Resolving plant transformation recalcitrance by Agrobacterium-mediated protein translocation

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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.