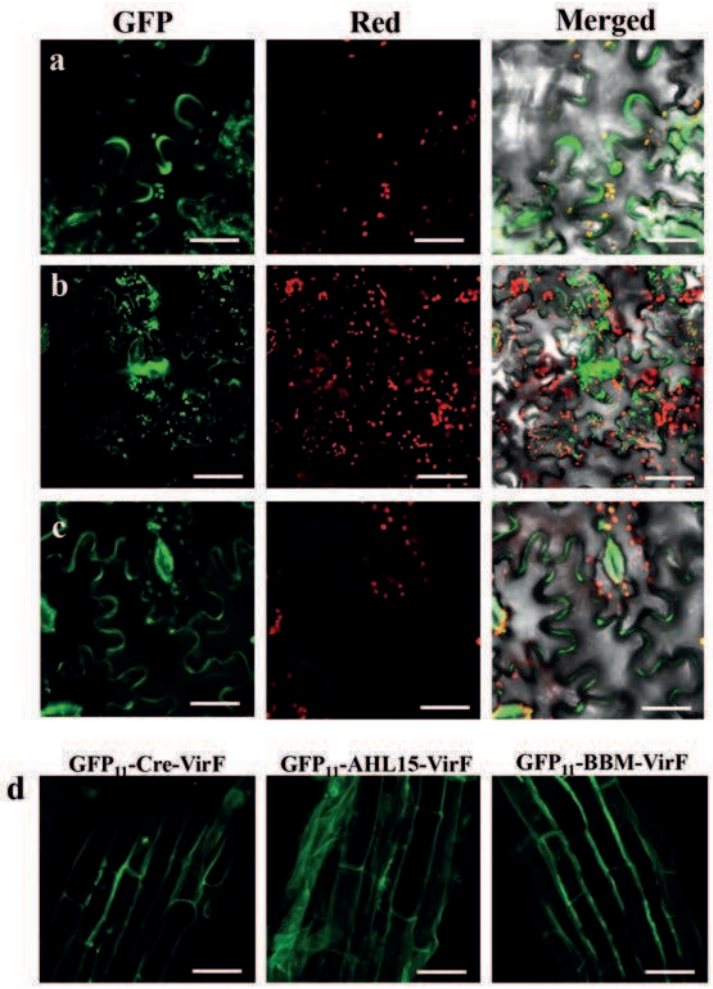

Figure 3. Visualization of Cre, BBM and AHL15 protein translocation via the split GFP assay in tobacco and *Arabidopsis* GFP1-10 marker lines. (a-c) Visualization by confocal microscopy of GFP1-10 complementation in leaf discs of *Nicotiana tabacum 35S::GFP*$_{1-10}$ line 3 days after co-cultivation with *Agrobacterium* AGL1 strain translocating GFP11-Cre-dVirF (a), GFP11-BBM-dVirF (b) or GFP11-AHL15-dVirF (c). Left panel shows the GFP signal in the green channel, middle panel showed the autofluorescence in the red channel, and right panel shows the merged image of the green, red and transmitted light channel. (d) Visualization by confocal microscopy of GFP1-10 complementation in the roots of *Arabidopsis thaliana 35S::GFP*$_{1-10}$ 3 days after co-cultivation with *Agrobacterium* AGL1 strain translocating GFP11-Cre-dVirF (left panel), GFP11-BBM-dVirF (middle panel) or GFP11-AHL15-dVirF (right panel). GFP signals were found mostly in the cytosol. Scale bar is 10 μm.

AHL15-VirF fusion protein translocation on shoot regeneration. In this experiment we did not observe shoot regeneration, indicating that the one week hormone treatment was too short to induce this process, and that translocation of either AHL15 or BBM could not compensate for this insufficiency. However, after two weeks on hormone free medium we observed that 100% of the leaf discs infiltrated with the GFP$_{11}$-BBM-VirF or GFP$_{11}$-AHL15-VirF fusion protein translocating bacterial strain, remained fresh and green (Fig. 4a and b), whereas the control

leaf discs turned yellow and necrotic (Fig. 4c). This is in line with previous observations that *BBM* or *AHL15* overexpression slows down leaf senescence in tobacco (Srinivasan et al., 2007 and Chapter 2, this thesis). No clear difference in senescence reduction was observed between strains with or without the additional T-DNA containing binary vector, suggesting that co-transfer of T-DNA does not have any observable effect on the efficiency of protein translocation.

In a second experimental set up, the leaf discs were incubated for two weeks (including the co-cultivation period) on shoot induction medium, and subsequently transferred to hormone free medium. This time, we observed a uniform shoots emergence within one month from all the leaf discs. The shoots induced on the GFP_{11}-AHL15-dVirF or GFP_{11}-BBM-dVirF fusion protein translocated samples (Fig. 4 d and e) looked slightly greener than those on the GFP_{11}-dVirF control leaf discs (Fig. 4f). After one month, the leaf discs and their shoots were transferred to big jars, and two weeks later we observed more and bigger shoots on the leaf discs induced with GFP_{11}-AHL15-dVirF or GFP_{11}-BBM-dVirF fusion protein translocation (Fig. 4 g and h) as compared to the negative control (Fig. 4i). To quantify this difference we counted all the small and big shoots (with meristem) per leaf disc and compared the different treatments. The GFP_{11}-AHL15-dVirF and GFP_{11}-BBM-dVirF fusions translocated leaf discs produced significantly more shoots compared to the control leaf discs, and co-transfer of a T-DNA did not significantly influence this number (Fig. 4j).

These results indicate that AMPT can be used to translocate plant developmental regulators to induce and regulate plant developmental processes such as senescence and regeneration. However, AMPT of the key developmental regulators AHL15 or BBM did not induce shoot regeneration from tobacco leaf discs by itself, but it did enhance hormone induced shoot regeneration, probably by reducing the explant senescence. This approach may be useful in the transformation or micro-propagation of important crop plants.

DISCUSSION

Previously, it was shown that *Agrobacterium* not only is able to transfer DNA to plants cells, but that in addition Vir proteins are translocated through the same T4SS pore to assist the process of T-DNA integration and subsequent tumor formation (Schrammeijer et al., 2003; Vergunst et al., 2000, 2005). To detect Vir protein translocation, Vir proteins have been coupled to the Cre recombinase or to subdomain 11 of GFP, allowing to detect protein translocation by recombinase-mediated restoration of a marker gene (Vergunst et al., 2000) or through split-GFP complementation (Sakalis et al., 2014), respectively. This at the same time showed that the *Agrobacterium* T4SS can be used to translocate heterologous proteins to plant cells, and that the translocation requires a signal peptide located at the C-terminus of these proteins. Here we showed that the 50 amino acids C-terminal part of VirF is sufficient to translocate plant developmental regulators, such as BBM and AHL15, to plant cells, and observe their effects on developmental processes such as senescence and regeneration. Plant transformation via genetically engineered *A. tumefaciens* is a common plant modification tool practicing in almost all plant molecular research labs (Shiboleth and Tzfira, 2012; Hwang et al., 2015). But regulating plant developmental processes through functional protein translocation via *A. tumefaciens* is a new tool that we introduced by this study.

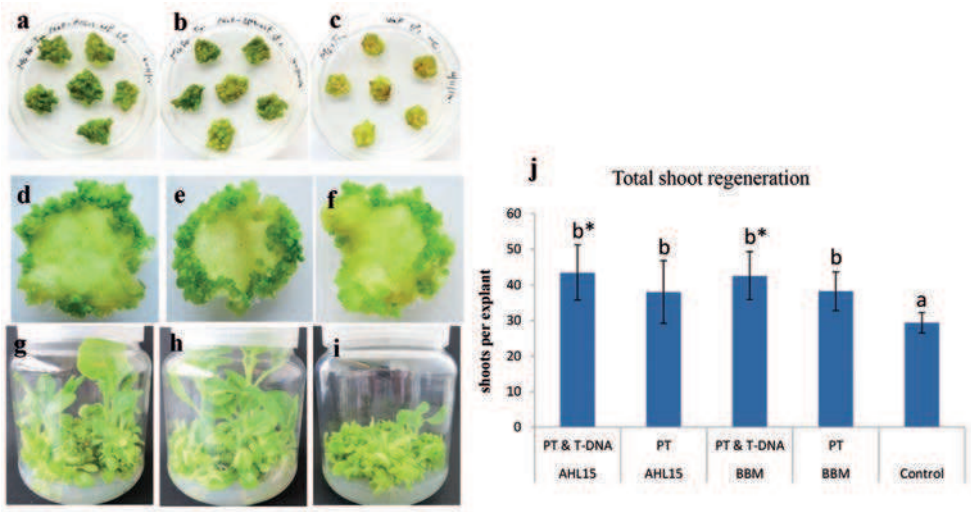

Figure 4. *Agrobacterium*-mediated translocation of AHL15-dVirF and BBM-dVirF fusions delay senescence and enhance shoot regeneration in *N. tabacum* leaf discs. (a-c) *N. tabacum* leaf discs after cocultivation with *Agrobacterium* strain AGL1 translocating AHL15-dVirF (a), BBM-dVirF (b) or dVirF (c) cultured for one week on shoot induction medium and pictures were taken after 3 weeks. (d-f) *N. tabacum* leaf discs after cocultivation with *Agrobacterium* strain AGL1 translocating AHL15-dVirF (d), BBM-dVirF (e) or dVirF (f) and cultured for two weeks on shoot induction medium and 2 week on hormone free medium. (g-i) The leaf discs from (d-f) transferred into jars with hormone free MS media for shoots development and the pictures were taken after 8 weeks. (j) Quantification of the regeneration efficiency (expressed as the number of shoots per explant) for leaf discs cocultivated with *Agrobacterium* strain AGL1, translocating GFP_{11}-dVirF (control), GFP_{11}-BBM-dVirF (BBM), or GFP_{11}-AHL15-dVirF (AHL15) (PT), or the latter two with cotransfer of the *35S::GFP*$_{1-10}$ construct (T-DNA). Values were statistically compared using the Student's *t*-test ($p<0,05$). Significantly different values are labelled with different letters. Asterisks (*) indicate significant difference.

The Cre recombinase protein translocation via AMPT has been used previously in *A. thaliana* roots explants to restore a disrupted kanamycin resistance gene by removal of a floxed insertion, thereby allowing recombinant cells to be selected on kanamycin (Vergunst et al., 2000; Schrammeijer et al., 2003). In our lab using *Arabidopsis* seedlings for GFP-based Cre recombinase assay via AMPT (Vergunst et al., 2005) we showed that the number of cells that received sufficient Cre protein to induce the recombination event via AMPT in the leaves cells was at least an order of magnitude lower than when the *35S::Cre* transgene is introduced via AMT. This difference might have several causes. First of all, although we showed that the GFP_{11}-Cre-dVirF fusion protein can mediate recombination at the *loxP* sites, we cannot exclude that this fusion protein has reduced activity compared to the Cre protein itself (Vergunst et al., 2000). However, control experiments in which we used the *35S::GFP*$_{11}$*-Cre-dvirF* constructs showed differences in efficiency based on reporter lines (Fig. S1), suggesting that the Cre recombination depends on the loci of the floxed marker *35S::GFP* gene in the reporter plant genome. Another possibility was that the GFP_{11}-Cre-dVirF fusion protein was not efficiently translocated to the nucleus.

Using the split-GFP assay with the *N. tabacum* and *Arabidopsis 35S::GFP*$_{1-10}$ reporter lines we found that most of the GFP signals were localized to the cytosolic region of the cell, which

might be because of availability of GFP_{1-10} part in the cytosol only, or because GFP_{11}-Cre/BBM/AHL15-dVirF fusion proteins cannot efficiently enter the nucleus. In fact, the AMPT efficiencies detected with the split-GFP reporter system were more constant and higher (20-30% of the cells) compared to what was observed using the Cre recombinase system (maximally 6-8% of the cells). The last possibility was that the amount of protein introduced by AMPT was lower compared to when an AMT introduced transgene is expressed from a constitutive promoter but that was also not the case as transient expression of *35S::GFP_{11}-Cre/BBM/AHL15-dVirF* in wild type tobacco leaf cell gave the same results with cytosolic localization of GFP signals but with bright fluorescence and higher efficiency (Fig. S3). The currently available experimental systems did not allow to accurately quantify the number of proteins translocated to host cells by AMPT. However, based on the sensitivity of the Cre recombinase and the split-GFP reporter assays this must range from 2 to 20 per cell (Shoura et al., 2012).

For the translocation of developmental regulator fusions to wild-type tobacco leaf tissues, we observed a significant effect on senescence and shoots regeneration. For BBM is well-established that overexpression of the protein delays plant senescence and enhances regeneration by slowing down developmental processes and by inducing somatic embryogenesis (Boutilier, 2002; Heidmann et al., 2011; Srinivasan et al., 2007). For AHL15 we observed similar effects (Karami et al., 2017; Chapter 2). So the enhanced shoot regeneration by the translocated AHL15-dVirF and BBM-dVirF fusion proteins might be a direct effect of the AHL15 and BBM proteins. However, it might also be an indirect effect of the delay in tissue senescence of the leaf discs. Alternatively, it is also possible that the translocated GFP_{11}-AHL15-dVirF and GFP_{11}-BBM-dVirF fusion proteins cause a general delay in development of the target cells, thereby providing opportunity to non-targeted cells to regenerate and develop faster, also leading to indirect shoot regeneration enhancement. Whatever the cause, the translocated fusion proteins were not able to induce hormone-independent regeneration (neither shoots, nor somatic embryos), suggesting that the amount of translocated protein is too low for this purpose. We did obtain proof of concept, however, that AMPT of plant developmental regulators such as AHL15 and BBM can induce detectable developmental changes in the infiltrated tissue explants. Although this technique clearly requires optimization, our findings present a new way to study the function of a gene without the need to make transgenic plant lines, and also hold promise for the development of marker free transformation protocols by inducing hormone-independent regeneration. In addition, this method may enhance transformation of important but recalcitrant crop species, such as sweet pepper, which is not only useful for their improvement by genetic modification, but also for research purposes to pretest the effect of specific genetic modifications that nowadays can be achieved in a non-GMO fashion by the CRISPR-CAS technology (Bortesi and Fischer, 2015).

MATERIALS AND METHODS

Arabidopsis and tobacco plant lines and growth conditions

The *Arabidopsis thaliana pcb1* transgenic line in C24 back ground was described before (Vergunst et al., 2005). The *Arabidopsis* GFP_{1-10} line was obtained by *Agrobacterium*-mediated

transformation of the pSDM3764 (*p35S::GFP*$_{1-10}$*-t35S*) construct (Sakalis et al., 2014) to the *Arabidopsis* Columbia ecotype (Col-0) by the floral dip method (Clough and Bent, 1998) using *Agrobacterium* strain AGL1. Transgenic lines were selected on hygromycin and one of the six independent lines with a single locus T-DNA insertion was selected in homozygous state for the use in the AMPT experiments. The *Nicotiana tabacum* SR1 *GFP*$_{1-10}$ plant line used for detection of AMPT by the split-GFP assay was described before (Sakalis et al., 2014). *N. tabacum* SR1 wild-type plants were used to detect the effects of AMPT of developmental regulators. *Arabidopsis* plants were grown in tissue culture at 21°C, 50% relative humidity and a 16 hours photoperiod, or on soil at 20°C, 70% relative humidity and 16 hours photoperiod. Tobacco plants were grown in tissue culture at 25°C, 50% relative humidity and a 16 hours photoperiod, or on soil at 25°C temperature, 75% relative humidity and 16 hours photoperiod.

Agrobacterium strains and culture conditions

The *A. tumefaciens* strains used in AMT and AMPT experiments are listed in Table 1. Plasmids were introduced into *Agrobacterium* by electroporation (Den Dulk-Ras and Hooykaas, 1995). To generate AGL1 strains containing both a binary plasmid for T-DNA transfer and a vector for protein translocation, the AGL1 strain already containing *p35S::GFP*$_{1-10}$ was electroporated with the appropriate plasmid for protein translocation. Agrobacteria containing both plasmids were selected on 20 µg/ml rifampicin (chromosomal marker), 75 µg/ml carbenicillin (disarmed binary vector), 100 µg/ml kanamycin (T-DNA plasmid) and 40 µg/ml gentamycin (AMPT vector).

Agrobacterium strains were grown in LC medium (10 g/l tryptone, 5 g/l yeast extract and 8 g/l NaCl, pH= 7.5) containing (if required) rifampicin, (20 µg/ml), gentamicin (40 µg/ml) and kanamycin (100 µg/ml). The cultures were incubated under continuous shaking (180 rpm) for two days at 30°C. One ml of the bacterial culture (OD_{600} around 1) was diluted in 50 ml AB minimal medium and incubated overnight (Gelvin, 2006). The overnight cultures were centrifuged in 50 ml falcon tubes for 20 minutes at 4000 rpm and 4°C. The pellets were washed with Milli-Q-water (MQ) and re-suspended and incubated overnight at room temperature in two volumes of induction medium (Gelvin, 2006) containing 100 µM acetosyringone. The overnight cultures were centrifuged as indicated above and the pellet was re-suspended in MA medium (Masson and Paszkowski, 1992) without FeNA-EDTA at an OD_{600} of 0.6.

Table 1. Agrobacterium strains used in this study

***Agrobacterium* strain**	**Specifications**	**Source / reference**
LBA1010	C58 containing pTiB6, Rif	Koekman et al., 1982
LBA1100	C58 containing pTiB6Δ (ΔT-DNA, Δ*occ*, Δ*tra*), Rif, Spc	Beijersbergen et al., 1992
LBA2587	*virD4* deletion in LBA1100, Rif, Spc	(Sakalis et al., 2014)
AGL1	C58, *RecA*, containing pTiBo542ΔT-DNA, Rif, Cb	Jin et al., 1987

D: deletion, *tra*: transfer region, *occ*: octopine catabolism, Antibiotic resistance: Rif: rifampicin, Spc: spectinomycin, Cb: carbenicillin.

Agro-infiltration and co-cultivation

For the Cre recombinase assay using *A. thaliana* line *pcb1,* 15 days old seedlings were vacuum infiltrated using the bacterial culture with an OD_{600} of 0.6 (described above) for 10 minutes at 250mm Hg pressure, after which they were blotted for 2-3 minutes on sterile tissue paper to remove the excess of *Agrobacterium*. Blotted seedlings were cultured for 3-4 days on MA medium containing 100 µM AS at 22°C in the dark.

For the split-GFP assay using the *Arabidopsis* and tobacco GFP_{1-10} marker lines, we syringe infiltrated sterile leaves or surface sterilized leaf discs respectively by keeping the leaves or leaf discs on a sterile tissue paper inside the down flow cabinet while the *Agrobacterium* culture with an OD_{600} of 0.6 (described above) was gently infiltrated into the abaxial side of the leaves or leaf discs using a 2 ml syringe (without needle). After blotting on sterile filter paper to remove excess of *Agrobacterium* culture, the leaves or leaf discs were co-cultivated on MS media plates containing 100µM AS for 3-4 days in the dark. For infiltration of intact *N. tabacum* plants, the fully developed 4th and 5th leaves of non-sterile 3 to 4 weeks old plants were syringe infiltrated at the abaxial side, using a 5 ml syringe (without needle) with an *Agrobacterium* culture of OD 0.8 to 1 (prepared by the same method as described above). The infiltrated plants were covered with plastic bags for a few hours, and co-cultivation occurred for 3 days in the growth room. For AMPT to *Arabidopsis* root cells, we used the same protocol that was developed for efficient transient transformation of *Arabidopsis* roots (Van Loock et al., 2010)

Stereo- and confocal microscopy

For the Cre recombinase assay, *A. thaliana* seedlings were observed under the Leica MZ16FA stereo fluorescent microscope (Leica Microsystems Singapore) with 16:1 zoom and 840Lp/mm resolution power, using the 1.0x plan apochromatic objective and the GFP plants (GFP3, excitation filter 450-490nm and emission filter 500-550nm) and DsRED (DSR, excitation filter 510-560nm and emission filter 590-650nm) filter sets. Confocal Laser Scanning Microscopy (CLSM) was performed on a Zeiss Imager (Zeiss, Oberkochen, Germany), equipped with an LSM 5 Exciter, using a 40x magnifying objective (numerical aperture1.4), and a 488 nm band pass excitation filter with a 505–530nm band pass emission filter to detect GFP. Chlorophyll fluorescence was detected by combining the 488nm band pass excitation filter with a 650nm long pass emission filter. Samples for microscopy analyses of co-cultivations were prepared by cutting 2 cm^2 pieces of infiltrated leaf tissue. A coverslip was placed on top of the sample with a drop of water to prevent drying. All images were taken in multiple focal planes (Z-stacks) and the selected optical sections were merged and analyzed using Image j software (Abramofff et al., 2005).

Tobacco leaf disc transformation

For leaf disc transformation, round leaf discs of 1.5cm diameter were collected from veinless parts of the fully expanded leaves of 4-5 weeks old tobacco plants. After surface sterilization in 10% (v/v) glorix for 20 minutes (Baltes et al., 2014), the leaf discs were syringe infiltrated (described above) and co-cultivated for three days in the dark on 40 mg/l acetosyringone (AS)

containing MS medium supplemented with 2 mg/l BAP and 0.2 mg/l NAA. For hormone-induced regeneration (up to one or two weeks), the leaf discs were transferred to the same co-cultivation medium (without AS) with 500 mg/l cefotaxime, after which they were transferred to hormone free MS medium with 500 mg/l cefotaxime. After 5 weeks, the leaf discs with regenerated shoots were transferred to jars containing MS medium for bigger shoots. Two weeks later the effect of APMT on tobacco leaf discs regeneration was quantified, by counting the number of shoots regenerated on all explants (n = 30 per experiment) (Tzfira et al., 2002). We calculated the total number of shoots per explant, and averages were compared for statistically significant differences using the Student's *t*-test ($p<0.05$).

Plasmid construction

All plasmids used and constructed in this study are listed in Table 2. Cloning steps were performed in *E. coli* strain DH5α. PCR amplifications were done with Phusion™ High-Fidelity DNA Polymerase and Table 3 lists all primers used for PCR amplifications.

Plasmid pSDM6500 [*pvirF::GFP*$_{11}$*-L2-dvirF2*] was obtained by modification of plasmid pSDM3760 [*pvirF::GFP*$_{11}$*-virF*] (Sakalis et al., 2014). First, the four restriction sites (*Sal*1, *Sma*1, *Bam*H1 and *Xba*1) in the backbone plasmid present in front of the C-terminus of *virF* were removed by restriction digestion with *Sal*1 and *Ssp*1 and subsequent self-ligation. The resulting plasmid was digested with *Bam*H1 and *Psp*14061 and a synthetic *Bam*H1---*Psp*14061 fragment named linker 2 (L2) was inserted, resulting in pSDM6500 [*pvirF::GFP*$_{11}$*-L2-dvirF*]. The *BBM*, *AHL15* and *Cre* genes (obtained from pSDM3155 [*pvirF::ATG-NLS-GSK-FLAG-BBM-virFdelta*, *pvirF::ATG-NLS-GSK-FLAG-AHL15-virFdelta* and *pvirF::ATG-NLS-GSK-FLAG-CRE-virFdelta* plasmids) were inserted using the *Sal*1, *Eco*R1 and *Pst*1 sites in L2. Correct ligations were checked by restriction analysis, PCR and sequencing.

To be able to test the functionality of the GFP$_{11}$-Cre-dVirF fusion protein, we replaced the *GFP*$_{1-10}$ coding region in pSDM3764 [*35S::GFP*$_{1-10}$] as *Nco*1-*BstE*II synthetic fragment (Eurofins) for the *GFP*$_{11}$*-L2-dvirF* coding region, resulting in plasmid pSDM6510 [*p35S::GFP*$_{11}$*-L2-dvirF*]. This plasmid was then used to insert the *Cre, BBM and AHL15* genes in the L2 linker, as described above, resulting in pSDM6511 [*p35S::GFP*$_{11}$*-Cre-dvirF*], pSDM6512 [*p35S::GFP*$_{11}$*-BBM-dvirF*] and pSDM6513 [*p35S::GFP*$_{11}$*-AHL15-dvirF*].

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Table 2. Plasmids used in this study

Name	Properties	Source / reference
pGreenMC007 [*p35S::Cre*]	pGreen backbone with the coding sequence of *Cre* under control of the *35S* promoter	Hellens et al., 2000
pSDM3760 [*pvirF::GFP$_{11}$-virF*]	pSDM3163 backbone with the coding sequence of *GFP$_{11}$-virF* under control of *virF* promoter	Sakalis et al., 2014
pSDM6500 [*pvirF::GFP$_{11}$-L$_2$-dvirF*]	pSDM3760 backbone with the coding sequence of *GFP$_{11}$-L2-dvirF* under control of the *virF* promoter (*L2* is the linker sequence having multiple unique restriction sites).	This study
pSDM6502 [*pvirF::GFP$_{11}$-Cre-dvirF*]	pSDM3760 backbone with the coding sequence of *GFP 11-Cre-virF* under control of the *virF* promoter	This study
pSDM6503 [*pvirF::GFP$_{11}$-AHL15-dvirF*]	pSDM3760 backbone with the coding sequence of *GFP$_{11}$-AHL15-virF* under control of the *virF* promoter	This study
pSDM6504 [*pvirF::GFP$_{11}$-BBM-dvirF*]	pSDM3760 backbone with the coding sequence of *GFP $_{11}$-BBM-virF* under control of the *virF* promoter	This study
pSDM3764 [*p35S::GFP$_{1-10}$*]	pCambia1302 backbone with the coding sequence of *GFP$_{1-10}$* under control of the *35S* promoter and the *CaMV* terminator.	Sakalis et al., 2014
pSDM6510 [*35S::GFP$_{11}$-L$_2$-dvirF*]	pCambia1302 backbone with the coding sequence of *GFP$_{11}$-L2-dvirF* under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6511 [*35S::GFP$_{11}$-Cre-dvirF*]	pCambia1302 backbone with the coding sequence of *GFP$_{11}$-Cre-dvirF* under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6512 [*35S::GFP$_{11}$-BBM-dvirF*]	pCambia1302 backbone with the coding sequence of *GFP$_{11}$-BBM-dvirF* under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6513 [*35S::GFP$_{11}$-AHL15-dvirF*]	pCambia1302 backbone with the coding sequence of *GFP$_{11}$-AHL15-dvirF* under control of the *35S* promoter and the *CaMV* terminator.	This study

Table 3. Synthetic sequences/primers used in this study

Primer name	Sequence (5'..... 3')
GFP11-L2-dVirF (*Nco*1--*Bst*EII)	***CCATGG****TTCGGGACCACATGGTGCTGCACGAGTACGTGAACGCCGCCGGCATCACAGGCGACGGCGGCAGCGGCGGCGG CAGCACTAGTGGATCCGAATTCCTGCAGGGTCGACTTAACGTTGCGGAACCGATTATGTTCAATGAAATCTCCGCTCTCGAG GTTATGGCAGAAGTTCGGCCCATAGCCCGATCCATTAAAACGGCTCACGACGATGCGCGAGCGGAATTAATGTCGGCGGACA GACCTCGATCAACGCGCGGTCTATGA****GGTGACC***
Oligo1.*Bam*H1--*Psp*14061	GATCCGAATTCCTGCAGGGTCGACTTAA
Oligo2.*Bam*H1--*Psp*14061	CGTTAAGTCGACCCTGCAGGAATTCG
*BamH*I-nls-oligo1-*EcoR*1	AATTCCAGCTCAACCTTCCTCTTCTTCTTAGGAGGCTCCATG
*Bam*HI-nls-oligo2-*EcoR*1	GATCCATGGAGCCTCCTAAGAAGAAGAGGAAGGTTGAGCTGG
*Xba*I-F2-Fwd	GC**TCTAGA**GGTCGACGGATCCCCGGGTT
*Nde*I-F2-Rev	CC**CATATG**GGTCGACGGATCCCCGGGTT
*Nco*I-GFP$_{1-10}$-Fwd	GC**CCATGG**TTTCGAAAGGCGAGGA
*Bst*EII-GFP$_{1-10}$-Rev	G**GGTCACC**TTATTTCTCGTTTGGGTCTT
*Xba*I-GFP$_{1-10}$-Fwd	GC**TCTAGAA**TGGTTTCGAAAGGCGA
*Xho*I-GFP$_{1-10}$-Rev	CC**CTCGAG**TTATTTCTCGTTTGGGT
*Nde*1-pVirF - Fwd	**ATAT**GCGGGACCACATGGTGCTGCA
*Eci*1-pVirF- Rev	**GATC**GAGGTCTGTCCGCCGACATTA
dVirF - Fwd	GGGCACCAATAACTGCCTTA
dVirF - Rev	TTCCTACTATACGATGCGCC
35Sgfp11-dVirF-Fr	TCATTTGGAGAGAACACGGG
35Sgfp11-dVirF-Rv	TAATCATCGCAAGACCGGCA

a, recognition sites for the restriction enzymes in the primer names are underlined.
b, linker sequences are annotated in italics.

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SUPPLEMENTARY FIGURES

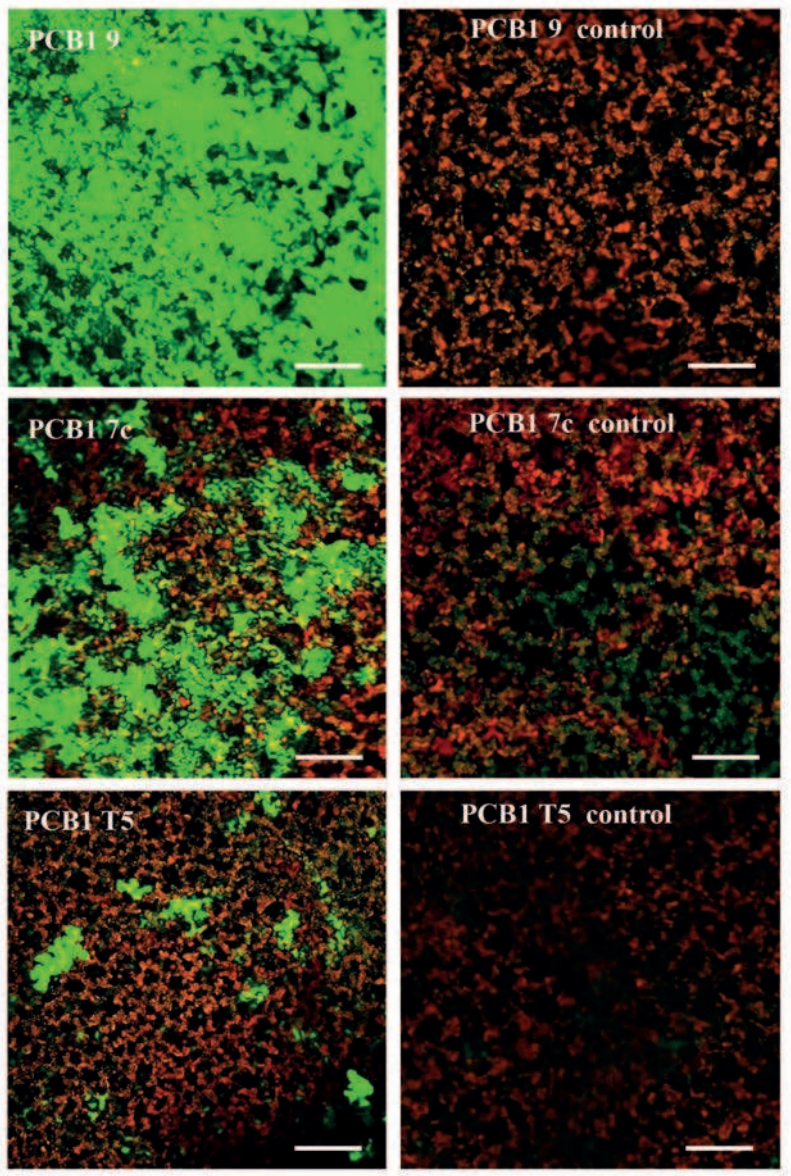

Figure S1. Independent *Arabidopsis pcb1* lines detect *Agrobacterium*-mediated *p35S::GFP11-Cre-dVirF* transfer with different sensitivities. Three plants of *pcb1* lines 9, 7c and T5 were infiltrated with AGL1 carrying the *p35S::GFP11-Cre-dvirF* construct, or with AGL1 carrying the *p35S::GFP*$_{1\text{-}10}$ construct as a control. The confocal images shown for pcb1-9 are representative for the fluorescence observed in all leaves of these plants. For pcb1-7 the fluorescent signal shown in the images was only observed in three leaves, and for pcb1-T5 the fluorescence shown was only observed in one leave. Plants in the control cocultivations, and three other *pcb1* lines tested didn't show any fluorescence. Lines *pcb1*-T5 and -7C were used for the experiments presented in Figure 1. Scale bar is 0.1 mm.

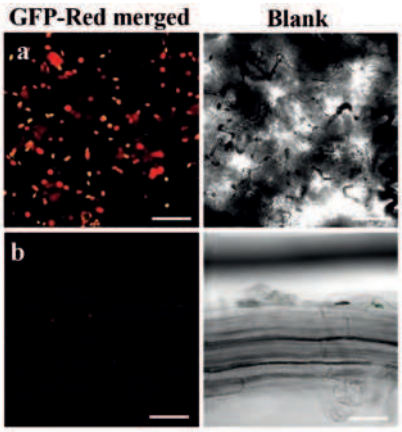

Figure S2. (a) Tobacco and (b) *Arabidopsis* lines GFP_{1-10} leaf epithelial and root cells respectively showed no GFP signal following cocultivation with *Agrobacterium* strain LBA2587 (*virD4* mutant) containing *pvirF::*GFP_{11}*-BBM-dvirF*. Scale bar is 10 μm.

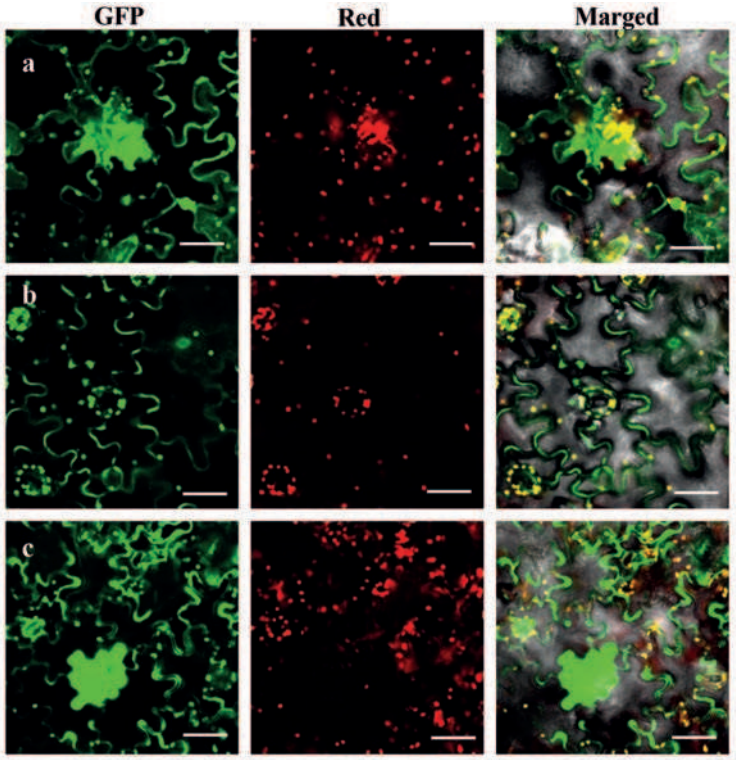

Figure S3. Visualization of *Agrobacterium*-mediated transient expression of *35S::GFP11-Cre-dvirF*, *35S::GFP11-BBM-dvirF* and *35S::GFP11-AHL15-dvirF* and their complementation with GFP11 in *Nicotiana tabacum* GFP1-10 marker line. (a-c) Visualization by confocal microscopy of GFP1-10 complementation in leaf discs of *Nicotiana tabacum 35S::*GFP_{1-10} line 3 days after co-cultivation with *Agrobacterium* AGL1 strain

harboring *35S::GFP11-Cre-dvirF* (a), *35S::GFP11-BBM-dvirF* (b) or *35S::GFP11-AHL15-dvirF* (c) construct. Left panel shows the GFP signal in the green channel, middle panel showed the autofluorescence in the red channel, and right panel shows the merged image of the green, red and transmitted light channel. Localization of GFP signals were found mostly in the cytosol. Scale bar is 10 μm.