Molecular engineering of plant development using Agrobacterium-mediated protein translocation

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Molecular engineering of plant development using *Agrobacterium*-mediated protein translocation

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Cover: The upper panel shows the generic split-GFP system in which *Agrobacterium* tumefaciens simultaneously transfers a GFP1-10 expressing T-DNA and a GFP11-tagged fusion protein to a wild-type plant cell. The lower panel shows the syringe infiltration of *Capsicum annuum* (sweet pepper) cotyledons with an *Agrobacterium* strain carrying the generic split-GFP system, and a confocal image of green fluorescent stomatal guard cell as a result of successful DNA and protein co-translocation.

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Molecular engineering of plant development using *Agrobacterium*-mediated protein translocation

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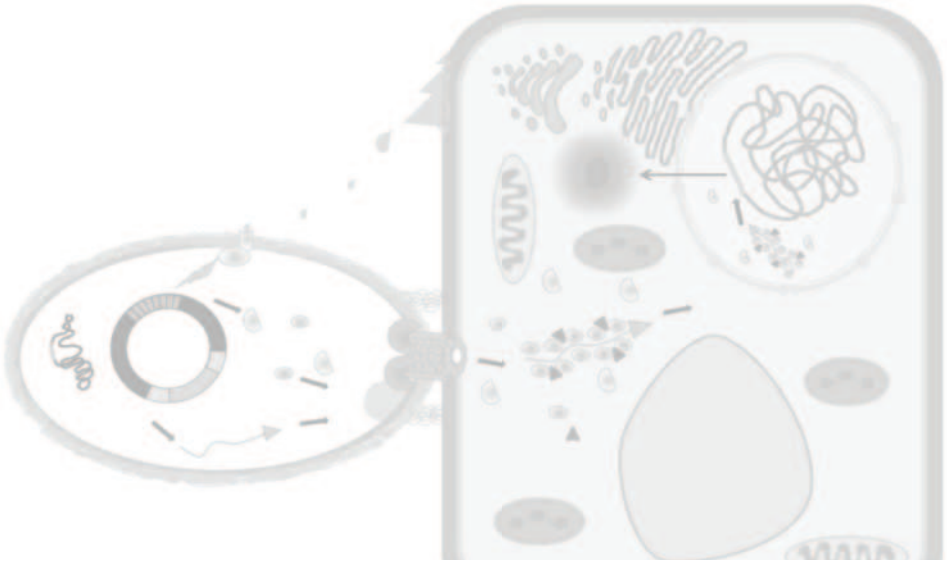
The richest poor man,

Abdul Sattar Edhi

No religion is higher than humanity
(Abdul Sattar Edhi)



GENERAL INTRODUCTION

CONTRIBUTION OF THE AGROBACTERIUM TRANSFORMATION MACHINERY TO PLANT DEVELOPMENTAL STUDIES

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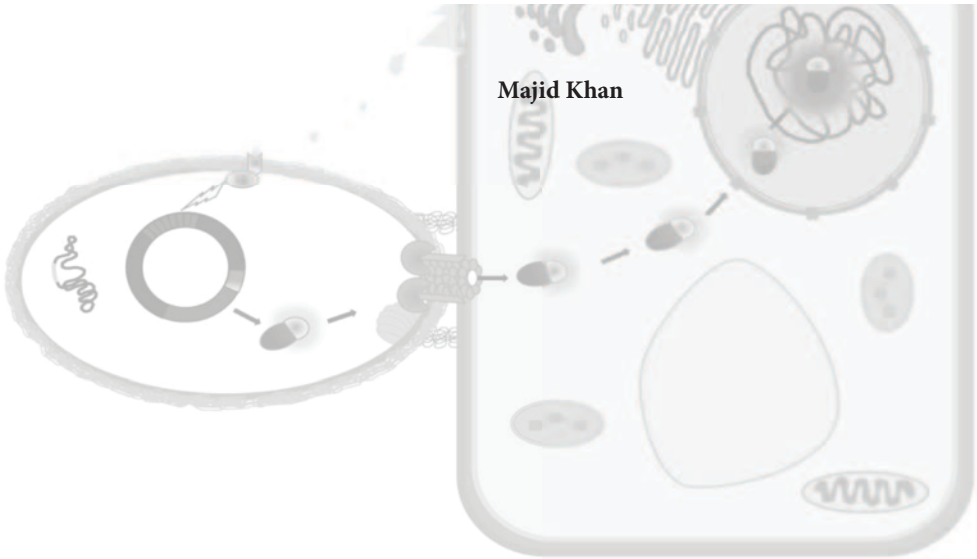

Majid Khan

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INTRODUCTION

The ancient book *Historia Plantarum* or *Enquiry into Plants* written by Aristotl's student Theophrastus around 350 BC is considered as the start of plant research, but the curiosity of humans in plant growth and development is even older. Plant morphology and alternation of generations were the first aspects investigated in these early studies in the process of plant domestication to maximize benefits (Morton, 1981). Especially the establishment of cereals with more and bigger grains that were easy to harvest and showed reliable germination led to the beginning of plant cultivation (Purugganan and Fuller, 2009). This changed the human life style from hunter-gatherer to farmer, and led to an explosion of new agricultural practices (Diamond, 2002). Norman Borlaug, known as the father of the green revolution, introduced for the first time high-yield disease resistant wheat varieties, and this introduction was accompanied by the use of chemical fertilizers, irrigation and mechanized agriculture (Patel, 2012). Further improvement of cultivation techniques and the application of insecticides and pesticides to control yield loss has maximized production and product quality. Due to the broad side effects of these agrochemicals, however, their use is currently being restricted, and plant breeders rely again on the available genetic potential of crop plants to generate resistant and tolerant varieties with improved yield and product quality (Tester and Langridge, 2010). The development of techniques for genetic modification (GM) of plants, of which *Agrobacterium*-mediated DNA transfer is currently the most versatile and widely used system, has allowed to introduce new traits across plant species borders. Unfortunately, GM of plants has met quite some public resistance, especially in Europe, and this has urged plant scientist to develop alternative non-GM methods to enhance crop productivity. The discovery that *Agrobacterium* can also translocate heterologous proteins to plant cells (Vergunst et al., 2000) has provided a non-GM method to modify the plant genome with site-specific recombinases. In this chapter I will focus on plant development, especially on switches or phase transitions during development of a plant, and how knowledge of the key regulators in these processes could be used to improve crops. In addition, I will discuss how non-GM tools for plant modification, such as *Agrobacterium*-mediated protein translocation, could be applied to target these phase transitions in plant development.

PLANT DEVELOPMENT

Plant development occurs in distinct phases. Following fertilization of the egg cell, the basic body plan of the plant is laid down during embryogenesis, which arrests with seed maturation (Yamaguchi and Nambara, 2007). Subsequent seed germination starts the vegetative phase, during which the change from juvenile to the adult vegetative development (vegetative phase change) enhances the photosynthetic capacity of the plant and at the same time signals the onset of flowering. Plant species that flower only once (monocarpic) show a complete transition from vegetative to reproductive development, and as a result seed set preludes senescence and death of the plant, whereas polycarpic species that flower multiple times retain some vegetative development, which allows them to repeat the transition to flowering multiple times (Huijser and Schmid, 2011).

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Compare to animals, plants are the most sensitive organisms to environment. As sessile organisms, they have evolved mechanisms to adapt to environmental constraints via developmental changes to guarantee their survival (Scutt et al., 2007). Plant development therefore involves the integration of the intrinsic genetic program, and exogenous environmental (extrinsic) signals, which together through the action of endogenous hormonal signals determine the final plant morphology (Huijser and Schmid, 2011). Hormones are defined as signaling molecules that after synthesis are transported to and act at a distant site. In plants, about nine classes of signaling molecules have been recognized as hormones that regulate plant development and are not only responsible for plant morphogenesis, but also affect processes such as seed germination, flowering time, fruit development and senescence (Sparks et al., 2013). Two of these classes of signaling molecules, the auxins and cytokinins, are considered as key regulators of plant development, since they regulate very basic processes such as the growth, division, and differentiation of plant cells. Other plant hormones such as abscisic acid (ABA), gibberellins (GAs) and ethylene are involved in more specific developmental processes, such as seed maturation and leaf abscission, seed germination and cell elongation, and plant stress signaling and fruit ripening, respectively, while jasmonic acid and salicylic acid are the most important defense hormones (Wang and Irving, 2011).

The mutual interaction of all these hormones at the right time and space assists the plant in completing a successful life cycle, by allowing plant development to adapt to environmental conditions, and at the same time by providing protection against various biotic and abiotic stresses. The plant hormone signaling pathways interact and merge with other signaling pathways at the gene expression level, resulting in a complex regulatory network (Spartz and Gray, 2008; Wang and Irving, 2011). The central hubs in this regulatory network are interesting targets for crop improvement in order to either enhance plant adaptations to extrinsic environmental stresses (plant tolerance to abiotic stresses and plant protection against biotic stresses) or to increase intrinsic yield potential in plants (manipulation of plant development and modification of plant architecture). Below, I will review the plant developmental stages starting from embryogenesis until plant senescence and will focus on phase change transitions in response to the interacting intrinsic and extrinsic signals.

EMBRYOGENESIS AND GERMINATION

During embryogenesis the basic body plan of the plant is laid down, comprising the shoot and root meristem that later form the shoot and root system of the plant, and the embryonic leaves that serve as primary storage and photosynthesis organs providing energy during the germination process (Bosca, 2011). Most of what we know on embryogenesis is from studies in the dicot model plant *Arabidopsis thaliana*. After fertilization of the egg cell and before the start of cell division the zygote elongates, which favors the first asymmetric cell division, producing a smaller apical and larger basal cell (ten Hove et al., 2015). The apical cell gives rise to the embryo proper and the basal cell after transverse divisions gives rise to a file of seven to nine cells named the suspensor that connects the embryo proper to the maternal tissue. In the globular embryo the shoot and root apical meristems (SAM and RAM) are established, and with the subsequent initiation of the two embryonic leaf- or cotyledon primordia flanking

the SAM the embryo becomes heart shaped (Yoshida et al., 2014). The specification of different cell identities during embryogenesis is tightly controlled by specific molecular pathways and is often marked by the onset of specific gene expression patterns. The SAM is established in the apical part of the early globular embryo by the cooperative action of the homeobox proteins SHOOTMERISTEMLESS (STM) and WUSCHEL (WUS) together with auxin signaling (Rademacher et al., 2011; Yoshida et al., 2014). For the SAM, WUS initiates the specification of the inner cells of the upper tier whereas for the RAM TARGET OF MONOPTEROS 5 (TMO5) and TMO7 initiate the specification of the inner cells of the lower tier of the embryo (Schlereth et al., 2010). Furthermore, the correct outer and inner cell fate separation is controlled by RECEPTOR-LIKE PROTEIN KINASE 1 (RPK1) and RPK2 (Nodine et al., 2007). RPK2 has also been shown to act downstream of CLAVATA3 (CLV3) in regulating the maintenance of meristem (Kinoshita et al., 2010) whereas the meristem size is restricted by the CLV1/3 regulatory loop. The establishment of the RAM ultimately requires the recruitment of the uppermost suspensor cell (hypophysis) into the embryo proper and mutations in components of auxin biosynthesis, transport, perception or response all cause defects in hypophysis division and RAM formation (Moller and Weijers, 2009). RAM initiation thus requires proper inductive auxin signaling from neighboring cells in the embryo proper.

The development of radicle and plumule by the activation of a dormant seed embryo as a result of positive interactions of exogenous environmental and intrinsic signals result in germination. Environmental parameters including salinity, acidity, temperature and light, can influence the germination process by changing the hormonal balance in the seed (Kucera et al., 2005; Ghaderi-Far et al., 2010). Abscisic acid (ABA) and ethylene in combination regulate many plant responses during stress conditions e.g. salinity, but during germination they have opposite effects on seed germination. ABA delays seed germination by negatively affecting endosperm softening and radicle expansion. Whereas ethylene, stimulated by Brassinosteroids (BR) and Indole-3-acetic acid (IAA), along with Gibberelline (GA) while antagonistically interacting the inhibitory effect of ABA, induces seed germination by rupturing the testa and softening of the endosperm (Arteca and Arteca, 2008; Finkelstein et al., 2008; Nonogaki, 2008; Graeber et al., 2010). Cytokinins also enhance the germination process by minimizing the effects of various stresses such as salinity, drought, heavy metals and oxidative stress (Peleg and Blumwald, 2011). Based on up to date research, Dekkers and Bentsink have summarized the dormancy and seed germination starting from embryogenesis until completion of germination process in the model plant *Arabidopsis thaliana*, showing that plant hormone ABA and the DELAY OF GERMINATION 1 (DOG1) gene are two key players for dormancy induction (Dekkers and Bentsink, 2015). Recently, it was shown that the embryonic regulator *FUSCA3* (*FUS3*) through hormonal regulation (modulating the ABA/GA ratio) along with biotic and abiotic factors, controls the embryonic-to-vegetative phase transition (Lumba et al., 2012).

VEGETATIVE PHASE

Following germination, and before plants become competent to flower and reproduce, they undergo a phase of vigorous growth and development involving a rapid increase in the size and mass of both the root and the shoot system, and accordingly an increase in photosynthetic

capacity of the plant. This phase is recognized as the vegetative phase. Based on the species-specific heteroblastic features this vegetative mode of growth can be further subdivided into a juvenile and an adult vegetative phase (Poethig, 2013).

The flowering incompetent phase of plants from immediately after germination until the appearance of adult features is known as the juvenile phase. The juvenile phase is usually shorter in annual plants as compared to perennials, and is characterized by some juvenile features of the shoot morphology. For example, in *Arabidopsis thaliana* the juvenile phase is characterized by rosette leaves with a long petiole, a small round/oval blade, smooth margins, and trichomes only on the adaxial (upper) side of the blade (Huijser and Schmid, 2011). The length of the juvenile phase is an important determinant of the final plant morphology, as the plants with a very short or no juvenile phase have a reduced number of shoots and flowers and senesce early. In contrast, plants with a long juvenile phase are highly branched and show an enhanced leaf initiation rate, late flowering, delayed senescence and sterility (Poethig, 2013). Environmental factors such as photoperiod and nutrient availability regulate intrinsic signals that maintain this vegetative phase, and only allow the change from juvenile to adult to occur when the conditions are suitable for the survival of the plant. This is the reason that the duration of the juvenile phase is not fixed and even in some plants flowering occurs independent of the juvenile to adult phase change (Poethig, 2013). Among the intrinsic signals especially the micro-RNAs (miRNAs) miR156 and miR157 extend the juvenile phase by repressing the expression of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Chien and Sussex, 1996; Wu and Poethig, 2006). SPL proteins restrict the length of the juvenile phase by promoting the transition into the adult phase and flowering (Huijser and Schmid, 2011). For maintaining the juvenility in plants, a sufficient amount of miR156 expression is required to completely suppress the SPL protein production. It has also been reported that in some plants the vegetative phase change and induction of flowering are independently inherited due to which the plants can flower either after a long adult phase or just after the transition from the juvenile to the adult phase or even during the juvenile phase. In most plants, however, the vegetative phase change is required for the plant to enter the reproductive phase (Poethig, 2013). As plants enter the vegetative phase, photosynthetic capacity of the plant increases with growth and development under standard environmental conditions, resulting in the increased production of sugars. These sugars, mainly sucrose, move from the site of production (leaves) to the sink tissues, such as young leaf primordia, where the sucrose is hydrolyzed into glucose. Glucose in turn is able to reduce the transcription of *MIR156* genes and to promote the breakdown of *MIR156A* and *MIR156C* primary messenger RNA transcripts (Yang et al., 2011; Yu et al., 2013). This gradual decrease in *miR156* expression results in a rise in SPL levels, which eventually leads to the disappearance of juvenile features and appearance of adult features such as short petioles, elongated leaves with serrated margins and abaxial trichomes (Wu et al., 2009; Poethig, 2010). The adult leaves produced after this so called vegetative phase change are more resistant to pathogens and pests, in part through the production of trichomes, and have a higher photosynthetic capacity (Hauser, 2014; Chondrogiannis and Grammatikopoulos, 2016).

REPRODUCTIVE PHASE

During the vegetative phase, the shoot apical meristem (SAM) produces leaves. For the vegetative to reproductive phase change, the vegetative meristem needs to be reprogrammed to become an inflorescence meristem, so that it will form the reproductive structures. The floral meristem identity genes *LEAFY (LFY)*, *APETALA1 (APT1)* and *CAULIFLOWER (CAL)* and the shoot meristem identity gene *TERMINAL FLOWER 1 (TFL1)* regulate this vegetative to reproductive switch. The floral identity genes induce flowering at lateral meristems in *Arabidopsis*, whereas *TFL1* represses the onset of flowering at the SAM, (Araki, 2001; Sablowski, 2010). The reproductive structures of the most successful and diverse group of plants, the angiosperms or flowering plants, characteristically develop flowers with stamens and a pistil as specialized male and female organs, respectively, generally surrounded by outer whorls of petals and sepals. As compared to other seed plants, in angiosperms the ovules are enclosed in the ovary, which later becomes the fruit. The timing of the transition from vegetative to reproductive phase is critical for reproductive success in the angiosperm life cycle, and is also of great importance from an agronomical point of view, because it has a direct effect on the biomass and number of seeds produced (Demura and Ye, 2010). Like the vegetative phase transition, the reproductive phase transition is regulated by various exogenous environmental factors such as photoperiod, temperature, and light intensity in combination with the endogenous signals derived from the nutritional status and developmental genetic network (Huijser and Schmid, 2011). Both of these transitions share some major connections regulated by the same endogenous developmental signals. For example, repression and induction of flowering during the juvenile and adult vegetative phases by miR156 and miR172, respectively (Poethig, 2013). As shown in figure 1 miR156 and miR172 are currently considered as two key regulators of the plant age-dependent flowering pathway (Li and Zhang, 2016).

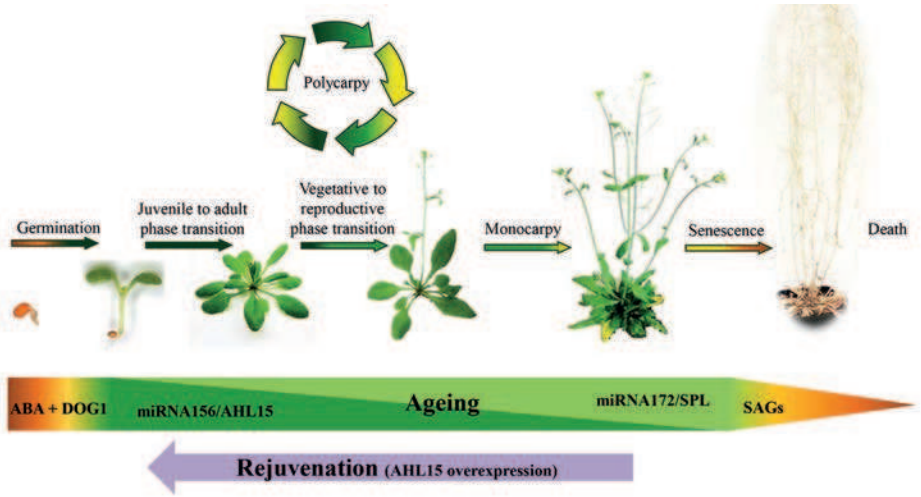

Figure 1. Schematic representation of plant ageing by transition from one to the next phase of development. The involvement of different key regulatory genes in ageing and rejuvenation is indicated.

Beside the microRNA-mediated pathway, flowering in plants is also controlled by vernalization, photoperiod, and GAs (Srikanth and Schmid, 2011). The MADS-domain transcription factor FLOWERING LOCUS C (FLC), a key player in the *Arabidopsis* vernalization response, has recently been shown to delay the juvenile-to-adult transition by directly acting on some of the same targets as the microRNAs. Several lines of evidence now indicate that in response to the photoperiod, a protein called FLOWERING LOCUS T (FT) interacting with the transcription factor FD at the shoot apex, is contributing to the floral induction by acting as a long distance signal between the leaves and the SAM (Kardailsky, 1999; Blázquez and Weigel, 2000). But it has also been reported that when miR156-targeted SPL activity continues to rise, plants will eventually flower without the requirement for photoperiod-dependent FT/FD activity (Wang et al., 2009). Similarly, Yu et al (2012) have shown that the age (miR156) and GA pathways are integrated through a direct physical interaction between SPL and the DELLA repressors of GA action. The binding of DELLA to SPLs attenuates SPL-mediated transcriptional activation of *FT* and *MADS box* genes, subsequently blocking the floral transition. The expression of *FT* in leaves is controlled by a number of proteins among which are the AT-hook containing nuclear protein *AHL22*, which is involved in chromatin remodeling, and a number of transcription factors that regulate *FT* expression in a more gene-specific manner (Yun et al., 2012).

MONOCARPY VERSUS POLYCARPY

With respect to entry into the reproductive phase, two life strategies can be distinguished in plants. Monocarpic plants (or monocarps) flower, produce seeds and then die, whereas polycarpic plants (polycarps) have the ability to produce flowers and fruits several times in successive years or seasons. Monocarps are usually annual or seasonal plants e.g. *Arabidopsis thaliana*, while polycarps are usually perennial plants that live from a few up to thousands of years, such as the red wood trees (Amasino, 2009). Both in monocarps and polycarps the flowers are produced after transition of the vegetative SAM to an inflorescence meristem (IM). In polycarps not all SAMs are converted to inflorescence meristems (IMs), thereby allowing the plants to maintain vegetative growth and to repeat the flowering cycle.

In many flowering plants such as winter annuals, biennials and perennials flowering is induced by a cold temperature treatment (vernalization) (Amasino, 2004). In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*), a gene encoding a MADS-domain transcription factor; is a flowering repressor that prevents the transition of the SAM to IM in the fall and creates a vernalization requirement. Wang et al (2009) showed that in *Arabis alpine* (a polycarpic relative of *Arabidopsis*) the vernalization-induced transition of SAMs to IMs is regulated by *PERPETUAL FLOWERING 1* (*PEP1*). Later it was shown that in *A. alpine* vernalization results in transient *PEP1* repression, whereas in *Arabidopsis*, vernalization results in a stable repression of the *Arabidopsis PEP1* ortholog *FLC*, which converts all SAMs into IMs (Wang et al., 2009). Both FLC and PEP1 action induce chromatin modifications during vernalization but after the arrival of warm temperature, in case of FLC these modifications increase while in case of PEP1 decreases (Bastow et al., 2004; Sung et al., 2006; Wang et al., 2009). The conversion of monocarpic to polycarpic growth in *Arabidopsis* can be achieved by mutations in two genes, *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *FRUITFULL* (*FUL*). Melzer et al (2008) showed

that in the *Arabidopsis soc1 ful* double mutant, under suitable environmental conditions some IMs revert to vegetative growth and other SAMs remain in the vegetative state producing new leaves after flowering. In monocarpic plants reproduction and leaf senescence are linked and beside floral-independent senescence, a correlation has been found between flowering- and the expression of senescence-associated genes (SAGs) in *Arabidopsis*. Recent evidence indicates that the synthesis of trehalose 6-phosphate (T6P)in the leaves in response to carbon availability plays a role in flowering and senescence regulation (Wingler et al., 2010; Wingler et al., 2012). Below I will discuss the senescence process, how this leads to plant death for monocarps, and how this process differs in polycarpic plants that are able to resume vegetative development following flowering.

SENESCENCE

In the final phase of the life cycle of a monocarpic plant, the energy stored in the organs is gradually made available for the progeny by senescence, and this programmed degradation process eventually leads to death of the plant (Figure 1). Senescence is a highly organized degradation and remobilization process controlled by both endogenous signals and several biotic and abiotic environmental stress signals. In both mono- and polycarps senescence is controlled by reproductive processes, especially seed development and maturation, but in polycarps senescence remains restricted to specific parts of the plant, such as the leaves (Gregersen et al., 2013). The genes involved in leaf senescence are collectively called senescence-associated genes (*SAGs*). Based on their activities and regulation by specific signaling pathways, the *Arabidopsis SAGs* are divided into six classes (Lim et al., 2003). Key regulators of the senescence process in *Arabidopsis* are transcription factors involved in the upregulation of *SAGs*, such as the WRKY transcription factor encoding genes *AtWRKY6* and *AtWRKY53*, and reversely proteins involved in the degradation of senescence regulators, such as the F-box protein ORE9 (Lim et al., 2003).

In monocarpic plants, senescence can also be induced by environmental signals, and especially in crop plants this can result in reduced productivity. For example, in annual plants the parent plant body is sacrificed under harsh conditions by early senescence to guarantee survival of the seeds (Buchanan-Wollaston, 2007). Similarly accelerated senescence of pathogen infected leaves leads to their removal thus lowering the risks of pathogen spread and increasing the chances of survival (Munne-Bosch and Alegre, 2004). In contrast, in small grain cereals such as wheat, delayed leaf senescence will allow active photosynthesis during seed set, which will increase the grain size (Gregersen et al., 2013).

The onset of senescence is marked by the enhanced expression of the *SAGs* (Figure 1), which are regulated by the increase in sugar levels in the photosyntheticaly active leaves (Quirino et al., 2000). Abiotic stresses, such as high light intensity and high temperature, damage the photosynthesis machinery and thus result in the production of reactive oxygen species that promote senescence (Suzuki et al., 2012; Bartoli et al., 2013). Biotic stresses, on the other hand, induce the production of the plant hormones ABA, jasmonic acid and salicylic acid that in turn promote senescence (Robert-Seilaniantz et al., 2011). In recent years a few factors repressing senescence by chromatin remodeling have been identified, such as the histone deacetylase HDA6, the histone methyltransferase SUVH2 and the AT-hook DNA binding protein ORE7/

ESC (Lim et al., 2007; Wu et al., 2008; Ay et al., 2009). The genes encoding these factors might be useful targets to delay senescence in crop plants to improve their biomass and productivity.

REVERSAL OF DEVELOPMENTAL PHASE CHANGES: REJUVENATION

As mentioned earlier, for most angiosperms the juvenile state is the phase in which a plant cannot flower even under favorable environmental conditions. In contrast, plants in the adult state produce flowers upon maturity under the proper environmental conditions. Each of these plant developmental states can be distinguished by specific morphological characteristics regulated by genetic signals and growth hormones (Sparks et al., 2013). The manipulation of these hormonal and genetic signals may lead to a reversal of the vegetative phase change or the reappearance of juvenile characteristics during the adult phase of a plants life cycle. For instance in maize, the *in vitro* culture of the adult shoot apices results in complete rejuvenation and the rejuvenated apices produce the same number of juvenile leaves and flowers as the seed-derived plants. This implies that the *in vitro* culture reverses the vegetative phase change in the SAM and the factors that regulate juvenile leaf identity act directly on leaf primordia, and can modify their identity even after they have been initiated (Poethig, 2013).

In animals, the transfer of a nucleus from the blastula stage of a frog embryo to an enucleated oocyte showed that the mystery of rejuvenation is hidden in the cytoplasm of the oocyte. The oocyte was able to reset the aging clock of the blastula nucleus to that of a zygotic nucleus (Briggs and King in 1952). At that time, these studies challenged the dogma that the process of aging and differentiation from a single fertilized egg to a mature adult involved the loss of genetic material, which would in essence be an irreversible process rendering the resulting nuclei incapable of recapitulating the embryological developmental program (Rando and Chang, 2012). Later, through somatic cell nuclear transfer (SCNT), Campbell et al (1996) developed the first cloned mammal from an adult somatic cell, the sheep Dolly, which confirmed that the oocyte cytoplasm can mediate reprogramming of genetic information in the mature nucleus, leading to rewinding of the aging clock of the nucleus.

In plants, however, rewinding of the nuclear clock to reach a totipotent zygotic state that allows cells to start the embryogenesis program does not require SCNT. Instead, this process, called somatic embryogenesis, can be induced in tissue culture by stress treatment or by culturing plant cells on medium containing growth regulators. The resulting somatic embryos are able to germinate and develop into juvenile plants exactly like zygotic embryos (Karami et al., 2009). Research in *Arabidopsis* has shown that somatic embryogenesis can also be obtained by overexpression of genes encoding transcription factors that are key regulators in zygotic embryogenesis, such as *WUSCHEL* (*WUS*), *LEAFY COTYLEDON 1 and 2* (*LEC1*, *LEC2*), *BABY BOOM (BBM)* and *AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 15 (AHL15)* (Srinivasan et al., 2007; Boutilier, 2002; Elhiti et al., 2013; Karami et al., 2017). Interestingly, the AT-hook protein AHL15 was found to generally delay or even revert developmental phase transitions in plants, including the vegetative phase change and flowering, and was therefore named REJUVENATOR (Figure 1) (Karami et al., 2017; chapter 2). AHL proteins have been reported to bind AT-rich motifs, and to act by chromatin modification (Aravind and Landsman,

1998; Ng and Ito, 2010). Recent observations suggest that AHL15 overexpression reprograms the cell by reducing the amount of heterochromatin in the nucleus (Karami et al., 2017).

Somatic embryogenesis requires the reprogramming of gene expression patterns comprising cascades of genetic signals that turn the expression of different gene groups on or off. Several proteins have been identified that function in early somatic embryogenesis. These proteins are predicted to be involved in hormone signal transduction, induction of epigenetic chromatin remodeling and cell cycle regulation (Elhiti et al., 2013). So far, several epigenetic mechanisms have been implicated in the control of SE, including DNA methylation, histone post-translational modifications, and micro RNA (miRNA) pathways (Smertenko and Bozhkov, 2014).

PLANT MODIFICATION VIA TRANSFORMATION

The most important and challenging issue of the world is food security, which is not possible to solve without crop improvement. To get maximum benefits, humans through time have improved crop productivity and product quality through plant domestication, breeding and more recently also through genetic modification. Transgenic plants with the desired genes, synthetic promoters and tunable transcription factors have been generated to meet the challenges of agri- and horticulture, but genetic modification has also been a very useful tool in studies to understand plant growth and developmental processes at the molecular and genetic level, (Liu et al., 2013). Here I will review various plant transformation techniques with a focus on the most generally applied method that uses the soil bacterium *Agrobacterium tumefaciens* to introduce foreign DNA into plant cells, its related problems especially recalcitrancy, and a possible solution which is answered in detail in chapter 3 of this thesis.

Plant transformation techniques are divided into two main categories: direct and indirect DNA transfer. Methods for direct DNA transfer use physical or chemical treatments, such as electric shock, particle bombardment, or poly ethylene glycol (PEG) treatment to introduce isolated DNA molecules into the target plant cells or tissues. In electroporation- or PEG-mediated DNA transfer, the permeability of the plasma membrane is enhanced by respectively an electric pulse or chemical shock, allowing the desired biomolecules such as DNA, RNA, proteins or lipids to enter the cells. In particle bombardment, plant cells or small tissues are bombarded with inert particles (usually tungsten or gold particles of approximately two microns in diameter) that are coated with the desired DNA. Direct DNA transfer methods have less limitations with respect to plant species or cell type; however, transformation efficiencies are generally low, and the DNA integration patterns complex (Rivera et al., 2012).

Indirect DNA transfer methods make use of the natural capacity of the soil bacterium *Agrobacterium tumefaciens* to transfer DNA to plant cells. Although *Agrobacterium*-mediated transformation (AMT) does have its limitations with respect to host specificity and regeneration recalcitrance, in general it is possible to obtain transgenic plant lines with a single intact T-DNA insert with a reasonable efficiency for many different plant species. Therefore, AMT is at the moment the most commonly used method of plant transformation (Hooykaas. 2010).

1 AGROBACTERIUM AS NATURAL DNA TRANSFER AGENT

Agrobacterium tumefaciens was first discovered as a soil born, rod shaped gram negative bacteria that was the causative agent of the crown gall disease in crop plants (Smith and Townsend, 1907). What makes *A. tumefaciens* harmful or useful is its tumor inducing (Ti) plasmid (Zaenen et al., 1974), which has the talent to transfer a part of DNA (transfer or T-DNA) to the host cell using the virulence (Vir) proteins that are expressed from its *vir* region (Figure 2a). T-DNA and *vir* region are the two most important parts of the Ti plasmid without which successful *Agrobacterium*-mediated transformation is not possible (Gelvin, 2003).

Agrobacterium attachment

Wounded plant cells secrete phenolic and sugar compounds that induce chemotaxis movement in *Agrobacterium* toward the wounded plant tissue (Bhattacharya et al., 2010). Attachment of *Agrobacterium* to the host cell is essential but how it is accomplished is still unknown (Matthysse, 2014). Cellulose fibrils are formed after initial binding and these give firm attachment of the bacterium to the plant cells (Matthysse, 1983). Vitronectine-like protein and rhicadhesin protein are considered as possible adhesives by which *Agrobacterium* attaches to host plant cells (Tzfira and Citovsky, 2002). The synthesis of extracellular polysaccharide, termed as unipolar polysaccharide (UPP) is induced at low calcium levels combined with phosphorus limitation and acidic pH, and this promotes polar adhesion of the *Agrobacterium* cells to the host cell surface (Figure 2a and b) (Matthysse, 2014).

Induction of Vir-region

The activation of the *Agrobacterium* transformation machinery starts with the induction of *vir* region by compounds produced by the host cells. The exudates of wounded plant cells contain phenolic compounds, such as acetosyringone, that are recognized by the *Agrobacterium* 'two-component' signal transduction system, consisting of the membrane-bound sensor VirA, which directly interacts with the plant wound signals and undergoes autophosphorylation, and subsequently transphosphorylates the transcriptional regulator VirG. VirG in turn activates the *vir* gene promoters at the *vir*-region (Figure 2a and b) (Lynn et al., 2008). Non-host plants, especially some monocots, were found to inhibit this sensory machinery by specific exudates containing 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA) (Zhang et al., 2000).

The *vir*-region contains approximately 35 virulence genes grouped into several operons including as *Vir*A, *Vir*B, *Vir*C, *Vir*D, *Vir*E, *Vir*F, *Vir*G and *Vir*H (Schrammeijer et al., 2000). These operons express various Vir proteins, which control the whole transformation process starting from *vir* genes induction to T-DNA production and transformation of the plant cell.

Production of T-DNA

The native T-region in most *Agrobacterium* strains is approximately 10 to 30kb in size and comprises 5-10% of the entire Ti plasmid. Some Ti plasmids have even multiple T-regions. The T-region is defined by two 25bp imperfect direct repeats called right border (RB) and

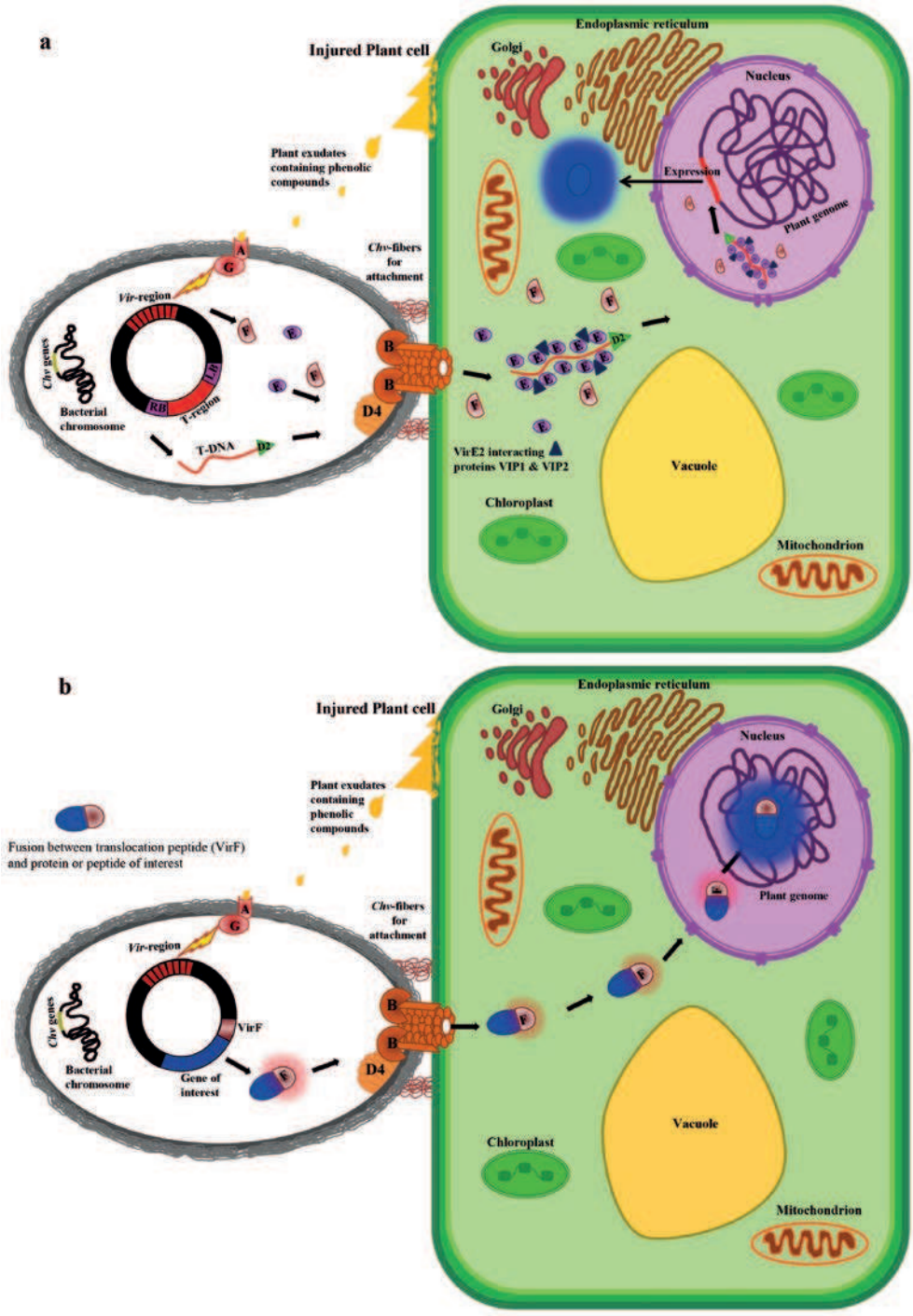

Figure 2. Schematic representation of (a) *Agrobacterium*-mediated transformation versus (b) protein translocation to a host plant cell.

left border (LB) (Gelvin, 2003). The T-DNA production process involves the recognition of the border repeats by the VirD1 and VirD2 relaxosome, and the subsequent introduction of a nick by VirD2 in the bottom strand. During the nicking process VirD2 remains covalently attached to the 5' end of the nick via its N-terminal tyrosine residue (Tyr29), (Mysore et al., 1998). Together with two other accessory Vir proteins VirC1 and VirC2, VirD1 enhances the binding and nicking at the T-region border sequences. VirD1 assist VirD2 in nicking on supercoiled double-stranded DNA, while VirC1 and VirC2 assist the relaxosome and DNA polymerase in finding the RB by attaching to the *overdrive* sequence located outside of the T-region close to the RB (Toro et al., 1989; Lu et al., 2009). Repair of the nicked strand by replacement synthesis by DNA polymerase releases the VirD2-T-strand complex (VTC), which is then subjected to transfer to the host cell through the translocation channel (Figure 2a).

Type-IV secretion system (T4SS)

The transfer of the VTC nucleoprotein complex from *Agrobacterium* to the host cell takes place through a conjugation channel called type-IV secretion system (T4SS). The *Agrobacterium* T4SS is composed of 12 proteins, 1-11 VirB proteins and VirD4 expressed from the *vir*B and *vir*D operons, respectively, due to which this system is also referred to as the VirB/D4 secretion system (Figure 2a) (Wallden et al., 2010). VirB1 is a periplasmic transglycosylase that makes holes in the peptidoglycan cell wall to allow the formation of the pilus structure (Zupan et al., 2007). VirB3 is a small inner membrane protein that is stabilized by VirB4 from cytoplasmic side and together they function in assembly of the pilus (Mossey et al., 2010). VirB2 and VirB5 form the elongated pilus structure extending outside the bacterial cell surface. VirB2 proteins are arranged in a tube-like form via head to tail peptide bonds making the major part of the 10nm diameter pilus , through which transportation of effectors and nuclear material takes place between *Agrobacterium* and the host cell (Eisenbrandt et al., 1999). VirB5 is only a minor component of the pilus and is considered to function in the adhesion to the recipient host cell (Backert et al., 2008). The rest of the proteins from VirB6 to VirB10 make the base of the conjugation channel, in which VirB6 and VirB8 generate the actual pore of the T4SS channel, while VirB7, VirB9 and VirB10 form the outer layer of the T4SS, covering the VirB pilus in the peptidoglycan layer of the cell wall (Wallden et al., 2010). Like VirB4, VirB11 and VirD4 are hexameric proteins, localized at the cytoplasm side of the inner membrane (Arechaga et al., 2008). These three inner membrane pheripheral proteins work as ATPases that energize the T4SS from the cytoplasm to accelerate the assembly of the T4SS and the transport of material from *Agrobacterium* to the host cell. VirD4 functions as a coupling protein that recruits substrate molecules (T-strand and effector proteins) to the T4SS (Alvarez-Martinez and Christie, 2009).

T-DNA transfer

VirD2 contains a translocation signal at the C-terminus that is essential for recruitment of the VTC by the VirD4 coupling protein (van Kregten et al., 2009). After recruitment by VirD4, the VTC is transferred to VirB11, and subsequently to the inner membrane proteins VirB6 and VirB8, to outer membrane protein VirB9, and finally to the pilus, which transmits it to the host cell cytoplasm. Virulence effectors proteins such as VirE2, VirE3 and VirF also

contain a C-terminal translocation signal that allows them to be recruited for translocation by the T4SS independent of the VTC (Vergunst et al., 2000; Sakalis et al., 2014). The VirE1 chaperonne prevents VirE2 from forming protein aggregates in *Agrobacterium* and thus assists in its independent translocation through the T4SS (Sundberg et al., 1996; Zhao et al., 2001).

VirE2 is a single stranded DNA binding protein that upon its translocation into the host cell is thought to bind and protect the T-strand from the nucleolytic degradation in the host cell (Figure 2a) (Gelvin, 2012). It has been suggested that dynein motor proteins are involved in transport of the T-complex along the microtubules toward the nucleus (Salman et al., 2005). The mature T-complex has a diameter of approximately 15.7nm, and its import through the nuclear pore complex having a diameter of 9nm requires an active mechanism mediated by the combined action of bacterial effectors (VirD2, VirE2 and VirE3) and the nuclear import machinery of the host plant cell (VirE2 interacting protein 1 (VIP1), cyclophilines and At KAPα) (Citovsky et al., 2007). VirD2 and VirE2 both contain nuclear localization signals and together with VIP1 (Tzfira et al., 2001) and *Arabidopsis* importin α isoform IMPa-4, which again interacts with VirE2 and VIP1 (Bhattacharjee et al., 2008), they allow the uptake of the T-complex into the nucleus (Figure 2a).

Integration

Integration is the last and most important step of AMT. Although the exact story of T-DNA travel toward the site of integration, its uncoating and subsequent integration in the host genome is not clear yet, with the advancement of fluorescent technology various plant and bacterial factors associated with the T-complex have been shown to accumulate inside the nucleus. Several lines of evidence suggest that the pilot protein VirD2 not only guides the T-DNA to the site of integration, but also is involved in its recruitment for integration in the host genome (Book:Tzfira et al, 2000-2013).

Chromatin targeting of the T-complex is proposed to be mediated by the interaction of VirD2 with two members of the plant RNA transcription machinery (i.e. CAK2M and TATA-box binding protein) (Bakó et al., 2003) and of VirE2 through VIP1 with core histones (H2A) (Tzfira et al., 2001; Loyter et al., 2005). In addition, the bacterial effector protein VirF has been suggested to function as the F-box protein part of a Skp-cullin-F-Box (SCF) E3 ubiquitin ligase complex, and to recruit VIP1 and possibly also VirE2 for proteolytic degradation by the proteasome, thereby releasing the T-strand from the T-complex just before integration (Schrammeijer et al., 2001; Tzfira et al., 2004b). Several reports indicate that the single stranded T-DNA uses the host DNA-repair machinery for integration (Ziemienowicz et al., 2000, 2008), either direct by a microhomology-mediated end joining (MMEJ) mechanism, or after its conversion to double stranded DNA by non-homologous enjoining (NHEJ)-mediated integration in double-strand breaks (DSBs) in the host genome (Tzfira et al., 2004a). Recently, it has been shown that the random integration of T-DNA in the plant genome is carried out by polymerase theta (Pol θ) which explain the genome break and repair mechanism using primer–template switching ability of Pol θ for T-DNA integration in plants (van Kregten et al., 2016).

DEVELOPING AGROBACTERIUM AS VECTOR SYSTEM

The genes on the T-DNA are divided into two sets of genes. One set, the oncogenes code for enzymes, such as tryptophan mono-oxygenase, indoleacetamide hydrolase and isopentenyl transferase, that are involved in the biosynthesis of auxin and cytokinin, plant hormones that drive tumor formation by inducing plant cell division. The second set of genes code for the enzymes involved in the production of opines, such as nopaline and octopine, which can serve as carbon and nitrogen source for *Agrobacterium* and make the tumor a suitable environment for bacterial proliferation and colonization (Gorden and Christie, 2015). The original T-region is very large and has no unique restriction sites that are suitable for cloning. Moreover, the presence of oncogenes prevents the regeneration of normal transgenic plants. In order to make the system more accessible for manipulation, a smaller T-region with unique restriction sites was constructed and placed on a separate wide host range replicon, a so called binary plasmid, which could replicate both in *Agrobacterium* and in *E. coli*. At the same time, the original T-region was deleted from the Ti-plasmid, resulting in a disarmed Ti plasmid that still carries the *vir* region (Hoekema et al., 1983). This binary vector system is currently the standard system used for *Agrobacterium*-mediated transformation (AMT), as it allows to efficiently construct the desired T-region on a binary vector in *E. coli*, and to subsequently introduce the resulting plasmid in a disarmed *Agrobacterium* strain by conjugation or electroporation. Based on experience, the genes of interest are nowadays usually placed near the RB end and the selection marker gene near the LB end, as the LB end seems more prone to degradation in the host cell, most likely because the RB end is protected by the VirD2 pilot protein (Rossi et al., 1996). In this way, by selecting for a functional selection marker, the chance is high that an intact T-DNA including the genes of interest is transferred to the host cell. Including the *overdrive* sequence next to the RB has been shown to increase the efficiency of T-DNA transfer (Peralta et al., 1986; van Haaren et al., 1987), and therefore these sequences are included in the binary vector.

APPLICATIONS OF AMT

AMT has been applied to different aspects of our human society, including agriculture and fundamental biological research. By increasing the yield and enhancing the quality of crops AMT has enabled remarkable improvements in the food and agriculture sector. Plants have been engineered for enhanced tolerance to biotic and abiotic stresses and pest resistance to increase crop productivity and reduce the use of harmful agrochemicals. Currently more than 181 million hectares of biotech crops are grown globally, which has reduced the chemical pesticide use by 37%, increased crop yield by 22% and increased the farmer profit by 68% (Klumper and Qaim, 2014). Maize, cotton and soybean are the main GM crops grown throughout the world, (James, 2014). The DroughtGard™ hybrid maize was planted for the first time in the US in 2013 at about 50,000 hectares, and in 2014 this area already increased to 275,000 hectares (James, 2014). Insect resistant crop plants have been produced by introduction of various *Bacillus thurengiensis* (Bt) toxin genes into maize, cotton, potato, chickpea, tomato, tobacco, rice and many other crop species (Kakkar and Verma, 2011). Moreover, through AMT plant resistance to viral pathogens has been achieved by introducing genes into plant cells producing viral antisense RNA or coat proteins (Smith et al., 1994).

Because of the practical, economical and safety advantages, nowadays the production of recombinant pharmaceutical and industrial proteins in crop plants are increasing and this new emerging technology/business is called molecular farming (Alvarez, 2014). *Agrobacterium*-mediated transformation has been used for genetic modification of plants for production of various useful proteins, such as recombinant antibodies (plantibodies) and edible vaccines (plantigens) (Daniell et al., 2001). Moreover, plants producing life-saving biopharmaceuticals such as anticoagulants, human epidermal growth factor, and interferons (Giddings et al., 2000; Daniell et al., 2001) are also the products of AMT technology.

Plants have been engineered for enhanced tolerance to biotic and abiotic stresses and pest resistance to increase crop productivity and reduce the use of harmful agrochemicals. Transgenic plants have also been used to detect environmental toxicity or to detoxify contaminated soil, water or air for example, the use of an *Arabidopsis* split GUS reporter line to detect the radioactivity around Chernobyl (Kovalchuk et al., 1998). Also transgenic *Arabidopsis* plants have been developed that can convert the highly toxic contaminant methyl mercury to the volatile and much less toxic elemental mercury (Bizily et al., 2000), or that can extract and accumulate arsenic from ground-water or methyl-seleno-cysteine from selenite-rich soils, or are tolerant to heavy metals such as cadmium and lead (Dixit et al., 2015).

Most importantly, the AMT technology has helped scientists to understand fundamental questions in plant biology at the cellular, subcellular or molecular level. For example, *Agrobacterium*-mediated transient transformation of *Nicotiana bentamiana* using a simple and fast method of leaf infiltration is widely used to visualize subcellular compartments such as nuclei, endoplasmic reticulum (ER), endosomes and cytoskeleton which are appropriately labeled by molecular markers containing GFP, YFP (yellow fluorescent protein) or RFP (red fluorescent protein). In addition, this allows to study biochemical properties , localization, and co-localization of and interaction between proteins (Krenek et al., 2015). AMT has also been used to generate collections of T-DNA insertion lines in model plants such as *Arabidopsis thaliana* or rice for gene function studies, or lines that express GFP fusion proteins to study the *in planta* dynamics of intracellular protein localization. Using the AMT system, the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) technology has been developed as an important new functional genomics approach for the site-directed mutagenesis of plant genomes by RNA-guided nucleases, such as Cas9 (Sander and Joung, 2014).

In conclusion, molecular plant biology and plant biotechnology have thrived and are very successful because of AMT, but still several economically important plants are recalcitrant to AMT. Below we will discuss which factors limit the use of the AMT technique.

FACTORS AFFECTING AMT

As discussed above, AMT is a highly complex process and beside the mutual compatibility between *Agrobacterium* and its host system, this process is also affected by various external factors. For example, conditions that can positively contribute to the AMT efficiency are cocultivation period (1-5days), *Agrobacterium* density (1-10^6 to 1-10^{10} cfu/ml), and medium composition. Especially the concentration of salts, sugars, and growth regulators is important. Addition of acetosyringone to the medium and a low pH enhance the induction of the *Agrobacterium vir*

genes. Antinecrotic agents such as sodium thiosulphate, melatonin, $AgNO_3$, and ascorbic acid may help to enhance the regeneration of transgenic shoots, especially in case of transformation and regeneration of recalcitrant plants such as sweet pepper. Low temperature (19-20°C for dicots and 24-25°C for monocots) during cocultivation is critical, as this probably prevents strong *Agrobcterium* proliferation, surfactants such as silwet L77 and Tween 20 enhance T-DNA delivery by eliminating *Agrobacterium* attachment inhibitors (Ziemienowicz, 2014). Many other external factors can also affect the efficiency such as the humidity around the leaves which can increase transformation efficiency (Kim et al., 2009). The developmental stage and the age of the transformed tissues are related to the levels of transient expression, but in general the highest transient expression levels are observed in younger cells that had completed cell division just before transformation (Krenek et al., 2015). Various antibiotics such as cefotaxime, kanamycine, and carbinicillin in the regeneration medium inhibit the infection of the explants caused by *Agrobacterium* overgrowth, but also affect the transformation efficiency by affecting the regeneration process.

SELECTION AND REGENERATION

During AMT generally only a relatively small number of cells are transformed, and therefore selection is applied during regeneration to be able to obtain transgenic plants. For plant species that are recalcitrant to AMT, in general the selection and regeneration of transformed cells are the main problems, and this is why more than 50 genes have been developed as markers for selection in AMT. Some of these selectable marker genes (SMG) promote (or do not affect) the growth and regeneration process (positive SMGs), while others are regeneration inhibitors (negative SMGs). Positive SMGs may be conditional (selection depends on the external substrate in the medium) or non-conditional (selection depends on the phenotypic effects as a result of marker gene expression) (Miki and McHugh, 2004). The substrate of a positive conditional SMG is toxic, such as an antibiotic or herbicide, whereas a substrate for a negative conditional SMG is non-toxic, such as D-valine or D-isoleucine. In AMT, positive conditional SMGs for kanamycin, hygromycin or phosphinothricin resistance have been extensively used, but are now replaced by reporter genes, such as GFP, which help to detect and select transgenic cells or tissue in the absence of antibiotic or herbicide pressure. This seems to enhance the regeneration process, and thus to overcome recalcitrance to AMT (Miki and McHugh, 2004; Yau and Stewart, 2013; Krenek et al., 2015).

Once transgenic plants have been obtained, the presence of a conditional positive SMG can be handy in crosses, but especially for antibiotic resistance genes that are considered unacceptable for biosafety reasons, and for a non-conditional positive SMG, such as the *Agrobacterium* cytokinin biosynthesis *ipt* gene, that causes unwanted pleotropic effects on plant development, removal of the SMG is preferable (Zubko et al., 2002; Kant et al., 2015). To eliminate these marker genes and get marker-free transgenic plants, various techniques have been developed, such as co-transformation, the use of transposable elements, Cre-*loxP* site-specific recombination, and more recently via site-specific DSBs introduced by meganucleases (e.g. I-SceI), TALENs, Zinc Finger Nucleases (ZFNs), or CRISPR-CAS9 (Scutt et al., 2002; Yau and Stewart, 2013; Sander and Joung, 2014). Cotransformation of a SMG with the construct of interest in theory

allows removal of the SMG by outcrossing, but the chance that the SMG integrates at the same locus is high (de Neve et al., 1997) which makes this method unreliable. Similarly, by making a construct with the SMG on an inactive *Ds* transposable element next to the gene of interest, it can be separated from the gene of interest by transposition after introduction of a *transposase* gene. This would then allow to remove both SMG and transposase by outcrossing, provided that the SMG is reinserted at an unlinked locus. However, since transposable elements are known to reinsert preferably close to the donor locus (Jones et al., 1990), this in most cases requires laborious screening for recombinants where the SMG is lost. Meganuclease-based removal of SMGs requires flanking of the SMG by nuclease recognition sequences, and has been very successful in plants, especially for vegetatively propagated crops. But additional unwanted deletions have been observed due to the presence of additional meganuclease recognition sites in the crop genome, or the occurrence of unwanted bigger deletions because of DSB-induced NHEJ repair (Salomon and Puchta, 1998). The TALEN- and ZFN-technologies are also not favored for removal of SMGs, because both methods are time consuming, laborious and complicated. The recently developed CRISPR-Cas9 system for the introduction of DSBs seems to provide an easier and more reliable alternative; however, also this system is prone to unwanted larger deletions due to imprecise repair by the NHEJ pathway. Although, like for meganucleases, the Cre-*loxP* recombination system requires flanking the SMG with *loxP* sequences, still this site-specific recombination system has until now been the most widely used and recommended method to make marker-free transgenic plants (Gidoni et al., 2008).

AGROBACTERIUM-MEDIATED PROTEIN TRANSLOCATION (AMPT)

All the methods for SMG removal described above require the introduction of a transposase or nuclease gene, either by crossing or by transformation, and their subsequent outcrossing to only keep the locus with the gene of interest. For the CRISPR-Cas9 system even two components (guide RNA and Cas9 nuclease) need to be expressed in the target cells. Alternatively, several reports describe the successful introduction of nucleases into plant cells by using positively charged cell-penetrating peptides (CPPs) (Bilichak et al., 2015; Ziemienowicz et al., 2015). A disadvantage of the CPP method is that the nuclease needs to be purified from *E. coli* or from other expression systems. The discovery that the *Agrobacterium* T4SS also translocates virulence proteins to plant cells has triggered investigations in using this system to modify the plant genome or to modulate processes in plant cells by translocation of proteins of interest (Vergunst et al., 2000). Recombinases Nucleases such as Cre or I-Sce have been successfully introduced into plant cells as active nuclease-VirE2, VirF or –VirD2 fusions via *Agrobacterium*-mediated protein translocation (AMPT) (Figure 2b), allowing to use this system for the removal of SMGs (Vergunst et al., 2000; van Kregten et al., 2009; Rolloos et al., 2015).

The initial discovery of AMPT focused on the virulence proteins VirF and VirE2, and used the Cre Recombinase Reporter Assay for Translocation (CRAfT) to show that both proteins have a C-terminal signal sequence that facilitates their translocation by the *Agrobacterium* T4SS system (Vergunst et al., 2000). Later it was discovered that several other virulence protein, such as VirD2, VirD5 and VirE3, also contain a C-terminal translocation signals and are translocated

by the T4SS to the host cell (Schammeijer et al., 2003; Vergunst et al., 2005). By applying the CRAfT assay for AMPT in *Arabidopsis*, it has been shown that at least 37 C-terminally located amino acids of VirE2 and VirF are required to give maximum translocation efficiency. As mentioned earlier, VirD2 is the most essential protein in AMT, and the CRAfT system allowed to test its translocation in the absence of T-DNA (Vergunst et al., 2005). It was found that the translocation of a Cre-VirD2 fusion was significantly less efficient in comparison with Cre-VirF fusion translocation (Vergunst et al., 2005), which suggested that the transfer of VirD2 in the form of VTC is its natural requirement to be efficiently recruited for translocation through T4SS (van Kregten et al., 2009; Cascales et al., 2013).

TRACKING OF AMPT USING THE SPLIT-GFP ASSAY

To be able to track the movement and localization of translocated *Agrobacterium* virulence proteins in the host cells, initially GFP-VirE2 and GFP-VirF were generated and tested. *Aequorea GFP* was the first cloned and expressed *GFP* gene extracted from the jelly fish *Aequorea victoria* (Prasher et al., 1992; Chalfie et al., 1994). GFP is composed of an 11 stranded beta-barrel like structure having a chromophore in the center of the β-barrel of a diameter 24Å and height 42 Å. The 11-strand beta-barrel of GFP is crucial for chromophore formation which takes 90 minutes to 4 hours after protein synthesis, so the appearance of fluorescence indicate that the 11-strand β-barrel has been formed (Zimmer, 2002). Unfortunately, because of this complex and rigid structural folding of GFP, the *Agrobacterium* T4SS was not able to translocate these fusion proteins.

In 2005 Cabantous et al (2005) introduced the split-GFP system. In this system the 11 GFP domains are split in two self-associating fragments (GFP1-10 and GFP11), each of which is unable to give fluorescence until their complementation happens. The advantage of this system is that the protein of interest can be tagged and detected either in soluble or insoluble form in living cells or cell lysates (Cabantous et al., 2005). Cabantous et al. developed and improved superfolding GFP1-10 having enhanced complementation with the GFP11 part. They also showed that the superfolding GFP takes only four minutes for folding and gives 95% fluorescence compared to wild-type GFP. The GFP11 part comprises only the 15 most C-terminal amino acids of superfolding GFP, which is considerably smaller than any other antecedently reported GFP fragment. The small size, minimal effect on solubility and function of the fused protein and efficient association with GFP 1-10 makes GFP11 a very attractive protein tag. Proteins tagged with intact GFP exhibit constitutive fluorescence, whereas GFP11-tagged proteins give fluorescence only in the presence of GFP1-10. So for visualizing the desired protein *in-vivo*, the target cells/tissues/organism has to express GFP1-10. This characteristic makes the split-GFP assay advantageous for visualizing protein transfer between two completely different cell-types, such as the translocation of effector proteins by the *Salmonella type III* secretion system (van Engelenburg et al., 2010). Unfortunately, however, the same advantage restricts the use of this assay only to host cells that are not recalcitrant to AMT. Still, the split-GFP system has allowed to track the movement and localization of GFP11-VirF and GFP11-VirE2 fusions in GFP1-10 marker lines of yeast, *Arabidopsis*, and tobacco (Li et al., 2014; Sakalis et al., 2014).

THESIS OUTLINE

Fundamental studies on plant development in model plants such as *Arabidopsis* and rice have benefited tremendously from the development of genetic tools such as AMT to generate transgenic lines and GFP as cell biology reporter. Unfortunately, developmental processes in several important food and ornamental crop plants such as sweet pepper and tulip respectively, cannot be studied in such detail, due to their recalcitrance to AMT. To solve these problems, I adopted the AMPT system for the co-transformation of regeneration-enhancing proteins, such as BBM and AHL15, to enhance regeneration. Alternatively, the AMPT of these key developmental regulators may be directly used to trigger developmental phase changes such as flowering, senescence, or the reversal of these phase changes.

In this thesis we studied the application of AMT and AMPT for the modulation of plant development.

In **Chapter 2** we first investigated the developmental effects of ectopic expression of a glucocorticoid-inducible AHL15-GR protein in *Nicotiana tabacum*. Our results showed that, upon DEX treatment, the *35S::AHL15-GR* tobacco seedlings or plants not only showed enhanced branching and seed number, but also a delay of developmental processes, such as seed germination, flowering and senescence. Interestingly, the activation of AHL15-GR by DEX treatment resulted in a reversal of the developmental phase transitions and rejuvenation, leading to polycarpy in tobacco.

In **Chapter 3** we studied translocation of AHL15 and BBM, another key developmental regulator, by AMPT to *Arabidopsis* and tobacco. Using the split-GFP system and GFP1-10 marker lines, we could show for the first time that such developmental regulators can be translocated to plants cells by *Agrobacterium*, and that they reduce senescence in and enhance regeneration from tobacco leaf discs.

In **Chapter 4** we report on the development of a new and generic split-GFP system for visualization and localization of AMPT in wild-type plants. Beside *Arabidopsis* and tobacco, we also show that this generic split-GFP system can be successfully used in AMT-recalcitrant plants, such as tulip and sweet pepper, not only to detect protein and DNA translocation, but also to select transgenic shoots.

1

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ARABIDOPSIS AHL15-INDUCED REJUVENATION PROMOTES LONGEVITY AND POLYCARPY IN NICOTIANA TABACUM

2

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2

ABSTRACT

Plant rejuvenation and senescence are interrelated and interdependent developmental processes. Early maturation and poor rejuvenation potential restricts flowering plants to a monocarpic life history strategy and thus to a seasonal life span, which is generally shortened by environmental factors such as biotic and abiotic stresses. In contrast, delay in senescence of adult shoots and rejuvenation of (axillary) shoot meristems may enhance plant longevity and allow plants to flower multiple times (polycarpy). Recently, we identified the AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 15/REJUVENATOR (AHL15/RJV) protein as a key switch between monocarpic and polycarpic life history strategy in *Arabidopsis thaliana*. Here we analyzed the effect of heterologous expression of an inducible version of AHL15 (AHL15-GR) on *Nicotiana tabacum* development. Early activation of AHL15-GR delayed seed germination and arrested seedling development, resulting in callus formation rather than the somatic embryogenesis observed in *Arabidopsis*. Late AHL15-GR activation enhanced plant longevity by reducing leaf senescence, delaying flowering, and by shoot meristem rejuvenation, leading to an increased number of branches, leaves and seeds produced per plant. But the quality of the produced seeds in polycarpic *35S::AHL15-GR* tobacco plants was affected at the cost of seed quantity. Our data indicates that the overall function of *AHL15* seems conserved in different families of flowering plants, but also points to specific differences that require further study before AHL15-induced polycarpy can be used as a generic tool to enhance biomass and seed production in monocarpic crop species.

Keywords: AHL15-GR. Rejuvenation. Senescence. Polycarpy. *Nicotiana tabacum*.

INTRODUCTION

2

The life span of a flowering plant depends on its genetic potential for longevity and on environmental conditions (Woo et al., 2013). The plants dying after one flowering and seed production period are called monocarpic. A monocarpic perennial plant lives for two or more years, then flowers once, sets seed and dies, while a polycarpic perennial lives for a number of years, often many years, flowering and setting seed annually throughout its life time. The removal of flowers is regarded to extend plant life up to 50% in monocarpic plants (Sadras et al., 2000; Pic et al., 2002). For annual herbaceous plant species, such as *Arabidopsis thaliana*, germination, vegetative growth, reproduction and senescence are the four main phases that can be distinguished during the life of these plants but their life is mainly limited by leaf senescence (Sharabi-Schwager et al., 2010). In contrast, woody perennials, such as deciduous trees rejuvenate themselves after passing through winter stress conditions by reactivation of shoot meristems and development of new leaves and branches (Andersson et al., 2004; Xu et al., 2016). Similarly, in herbaceous perennial plants some meristems remain vegetative and initiate new shoots after a short dormant stage, producing new leaves and branches throughout the year, even at advanced age (Munne-Bosch, 2008; Xu et al., 2016).

Developmental processes such as organogenesis and developmental phase changes in plants are orchestrated by complex regulatory networks comprising hormone- or peptide signaling and downstream transcription factors that change gene transcription through direct (in)activation, or by inducing epigenetic changes involving chromatin remodeling (Sparks et al., 2013). For example, the SQUAMOSA promoter binding protein-like (SBP or SPL) family of transcription factors regulated by microRNAs miR156 and miR157 are responsible for various plant developmental processes like heteroblasty (juvenile to adult vegetative phase change), apical dominance, inflorescence branching, flowering time and fruit ripening in various flowering plants such as *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum* (Preston and Hileman, 2013). Similarly, phytohormones working as upstream regulators in plant developmental pathways have multiple effects in interconnecting different signaling pathways (Jibran et al., 2013). The plant hormone cytokinin can extend the plant life by delaying senescence and enhancing production of multiple new shoots (Wang and Irving, 2011). The delay in maturation and formation of juvenile shoots is of great importance for longevity in plants, especially in clonal forestry where different physical methods such as serial propagation, micropropagation and serial grafting are used to maintain juvenility (Wendling et al., 2014).

Senescence occurs at the final stage of plant development, and is defined as the age-dependent programmed degradation and degeneration process of the cells, organs or the entire organism, leading to death (Lim et al., 2007a). Sometimes this normal developmental process is induced by various biotic and abiotic stresses, which by increasing the level of reactive oxygen species negatively affect the developmental processes and leads to early maturation and death of the plants resulting in decrease of productivity (Petrov et al., 2015). Leaf senescence is a highly complex genetic and epigenetic program that is controlled by interconnected regulatory pathways at the level of chromatin and transcription, as well as by post-transcriptional, translational and post-translational regulation (Woo et al., 2013; Ay et al., 2014). Dark-induced senescence of *Arabidopsis* leaves, for example, involves 137 miRNAs that control many genes

(Huo et al., 2015), and the expression of *SENESCENCE-ASSOCIATED GENES* (*SAGs*) is regulated at the chromatin level by HISTONE DEACETYLASE 3 (HDA6), the AT-Hook motif nuclear Localized (AHL) protein ORE7 and other members of AHL family (Woo et al., 2013). Lim et al. (2007) have also shown that increased expression of the AHL protein encoding gene *ORE7* markedly extends the leaf longevity.

AT-hook is a small protein motif that binds the minor groove of the DNA at AT-rich regions and is associated with High Mobility Group (HMG) proteins in animal cells (Aravind and Landsman, 1998). The HMG proteins influence gene transcriptional regulation by participating in the formation of multi-protein complexes on the promoter regions of the genes they regulate (Bustin et al., 1990; Reeves and Nissen, 1990; Tjian and Maniatis, 1994). It is shown that HMGI/Y proteins mostly play a role as a cis-acting enhancers in the enhancement of gene activation by regulating both specific protein-DNA and protein-protein interactions at the promoter region (Reeves and Beckerbauer, 2001). Aravind and Landsman, (1998) extracted these AT-hook motifs from a non-redundant protein sequence database at NCBI and classified these motifs into three types according to their sequence similarity and found that they are prevalent in many eukaryotic nuclear proteins in single or multiple copies.

AHL proteins have been shown to have key roles in growth and development and act by modifying the chromosomal architecture to co-regulate transcription of a set of genes. In *Arabidopsis thaliana*, AHL proteins are encoded by a family of 29 genes, and they contain two conserved structural units, the AT-hook motif and the plant- and prokaryote-specific (PPC) domain. *Arabidopsis* AHL protein evolved in two clades: Clade A proteins contain a type 1 AT-hook motif, while clade B AHL proteins contain a type 2 and some also a type 1 AT-hook motif (Zhao et al., 2013). Previous analysis has shown that AHL proteins function in a large variety of processes, modulating plant size and biomass, yield and size of seeds, senescence and life cycle, ploidy and branching, immunity and stress resistance, production of secondary metabolites, tissue patterning, somatic embryogenesis, rejuvenation, regeneration and floral induction (Cai-Zhong, 2004; Zhao et al., 2013; Zhou et al., 2013; Karami, 2015).

The *Arabidopsis* AT-Hook nuclear Localized protein AHL15/REJUVENATOR (RJV) was previously identified as a DNA binding factor in a yeast one-hybrid screen (Hooykaas and Jacobus, 2004). Our recent functional analysis of this gene has revealed that *AHL15/RJV* overexpression maintains juvenile traits in the adult reproductive phase, thereby inducing polycarpic behavior in *Arabidopsis* (Karami et al., 2017). Here, we analyzed the effect of heterologous expression of a Dexamethasone (DEX)-activatable version of *Arabidopsis* AHL15 (AHL15-GR) in *Nicotiana tabacum* SR1 (tobacco), and showed that DEX activation of this fusion protein significantly increased shoot branching, delayed flowering and leaf senescence, and maintained axillary meristems in the vegetative state with juvenile features, thereby allowing the monocarpic tobacco plants to become polycarpic.

RESULTS

Arabidopsis AHL15 delays seed germination and arrest seedling development in tobacco

Previous research has shown that overexpression of *AHL15/RJV* can slow down various developmental processes such as germination, vegetative phase change, flowering and senescence in *Arabidopsis thaliana* (Karami et al., 2017). To demonstrate that this effect of AHL15/RJV overexpression was not specific for *Arabidopsis*, we generated *AHL15/RJV* overexpression lines for another annual plant species from a different family, being *Nicotiana tabacum* SR1. Initial attempts to generate lines with the *35S::AHL15/RJV* construct did not lead to transformants. We therefore transformed tobacco cells with the *35S::AHL15-GR* construct, expressing a translational fusion between the AHL15 protein and the glucocorticoid receptor (GR), allowing conditional activation of the fusion protein through its dexamethasone (DEX)-dependent nuclear import. In total 11 independent transgenic lines were obtained, of which we selected four single locus T-DNA insert lines based on their segregation for the phosphinothricin (ppt) resistance marker present on the T-DNA construct.

Germination of segregating *35S::AHL15-GR* T1 seeds on medium with or without 10 μM DEX and/or ppt showed that the development of the transgenic seedlings on DEX containing medium was significantly delayed compared to wild type seedlings. Whereas two weeks old SR1 wild-type and non DEX treated *35S::AHL15-GR* seedlings already developed one or two leaves next to normal looking cotyledons (Fig. 1b and c), the DEX treated *35S::AHL15-GR* transgenic seedlings showed comparatively short cotyledons and no leaves after two weeks of germination (Fig. 1d and e), suggesting that heterologous expression of *AHL15-GR* significantly delayed germination, and also reduced cotyledon growth. When we tested T3 seeds homozygous for the *35S::AHL15-GR* construct, germination on DEX-containing medium was again delayed, but now root and shoot development completely stopped in early seedling stage. Detailed studies showed that *35S::AHL15-GR* seedlings had lost the root and shoot meristem function and were converted to callus like structures (Fig. 1f and g). Also the cotyledons were thickened and produced callus on the upper and lower surfaces (Fig. 1h). Transfer of the root and shoot parts with callus to DEX-free medium neither recovered development nor resulted in shoot or root regeneration, suggesting that stem cell activity was permanently lost. These results also explain why no transgenic lines could be obtained with the non-inducible *35S::AHL15* construct.

Arabidopsis AHL15 delays flowering and leaf senescence in tobacco

Non DEX-treated *35S::AHL15-GR* plants slightly lagged behind in development and had greener leaves compared to wild-type plants (Fig. 2a), probably because of leaky nuclear import of the AHL15-GR fusion protein in the absence of DEX. To study the effect of *35S::AHL15-GR* expression on flowering, we DEX-sprayed (see schedule in Table. 1) 6 weeks old wild-type and *35S::AHL15-GR* tobacco plants just before flowering (Fig. 2a). One week after DEX treatment, wild-type plants developed elongated inflorescences with open flowers, whereas the *35S::AHL15-GR* plants showed a short inflorescence with closed flower buds (Fig. 2b). Two weeks after DEX treatment, the elongated *35S::AHL15-GR* inflorescences carried opened flowers, whereas the wild-type plants were already in the fruit ripening stage (Fig. 2c). These results suggested

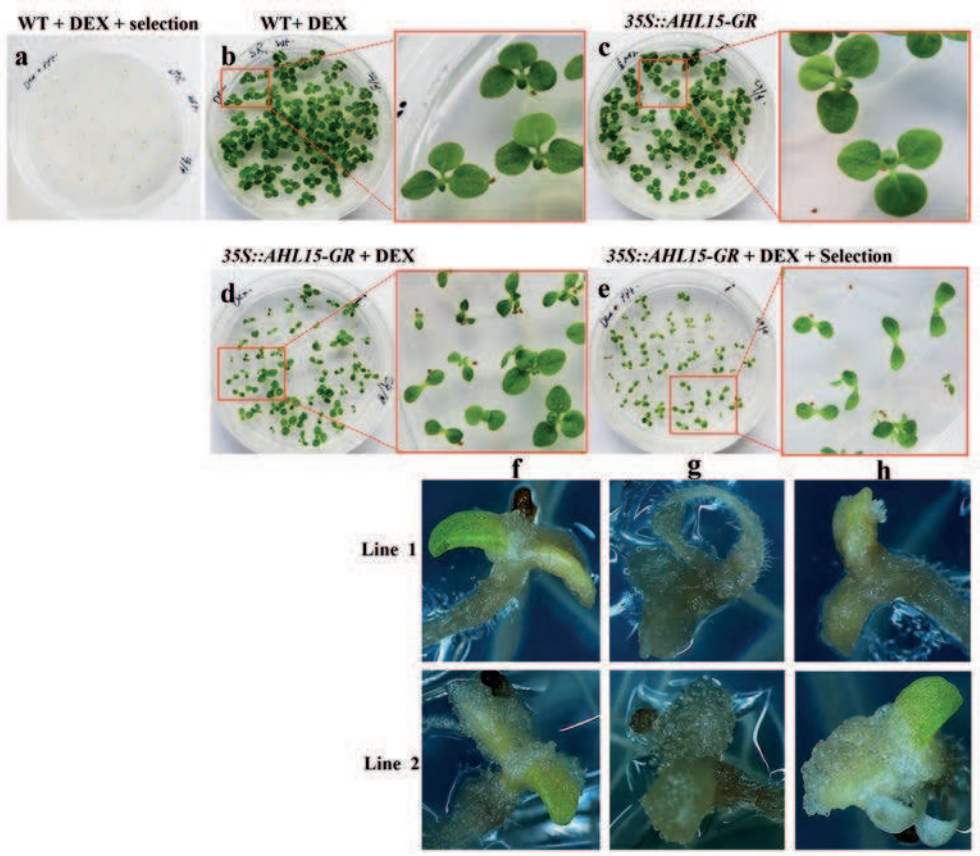

Figure 1. DEX activation of AHL15-GR delays seed germination and arrests seedling development in *Nicotiana tabacum*. (a-e) Germination of *Nicotiana tabacum* wild-type (a,b) and heterozygous *35S::AHL15-GR* (c-e) seeds on medium with DEX and phosphinothricin (selection) (a,e), with DEX but without selection (b,d) and without DEX and selection (c). Red boxed right panel in (b-e) shows enlarged part of the left panel. (f-h) Two weeks old seedlings from two homozygous *35S::AHL15-GR* lines (1 and 2) after germination on DEX containing MS medium, showing loss of meristem function and formation of callus-like structures.

that *AHL15* overexpression increased the flowering time in tobacco, just like overexpression of this gene or the *AHL22* gene did in *Arabidopsis* (Yun et al., 2012).

We noticed that leaves of DEX-sprayed *35S::AHL15-GR* plants generally stayed greener compared to the DEX-sprayed wild-type leaves (Fig. 2c), suggesting that *AHL15* overexpression also reduced leaf senescence. When we left these plants growing for 3 more weeks, we observed an even more significant difference in leaf senescence. On wild-type plants, leaves had already senesced or showed strong signs of senescence, whereas most leaves on DEX-treated *35S::AHL15-GR* plants remained green (Fig. 2d). After the seed pods of these plants were harvested, plants were cut back so that only a 6 inch main stem with attached leaves was left behind (Fig. S1a). Repotting of these remaining stems and root systems to fresh soil led to renewed shoot growth, which was more vigorous and branched for the DEX sprayed *35S::AHL15-GR* stems than for the wild-type stems (Fig. S1b).

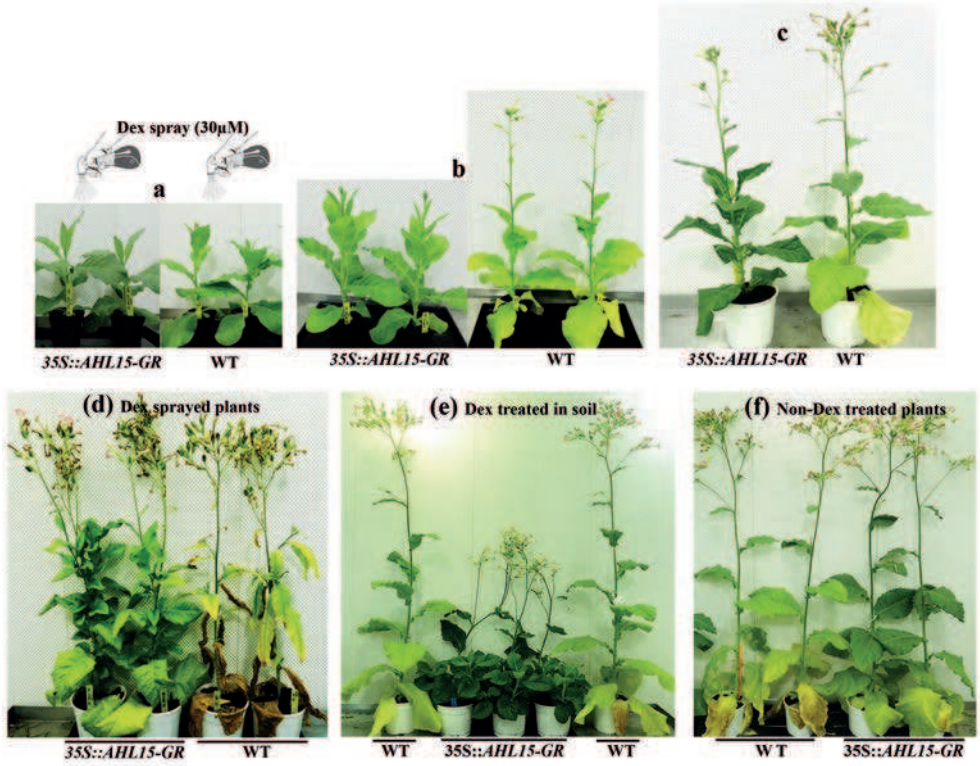

Figure 2. AHL15-GR activation delays flowering and senescence and enhances branching in *Nicotiana tabacum*. (a-c) 6 weeks old *Nicotiana tabacum* wild-type (WT) and *35S::AHL15-GR* plants were sprayed with 30 µM DEX before flowering (a), and subsequently photographed one week (b), two weeks (c), or 5 weeks (d) after spraying. (e, f) 2 weeks old *Nicotiana tabacum* wild-type (WT) and *35S::AHL15-GR* plants treated with DEX in the soil and spray (e), or non-DEX treated (f) and photographed after 6 weeks.

In another experiment, 2 weeks old wild-type or *35S::AHL15-GR* plants were DEX-treated by watering and spraying according to the schedule in table. 2. After 6 weeks, the *35S::AHL15-GR* plants showed a strong delay in development with short and branched stems and smaller dark green leaves, whereas wild-type plants were fully developed and started to show senescence of the bottom leaves (Fig. 2e), similar to the non-DEX treated control plants (Fig. 2f). The leaves of non-treated *35S::AHL15-GR* plants remained darker green compared to wild type, in line with the earlier observed slight leakiness of the AHL15-GR system. Also *35S::AHL15-GR* plants cultured *in-vitro* on DEX medium showed delayed development compared to DEX-treated wild-type or non-treated *35S::AHL15-GR* plants. Moreover, plants were very bushy and showed a strong delay in senescence compared to the controls (Fig. S2). These phenotypic effects of induced *Arabidopsis* AHL15 activity in tobacco corroborate our observations in *Arabidopsis* and indicate that this protein has a general effect in delaying plant development including germination, flowering time and leaf senescence.

Arabidopsis AHL15 induces rejuvenation in young and adult tobacco plants

2

Like *Arabidopsis*, tobacco plants are heteroblastic. In the juvenile vegetative phase the leaves are smaller and round with a relatively long petiole (Fig. S3a), while after the vegetative phase change the adult leaves are much larger with a shorter petiole and a clear central midrib (Fig. S3b).

To see the effect of heterologous AHL15 expression on the timing of the vegetative phase change in tobacco, both wild-type and *35S::AHL15-GR* seeds were germinated on MS medium (Murashige and Skoog, 1962) with or without DEX. Wild-type plants on DEX containing medium or *35S::AHL15-GR* plants on medium without DEX developed normally, showing the same timing of the vegetative phase change (Fig. 3a and 3b), while the DEX grown *35S::AHL15-GR* plants extended their juvenile phase with 5 to 6 leaves (Fig. 3c), or even stayed in the juvenile phase (Fig. S2a). When adult *35S::AHL15-GR* plants grown on normal medium were transferred to DEX medium, the newly formed leaves showed juvenile traits (Fig. 3d and S4c) while after 4 months, it was seen on the same plants that all the adult leaves stopped further development and new shoots with juvenile leaves appeared from the lateral buds (Fig. S5a). In reverse, transfer of DEX grown plants to medium without DEX resulted in an immediate shift of the newly formed leaves from juvenile to adult morphology (Fig. S4d,f). While the plants developed on non-DEX medium and after 40 days of development DEX-induced for only 15days and then transferred back to non-DEX medium, gave rise a thick branched bushy appearance (Fig. S5b). Whereas the plants continuously staying on DEX-containing medium produced branches with minute juvenile leaves and then completely stopped further development (Fig. S5a). These results indicated that, like in *Arabidopsis*, AHL15 is not only able to slow down but also to reverse development in tobacco.

To test whether activation of AHL15 would also lead to rejuvenation of senesced tobacco plants, we transferred 3 months old wild type and *35S::AHL15-GR* plants from which seeds had been harvested (approximately 60 cm long stem with root system) to bigger pots with fresh soil (Fig. 4a). One set of plants (wild type and *35S::AHL15-GR*) were DEX-treated in the soil, whereas a control set of plants (*35S::AHL15-GR*) was just treated with water. Only the DEX-treated *35S::AHL15-GR* plants developed lateral shoots form the axillary meristems producing adult leaves, and a few shoots from the transition area between roots and shoots producing juvenile leaves (Fig. 4b middle plant). The DEX-treated wild-type and mock-treated *35S::AHL15-GR* plants showed no shoot production (Fig. 4b right and left plants). These results indicate that apart from the ability to rejuvenate active meristems, AHL15 is also capable to reactivate dormant axillary meristems after the plant has senesced.

Arabidopsis AHL15 overexpression significantly increases branching and leaf number in tobacco

The biomass and yield of crop plants mostly depends on the plant architecture, which is determined for an important part by the level of apical dominance (Reinhardt and Kuhlemeier, 2002). Wild-type tobacco plants generally show a strong apical dominance, producing a main stem with a single inflorescence. For tobacco *35S::AHL15-GR* plants, however, we observed that induction of AHL15 activity significantly induced branching, by breaking the apical dominance

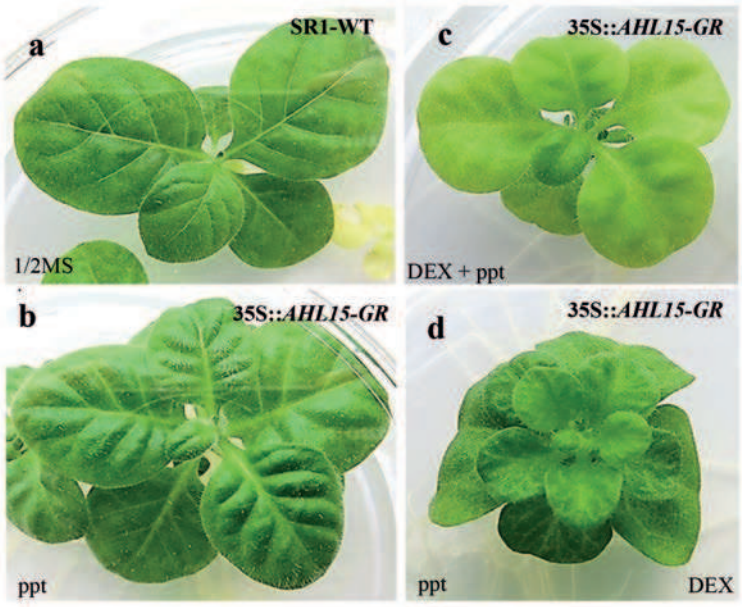

Figure 3. DEX induction of *Nicotiana tabacum 35S::AHL15-GR* plants extend juvenile phase and rejuvenate the adult plants. (a-d) 3 weeks old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants development on non-inducible (a,b) DEX inducible (c,d) MS medium. WT (a) and *35S::AHL15-GR* (b) plants show 4-5 adult leaves in the same developmental stage. *35S::AHL15-GR* plant (c) shows 4-5 juvenile leaves while *35S::AHL15-GR* plant (d) after transferring to MS medium with DEX shows switch from adult to juvenile leaf morphology.

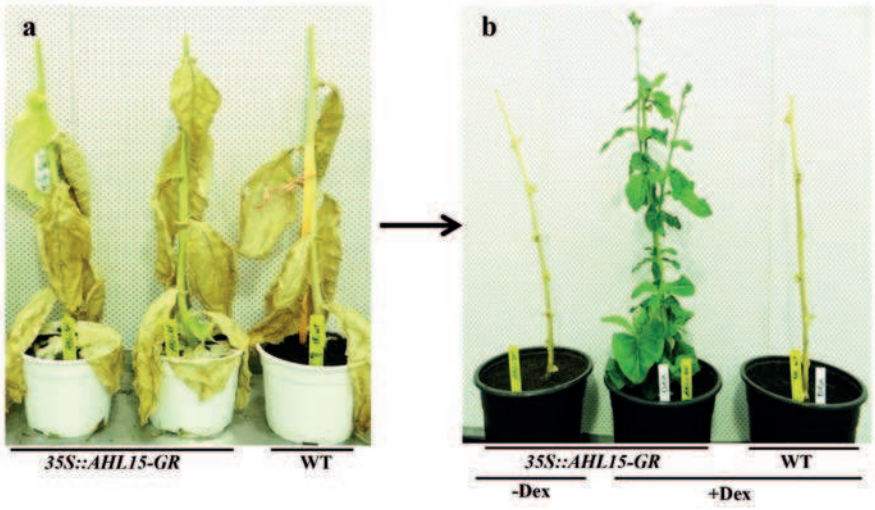

Figure 4. AHL15 triggers activation/rejuvenation of axillary meristems in *N. tabacum 35S::AHL15-GR* plants upon DEX induction. 3 months old *Nicotiana tabacum* (a) non DEX-treated wild type (one on the right side) and *35S::AHL15-GR* (two on the left side) plants with two feet stem along with roots transferred to fresh soil with DEX-treatment (only white tagged plants). (b) After 3 weeks only DEX-treated *35S::AHL15-GR* (middle) plant shows development of all lateral shoots along with juvenile shoots from transition area between stem and roots.

and by induction of lateral shoots, and that this completely changed the morphology of the plant as compared to wild type (Fig. 5a and 6a). The number of branches and leaves were significantly enhanced in *35S::AHL15-GR* plants grown *in vitro* on DEX medium as compared to wild-type or non-DEX-treated *35S::AHL15-GR* plants (Fig. 5a-c). Also for soil grown DEX-treated *35S::AHL15-GR* plants the number of leaves and branches was significantly enhanced (Fig. 6b and 6c) compared to DEX-treated wild-type or non-treated *35S::AHL15-GR* control plants (Fig. 6a). These results indicate that, like in *Arabidopsis*, enhanced expression of *AHL15* results in loss-of-apical dominance, and enhanced biomass production by increased shoot initiation.

Heterologous expression of *Arabidopsis AHL15* induces polycarpy in tobacco in the presence of sufficient nutrients.

Tobacco is an herbaceous annual plant that is very sensitive to temperature, light and humidity. Tobacco plants therefore complete their life cycle by flowering and producing offspring once in a single growing season (around 6 months). In contrast, our transgenic tobacco *35S::AHL15-GR* plants after DEX-treatment not only extended their life by delaying flowering time and leaf senescence, but also converted from seasonal monocarpic to polycarpic plants.

To further show that AHL15 induces perenniality and polycarpy in tobacco, we DEX-treated *35S::AHL15-GR* plants (either grown in tissue culture or on soil) and followed them

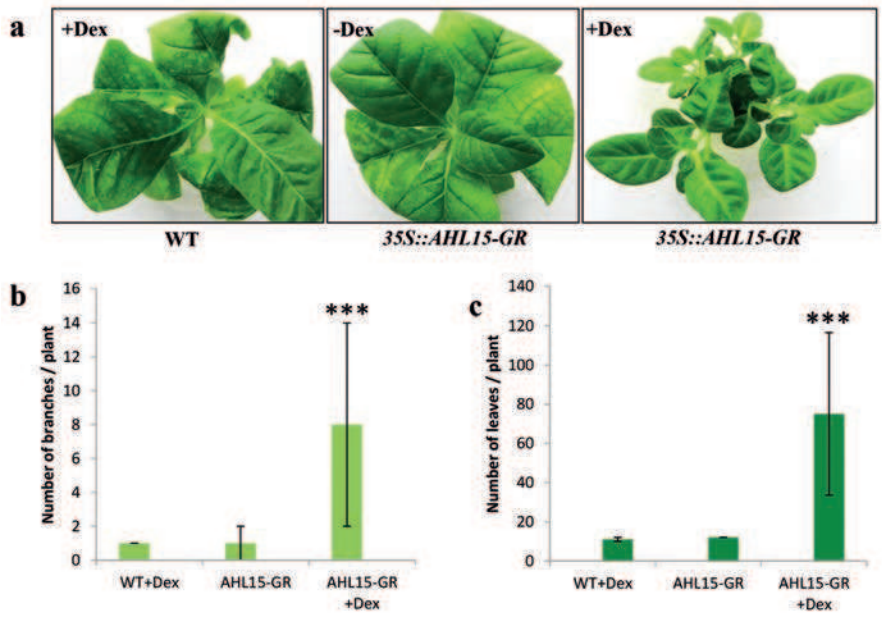

Figure 5. DEX induced *in vivo Nicotiana tabacum 35S::AHL15-GR* plants significantly enhances branching and leaf number. (a) 2 months old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants developed on DEX-inducible and non-inducible MS medium. WT (right) and *35S::AHL15-GR* (middle) plants with normal morphology and *35S::AHL15-GR* (left) plant with branched morphology on DEX-inducible medium. The graphs show significant enhancement in (c) branching and (d) leaves number per plant in *35S::AHL15-GR* plants on DEX medium. Significant increase is indicated by asterisks (*) ($p < 0.05$).

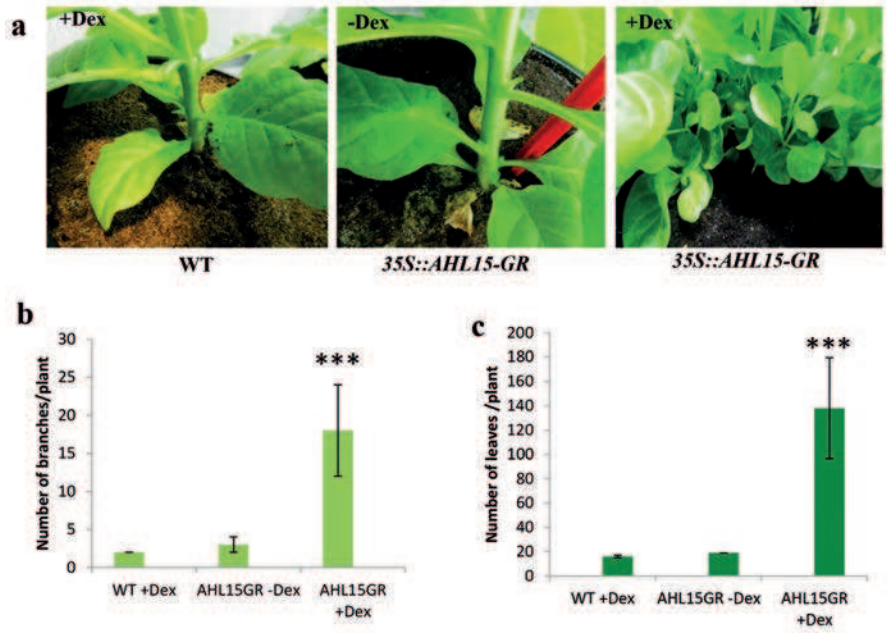

Figure 6. AHL15 induces enhanced branching and leaves number in *Nicotiana tabacum 35S::AHL15-GR in vitro* plants. (a) 2 months old *Nicotiana tabacum* wild type and *35S::AHL15-GR* plants developed in the green house. WT (right plant) with DEX treatment and *35S::AHL15-GR* (middle plant) without DEX shows normal morphology while *35S::AHL15-GR* plant on soil DEX-treatment shows branched morphology. The graphs show significant enhancement in (c) branching and (d) leaves number per plant in DEX-treated *35S::AHL15-GR* plants. Significant increase is indicated by asterisks (*) ($p < 0.05$).

for one year next to non-treated *35S::AHL15-GR* and DEX-treated wild-type control plants. After one year of *in vitro* culture (without media refreshment) the DEX-treated *35S::AHL15-GR* plants were still alive, having some green leaves near the shoot apex, whereas the control plants were completely dried out and dead (Fig. 7a). Also the soil-grown DEX-treated *35S::AHL15-GR* plants continuously produced multiple lateral and new juvenile shoots (Fig. 7b), and even after one year and three rounds of seeds harvesting just by soil refreshment and DEX-treatment the plants remained green and healthy with multiple shoots producing flowers and fruits (Fig. 7c). In contrast, the control plants completed their life cycle in less than six months and died after a single fruit set even when the soil was refreshed (Fig. 4). But DEX-treated *35S::AHL15-GR* plants even without soil refreshment remained green and produced flowers and seeds along with developing lateral and juvenile shoots, only some of the leaves turned yellow (Fig. 8a). By transferring the plants (seedpods, flowers and extra branches removed) to fresh soil, they completely rejuvenated producing fresh leaves and new shoots (Fig. 8b), suggesting that the yellowing of leaves was because of nutrient deficiency. To confirm this, stems of first generation DEX-treated *35S::AHL15-GR* plants were allowed to grow and regenerate new shoots without soil refreshment. We observed that all the newly developed shoots produced whitish yellow leaves (Fig. 8c); however, when 500 ml MS medium was added to the pots, we found that after two weeks the plants developed multiple new green shoots (Fig. 8d) in

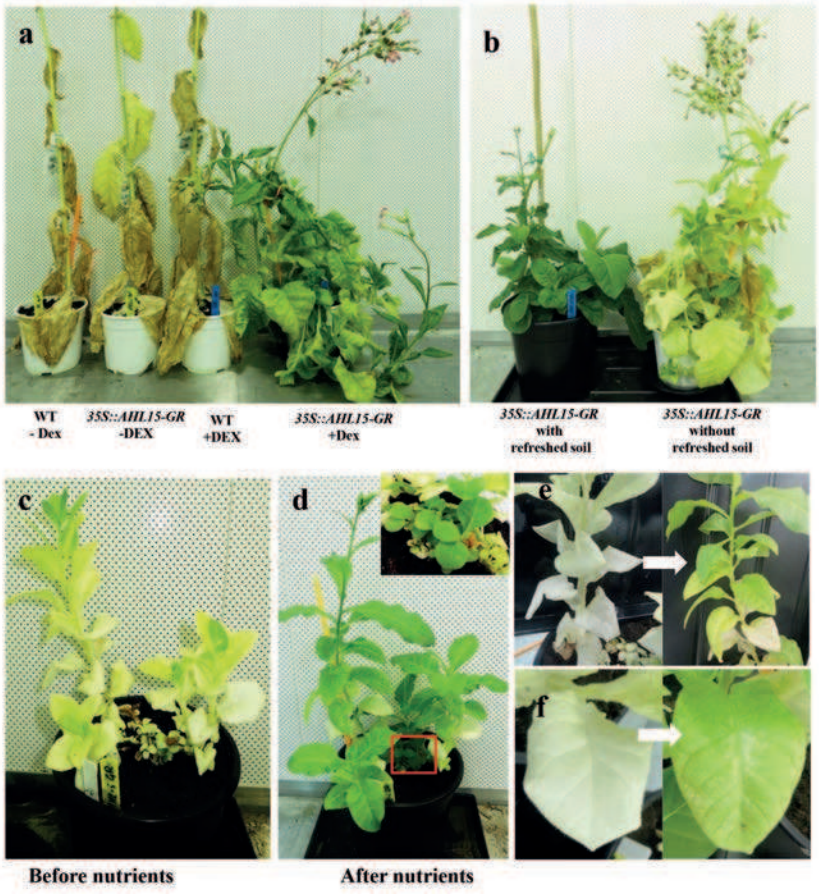

Figure 7. AHL15 induces perenniality and polycarpy in *35S::AHL15-GR* plants upon DEX treatment. (a) *in vivo Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants developed on 100 ml DEX-inducible and non-inducible MS medium. The picture taken after one year shows WT (right) on inducible medium and *35S::AHL15-GR* (middle) plant on non-inducible medium completely died while *35S::AHL15-GR* (left) plant on inducible medium alive with some green leaves. While (b) *35S::AHL15-GR* plant maintained *in-vitro* by periodically soil refreshment and DEX-treatment shows juvenile (highlighted part) and (c) multiple lateral shoots with flowers and fruits after three rounds of seed harvesting.

a similar way as when the DEX-treated *35S::AHL15-GR* plants were transferred to fresh soil (Fig. 4 and 8b) and the whitish-yellow leaves became green (Fig. 8e and f) indicating nutrients restoration. Like the previous observations, these plants were continuously producing new shoots, flowers and fruits but only because of deficiency of nutrients the leaves were turning whitish-yellow. This data shows that heterologous expression of *Arabidopsis AHL15* in tobacco (*35S::AHL15-GR* plants) in the presence of sufficient nutrients maintains the plants in a polycarpic and perennial-like state via delay of senescence, rejuvenation, and enhanced regeneration of lateral shoots throughout the year.

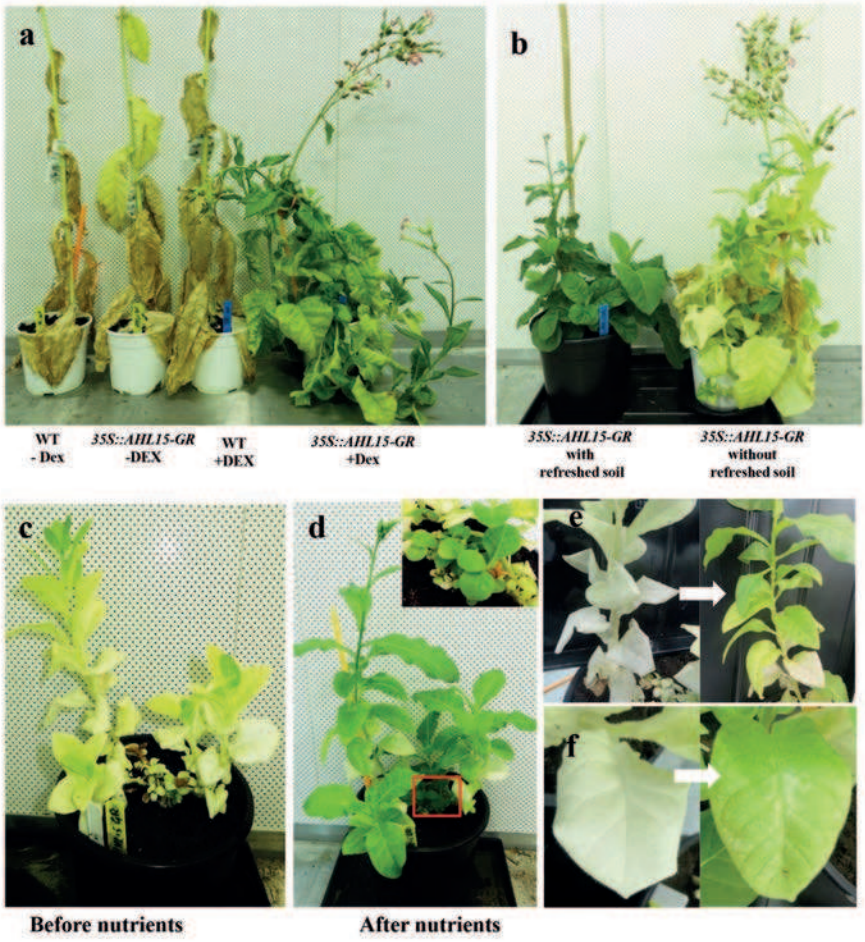

Figure 8. DEX-treatment with sufficient nutrients availability leads to perenniality longevity and rejuvenation in *35S::AHL15-GR* tobacco plants. (a) 3 months old *Nicotiana tabacum* wild type (WT) and *35S::AHL15-GR* plants with and without DEX-treatment. Non-induced WT and *35S::AHL15-GR* plants (with yellow tag) and induced WT plant (with blue tag) show normal senescence at the same time. Whereas DEX-induced *35S::AHL15-GR* plant (with blue tag) shows delay in senescence with development of lateral shoots. (b) *35S::AHL15-GR* DEX-induced plants without soil refreshment shows whitish-yellow leaves (right plant) and slow aging process while with refreshed soil (left plant) shows green leaves with lateral and juvenile shoots. (c) *35S::AHL15-GR* second generation plants (DEX-induction during 1st generation only) developed new shoots with whitish-yellow leaves from the 5 inches stem. (d) After two weeks of adding 100 ml MS medium into the soil the whole plant turned green with the development of new shoots (highlighted part) and already present (e) whitish-yellow and wrinkled shoots turned green and fresh. (f) Shows the changing of white leaf to green upon nutrients availability.

Heterologous *AHL15* expression increases seed quantity at the cost of seed quality in tobacco

Survival at the cost of reproduction is common both in plants and animals (Obeso, 2002; Tabatabaie et al., 2011). Aragon et al. (2009) have shown that the survival rate of perennial plants significantly increases when flowers are removed. This suggests that the high reproduction

burden often observed in annual plants restricts them to a monocarpic mode of life (Suzuki et al., 2012). Like *35S::AHL15 Arabidopsis* plants, the DEX-treated *35S::AHL15-GR* tobacco plants continuously produced many lateral branches that flowered and produced seeds, causing the plants to carry new shoots, flower buds, opened flowers, green fruits and ripened fruits at the same time (Fig. S6a). To quantify the effect of *AHL15* expression on seed production we harvested seeds from all the ripened fruits from DEX-treated and non-treated wild-type and *35S::AHL15-GR* plants. The average weight of individual seed from all four samples was determined by weighing 300 seeds. The total number of seeds produced by a plant was calculated by dividing the total weight of all the seeds of a plant by its average single seed weight. After statistical analysis we found that the total number of seeds produced per DEX-treated *35S::AHL15-GR* plant was two times more than the control plants (Fig. 9a). But when we compared the total weight of seeds of all four types of plants, surprisingly there was no significant difference (Fig. 9b). We also compared the volume of four types of seeds by putting them in 50ml falcon tube, interestingly the volume of DEX-induced *35S::AHL15-GR* seeds was more than the volume of other three types of seed (Fig. S6b). This discrepancy between the calculated seed number, and the quantified total seed weight and volume per plant led us to look into the seed morphology and viability. Electron micrographs of the four types of seeds showed clear shape and size abnormalities in the seeds harvested from DEX-treated *35S::AHL15-GR* plants (Fig. 10 d1), whereas seeds harvested from the other plants (non-treated wild type and *35S::AHL15-GR* and DEX-treated wild type) were normal round and oval shaped (Fig. 10 a1, b1 and c1). Observation of the seeds with a stereomicroscope using dark field lighting showed that most seeds from DEX-treated *35S::AHL15-GR* plants were empty (Fig. 10 d2). Germination of 500 seeds per seed batch showed that the seeds from the control plants germinated normally (Fig. 10 a3, b3, and c3), but that the germination efficiency of the seeds collected from the DEX-treated *35S::AHL15-GR* plants was reduced to 2-3 percent (Fig. 10 d3). These results suggest that the abundant and repeated flowering induced by DEX treatment of the *35S::AHL15-GR* tobacco plants affected their reproduction efficiency. At this moment we cannot exclude,

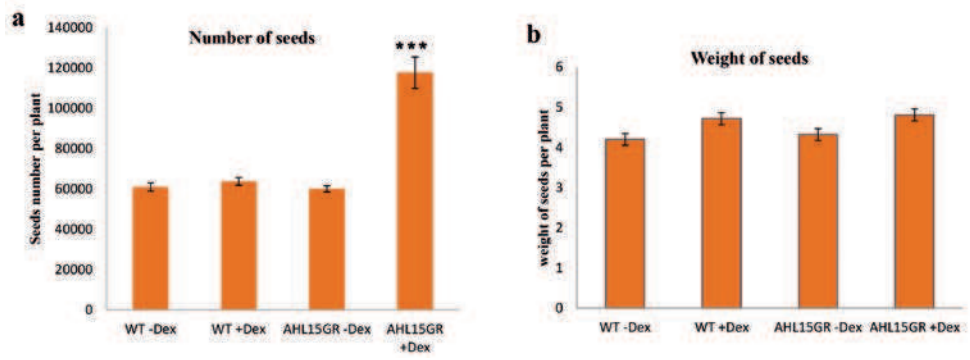

Figure 9. AHL15 induction in *Nicotiana tabacum 35S::AHL15-GR* plants significantly enhances total seed number without affecting total seeds weight. The total number of seeds per plant is calculated by dividing the total weight of seeds of a plant by the average weight of a single seed of that plant. The comparison of (a) total seed number per plant shows that AHL15 induction in *35S::AHL15-GR* plants significantly enhances number of seeds per plant as compared to induced wild type plants and non-induced wild type and *35S::AHL15-GR* plants. The comparison of seed weight (b) shows no significant difference in total seed weight per plant. Significant increase in indicated by asterisks (*) ($p < 0.05$) and (***) indicate 2x difference.

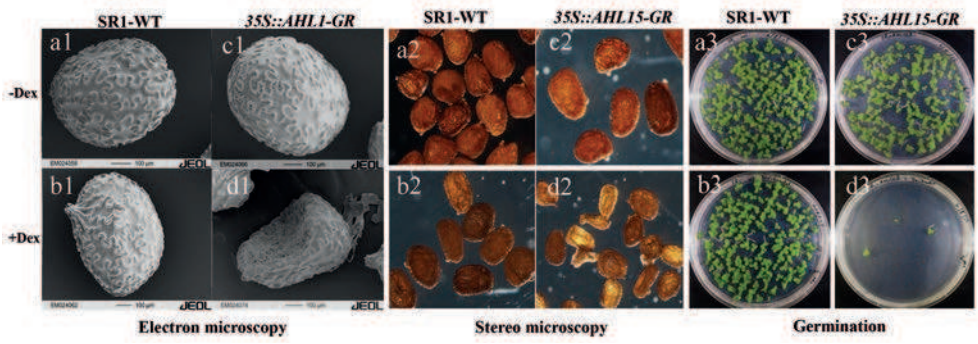

Figure 10. AHL15-induction in *Nicotiana tabacum 35S::AHL15-GR* plants affects seed quality. Electron microscopy of the *Nicotiana tabacum* seeds harvested from non DEX-treated wild type (a1) and *35S::AHL15-GR* (c1) plants and DEX-treated wild type (b1) plants show normal oval/round morphology and that of *35S::AHL15-GR* DEX-treated (d1) plants with irregular shapes. Stereomicroscopy shows all the seeds with normal and oval/round morphology (a2, b2, c2 and some in d2) compact while all the abnormal seeds (d2) with irregular morphology are empty from inside. The germination of the seeds from non-induced wild type (a3) and *35S::AHL15-GR* (c3) and induced wild type (b3) plants on MS medium without DEX and selection shows 100% efficiency while only 2-3% of the seeds from induced *35S::AHL15-GR* plants (d3) show germination.

however, that the poor seed quality was induced by nutrient deficiency, which was observed previously for DEX-treated *35S::AHL15-GR* tobacco plants that were allowed to rejuvenate without refreshing the soil (Fig. 8).

DISCUSSION

The annual and perennial life cycles are two different life history strategies of flowering plants that allow them to battle against harsh environmental conditions. Common in both types of life history strategies is the developmental transition from vegetative to reproductive phase (Friedman and Rubin, 2015). In annual plants, the reproductive phase is followed by senescence and death of the plant while survival of the species is guaranteed by the seed. In contrast, in perennials the hardy part of the vegetative plant body is maintained, which allows the plant itself to survive the harsh winter conditions and re-enter the reproductive phase again and again (Albani and Coupland, 2010). So polycarpy and longevity in flowering plants depend on maintaining the vegetative plant body and development of lateral or new shoots with juvenile characteristics having the potential to pass through the reproductive phase. In this study we showed that heterologous expression of the *Arabidopsis AHL15* gene changes the monocarpic tobacco into a polycarpic plant, inducing branching by activating axillary meristems and by keeping these meristems in the vegetative state. Other phenotypes observed in DEX-treated *35S::AHL15-GR* plants were delayed germination and seedling development even leading to callus formation, and delayed flowering and leaf senescence. All these observations indicated that *AHL15* overexpression has the same effects on tobacco as it has on *Arabidopsis* (Karami et al., 2017), and that it generally is able to revert developmental transitions, thereby keeping plant tissues in a juvenile state.

However, there are also clear differences in the phenotypes induced by *AHL15* overexpression in *Arabidopsis* or tobacco. For example, in *Arabidopsis*, *AHL15* overexpression, similar to *BABY BOOM* (*BBM*) overexpression, induces somatic embryos on cotyledons and also callus-like structures that later convert to somatic embryos (Boutilier, 2002; Karami et al., 2017). In tobacco, however, *AHL15* overexpression only led to the formation of callus, whereas *BBM* overexpression leads to shoot and root induction. *BBM* overexpression requires cytokinin to be added to the medium to induce somatic embryogenesis (Srinivasan et al., 2007), and possibly the same hormonal treatment is required before *AHL15* overexpression can induce somatic embryos on tobacco seedlings, but this needs further testing. In any case, it is clear that activation of AHL15-GR by DEX-treatment in homozygous *35S::AHL15-GR* seedlings completely arrests development, probably by converting organized stem cell zones into undifferentiated callus tissues. This suggests that in tobacco *AHL15* expression completely inhibits cell differentiation and organogenesis, which might explain why *35S::AHL15* plants that constitutively overexpress *AHL15* could not be obtained. This difference in phenotypes for *AHL15* or *BBM* overexpression in tobacco and *Arabidopsis* suggests that there is a significant difference in the set of target genes that are up- or downregulated by AHL15 or BBM in these plant species. The fact that for BBM this can be restored by adding cytokinin to the medium, hints that a major difference might lie in plant hormone-related genes.

35S::AHL15-GR tobacco plants first grown in the absence of DEX developed normally like wild-type plants in the presence or absence of DEX. DEX-treatment in *35S::AHL15-GR* plants resulted in the activation of axillary meristems, not only delaying senescence and enhancing branching, but also leading to the production of leaves with juvenile features. Similar to *Arabidopsis*, AHL15 can rejuvenate development in tobacco, as *in-vitro* induction experiments showed that AHL15 activation brings the adult plant morphology back to juvenile state (Fig. 3d & S4), and shoots with juvenile features could be induced on soil-grown plants (Fig. 7b and 8d). Similarly, we observed that the DEX activation of AHL15 in *35S::AHL15-GR* plants, delayed leaf senescence and caused a delay in flowering time. This suggests that, like in *Arabidopsis*, the vegetative phase change is important for flower initiation and also preludes the leaf senescence (maturation) process.

DEX-treated *35S::AHL15-GR* tobacco plants continued to grow for at least 2 years. Continuous refreshment of the soil was essential for their proper development. If not, leaves turned yellow and eventually white. But this could be restored by transferring them to fresh soil again. Like in *Arabidopsis*, *AHL15* overexpression resulted in polycarpy in tobacco, giving rise to more seeds. Interestingly, in tobacco this came at the cost of reduced seed quality, which could be because the *35S* promoter driving the *AHL15-GR* expression is more active in the seeds during embryogenesis in tobacco. Alternatively, the more branched growth of th DEX treated *35S::AHL15-GR* tobacco plants might limit nutrient availability. Tobacco is considered as heavy feeder, and the refreshment of soil alone might not provide sufficient nutrients, as additional nutrient supply resulted in a rapid but transient conversion of the leaves from yellow to dark green (Fig. 8b,d). Moreover, in contrast to *Arabidopsis*, *AHL15* overexpression in tobacco might not result in the production of photosynthetic leaves that produce sufficient energy to support the renewed seed set. This might explain why polycarpy in this plant species comes at the cost

of reduced seed quality, which is typically a seed size versus seed number trade-off in larger-seeded perennial species (Leishman and Westoby, 2000). For example, the average number of seeds produced per plant in *Arabidopsis thaliana* is much more than its perennial relative *Arabis alpina* that produces significantly larger seeds (Boyes et al., 2001; Andrello et al., 2016). Like with our *35S::AHL15-GR* tobacco plants, it is generally observed that in evolution plants adopt the polycarpic life history at the cost of reproduction (Friedman and Rubin, 2015). That this is not the case in *Arabidopsis* plants overexpressing *AHL15* is quite surprising (Karami et al., 2017). Our observations on the *35S::AHL15-GR* tobacco lines suggest that the outcome of a switch from monocarpic to polycarpic growth probably depends on how efficient a plant uses its resources. At the same time *AHL15* overexpression in *Arabidopsis* and tobacco due to enhanced number of branches resulted in more seeds production.

Like the effect of ipt gene on axillary buds in tobacco (Hewelt et al., 1994), *35S::AHL15-GR* induction strongly reduces apical dominance which results in branched morphology and smaller leaf size (Fig. 6a and S7). In case of soil-DEX treatment of *35S::AHL15-GR* plants, leaves kept the juvenile characteristics and new juvenile shoots appeared from the transitional area between shoots and roots (Fig. 6a, 7b & 8d) while the non-DEX treated *35S::AHL15-GR* and wild type plants passed normally through the developmental processes and died after one flowering period with a single main stem. A comparison of flowers, inflorescence and fruits morphology of wild type and induced *35S::AHL15-GR* plants did not show any significant differences in their size and shape (Fig. S8). Only the inflorescence of *35S::AHL15-GR* was more scattered as compared to wild type which could be because of the branched morphology induced by *35S::AHL15-GR* induction.

Our data add to the general picture that *AHL* genes are key regulators of plant developmental processes. Our results show that ectopic expression of *Arabidopsis AHL15* leads to similar phenotypic changes in *Arabidopsis* (Karami et al., 2017) and tobacco, suggesting that *AHL15* could be a generic switch between monocarpic and polycarpic life history strategy in flowering plants. However, our results also indicate that the strategy to use *AHL15* overexpression to enhance seed production will not work in all plant species. It is important to understand what is at the basis of this difference, and also to determine the molecular mechanism underlying action of *AHL15* so that we can successfully apply this knowledge to enhance the yield of important crop plants.

MATERIAL AND METHODS

Plant material and growth conditions

Nicotiana tabacum SR1 (tobacco) plants were grown axenically on MS medium (Murashige and Skoog, 1962) in 1l glass jars. To establish this axenic plant culture, seeds were surface sterilized by a first wash with sterile Milli-Q water (MQ), followed by one minute incubation in 70% ethanol, a wash with sterile MQ, 10 minutes incubation in 50% Glorix (commercial solution containing 4.5% active chlorine, and <5% sodium hypochlorite) with periodically shaking, and finally 4 to 5 washes with sterile MQ.

For *in-vitro* seed germination, half strength MS medium with 0.8% agar (w/v) (Diachin agar) and 1.5% sucrose was used, while for transformation and regeneration normal MS medium

with 0.7% agar, 3% sucrose 2 mg/l BAP and 0.2 mg/l NAA was used. *In-vitro* germination, plant growth and regeneration were carried out at 24°C and 16 hours photoperiod, while plants were grown on soil in a growth room at 25°C, 75% relative humidity and 16 hours photoperiod.

Generation of *35::AHL15-GR* tobacco lines

The *35S::AHL15-GR* construct was obtained by replacing *BBM-GR* fragment in a binary vector pSRS031 with a synthetic *Pst*I-*Xho*I fragment containing the *AHL15-GR* fusion (Passarinho et al., 2008). Fresh single *A. tumefaciens* colonies were obtained from a -80°C stored glycerol stock by making a pure streak on LC plates (10 g/l tryptone, 5 g/l yeast extract, 8 g/l NaCl, 0.8 % agar) containing 20 µg/ml rifampicin and 75 µg/ml carbenicillin and 250 µg/ml spectinomycin to select the *A. tumefaciens* AGL1 strain (Jin et al., 1987) containing the *35::AHL15-GR* construct. Plates were incubated at 29°C for two days. For liquid culture, a single colony was inoculated in 25 ml LC medium (without agar) in a 100 ml flask that was incubated at 30°C with 180 rpm rotation for two days. The liquid cultures were transferred to 50 ml falcon tubes (SATSTED) and centrifuged at 4000 rpm for 20 minutes. The supernatant was discarded and after washing with MQ, the pellet was re-suspended in induction medium (Gelvin, 2006) with 100 µM acetosyringone and induced for overnight in the dark on 50 rpm rotator at room temperature.

For *Agrobacterium* leaf disc infiltration, round leaf discs were cut from the 3rd and 4th leaf of non-sterile 4 to 5 weeks old greenhouse grown wild type tobacco plants with the help of a blue cap tube of 5 cm diameter. The non-sterile round leaf discs were surface sterilized with a first wash in sterile MQ followed by 15 minutes incubation in 10% Glorix (commercial solution containing 4.5% active chlorine, and <5% sodium hypochlorite) with gentile rotation and finally 4-5 washes with sterile MQ (Baltes et al., 2014). The surface sterilized leaf discs were infiltrated with overnight induced *A. tumefaciens* culture of OD_{600} 0.6-0.8. The infiltrated leaf discs were blotted for 2 to 3 minutes and then transferred to co-cultivation plates containing 25 ml MS medium supplemented with 2 mg/l BAP, 0.2 mg/l NAA and 40 mg/l acetosyringone. The cocultivation was carried out for three days in the dark at 24°C. Selection and regeneration was carried out on MS medium without acetosyringone using 15 mg/l phosphinothrycine (ppt) for selection and 500 mg/l cefotaxime for killing *Agrobacterium*. Ppt resistant shoots were transferred to 1l jars with hormone free MS medium containing 15mg/l ppt and 500mg/l cefotaxime for rooting. The rooted transformed plants were transferred to soil and grown in growth rooms at 25C° with 75% relative humidity and 16 h photoperiod.

T2 seeds were germinated on selection medium to identify single locus T-DNA insertion lines based on their 3:1 segregation ratio. Four lines were selected and used in the subsequent experiments. To check for the presence of the *35S::AHL15-GR* construct, genomic DNA was isolated from leaf tissue of T2 plants of the transgenic lines via CTAB method (Doyle and Doyle, 1990) and PCR was performed using the primers A-GR-fw CATTTGGAGAGGACTCGAGCTCAT and A-GR-rev CGCTGTACCATGCATGATCTGGAT. Homozygous T3 plants were selected by segregation analysis.

Phenotypic analysis and morphometry on wild-type and *35S::AHL15-GR* plants

To compare phenotypes, generally 3 plants per line were grown and images of only representative plants are shown. To quantify the effect of induced AHL15 activity on number of leaves and branches, 3 representative plants were grown each *in vitro* on MS medium with or without 10μM DEX and on soil of same quality and quantity with 30μM DEX-treatment via spray or watering. To quantify the seed number and weight per plant, one plant of each lines was grown and the DEX treatment was done via spray and watering in soil (see Table. 2). All the seeds were harvested from completely senesced tobacco control plants while in positive tobacco plants the seeds were harvested 2-3 weeks later only from ripe fruiting bodies only.

Photography, Stereo- and scanning electron microscopy

Plants photography was done with Canon camera (model: pc1742) with 12.1 mega pixels and 20X zooming power. Stereomicroscopy of seeds was done with Leica MZ16FA stereo fluorescent microscope with Leica DFC 420 Camera and cable -5 megapixel,6 pin firewire and seedlings and callus pictures were taken with Leica MZ12 with LEICA DC 500 microscope CCD Camera Head 12447108 12-33 vdc Firewire. For scanning electron microscopy dry clean seeds were fixed to specimen stubs with adhesive and placed on the revolving discs of a sputter coater E5100 (Polaron Equipment ltd) where each seed was uniformly coated with gold. These specimen tubes were then fixed to the specimen holder of scanning electron microscope (Joel JSM6400) maintained at an accelerating potential of 10KV. The images were taken at different angles and magnifications. Images were modified assembled in PowerPoint (Microsoft office 2010).

DEX treatment

A stock solution of 30 mM DEXamethasone (Sigma-Aldrich®) was prepared in 70% alcohol. For *in-vitro* treatments, DEX was added at a final concentration of 10 μM to sterile MS medium. For *in-vivo* treatments, DEX was added to water at a final concentration of 30 μM and directly sprayed on plants or 100ml DEX-water was added to the pots per soil treatment, according to the schedules in Tables 1 and 2.

Table 1. Three days DEX spray schedule for 50 days old plants

Spray	Day	Time
1st	1st day	16:00 pm
2nd	2nd day	10:00 am
3rd	3rd day	10:00 am

Table 2. Combined soil DEX-treatment and DEX-spray schedule for two weeks old plants

DEX treatment	Soil DEX treatment	DEX spray
In-vitro seedlings transferred to soil	100 mL (Friday)	No spray
1st Week	100 mL (Tuesday)	1st Tuesday 2nd Friday
2nd Week	100 mL (Tuesday)	3rd Tuesday 4th Friday
3rd Week	-------	5th Tuesday 6th Friday

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SUPPLEMENTARY FIGURES

2

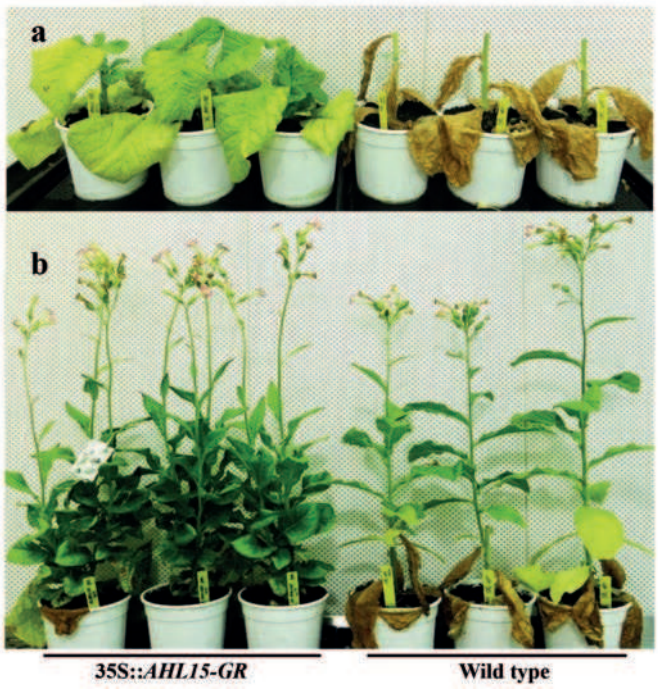


Figure S1. AHL15-induction maintains the delay in senescence in *35S::AHL15-GR* tobacco plants during the second generation. (a) 4 months old *Nicotiana tabacum* first generation DEX-treated wild type (senesced) and *35S::AHL15-GR* (delayed senescence) plants 6 inches stems DEX sprayed. (b) After 5 weeks wild type stems show development of single shoot with early senescence and *35S::AHL15-GR* plants show development of multiple lateral shoots with delayed senescence.

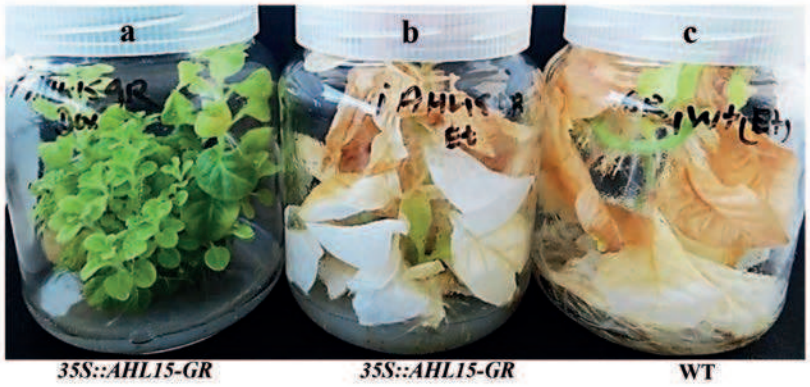


Figure S2. 4 months old *Nicotiana tabacum*. (a) *35S::AHL15-GR* plant developed on DEX-induced medium shows delayed senescence with multiple juvenile and some adult shoots (b) *35S::AHL15-GR* plant on non-DEX medium and (c) wild type on DEX medium shows completely senesced adult leaves and having no juvenile shoots or leaves.

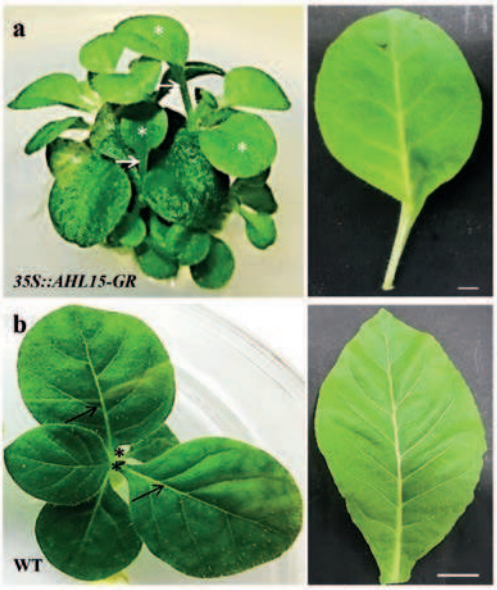


Figure S3. Adult and juvenile leaf morphology in *35S::AHL15-GR* and wild type *Nicotiana tabacum*. *Nicotiana tabacum* (a) DEX-treated *35S::AHL15-GR* plant shows small round leaves (white asterisks) with long petiole (white arrows) and no clear veins as juvenile characteristics and (b) wild type plant shows adult morphology having large elongated leaves with short petiole (black asterisks) and a clear central midrib (black arrows). The right panel shows the juvenile and adult leaf morphology difference in *in-vivo* plants. Scale bar is 10 mm.

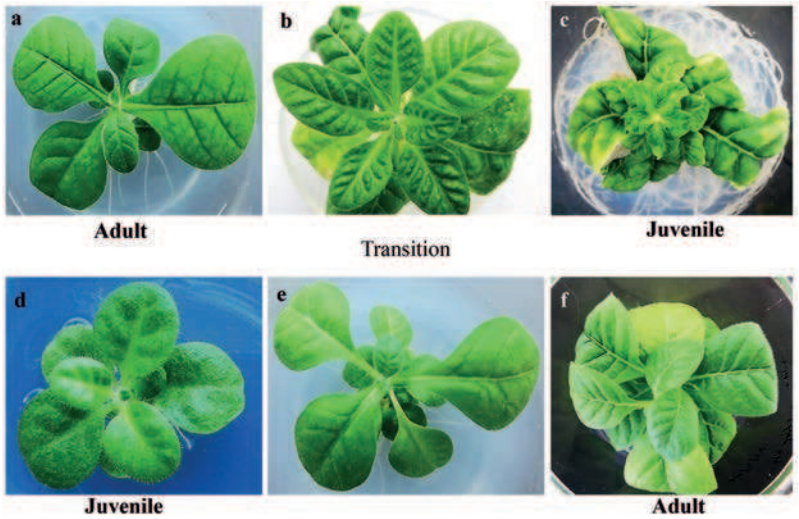


Figure S4. *Nicotiana tabacum 35S::AHL15-GR* plants showing phase change from adult to juvenile and juvenile to adult morphology. 40 days old *Nicotiana tabacum* (a) *35S::AHL15-GR* adult plant developed on MS medium. (b) After 15 days of transfer to DEX containing medium, the leaves show transition phase while (c) after 40 days all the new leaves show juvenile characteristics. (d) *35S::AHL15-GR* plant on DEX containing MS medium shows juvenile morphology (e) after 15 days of transfer to non-DEX medium shows transition phase while (f) after 40 days the plant shows completely adult morphology.

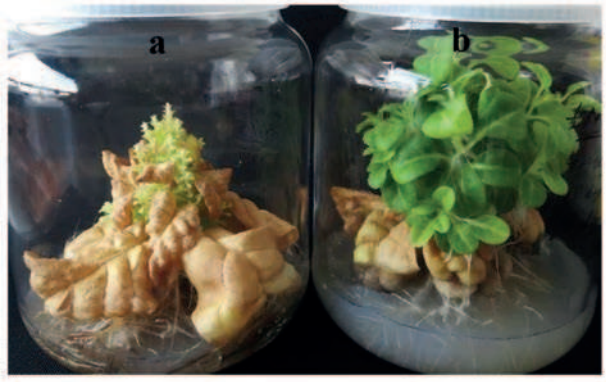


Figure S5. 4 months old *Nicotiana tabacum 35S:AHL15-GR* plants developed on MS medium. After 40 days the plants were transferred to DEX containing MS medium. After 15 days of induction, plant (b) is transferred back to MS medium without DEX showing the new shoots with juvenile characteristics and branched morphology while plant (a) left on inducible medium shows many small branches in the axil of the dried adult leaves with minute juvenile leaves.

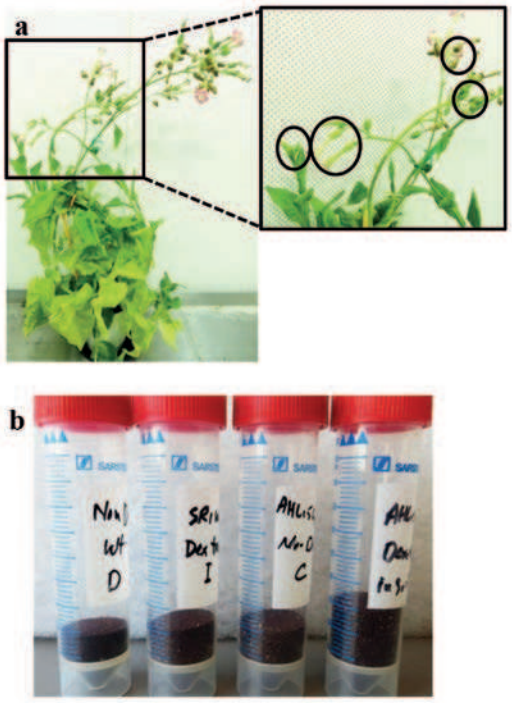


Figure S6. AHL15 induces continuous reproductive phase in *Nicotiana tabacum 35S::AHL15-GR* plants. (a) 3 months old DEX-treated *N. tabacum 35S::AHL15-GR* plant having flower buds, mature flowers, green fruits and ripened fruits (encircled in highlighted part) during the same phase of plant development. (b) Volume of total seeds collected from DEX-treated and non DEX-treated wild type and *35S::AHL15GR* plants. The right tube shows the volume of the seeds collected from only ripened fruits of DEX-treated *35S::AHL15-GR* plants.

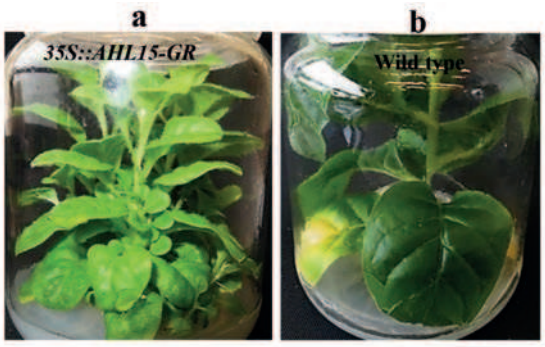


Figure S7. 3 months old DEX-treated *Nicotiana tabacum*. (a) *35S::AHL15-GR* plant shows breaking of lateral shoots dormancy while in (b) wild type plant the lateral shoot dormancy is maintained showing no effect of DEX on lateral shoot development.

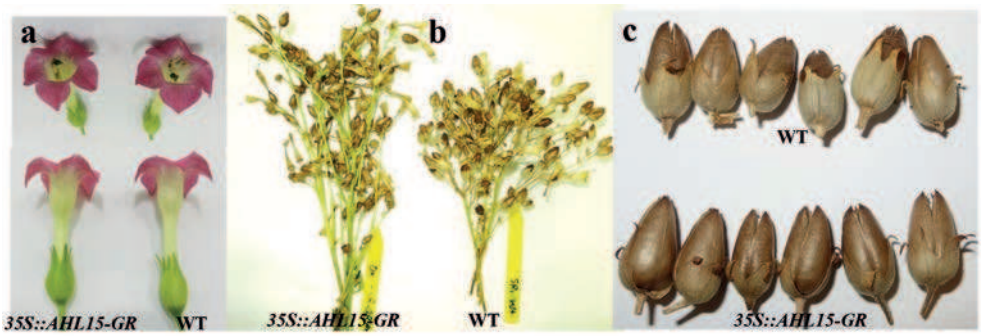
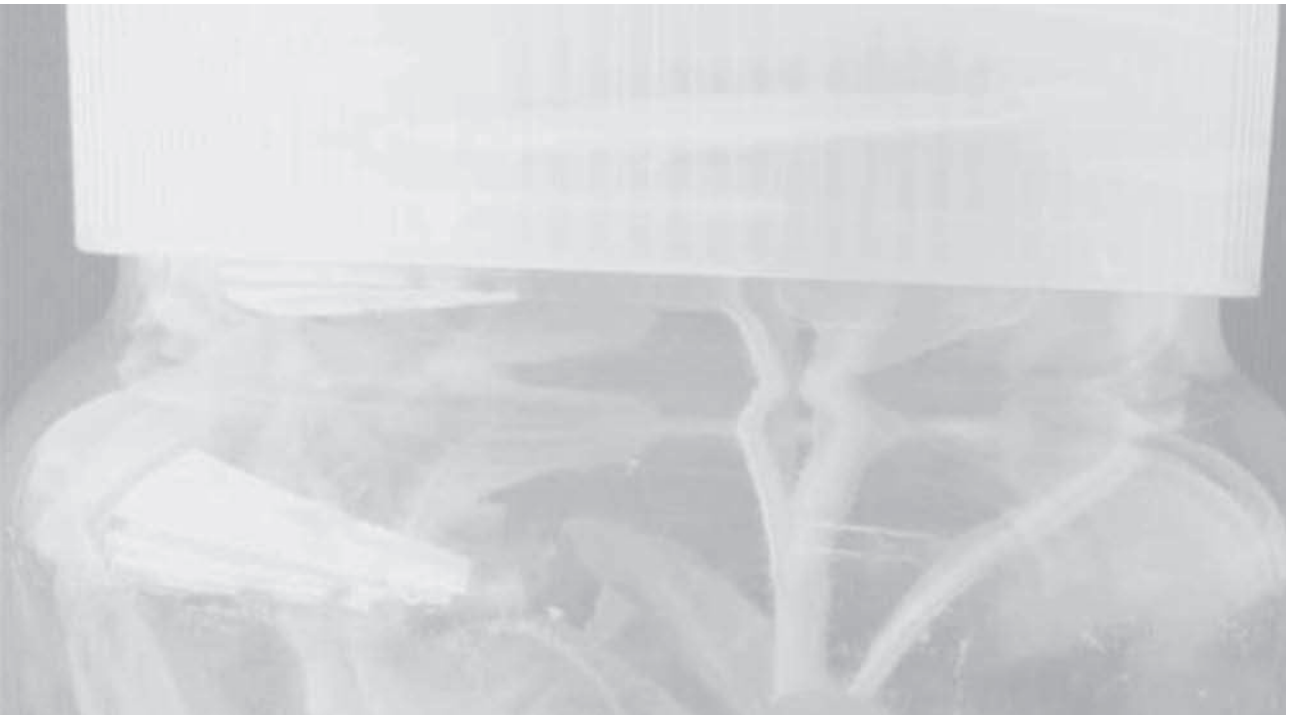
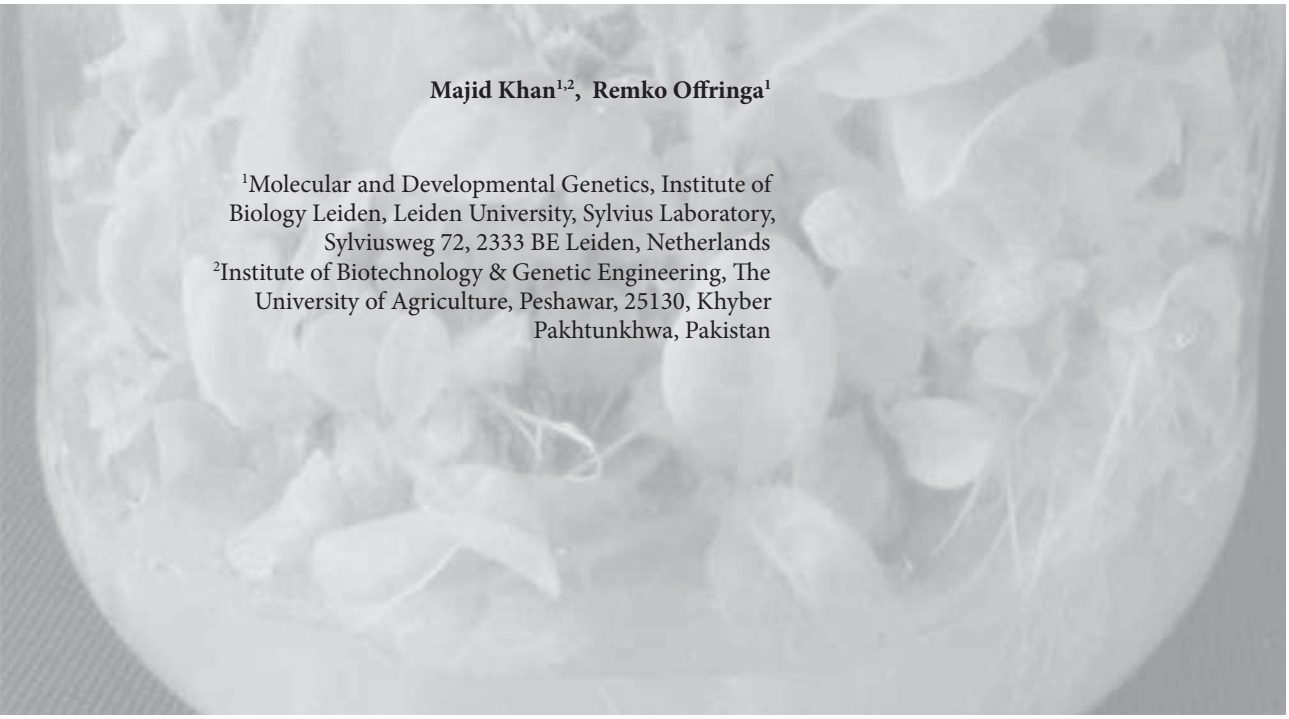


Figure S8. Flower, inflorescence and seed pods morphology comparison between wild type and *35S::AHL15-GR* tobacco plants. *Nicotiana tabacum* wild type and *35S::AHL15-GR* DEX-treated plants show similar (a,c) flower and seed pod morphology while *35S::AHL15-GR* inflorescence (b) is more dispersed compared to wild type.



AGROBACTERIUM-MEDIATED TRANSLOCATION OF PLANT DEVELOPMENTAL REGULATORS TO PLANT CELLS

3



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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

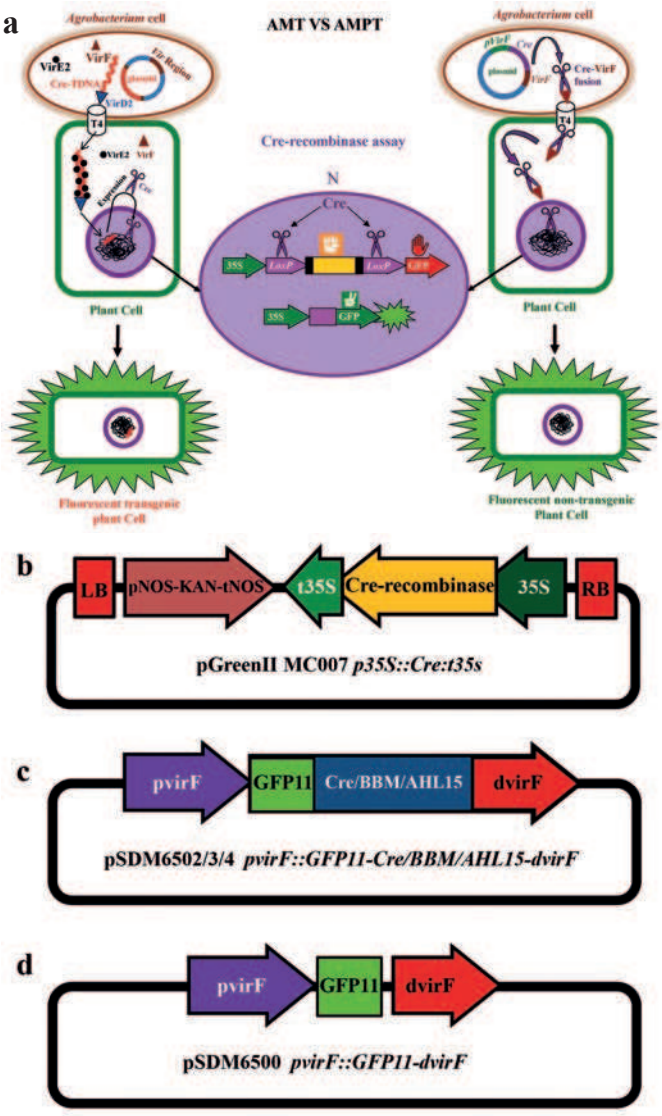

Figure 1. General strategy and DNA constructs for detection of *Agrobacterium*-mediated T-DNA transfer (AMT) or protein translocation (AMPT) to plant cells. (a) Schematic representation of the Cre-recombinase assay for detection of *Agrobacterium*–mediated T-DNA transfer or protein translocation. The *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.

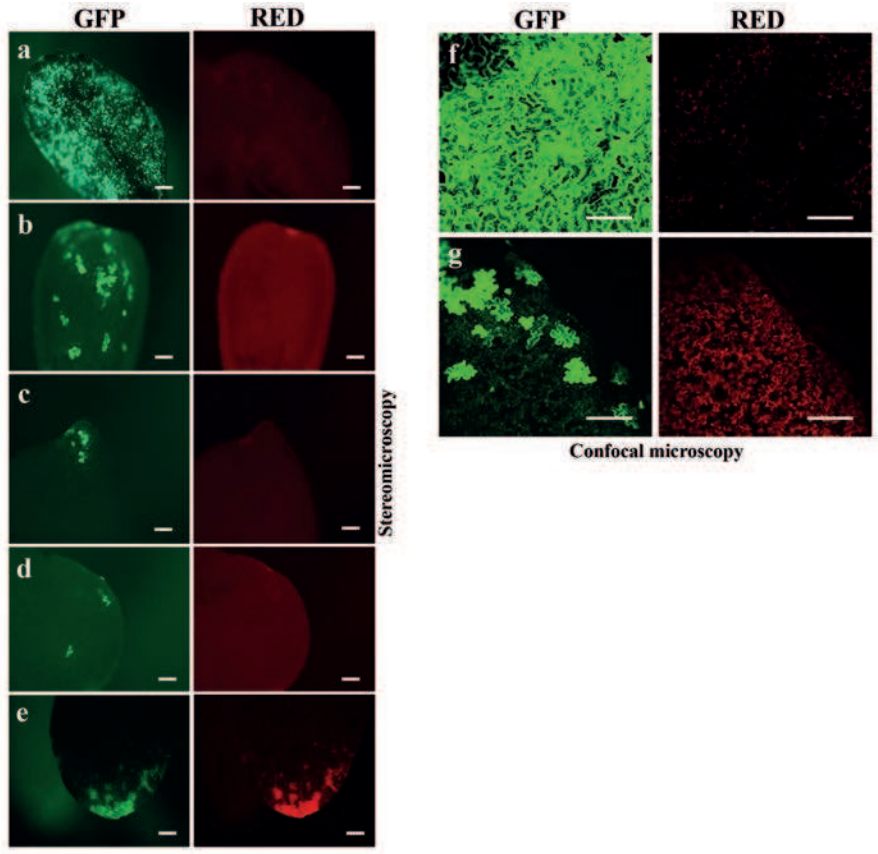

Figure 2. GFP based Cre-recombinase assay via AMT or AMPT in leaf tissues of *Arabidopsis thaliana pcb1* seedlings. (a-e) Detection of GFP expression by fluorescent stereomicroscopy following Cre-mediated restoration of the *GFP* reporter gene in *Arabidopsis pcb1* seedlings after vacuum infiltration 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

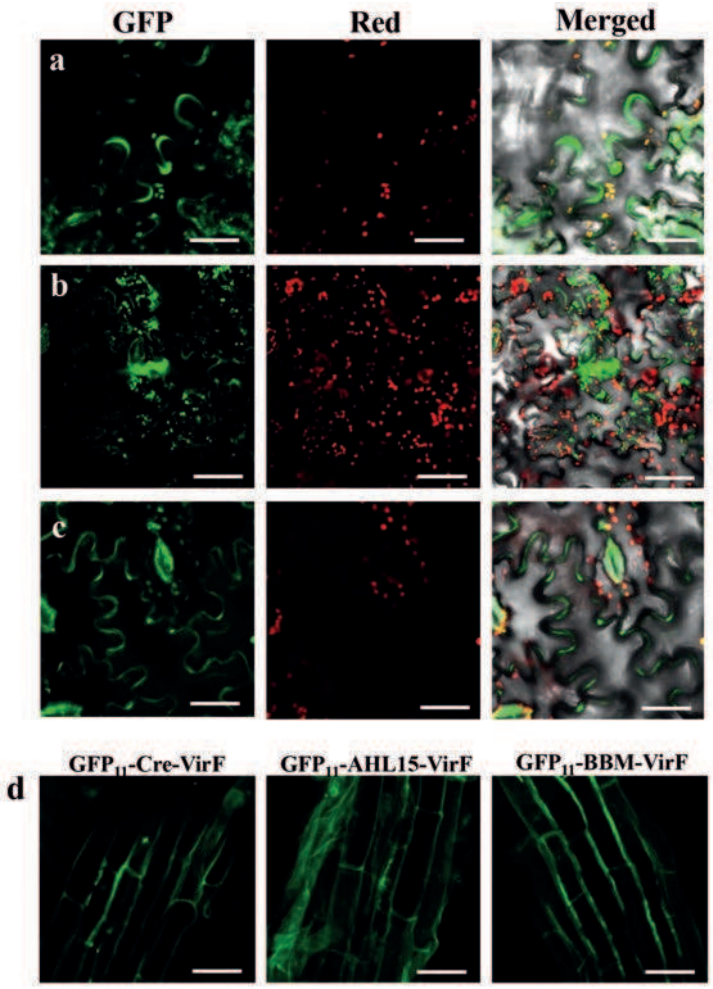

Figure 3. Visualization of Cre, BBM and AHL15 protein translocation via the split GFP assay in tobacco and *Arabidopsis* GFP1-10 marker lines. (a-c) Visualization by confocal microscopy of GFP1-10 complementation in leaf discs of *Nicotiana tabacum 35S::GFP*$_{1-10}$ line 3 days after co-cultivation 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.

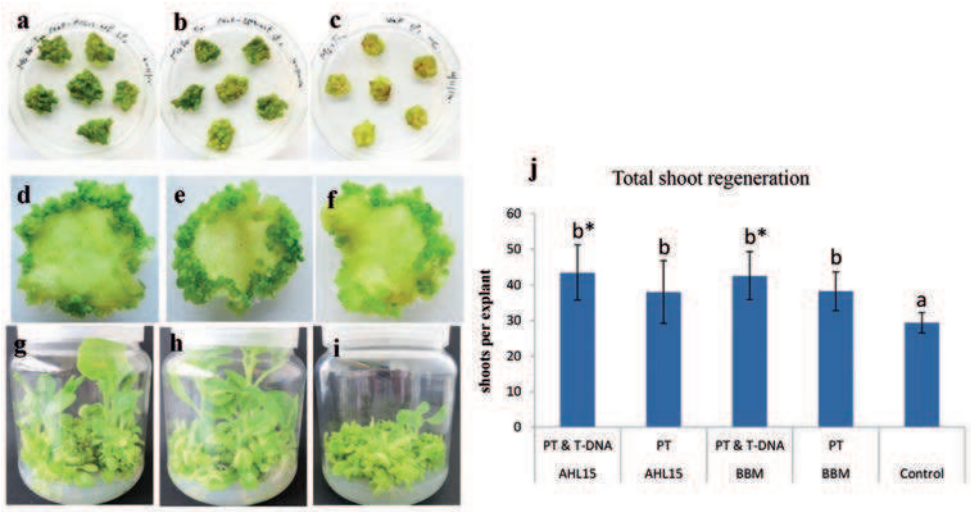

Figure 4. *Agrobacterium*-mediated translocation of AHL15-dVirF and BBM-dVirF fusions delay senescence and enhance shoot regeneration in *N. tabacum* leaf discs. (a-c) *N. tabacum* leaf discs after cocultivation with *Agrobacterium* strain AGL1 translocating AHL15-dVirF (a), BBM-dVirF (b) or dVirF (c) 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

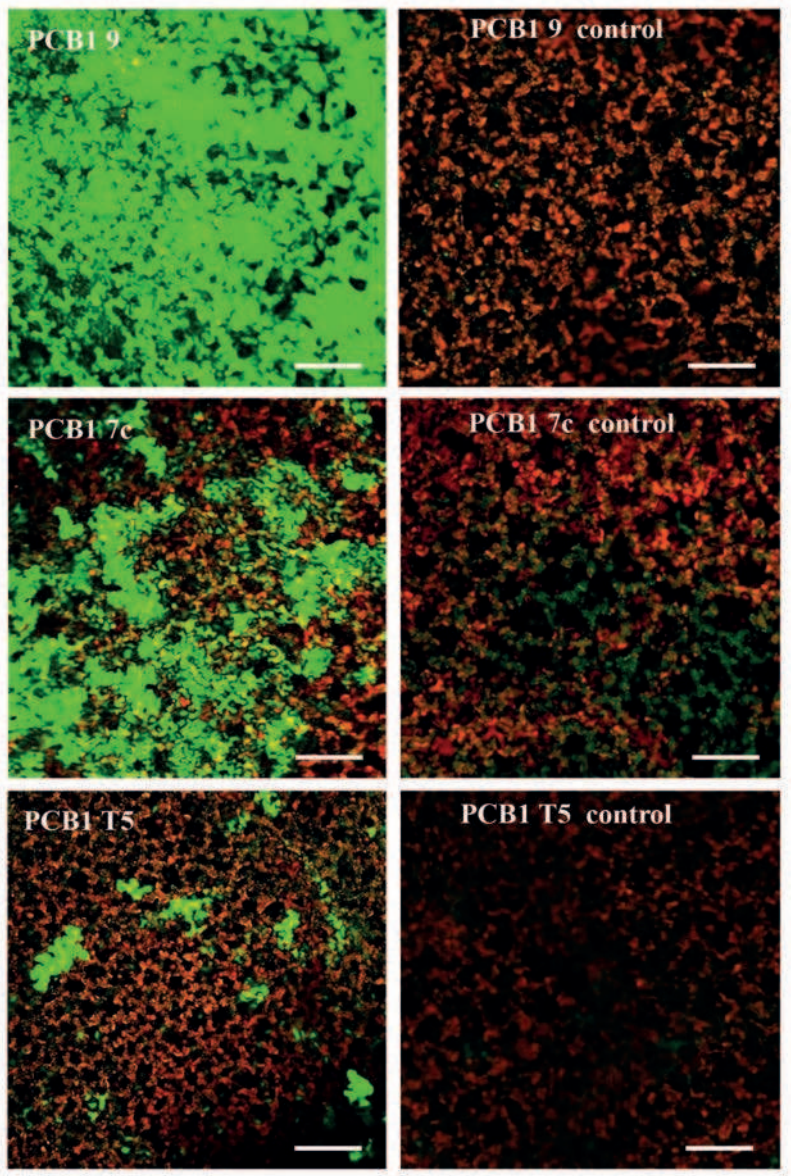

Figure S1. Independent *Arabidopsis pcb1* lines detect *Agrobacterium*-mediated *p35S::GFP11-Cre-dVirF* transfer with different sensitivities. Three plants of *pcb1* lines 9, 7c and T5 were infiltrated with AGL1 carrying the *p35S::GFP11-Cre-dvirF* construct, or with AGL1 carrying the *p35S::GFP*$_{1\text{-}10}$ 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.

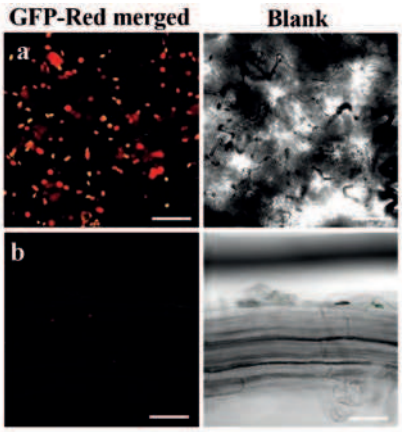

Figure S2. (a) Tobacco and (b) *Arabidopsis* lines GFP_{1-10} leaf epithelial and root cells respectively showed no GFP signal following cocultivation with *Agrobacterium* strain LBA2587 (*virD4* mutant) containing *pvirF::*GFP_{11}*-BBM-dvirF*. Scale bar is 10 μm.

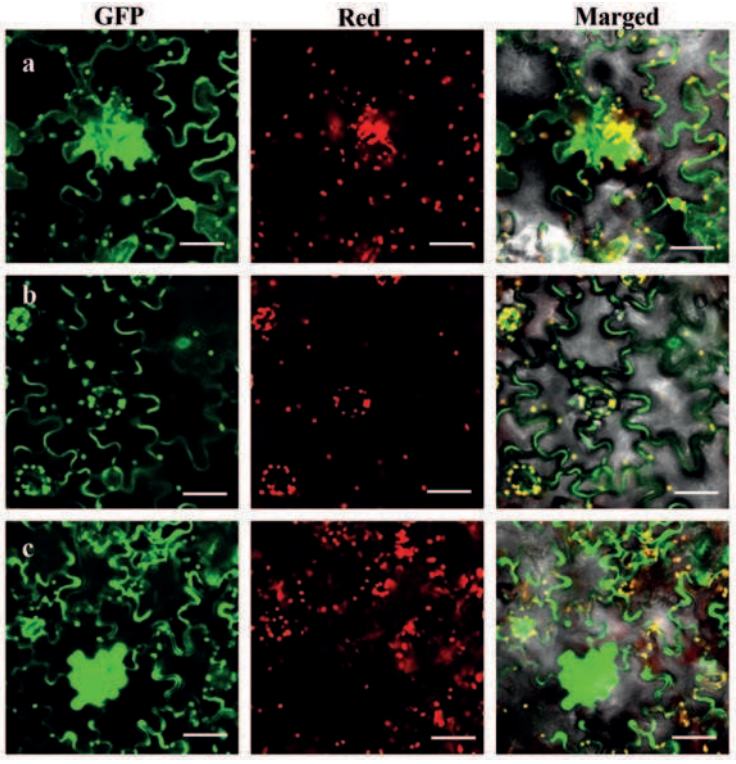

Figure S3. Visualization of *Agrobacterium*-mediated transient expression of *35S::GFP11-Cre-dvirF*, *35S::GFP11-BBM-dvirF* and *35S::GFP11-AHL15-dvirF* and their complementation with GFP11 in *Nicotiana tabacum* GFP1-10 marker line. (a-c) Visualization by confocal microscopy of GFP1-10 complementation 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.

A GENERIC SPLIT-GFP-BASED REPORTER SYSTEM FOR AGROBACTERIUM-MEDIATED PROTEIN TRANSLOCATION IN PLANTS

4

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ABSTRACT

Agrobacterium tumefaciens is well characterized for its ability to transfer DNA to plant and fungal cells, but the fact that it also translocates proteins to its host cells was only revealed more recently. *Agrobacterium*-mediated protein translocation (AMPT) was first detected by restoration of a resistance marker or GFP reporter following translocation of a Cre recombinase-VirF/VirE2 protein fusion. Later the split-GFP system was used to detect translocation of GFP_{11}-Vir fusions to recipient reporter lines overexpressing GFP_{1-10}. Unfortunately, these translocation reporter systems are not easily applicable to *Agrobacterium*-mediated transformation (AMT) resistant and regeneration recalcitrant plants such as sweet pepper and tulip, for which the generation of reporter lines are difficult. Here, we designed a generic split-GFP-based reporter system for AMPT to be used directly in wild-type plants. In this system, the GFP_{1-10} part is transiently expressed from a T-DNA that is co-transferred with a fusion protein comprising the GFP_{11} part and the C-terminal translocation signal of VirF from the same *Agrobacterium* to any desired wild-type recipient cell. This modified generic protein translocation reporter system was successfully tested in a variety of tissues of different plant species, such as *Nicotiana benthamiana*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Capsicum annum* and *Tulipa gesneriana*. The system reported efficient AMPT to these plant species, and also appeared to be useful for optimization of AMT of tulip, and for the visual selection of transgenic tulip shoots.

Keywords: *Agrobacterium tumefaciens*, Protein translocation, Split-GFP, Generic system, Tulip

INTRODUCTION

The soil born gram negative plant pathogenic bacterium *Agrobacterium tumefaciens* is the most common and successful tool for plant transformation (Ziemienowicz, 2014). The capacity of *A. tumefaciens* to transfer DNA to plant cells is determined by an extrachromosomal circular DNA molecule called tumor inducing (Ti) plasmid, which the bacterium normally uses to induce crown gall tumors on its host plants (Larebeke et al., 1974). After the discovery that the crown gall disease was caused by transfer of a copy of a specific region of the Ti plasmid, the transfer or T-region, to the host cells, *A. tumefaciens* became a tool for plant transformation (Ziemienowicz, 2014).

The process of *Agrobacterium*-mediated DNA transfer starts with the induction of genes in the *vir*-region on the Ti plasmid by signaling molecules exuded by wounded plant tissues (Subramoni et al., 2014). Phenolic compounds, such as acetosyringone, are the most potent inducers of *vir* gene expression in wounded plant exudates (Stachel et al., 1985). Other signals, such as temperature, low pH and certain aldose-type monosaccharides, can enhance *vir* gene induction (Melchers et al., 1989). All these signals are perceived by the transmembrane receptor VirA (Melchers et al., 1989), which subsequently activates the transcriptional regulator VirG through a typical bacterial two-component phosphorylation system (Jin et al., 1990). VirG subsequently activates the transcription of the *vir* operons, resulting in the production of 11 VirB proteins that together with VirD4 assemble into the type-4 secretion system (T4SS) translocation pilus (Chandran Darbari and Waksman, 2015). The VirB proteins make up the T-pilus through which T-DNA and Vir proteins are translocated into the host cell, while VirD4 acts as a coupling protein that recognizes DNA and proteins that are to be translocated (Lai and Kado, 2000; Kumar and Das, 2002; Zupan et al., 2007). Among the Vir proteins, VirD1 and VirD2 form a relaxase that introduces a nick in the bottom strand of the Ti plasmid at the position of imperfect border repeats that delineate the T-region (Wang et al., 1987; Vogel and Das, 1992). During this nicking, VirD2 becomes covalently attached to the 5′ end of the single stranded T-DNA where it serves to recruit and guide the T-DNA during translocation by the T4SS to the nucleus of the host cell (Lacroix et al., 2006). By using a Cre recombinase Reporter Assay for Translocation (CRAfT) it was demonstrated that the T4SS system can also mediate transfer of Vir proteins, such as VirE2 and VirF, to the host cells independent of the T-DNA, and that the signal peptide responsible for protein translocation is located in the C-terminal part of these Vir proteins (Vergunst et al., 2000). The function of these translocated Vir proteins is to maintain the integrity of T-DNA inside the host cell and help its integration in the host genome (Lacroix and Citovsky, 2013).

Detection of protein translocation by the CRAfT system involved Cre-Vir fusion protein-mediated excision of a *lox*-flanked (floxed) DNA segment that disrupted a kanamycin resistance selection marker or a *GFP* reporter gene (Vergunst et al., 2000; Vergunst et al., 2005). However, the CRAfT system did not allow to follow translocated proteins in the recipient cells. As GFP-VirF or GFP-VirE2 fusions were not translocated by the *Agrobacterium* T4SS, probably because of the complex structural folding of the GFP protein (Vergunst et al., 2005), the split-GFP system was adapted for visualization of *Agrobacterium*-mediated protein translocation (AMPT) (Li et al., 2014; Sakalis et al., 2014). This split-GFP system was specifically developed to detect protein

translocation by the type III secretion system (T3SS) of *Salmonella enterica* into human cells (Van Engelenburg et al., 2010). Adaptation of this system for the visualization of AMPT to yeast and plant cells involved expression of GFP_{11}-Vir fusion proteins in *Agrobacterium* cells and generation of yeast or plant reporter lines that stably express the complementary GFP part (GFP_{1-10}) in the recipient cells (Li et al., 2014; Sakalis et al., 2014). Upon its translocation to recipient cells, the GFP_{11}-Vir fusion will recreate a functional GFP protein by interacting with the GFP_{1-10} part in the reporter line and thus result in fluorescent signals (Sakalis et al., 2014).

Visualization of AMPT is not only important for fundamental studies on the bacterial protein translocation process itself, but also for application of AMPT to check the efficiency of protein translocation, and whether the protein of interest is correctly localized in the target recipient cells. One drawback of the split-GFP system is that the recipient organism must be transformed *a priori* with a construct that expresses the GFP_{1-10} part. Especially for transformation resistant or regeneration recalcitrant plants this step can be time consuming and difficult. Here we report on the construction of a new generic split-GFP system for visualization of AMPT in wild-type plants. In the new system the GFP_{1-10} part and the GFP_{11} part are delivered into the host cell from the same *Agrobacterium* strain, with GFP_{1-10} expressed from the T-DNA following *Agrobacterium*-mediated transformation (AMT) and GFP_{11} as part of a Vir protein fusion by AMPT. The GFP_{1-10} coding region was modified with an intron sequence (Pang et al., 1996) to prevent GFP_{1-10} expression in *Agrobacterium*. Using this new generic reporter system we successfully visualized AMPT in a variety of tissues from different plant species, among which the recalcitrant crop species *Capsicum annuum* and *Tulipa gesneriana*.

RESULTS

Testing *GFP-intron* versions and *Agrobacterium* strains in tobacco leaf infiltration

As a first step in developing a generic split-GFP system for visualization of AMPT, we verified that the GFP version that was originally used for the split-GFP assays (GFP) was sufficiently bright by comparing it to the previously reported plant-enhanced GFP (pGFPi) version (Pang et al., 1996). The *pGFPi* coding sequence was disrupted by the *intron IV* sequence of the potato *ST-LS1* gene introduced at a splicable position (Pang et al., 1996). Synthetic intron-containing coding regions *GFP(i)* and *pGFP(i)* placed on a T-DNA under control of the *CaMV 35S* promoter were introduced into *A. tumefaciens* strains LBA1100 and AGL1. As expected, the intron-containing reporters *p35S::GFP(i)* and *p35S::pGFP(i)* did not lead to GFP expression in *Agrobacterium* (Fig. S1). When the resulting strains were used to infiltrate intact leaves on *N. benthamiana* plants, there was no obvious difference in the number of plant cells that showed GFP expression or the intensity of the GFP fluorescence (Fig. 1 a and b). As this indicated that both constructs were equally suited as transformation reporter, we continued to use the *GFP(i)* gene construct for our experiments. A comparison of *A. tumefaciens* strains AGL1 *35S::GFP(i)* and LBA1100 *35S::GFP(i)* in *N. benthamiana* and *N. tabacum* leaf infiltration experiments showed that the super virulent AGL1 strain was more efficient in AMT than LBA1100 (Fig. 1c and d). The absence of green fluorescence in leaves infiltrated with the *virD4* mutant strain

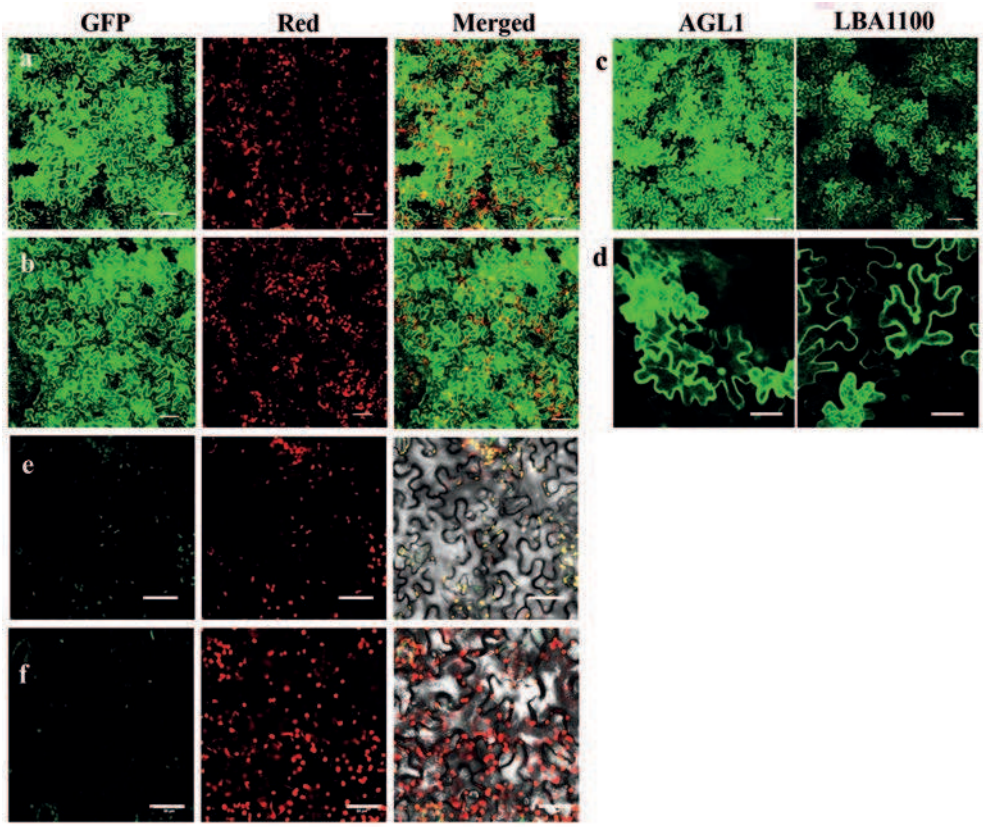

Figure 1. Comparison of AMT efficiencies using different *Agrobacterium* strains and GFP-intron versions in tobacco leaf infiltration experiments. a-f) Representative confocal laser scanning microscopy images of *Nicotiana benthamiana* (a, b, c, e) or *Nicotiana tabacum* (d, f) leaf tissues 3-4 days after infiltration with *Agrobacterium* strains AGL1 *p35S::GFP(i)* (a, c and d left panel), AGL1 *p35S::pGFP(i)* (b), LBA1100 *p35S::GFP(i)* (c and d right panel) or LBA2587 (*virD4* deletion mutant) *p35S::GFP(i)* (e, f). Left, middle and right panel in (a, b, e, f) show GFP channel, red channel and merged image of GFP, red and transmitted light channels, respectively. GFP channel images are shown in (c, d). Scale bar is 0.1 mm.

LBA2587 (Sakalis et al., 2014) containing *35S::GFP(i)* (Fig. 1e and f) showed that the GFP signals observed with the other strains were the result of AMT.

Construction of a generic split-GFP system for visualization of AMPT

After the confirmation that the *35S::GFP(i)* construct was an effective reporter in plant cells, we modified the original split-GFP system (Fig. 2a) (Sakalis et al., 2014) by generating an intron-containing version of GFP_{1-10}. In addition, we fused a nuclear localization signal (NLS) to the N-terminus of the GFP_{1-10} part, as we expected that nuclear accumulation would facilitate the detection of the generally weak fluorescent signals in the nuclei after AMPT. The resulting plasmid *35S::NLS-*GFP_{1-10}*(i)* was introduced into *Agrobacterium* strain AGL1 already containing construct *pvirF::*GFP_{11}*-dvirF*. In this bacterium, expression of the GFP_{11} part fused to the 50

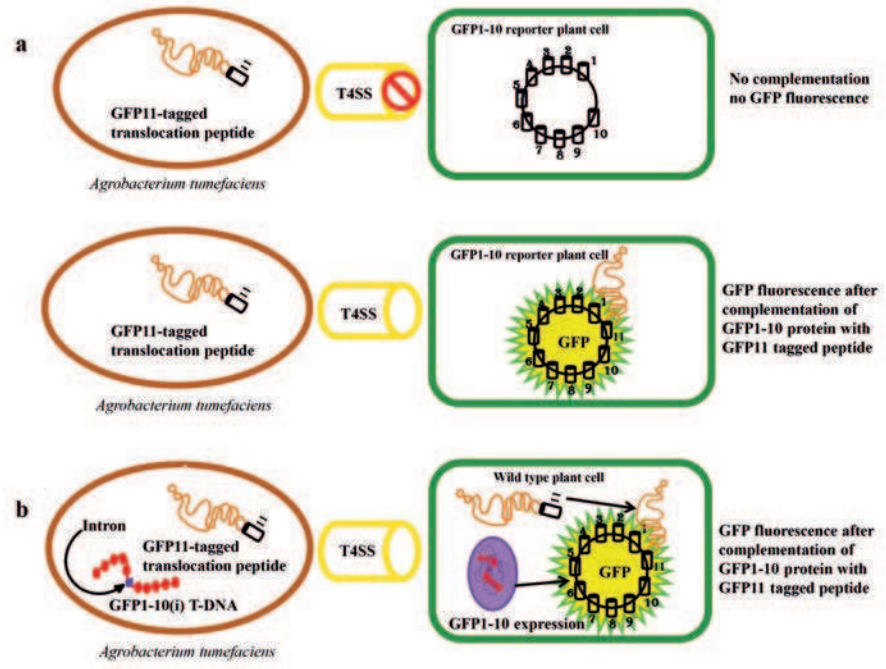

Figure 2. A schematic representation of the original and the generic split-GFP-based reporter system for AMPT in plant cells. a) The original split-GFP system for visualization of AMPT. *Agrobacterium tumefaciens* strains AGL1 *pVirF::GFP*$_{11}$*-dVirF* (lower) and LBA2587 (VirD4 mutant) *pViF::GFP*$_{11}$*-dVirF* (upper) both express a GFP$_{11}$-tagged protein fused with the C-terminal VirF translocation signal. Strain LBA1143 will not translocate the fusion protein because it lacks a functional T4SS, and therefore no GFP$_{1-10}$ complementation and GFP fluorescence will be observed in cells of the GFP$_{1-10}$ reporter plant line (upper). Translocation of the GFP$_{11}$-protein-VirF fusion by the AGL1 strain will result in complementation of GFP$_{1-10}$ in the recipient GFP$_{1-10}$ reporter plant cells that thus become green fluorescent (b). In the new generic split-GFP-based reporter system *Agrobacterium* strain AGL1 containing *pVirF::GFP*$_{11}$*-dVirF* and *35S::GFP*$_{1-10}$*(i)* will translocate both the T-DNA containing the *35S::GFP*$_{1-10}$*(i)* gene (with a splicable intron (i)) and the GFP$_{11}$-tagged fusion protein to cells of wild-type plants. The translocated GFP$_{11}$-tagged fusion protein will complement the partial GFP$_{1-10}$ protein that is expressed from the T-DNA in the plant cell nucleus, thus leading to GFP fluorescence.

C-terminal amino acids of VirF (dVirF) was controlled by the *virF* promoter. Cocultivation with this *Agrobacterium* strain should only result in GFP positive cells if both the *35S::NLS-GFP*$_{1-10}$*(i)* T-DNA and the GFP$_{11}$-dVirF fusion are translocated to the same plant cell (Fig. 2b). This modified split-GFP system was tested by infiltrating leaves of *Nicotiana benthamiana, Nicotiana tabacum* and *Arabidopsis thaliana* (Fig. 3). For all three plant species the generic split-GFP system allowed us to detect successful AMPT to leaf epithelial cells, but surprisingly in *Arabidopsis* no nuclear GFP signals were observed, even in cells expressing the NLS-GFP(i) protein. This suggests that GFP fusions are inhibited or not able to cross the nuclear pore in *Arabidopsis* (Fig. 3d-f). In contrast, simultaneous expression of the NLS-GFP$_{1-10}$ fusion protein and translocation of GFP$_{11}$-dVirF resulted in a strong fluorescent signal in nuclei of the recipient cells in tobacco plants, indicating that NLS-GFP$_{1-10}$ was able to efficiently recruit the complementing

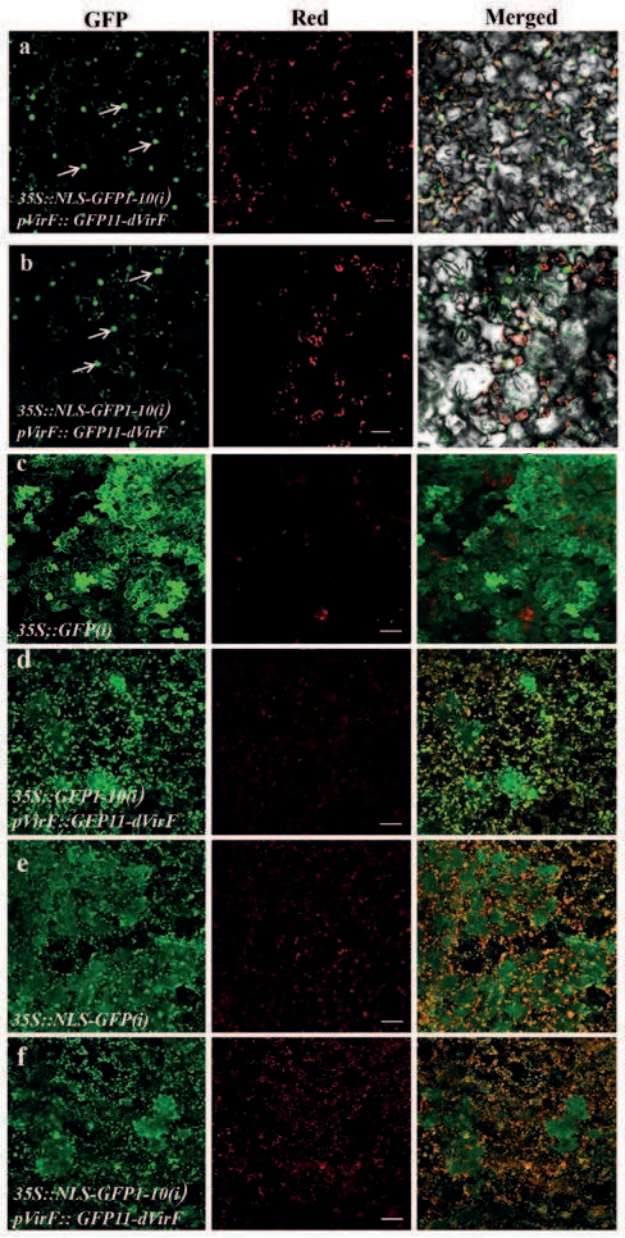

Figure 3. Visualization of AMPT using the new generic split-GFP system in leaves of wild-type tobacco and *Arabidopsis* plants. a,b) Representative confocal laser scanning microscopy images of *Nicotiana benthamiana* (a) and *Nicotiana tabacum* (b) leaf tissues 3-4 days after infiltration with *Agrobacterium* strain AGL1 containing *pVirF::GFP*$_{11}$*-dVirF* and *35S::NLS-GFP*$_{1-10}$*(i)*. c-f) Representative confocal laser scanning microscopy images of *Arabidopsis thaliana* leaf tissues 3-4 days after infiltration with *Agrobacterium* strain AGL1 containing *35S::GFP(i)* (c), *pVirF::GFP*$_{11}$*-dVirF* and *35S::GFP*$_{1-10}$*(i)* (d), *35S::NLS-GFP(i)* (e) and *pVirF::GFP*$_{11}$*-dVirF* and *35S::NLS-GFP*$_{1-10}$*(i)* (f). Left, middle and right panel in (a-f) show the GFP channel, the red channel and a merged image of the GFP, red and transmitted light channels, respectively. Arrows indicate GFP fluorescent nuclei. Scale bar is 0.1 mm.

GFP_{11} part to the nucleus. This nuclear localization enabled us to easily score the number of GFP positive cells and calculate and compare AMPT and AMT efficiencies (see below).

Optimization and efficiency of the new generic split-GFP system

A disadvantage of the new generic split-GFP-based reporter system for AMPT is that both components are located on separate plasmids that are maintained in *Agrobacterium* by antibiotic selection. Since the bacteria are not under antibiotic selection during the infiltration/cocultivation period, plasmids might be lost, leading to a reduced efficiency of T-DNA and/or protein translocation. To simplify the new generic split GFP system and possibly also to enhance the efficiency of this new system, we generated a single plasmid containing both the *p35S::NLS-GFP$_{1-10}$(i)* and the *pvirF::GFP11-dvirF* part. Protein translocation via this 'fused' generic split-GFP system was confirmed through *N. benthamiana* leaf infiltration (Fig. 4c). In the same experiment we also infiltrated *N. benthamiana* leaves with strain AGL1 *35S::NLS-GFP(i)* to monitor AMT efficiency, and with strain AGL1 containing *p35S::NLS-GFP$_{1-10}$(i)* and *pvirF::GFP11-dvirF* to monitor AMPT from the separate system (Fig. 4a,b). Quantification of the percentage of fluorescent recipient cells showed that there was no significant difference between AMPT via the separate or fused generic split-GFP system, while AMT resulted in two-fold more GFP positive nuclei (Fig. 4d). This difference between AMT and AMPT might relate to the fact that in the case of AMPT two components have to be translocated simultaneously to the recipient plant cell. Alternatively, the possibly low number of translocated GFP_{11} proteins might limit the detection of protein translocation in some of the recipient plant cells.

AMPT of potential regeneration-enhancing proteins to *Capsicum annuum*

Capsicum annuum (sweet pepper) is regarded as one of the crop plants that is most recalcitrant to AMT (Kotharet al., 2010). Previously, it was shown that DEX-mediated activation of a BABY BOOM-Glucocorticoid Receptor (BBM-GR) fusion protein enabled the selection of transgenic shoots after AMT by enhancing the shoot regeneration process (Heidmann *et al.*, 2011). A disadvantage of this method is that first a transgenic line with the *35S::BBM-GR* construct has to be generated that can subsequently be used for AMT by BBM-enhanced regeneration of transgenic shoots. Here we tested the possibility of AMPT of BBM and AT-HOOK CONTAINING NUCLEAR PROTEIN-LIKE 15 (AHL15) to tissues of *C. annuum.* In Chapter 3 we showed that both of these proteins enhanced shoot regeneration from tobacco leaf discs. If successful, this would allow the use of AMPT as an alternative method to enhance regeneration and selection of transgenic *C. annuum* plants. Our new generic split-GFP reporter system was used to visualize the translocation of GFP_{11}-BBM-VirF or GFP_{11}-AHL15-VirF fusion proteins to *C. annuum* cells. Cotyledon explants were syringe infiltrated with *Agrobacterium* strain AGL1 containing either the *pvirF::GFP$_{11}$-virF*, *pvirF::GFP$_{11}$-BBM-virF* or *pvirF::GFP$_{11}$-AHL15-virF* construct together with the *35S::NLS-GFP$_{1-10}$(i)* T-DNA construct on another plasmid. Confocal microscopy of the infiltrated cotyledon tissues showed nuclear GFP signals in the epithelial cells for all three *Agrobacterium* strains (Fig. 5), indicating successful AMPT. Because the background fluorescence was quite high, it was difficult to determine the efficiency of AMPT in *C. annuum*. Although we did not score for an immediate positive effect on shoot

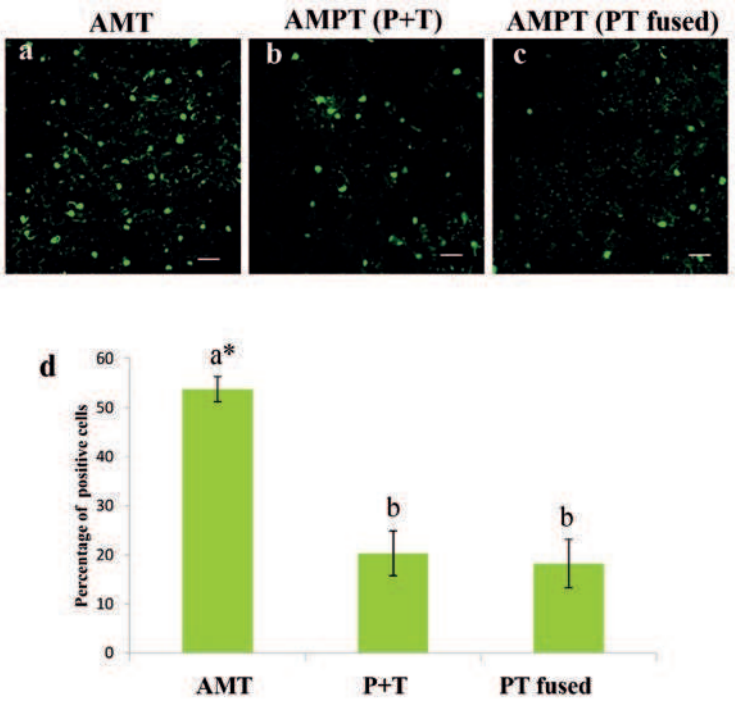

Figure 4. The efficiency of AMT in infiltrated tobacco leaves is two-fold higher compared to AMPT. a-c) Representative confocal laser scanning microscopy images of wild-type *Nicotiana benthamiana* leaves three days after infiltration with *Agrobacterium* strain AGL1 containing *35S::NLS-GFP(i)* for detection of AMT (a), or AGL1 *35S::GFP*$_{1\text{-}10}$*(i)* with *pVirF::GFP*$_{11}$*-dVirF* (P+T, b)), or AGL1 *35S::GFP*$_{1\text{-}10}$*(i)-pVirF::GFP*$_{11}$*-dVirF* (PT fused, c) for detection of AMPT. Scale bar is 0.1mm. d) Graph showing the AMT or AMPT (P+T or PT fused) efficiencies as percentage of positive cells. The efficiency was calculated based on the number of GFP-positive nuclei over the total number of nuclei in a single image. Bars represent average percentages determined from confocal images from three different parts of three leaves (n =9) of an infiltrated plant. Error bars depict the SEM. Significantly different values are differently labeled with a and b (Post ANOVA Tukey's test, $p<0.05$).

regeneration in our experiments, these results show that our new generic split-GFP-based reporter system can be used in crop plants to optimize the conditions for enhanced selection and regeneration of transgenic plants by combined AMPT and T-DNA delivery.

Successful protein translocation and T-DNA transfer to tulip cells

The AMT of monocot plant species is generally more difficult than that of dicot plant species. Different methods other than *A. tumefaciens* transformation can be used, such as the gene gun, but generally these result in lower transformation efficiencies (Barampuram and Zhang, 2011). After confirmation and successful application of the GFP(i)-based generic split-GFP AMPT reporter system in tobacco and *C. annuum*, it was also applied on one of the most recalcitrant monocot species *Tulipa gesneriana* (tulip). DNA transformation to tulip has previously been reported, but with low efficiency (Wilmink et al., 1992). Based on these results, we expected to see only low AMPT efficiencies in tulip using the generic split-GFP reporter system. To our

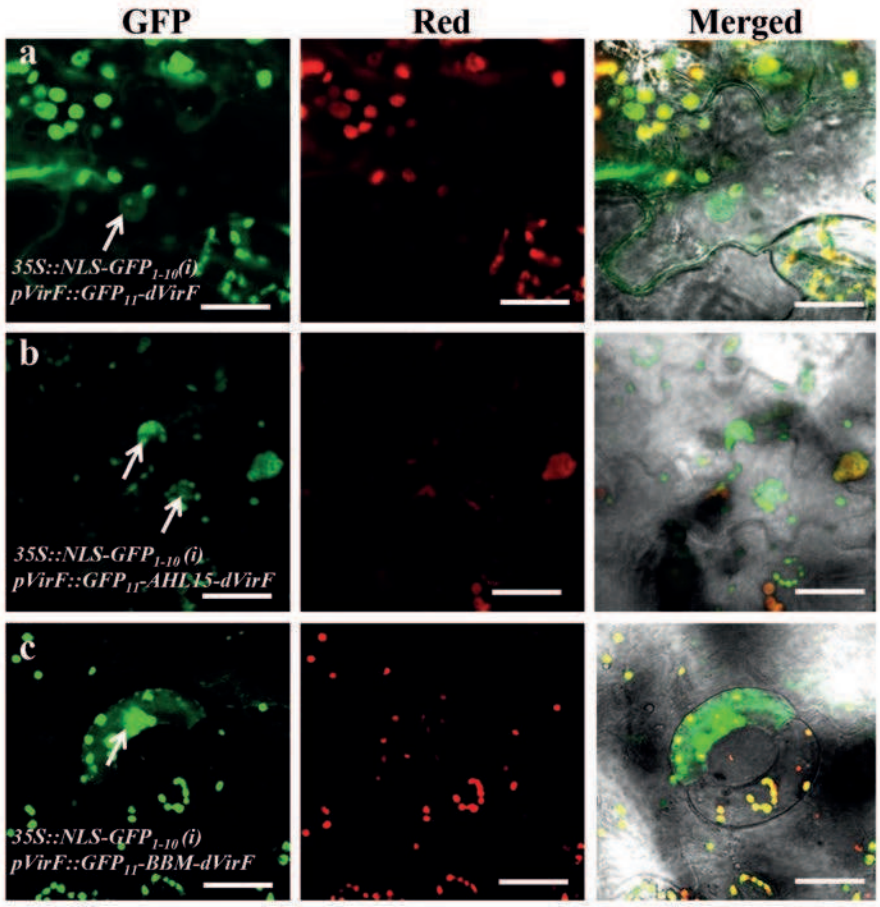

Figure 5. Visualization of AMPT of plant developmental regulators to *Capsicum annuum* cotyledon cells using the generic split-GFP system. a-c) Syringe infiltrated cotyledons of *Capsicum annuum* visualized under a confocal microscope after 3 days of cocultivation with *Agrobacterium* strain AGL1 containing *35S::NLS-GFP$_{1-10}$(i)* along with either construct *pVirF::GFP$_{11}$-dVirF* (a), *pVirF::GFP$_{11}$-AHL15-dVirF* (b) or *pVirF::GFP$_{11}$-BBM-dVirF* (c). GFP fluorescent nuclei of the recipient cells are indicated by arrows. Left panel shows the GFP channel, middle panel shows the autofluorescence in the red channel, and right panel shows the merged image of the green, red and transmitted light channels. Scale bare is 30μm.

surprise, however, nuclear GFP fluorescence was observed at an unexpectedly high efficiency either after AMT or after AMPT, especially in vertically cut thin layer sections of regenerated shoot explants (Fig. 6a,b). These results of T-DNA and protein translocation to tulip cells stimulated us to investigate possibilities to optimize the protocol for genetic modification of this economically important plant species.

As there is no standard protocol available yet for the *Agrobacterium*-mediated transformation of tulip, we performed a time-lapse experiment where the optimum duration of co-cultivation was investigated by multiple observations using confocal laser scanning microscopy. *In vitro* tulip tissues were co-cultivated for up to 9 days with the *Agrobacterium* strain AGL1 containing

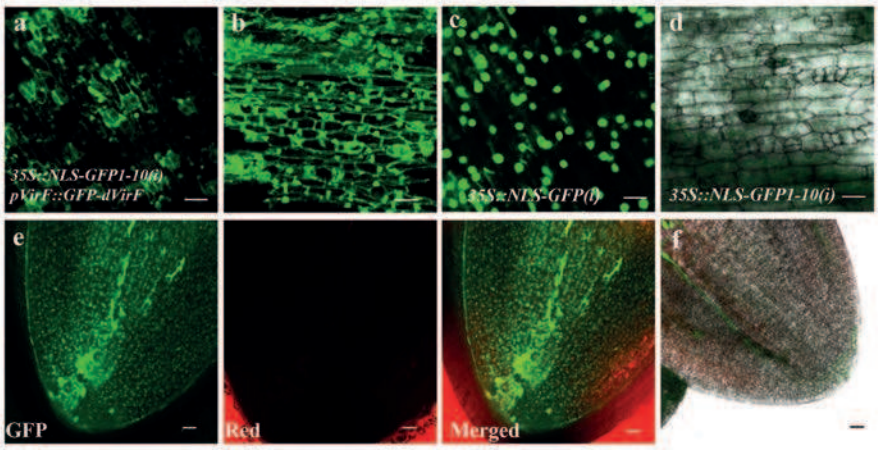

Figure 6. Visualization of AMPT and stable AMT in tulip cells. a,b) Representative confocal laser scanning microscopy images of transverse (a) or vertical (b) sections of tulip explants after 7 days of cocultivation with *Agrobacterium* strain AGL1 containing *35S::GFP*$_{1-10}$*(i)* and *pVirF::GFP*$_{11}$*-dVirF* to detect AMPT. The transverse section shows GFP fluorescence in nuclei of the cells and also in stomatal cells (a), while in the vertical section most of the nuclei are brightly fluorescent along with cytosolic signals (b). c,d) Confocal laser scanning microscopy images of transverse sections of tulip explants after 7 days of cocultivation with *Agrobacterium* strain AGL1 *35S::NLS-GFP(i)* to detect AMT (c) or with AGL1 *35S::NLS-GFP*$_{1-10}$*(i)* as negative control (d). e,f) Representative confocal laser scanning microscopy images of regenerated tulip shoots 4-5 weeks after cocultivation with AGL1 *35S::NLS-GFP(i)* (e), or with negative control strain AGL1 *35S::NLS-GFP*$_{1-10}$*(i)* (f). (Left, middle and right panels in (e) show the GFP channel, the red channel, and a merged image of the GFP and red channel while pannel (f) shows a merged image of GFP, red and transmitted light channels, respectively). Scale bar in all images is 0.1mm.

35S::NLS-GFP$_{1-10}$*(i)* and *pvirF::GFP*$_{11}$*-dvirF*. The tulip tissues were observed at different time points (5, 7 and 9 days). The results showed increased GFP fluorescence after 7 days of co-cultivation (Fig. S2). Extending the co-cultivation period up to 9 days resulted in overgrowth of bacteria and in tissue necrosis accompanied by high autofluorescence, which interfered with observation of GFP signals. The optimized conditions of 7 days co-cultivation of tulip explants on hormone free MS medium with 40mg/L acetosyringone, resulted in enhanced protein translocation, whereas longer co-cultivation damaged the explant tissues as a result of the bacterial overgrowth. Strikingly, the use of growth hormone containing medium with acetosyringone during co-cultivation prevented this overgrowth of *Agrobacteria* for up to two weeks, but resulted in reduced AMPT efficiencies, most likely because the proliferating cells on hormone medium produced antibacterial compounds that prevented *Agrobacterium* growth.

In the time lapse experiments we also infiltrated tulip tissues with *A. tumefaciens* containing *35S::NLS-GFP(i)* for T-DNA transfer as positive control. After 7 days of co-cultivation, some of the tissues were transferred to hormone containing medium without selection. Some of the regenerated shoots obtained after 4-5 weeks on this medium showed nuclear GFP signals in all cells (Fig. 6e), suggesting stable integration and expression of *35S::NLS-GFP(i)*. DAPI staining confirmed that the observed GFP signals were located in the nuclei of tulip cells (Fig. S3). Although, the regeneration efficiency of tulip varies per explant, these experiments

showed that efficient AMPT and AMT can be achieved in tulip by using the *35S::NLS-GFP(i)* construct as a reporter for stably transformed regenerating shoots. These first results pave the way for the establishment of a stable AMT system for tulip.

DISCUSSION

Previous studies have shown that the most acceptable tool for generating transgenic plants, *Agrobacterium tunefaciens*, can also be used to translocate desired proteins into plant or yeast cells (Vergunst et al., 2000; Schrammeijer et al., 2003). Two reporter systems have been developed to study and optimize this AMPT, but until now these methods relied on the construction of stably transformed reporter lines, either containing a marker gene disrupted by a floxed insert to detect translocation of the Cre recombinase, or a split-GFP-based system where a stable line is generated that expresses the non-fluorescent GFP_{1-10} to detect translocation of the complementing GFP_{11} part (Li et al., 2014; Sakalis et al., 2014). The advantage of the latter system is that it allows to follow the translocated protein in the recipient cells to its predominant final localization (Li et al., 2014; Sakalis et al., 2014). Here we generated a generic split-GFP-based reporter system for direct visualization of AMPT in wild-type recipient cells. The advantage of this generic split-GFP system is that both the GFP_{1-10} and the GFP_{11} parts are transferred by the same *Agrobacterium* strain, thereby circumventing the time consuming step to generate a GFP_{1-10} expressing reporter line, but also to make the method applicable to transformation resistant plants species. This new generic split GFP system has been successfully used to show AMPT to cells of tobacco and *Arabidopsis*, and to the transformation recalcitrant crops sweet pepper and tulip.

Using the original version of split-GFP, most of the observed GFP signals localized to the cytosol of the plant cells (Sakalis *et al.*, 2014; Chapter 3). The cytosolic signals were generally weak and difficult to score because they were hard to distinguish from background fluorescence. To avoid this problem, a nuclear localization signal (NLS) was added to the GFP_{1-10} moiety. Using this, we could show that AMPT is only 2-fold less efficient than AMT, which is still surprisingly high in view of previously published efficiencies (Sakalis et al., 2014). One has to keep in mind, however, that the observed AMPT efficiency is likely to be an underestimation, since detection of AMPT with the generic system requires translocation of both T-DNA and protein and might be limited by the number of GFP_{11}-dVirF fusion proteins translocated. Having the *35S::NLS-GFP_{1-10}(i)* and *pvirF::GFP_{11}-dvirF* parts at separate or at one construct did not change the AMPT efficiency. However, in some tissue explants we even obtained more GFP positive cells with AMPT than with AMT, suggesting that infiltration handling, leaf tissue damage and tissue type also considerably contribute to the AMPT efficiency, and that in fact the AMT and AMPT efficiencies do not differ that much.

Application of our new generic split-GFP-based AMPT reporter system to the AMT resistant crops sweet pepper and tulip showed that both crop species are not resistant to AMT or AMPT, but rather that efficient regeneration of the transformed cells into transgenic plants is problematic. For sweet pepper this was already reported previously (Heidmann et al., 2011), but for tulip the high AMT and AMPT efficiencies came as a surprise. For sweet pepper, usually cotyledon explants are used for AMT (Heidmann et al., 2011), and by applying our syringe infilteration

technique on these tissues, we observed many cells with GFP signals in both the nucleus and cytosol. However, due to the sensitivity of the sweet pepper tissues to *Agrobacterium*-infiltration induced necrosis, the high intensity of autoflourescence made it also difficult to detect GFP signals. Our results showed that it is possible to introduce regulatory proteins, such as BBM and AHL15, to sweet pepper cells via AMPT, and besides the enhancement of regeneration, as has been shown for BMM in sweet pepper (Heidmann et al., 2011), this method could be used to translocate proteins that reduce the tissue necrosis reaction.

Tulip inflorescence stem explants harvested from bulbs appeared to be relatively insensitive to cocultivation with *Agrobacterium*, and leaving the co-cultivation at hormone free medium for an extended period of 7 days led to high AMT and AMPT efficiencies. Subsequent transfer to hormone-containing medium not only started the regeneration, but at the same time induced the production of some anti-bacterial activity that prevented tissue overgrowth by *Agrobacterium*. This has been reported previously for *Centella asiatica* (Bibi et al., 2011). Explants co-cultivated with the AGL1 *35S::NLS-GFP(i)* strain produced homogenous GFP positive shoots after 4 to 5 weeks on hormone medium, suggesting that it is possible to obtain stably transformed tulip plants without selection, purely based on GFP visualization. Our results indicate that this new generic split-GFP system can not only be used to report AMPT, but also to optimize the co-cultivation and tissue culture conditions to efficiently generate and obtain mutant or transgenic lines from plant species that are currently considered to be recalcitrant to AMT or AMPT.

MATERIAL AND METHODS

Bacterial strains and media

Agrobacterium tumefaciens strains are listed in Table 1. All 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, (rif, 20 µg/ml), spectinomycin, (spc, 250 µg/ml), carbenicillin, (cb, 75µg/ml) gentamicin (gent, 40 µg/ml) and kanamycin (km, 100 µg/ml). Ten ml cultures were inoculated with a single colony and incubated under continuous shaking (180 rpm) in 200 ml flasks for two days in an incubator at 30˚C. 10-20 ng of plasmids DNA was electroporated into 50 µl electrocompetent cells (Den Dulk-Ras and Hooykaas, 1995; McCormac et al., 1998) of *A. tumefaciens* strains LBA1100 (Beijersbergen et al., 1992) and AGL1 (Lazo et al., 1991) using prechilled cuvettes and by applying electric pulse at 12.5 kv/cm with a constant time of approximately 4.7 msec (Mersereau et al., 1990). For cloning *Escherichia coli* strain DH5α was used and grown at 37°C in LC medium containing (if required) 100 µg/ml cb, 25 µg/ml gent, and 25 µg/ml km.

Plant material

The plant lines used in this study were: *Nicotiana tabacum* streptomycin resistance-1 (SR-1), *Nicotiana benthamiana, Arabidopsis thaliana* Col-0, *Capsicum annuum* (Bruinsma wonder and Fire flame) and tulip cultivar 'Strong Gold'. *Arabidopsis* seedlings and tulip bulb explants were cultured at 21°C, 50% relative humidity and at 16 hours photoperiod. Nicotiana and Capsicum plants were grown at 25°C, 50% relative humidity and 16 hours photoperiod.

Table 1. List of *Agrobacterium* strains used in this study for *Agrobacterium* infiltration.

Strains	Chromosomal background	Antibiotic resistance	Source
AGL1	C58	rif cb	Jin et al., 1987
LBA1100	C58	rif spc	Beijersbergen et al., 1992
LBA2587(*ΔvirD4*)	C58	rif spc	(Sakalis et al., 2014)

rif: rifampicin; spc: spectinomycin; cb: carbenicillin; Δ: deletion.

Agro-infiltration and co-cultivation

Agrobacterium strains were grown in LC medium as described above and subsequently diluted in AB-sucrose minimal medium containing appropriate antibiotics and grown for overnight at 30°C. Bacterial cultures were induced at gentle rotation (50rpm) and room temperature for 14-24 hours by adding 3,5-dimethoxy-4-hydroxy-acetophenone (acetosyringone AS) [Sigma Aldrich] dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100μM, as described (Gelvin et al ,2006). Fifteen days old *Arabidopsis* seedlings were infiltrated with the bacterial culture (OD_{600} 0.6) using vacuum infiltration (Rossi *et al.*, 1993) or bacterial cultures were directly injected (Wroblewski et al., 2005) into leaves using a blunt tipped plastic 10 ml syringe (Nissho NIPRO Europe N.V., Zaventem, Belgium). *N. tabacum* and *N. benthamiana* non-sterile leaves of 3-4 weeks old soil grown plants were syringe infiltrated with bacterial cultures of OD_{600} 0.6-0.8. *N. tabacum* surface sterilized leaf discs (Baltes et al., 2014) and *C. annuum* cotyledons from 2 weeks old *in vitro* grown seedlings were also syringe infiltrated (Fig. S4) with bacterial cultures of OD_{600} 0.3-0.4, in the sterile environment of a down flow laminar flow hood and cultivated for 3 days on 40mg/L AS containing MS medium. The tulip tissues were inoculated with AS-induced bacterial culture of OD_{600} 0.6 for overnight in the dark and, after wiping excess bacteria with sterile tissue paper, co-cultivated for up to 9 days on 40mg/L AS containing hormone free MS medium at 21°C, 50% relative humidity.

Leaf samples of *N. tabacum, N. benthamiana* and *A. thaliana* were analyzed 3-4 days after infiltration while tulip samples were analyzed at day 5, day 7 and day 9 of co-cultivation period. For DAPI staining samples were incubated for one hour in 1 mg/l DAPI solution.

Tulip transformation

The sterile tulip bulb (Podwyszyńska and Sochacki, 2010) stem explants (10-12 mm thick), pre-cultured for 3-4 weeks on MS medium supplemented with 1mg/l thidiazuron (TDZ) and 1mg/l 1-naphthalene acetic acid (NAA), were cut into 2-3 mm thin layer explants. After bacterial inoculation and 7 days of co-cultivation (as described above) on hormone free MS medium the explants were transferred back to the pre-culture medium for shoot induction. After 4-5 weeks the regenerated shoots were analyzed for transformation.

Microscopy

All fluorescent microscopy was performed using a Zeiss Imager M1 or a Zeiss observer confocal laser scanning microscopy (CLSM) both equipped with an LSM 5 Exciter scanning module

(Zeiss, Oberkochen, Germany) and 10x, 20x and 40x objectives (numerical aperture 1, 0.8 and 0.65, respectively). GFP signals were detected using an argon 488 nm laser and a 505-530 nm band pass (BP) emission filter. Chloroplast- and other auto-fluorescence were detected using a 650 nm long pass (LP) emission filter following excitation at 488 nm. DAPI was excited using a 405 nm diode laser and emission detected using a 420-480 nm (blue) BP filter.

Plasmid construction

All plasmids used in this study are listed in Table 2. For the T-DNA transfer a modified version of the plasmid pSDM3764 was used which harbors a GFP_{1-10} sequence under control of the *35S CaMV* promoter and the *35S CaMV* terminator (Sakalis et al., 2014). For the intron splicing validation and GFP sequence fluorescence comparison, synthetic fragments (Eurofin) of the full length original GFP (*GFP(i)*) and a plant enhanced full length GFP (*pGFP(i)*) (Appendix 1) coding sequences, containing a splicable 84 nucleotide *intron IV* sequence of the potato *ST-LS1* gene (Pang et al., 1996), were ligated into the *Bst*EII and *Nco*I digested pSDM3764 vector backbone, thereby replacing the GFP_{1-10} sequence (Cabantous et al., 2005) and generating *p35S::GFP(i)* (pSDM6506) and *p35S::pGFP(i)* (pSDM6505). For the new generic split-GFP system, synthetic fragments (Eurofin) containing the GFP_{1-10}*(i)* sequence harboring the intron with and without *NLS* (Appendix 2) were cloned into *Bst*EII and *Nco*I digested pSDM3764, thereby generating *p35S::NLS-*GFP_{1-10}*(i)* (pSDM6509) and *p35S::*GFP_{1-10}*(i)* (pSDM6508). For the translocation of fusion proteins a modified version of the plasmid pSDM3760 (Sakalis et al., 2014) was used, containing the GFP_{11} coding region under the *virF* promoter and fused to the N-terminal part of *dvirF* (Chapter 3). Additionally, for AMPT of plant developmental regulators AHL15 and BBM, *AHL15* and *BBM* coding regions were cloned as translational fusions between GFP_{11} and *dvirF* (Table 2). All cloning steps were performed in *E.coli* strain DH5α (Bethesda Research Laboratories, 1986). Ligations were checked by restriction enzyme digestion and confirmed by PCR analysis using 1μg plasmid DNA and 0.3μl dream taq polymerase (for primers see Table 3). All constructed plasmids were sequenced for verification (Macrogen, Amsterdam, Netherlands).

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Table 2. List of binary vectors used for *Agrobacterium* infiltration.

Name	Properties	Source
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).	Chapter 3
pSDM6503[*pvirF::GFP$_{11}$-AHL15- ΔvirF*]	pSDM3760 backbone with the *GFP$_{11}$-AHL15-ΔvirF* coding sequence under control of the *virF* promoter	Chapter 3
pSDM6504[*pvirF::GFP$_{11}$-BBM- ΔvirF*]	pSDM3760 backbone with the *GFP$_{11}$-BBM-ΔvirF* coding sequence under control of the *virF* promoter	Chapter 3
pSDM3764[*35S::GFP$_{1-10}$*]	pCambia1302 with *GFP$_{1-10}$* coding sequence under control of the *35S* promoter and the *CaMV* terminator.	(Sakalis et al., 2014)
pSDM6506[*35S::GFP(i)*]	pCambia1302 with *GFP(i)* coding sequence under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6507[*35S::NLS-GFP(i)*]	pCambia1302 with *NLS-GFP(i)* coding sequence under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6505[*35S::pGFP(i)*]	pCambia1302 with the *pGFP $_{1-11}$(i)* coding region under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6508[*35S::GFP$_{1-10}$(i)*]	pCambia1302 with the *GFP$_{1-10}$(i)* coding region under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6509[*35S::NLS-GFP$_{1-10}$(i)*]	pCambia1302 with *NLS-GFP$_{1-10}$(i)* coding region under control of the *35S* promoter and the *CaMV* terminator.	This study
pSDM6514 [*35S::NLS-GFP$_{1-10}$(i)-pvirF::GFP$_{11}$-L2- ΔvirF*]	pCambia1302 with *NLS-GFP$_{1-10}$(i)* coding region under control of the 35S promoter and the CaMV terminator and pSDM3760 backbone with *GFP$_{11}$-L2-ΔvirF* coding sequence under control of the *virF* promoter. (*L2* is the linker sequence having multiple unique restriction sites).	This study
pEX-A2[*GFP$_{1-10}$(i)*]	*GFP$_{1-10}$(i)* coding sequence	Eurofins
pEX-A2[*NLS-GFP$_{1-10}$(i)*]	*NLS-GFP$_{1-10}$(i)* coding sequence	Eurofins

Table 3. List of primers used in this study

Primer name	Sequence (5' → 3')
XbaI-GFP1-10 Fw	GCTCTAGAATGGTTTCGAAAGGCGA
XbaI-GFP1-10 Rv	CCCTCGAGTTATTTCTCGTTTGGGT
NcoI-GFP_{1-10}-Fw	GCCCATGGTTTCGAAAGGCGAGGA
BstEII-GFP_{1-10}-Rev	GGGTCACCTTATTTCTCGTTTGGGTCTT
Plant GFP Fw	CGAGAATATTCGGATCCCATGGGCAA
Plant GFP Rev	TGAATTCGCTGCAGGTCACCTCACTT
pEX-A2-Fw	GGAGCAGACAAGCCCGTCAGG
pEX-A2-Rev	GCCGGAAGCATAAAGTGTAAAGCCTG
FW GFP1-11nls	TCATTTGGAGAGAACACGGGGG
Rev GFP1-11nls	GGAAATTCGAGCTGGTCACCTTA
35Sgfp11-dVirF-Fr	TCATTTGGAGAGAACACGGG
35Sgfp11-dVirF-Rv	TAATCATCGCAAGACCGGCA

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SUPPLEMENTARY FIGURES

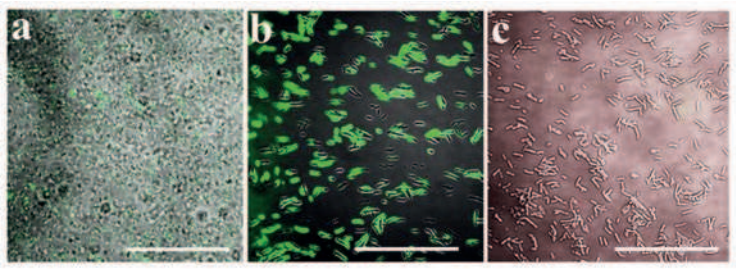

Figure S1. An intron in the *GFP*$_{1-10}$ coding sequence effectively prevents bacterial GFP expression in the generic split-GFP system. a,b) Confocal images of *Agrobacterium tumefaciens* strain AGL1 containing *p35S::GFP*$_{1-10}$ and *pVirF::GFP*$_{11}$*-dVirF* after overnight induction with acetosyringone (a) and after co-cultivation with *N. tabacum* leaf discs (b). c) Confocal images of *Agrobacterium tumefaciens* strain AGL1 containing *p35S::GFP*$_{1-10}$*(i)* and *pVirF::GFP*$_{11}$*-dVirF* where the *GFP*$_{1-10}$ coding region is disrupted by an intron *(i)*. Shown are merged images of the GFP channel and the transmitted light channel. Scale bar is 10 µm.

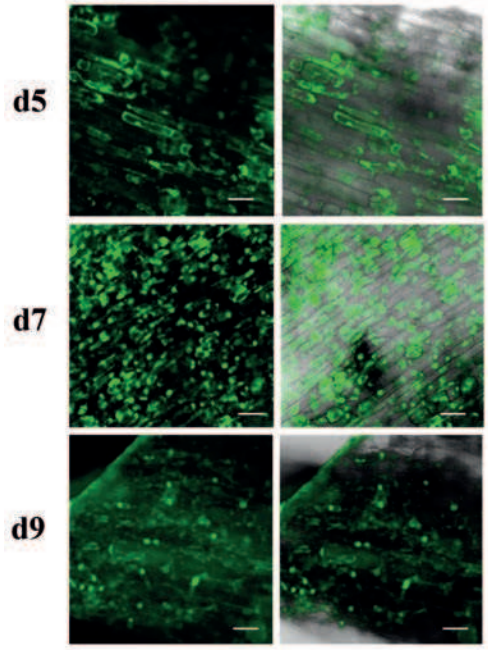

Figure S2. AMPT to tulip cells peaks after 7 days of cocultivation with *Agrobacterium*. a-c) Confocal images of *in vitro* regenerated shoot tissues (from tulip bulb stem explants) cocultivated with *Agrobacterium tumefaciens* strain AGL1 containing *p35S::GFP*$_{1-10}$*(i)* and *pVirF::GFP*$_{11}$*-dVirF* and cocultivated for 5 (a), 7 (b) or 9 days (c) on hormone free medium. Left panel shows the GFP channel, right panel shows a merged image of the GFP channel and the transmitted light channel. Scale bar is 0.1 µm.

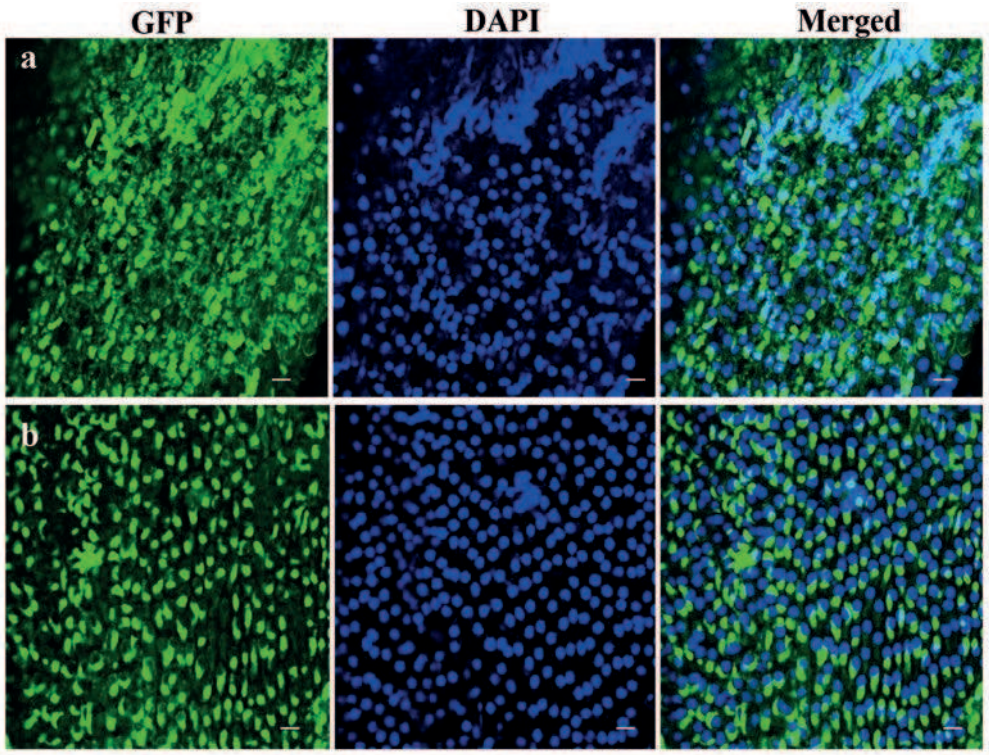

Figure S3. DAPI staining confirms nuclear GFP fluorescence in tulip cells after AMPT or AMT. a,b) Confocal laser scanning microscopy images of DAPI stained tulip tissues shown in figure 5 after cocultivation for 7 days with *Agrobacterium* strain AGL1 containing *p35S::GFP*$_{1\text{-}10}$*(i)* and *pVirF::GFP*$_{11}$*-dVirF* (a) or with strain AGL1 *35S::NLS-GFP(i)* and subsequent regeneration for 4-5 weeks (b). Left panel shows GFP channel, middle panel shows DAPI stained nuclei in blue channel, and the right panel shows a merged image of the GFP and DAPI channels. Scale bar is 0.1mm.

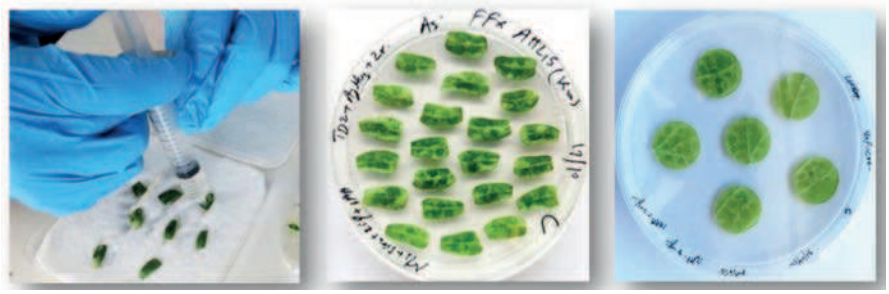

Figure S4. Syringe infiltration method of sterile *Capsicum annum* cotyledons (left and middle panel) or *Nicotiana tabacum* leaf discs (right panel).

Appendix 1. a,b) DNA sequences of the original GFP (*GFP(i)*) (a) and the plant-optimized GFP (*pGFP(i)*) (b) showing the inserted intron (i) sequence in red. c) Alignment of *GFP(i)* sequence with *pGFP(i)* sequence shows intron position and DNA sequence differences.

a). Full length $GFP_{1\text{-}10+11}$(i) (GFP(i)); intron sequence is RED and underlined

ATGGTTTCGAAAGGCGAGGAGCTGTTCACAGGCGTGGTGCCAATCCTGGTGGAGCT
GGACGGCGACGTGAACGGCCACAAATTCAGCGTGAGAGGCGAGGGCGAGGG
CGACGCCACAATCGGCAAACTGACACTGAAATTCATCTGCACAACAGGCAAACTGC
CAGTGCCCTGGCCAACACTAGTGACAACACTGACATACGGCGTGCAGTGCTTCAG
CAGATATCCGGACCACATGAAAAGACACGACTTCTTCAAAAGCGCCATGCCAGAG
GGCTACGTGCAGGAGAGAACAATCAGCTTCAAAGACGACGGCAAATACAAAA
CAAGAGCCGTGGTGAAATTCGAGGGCGACACACTGGTGAACAGAATCGAGCT
GAAG**GTATGACAATTTACTCGAACTTCCTTTTTTAACTCGAACTATGTATATACA**
CAACAACGTTAATAATTAAGTCGTACTCATTTTGAATCTACTGACTCTAGATCCT
GATTCACACATGTAATATAATTGCAGGGCACAGACTTCAAAGAGGACGGCAA
CATCCTGGGCCACAAACTGGAGTACAACTTCAACAGCCACAACGTGTACATCA
CAGCCAACAAACAGAAAAACGGCATCAAAGCCAACTTCACAGTGAGACACAACGT
GGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACACCAATCGG
CGACGGCCCAGTGCTGCTGCCAGACAACCACTACCTGAGCACACAGACAGTGCT
GAGCAAAGACCCAAACGAGAAACGGGACCACATGGTGCTGCACGAGTACGT
GAACGCCGCCGGCATCACATAA

b). Full length plant enhanced $pGFP_{1\text{-}11}$ (pGFP(i)) ; intron sequence is RED and underlined

ATGGGCAAGGGCGAGGAACTGTTCACTGGCGTGGTCCCAATCCTGGTGGAACTG
GATGGTGATGTGAACGGGCACAAGTTCTCCGTCAGCGGAGAGGGTGAA
GGTGATGCCACCTACGGAAAGCTCACCCTGAAGTTCATCTGCACTACCG
GAAAGCTCCCTGTTCCGTGGCCAACCCTCGTCACCACTTTCACCTACGGTGT
TCAGTGCTTCTCCCGGTACCCAGATCACATGAAGCAGCATGACTTCTTCAAGAG
CGCCATGCCCGAAGGCTACGTGCAAGAAAGGACTATCTTCTTCAAGGATGACGG
GAACTACAAGACACGTGCCGAAGTCAAGTTCGAAGGTGATACCCTGGTGAACCG
CATCGAGCTGAAAG**GTATGACAATTTACTCGAACTTCCTTTTTTAACTCGAAC**
TATGTATATACACAACAACGTTAATAATTAAGTCGTACTCATTTTGAATCTACT
GACTCTAGATCCTGATTCACACATGTAATATAATTGCAGGCATCGATTTCAAG
GAAGATGGAAACATCCTCGGACACAAGCTGGAGTACAACTACAACTCCCACAACG
TATACATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGAT
CAGGCACAACATCGAAGATGGAAGCGTGCAACTGGCGGACCACTACCAGCAGAA
CACGCCCATCGGCGATGGCCCTGTCCTGCTGCCGGACAACCATTACCTGTCCACG
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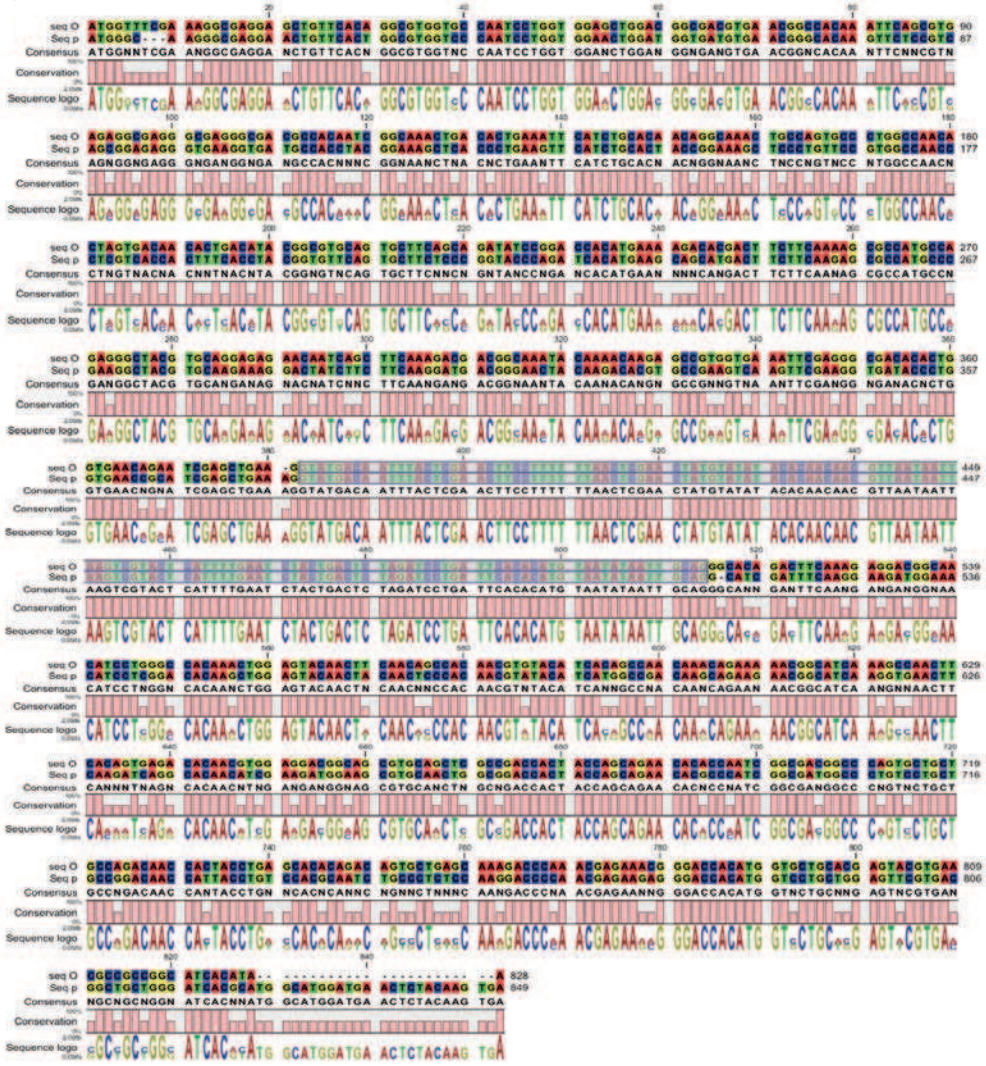
GAGTTCGTGACGGCTGCTGGGATCACGCATGGCATGGATGAACTCTACAAGTGA

c).



Appendix 2. a,b) DNA sequences of *GFP$_{1\text{-}10}$(i)* (a) and *NLS-GFP$_{1\text{-}10}$(i)* (b) showing inserted intron in red and the nuclear localization signal (NLS) in purple. c) Alignment of *GFP$_{1\text{-}10}$(i)* with *NLS-GFP$_{1\text{-}10}$(i)* sequence shows intron and NLS positions and DNA sequence differences.

a). GFP$_{1\text{-}10}$(i); intron sequence is RED and underlined.

ATGGTTTCGAAAGGCGAGGAGCTGTTCACAGGCGTGGTGCCAATCCTGGTGGAGCT
GGACGGCGACGTGAACGGCCACAAATTCAGCGTGAGAGGCGAGGGCGAGGG
CGACGCCACAATCGGCAAACTGACACTGAAATTCATCTGCACAACAGGCAAACTGC
CAGTGCCCTGGCCAACACTAGTGACAACACTGACATACGGCGTGCAGTGCTTCAG
CAGATATCCGGACCACATGAAAAGACACGACTTCTTCAAAAGCGCCATGCCAGAG
GGCTACGTGCAGGAGAGAACAATCAGCTTCAAAGACGACGGCAAATACAAAACAA
GAGCCGTGGTGAAATTCGAGGGCGACACACTGGTGAACAGAATCGAGCTGAAA
G**GTATGACAATTTACTCGAACTTCCTTTTTTAACTCGAACTATGTATATACACAA
CAACGTTAATAATTAAGTCGTACTCATTTTGAATCTACTGACTCTAGATCCTGAT
TCACACATGTAATATAATTGCAG**GCACAGACTTCAAAGAGGACGGCAACATCCT
GGGCCACAAACTGGAGTACAACTTCAACAGCCACAACGTGTACATCACAGC
CAACAAACAGAAAAACGGCATCAAAGCCAACTTCACAGTGAGACACAACGTG
GAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACACCAATCGG
CGACGGCCCAGTGCTGCTGCCAGACAACCACTACCTGAGCACACAGACAGTGCT
GAGCAAAGACCCAAACGAGAAATAA

b). NLS:GFP$_{1\text{-}10}$(i); NLS is purple and intron sequence is RED and underlined.

ATGGAGCCTCCTAAGAAGAAGAGGAAGGTTGAGCTGATGGTTTCGAAAGGC
GAGGAGCTGTTCACAGGCGTGGTGCCAATCCTGGTGGAGCTGGACGGCGACGT
GAACGGCCACAAATTCAGCGTGAGAGGCGAGGGCGAGGGCGACGCCACAATCGG
CAAACTGACACTGAAATTCATCTGCACAACAGGCAAACTGCCAGTGCCCTGGC
CAACACTAGTGACAACACTGACATACGGCGTGCAGTGCTTCAGCAGATATCCG
GACCACATGAAAAGACACGACTTCTTCAAAAGCGCCATGCCAGAGGGCTACGTG
CAGGAGAGAACAATCAGCTTCAAAGACGACGGCAAATACAAAACAAGAGCCGT
GGTGAAATTCGAGGGCGACACACTGGTGAACAGAATCGAGCTGAAAG**GTATGA
CAATTTACTCGAACTTCCTTTTTTAACTCGAACTATGTATATACACAACAACGT
TAATAATTAAGTCGTACTCATTTTGAATCTACTGACTCTAGATCCTGATTCA
CACATGTAATATAATTGCAG**GCACAGACTTCAAAGAGGACGGCAACATCCTG
GGCCACAAACTGGAGTACAACTTCAACAGCCACAACGTGTACATCACAGCCAA
CAAACAGAAAAACGGCATCAAAGCCAACTTCACAGTGAGACACAACGTGGAG
GACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACACCAATCGG
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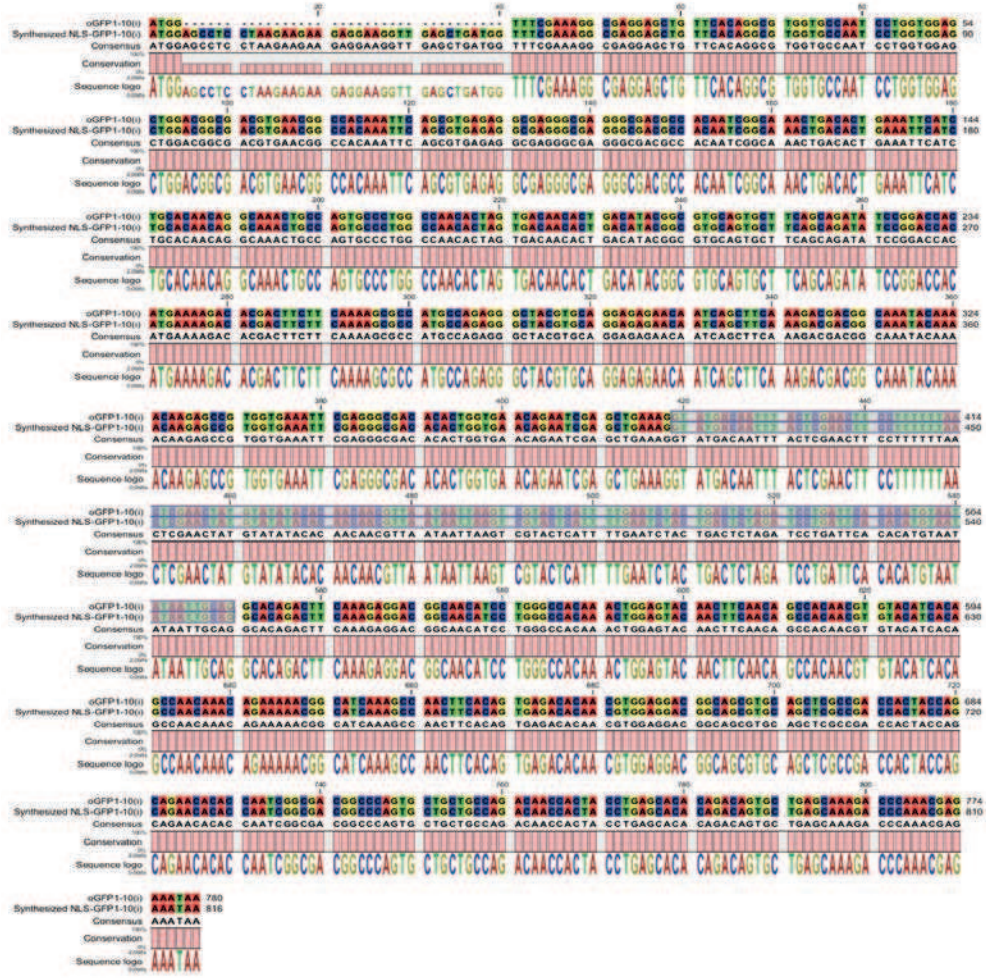
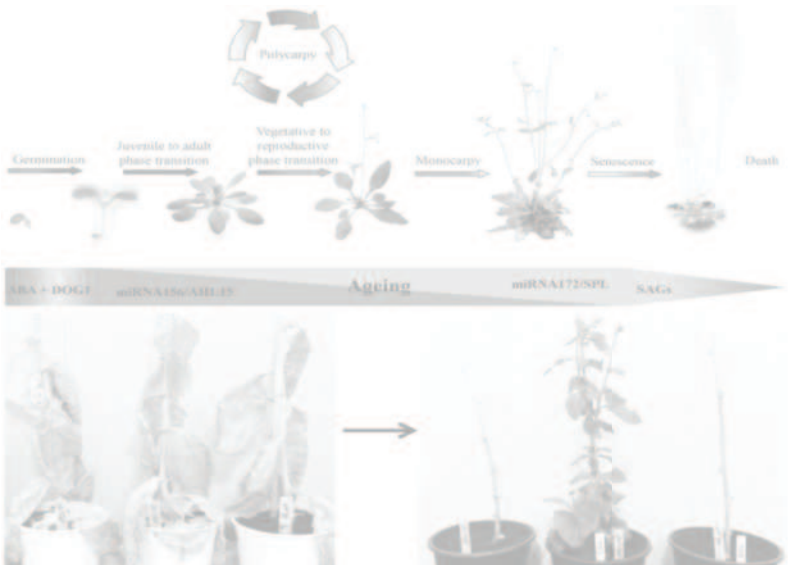
GAGCAAAGACCCAAACGAGAAATAA

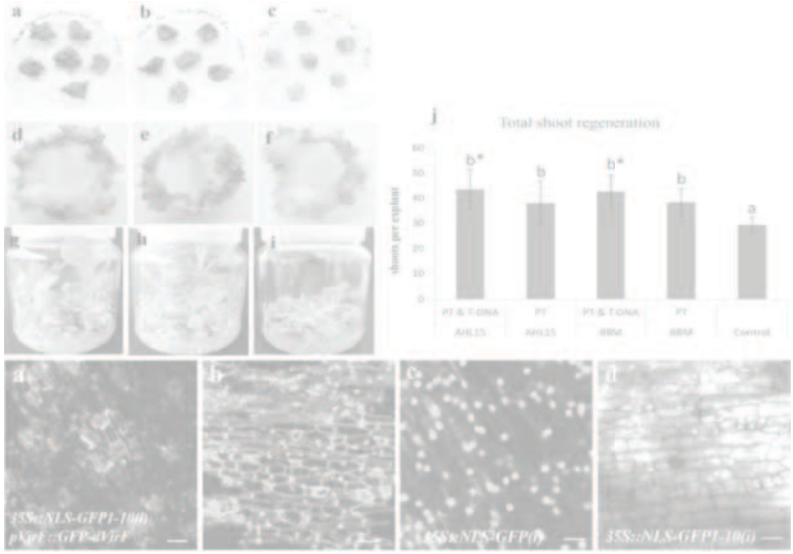
c).


oGFP1-10(i)
Synthesized NLS-GFP1-10(i)
Consensus
Conservation
Sequence logo



5

SUMMARY AND SAMENVATTING



SUMMARY

The development of methods for the genetic modification of plants a few decades ago has provided a tremendous boost for molecular plant science. Crop plants have been generated that are resistant to insects or herbicides, or that produce useful sugars or healthy nutrients. Although the ban on growing GM crops in Europe has considerably limited the application of GM technologies, they have still contributed considerably to fundamental plant science. Especially by using the natural and very efficient mechanism of DNA transfer by the soil born bacterium *Agrobacterium tumefaciens,* many collections of mutant lines of model plant species such as *Arabidopsis* and rice have been generated, in which genes are disrupted or overexpressed by the insertion of an *Agrobacterium* transfer DNA (T-DNA) construct. These collections have been used in forward or reverse genetics studies to unravel the function of a gene or a family of genes in plant defense or development, and to identify the key regulators in these processes. The study described in this thesis focused on the use of one of these key regulators, the *Arabidopsis* AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 15/REJUVENATOR (AHL15/RJV), to alter developmental processes such as flowering, senescence and regeneration.

C**hapter 1** of this thesis reviews our current knowledge on the molecular mechanisms of plant development, and how environmental, hormonal and genetic signals interact and affect plant phenotypes and life history strategies. GM technologies have been used to unravel these plant developmental signals, and the second part of **Chapter 1** therefore discusses different plant transformation techniques, specifically focusing on *Agrobacterium*-mediated transformation (AMT) and describing the type-IV secretion system (T4SS) that the bacterium uses to translocate the T-DNA and virulence (Vir) proteins into the host plant cells. For some of these Vir proteins, such as VirE2 and VirF, it has been shown that they are translocated independent of T-DNA to the host cell. The translocation signals of these proteins can be used to translocate heterologous proteins, such as the Cre-recombinase or domain 11 of Green Fluorescent Protein (GFP11). The latter allows visualizing the translocation event, as it results in a green fluorescent signal when combined in the recipient cell with the complementing GFP1-10 part (Split-GFP system).

C**hapter 2** describes the effect of overexpression of the AHL15/RJV protein on *Nicotiana tabacum* (tobacco) development. Tobacco lines were generated by AMT that overexpress a DEX inducible version of AHL15 (AHL15-GR). Early activation of AHL15-GR by germinating seeds on DEX-containing medium delayed seed germination and arrested seedling development, resulting in callus formation rather than the somatic embryogenesis observed in *Arabidopsis*. Late AHL15-GR activation by DEX treatment of flowering tobacco plants enhanced plant longevity by reducing leaf senescence, delaying flowering and, like in *Arabidopsis,* by rejuvenation of the axillary shoot meristems, resulting in a renewed cycle of vegetative development, flowering and seed set. By repeated DEX treatment tobacco plants could be maintained for more than three years, producing flowers and seeds following every DEX treatment. Seeds produced by DEX-treated plants did not germinate and contained defective embryos, suggesting that tobacco plants are limited in their polycarpic behavior either due to nutrient deficiency or by unknown genetic factors.

Chapter 3 provides evidence that developmental key regulators such as AHL15/RJV or the AP2- domain transcription factor BABY BOOM (BBM) can successfully be introduced into

plants cells by *Agrobacterium*-mediated protein translocation (AMPT)**.** The proteins were fused at the C-terminus to the 50 amino acid translocation signal of VirF, and at the N-terminus to GFP11. The latter allowed to visualize protein translocation into reporter lines of tobacco and *Arabidopsis* expressing GFP1-10. AMPT of the GFP10-AHL15-dVirF or GFP10-BBM-dVirF fusion proteins slowed down the senescence of infiltrated leaf discs and also significantly enhanced tobacco shoot regeneration. These results show that AMPT can be used as a non-GMO approach to induce developmental changes in plant cells.

In the above experiments with *Arabidopsis* and tobacco AMPT could be visualized because a transgenic GFP1-10 marker line was available. However, for plant species that are recalcitrant to transformation, such as sweet pepper or tulip, generating such a marker line is time consuming, if not impossible. **Chapter 4** describes the development and testing of a generic split GFP-based reporter system for AMPT that does not require the availability of GFP1-10 marker lines. In this system the GFP1-10 part was expressed from a T-DNA construct that was translocated to plant cells together with a GFP11- and VirF-tagged fusion protein by the same *Agrobacterium* strain. The generic split GFP-based system was successfully tested in a variety of tissues of different plant species such as *Nicotiana tabacum*, *Nicotiana benthamiana*, *Arabidopsis thaliana*, *Capsicum annuum* and *Tulipa gesneriana*.

SAMENVATTING

De ontwikkeling van methoden voor de genetische modificatie (GM) van planten een paar decennia geleden heeft een enorme stimulans gegeven aan de moleculaire plantenwetenschappen. Door GM werd het mogelijk transgene gewassen te maken die resistent zijn tegen insecten of herbiciden, of die nuttige suikers of gezonde voedingsmiddelen produceren. Hoewel het verbod op het kweken van genetisch gemodificeerde gewassen in Europa de toepassing van de GM technologieën sterk heeft beperkt, hebben transgene planten wel enorm bijgedragen aan de vooruitgang binnen het fundamentele onderzoek aan planten. Vooral met het natuurlijke en zeer efficiënte mechanisme van DNA-overdracht door de bodembacterie *Agrobacterium tumefaciens* zijn vele collecties van mutante lijnen van modelplanten zoals *Arabidopsis* en rijst gegenereerd, waarbij genen worden uitgeschakeld of tot overexpressie worden gebracht door het inbrengen van een *Agrobacterium* transfer DNA (T-DNA) construct. Deze collecties zijn gebruikt in voorwaartse of omgekeerde genetische studies om de functie van een gen of een familie van genen in plantenafweer of -ontwikkeling te ontrafelen, en de sleutelregulatoren van deze processen te identificeren. Het in dit proefschrift beschreven onderzoek richtte zich op het gebruik van één van deze belangrijke regulatoren, de *Arabidopsis* AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 15/REJUVENATOR (AHL15/RJV), om ontwikkelingsprocessen zoals de bloei, veroudering en regeneratie te veranderen.

Hoofdstuk 1 van dit proefschrift geeft een overzicht van onze huidige kennis over de moleculaire mechanismen van de ontwikkeling van planten, en hoe hormonale, genetische en omgevingssignalen met elkaar interacteren en invloed hebben op plantfenotypes en levensgeschiedenisstrategieën. GM-technologieën zijn gebruikt om deze ontwikkelingssignalen in planten te ontrafelen, en in het tweede deel van **Hoofdstuk 1** worden daarom de verschillende transformatietechnieken voor planten bediscussieerd, met een specifieke focus op *Agrobacterium*-gemedieerde transformatie (AMT) en het type-IV secretie systeem (T4SS) dat de bacterie gebruikt om het T-DNA en virulentie (Vir) eiwitten naar gastheerplantencellen over te brengen. Voor sommige Vir eiwitten, zoals VirE2 en VirF, is aangetoond dat zij onafhankelijk van T-DNA naar de gastheercel kunnen worden overgebracht. De translocatiesignalen van deze eiwitten kunnen worden gebruikt om heterologe eiwitten, zoals het Cre-recombinase of domein 11 van het groen fluorescente eiwit (GFP11) over te brengen. Dit laatste eiwitje stelt ons in staat om eiwittranslocatie direct zichtbaar te maken, aangezien GFP11 kan leiden tot een groen fluorescent signaal wanneer het in de ontvangende cel gecombineerd wordt met het complementerende GFP1-10 deel (Split-GFP systeem).

Hoofdstuk 2 beschrijft het effect van overexpressie van het AHL15 / RJV eiwit op *Nicotiana tabacum* (tabak) ontwikkeling. Met behulp van AMT zijn er tabakslijnen gegenereerd die een DEX-induceerbare versie van AHL15 (AHL15-GR) tot overexpressie brengen. Vroege activering van AHL15-GR door zaden op DEX-bevattend medium te ontkiemen vertraagde de zaadkieming en stopte de zaailingontwikkeling, wat uiteindelijk leidde tot de vorming van callus in plaats van de somatische embryogenese die in *Arabidopsis* is waargenomen. AHL15-GR activatie door DEX behandeling van bloeiende tabaksplanten leidde tot een significante verhoging van hun levensduur, door verminderde bladveroudering, uitgestelde bloei en, zoals in *Arabidopsis*, door verjonging van de okselmeristemen, resulterend in een hernieuwde

cyclus van achtereenvolgens vegetatieve ontwikkeling, bloei en zaadvorming. Door herhaalde behandeling met DEX konden de AHL15-GR tabaksplanten gedurende meer dan drie jaar in leven gehouden worden, waarbij er na elke DEX behandeling bloemen en zaden geproduceerd werden. Zaden geproduceerd door DEX-behandelde planten bleken niet te ontkiemen doordat ze defecte embryos bevatten. Dit suggereert dat tabaksplanten beperkt zijn in hun polycarpe gedrag, mogelijk door een tekort aan nutriënten of door nog onbekende genetische factoren. **Hoofdstuk 3** levert het bewijs dat sleutelregulatoren van plantenontwikkeling, zoals AHL15/RJV of de AP2-domein transcriptiefactor BABYBOOM (BBM), met succes in plantencellen kunnen worden geïntroduceerd door *Agrobacterium*-gemedieerde eiwit translocatie (AMPT). Hiertoe werden de eiwitten aan de C-terminus gefuseerd met het 50 aminozuren lange translocatiesignaal van VirF, en aan de N-terminus met GFP11. Deze laatste tag stelde ons in staat om AMPT van de GFP10-AHL15-dVirF of GFP10-BBM-dVirF fusie-eiwitten te visualiseren in reporterlijnen van tabak en *Arabidopsis* die het complementerende GFP1-10 tot expressie brachten. Tevens konden we aantonen dat AMPT van deze eiwitten de veroudering van geïnfiltreerde tabaksbladschijven vertraagde, en ook de scheutregeneratie uit deze weefsels significant verbeterde. Deze resultaten tonen aan dat AMPT kan worden gebruikt als niet-GMO benadering om ontwikkelingsveranderingen in plantencellen of -weefsels te induceren.

In de bovenstaande experimenten met *Arabidopsis* en tabak was het mogelijk om AMPT te visualiseren omdat transgene GFP1-10 markerlijnen voor deze plantensoorten beschikbaar waren. Echter, voor plantensoorten die recalcitrant zijn voor transformatie, zoals paprika of tulp, is het genereren van dergelijke markerlijnen een tijdrovend, zo niet onmogelijk proces. **Hoofdstuk 4** beschrijft de ontwikkeling en het testen van een generiek split-GFP gebaseerd reportersysteem voor AMPT, dat niet de beschikbaarheid van GFP1-10 markerlijnen vereist. In dit systeem werd het GFP1-10 deel tot expressie gebracht vanaf een T-DNA construct dat samen met een GFP11- en VirF-gelabeld fusie-eiwit door dezelfde *Agrobacterium* stam naar plantencellen werd getransloceerd. Dit generieke split-GFP reportersysteem werd met succes getest in verscheidene weefsels van verschillende plantensoorten, zoals *Nicotiana tabacum, Nicotiana benthamiana, Arabidopsis thaliana, Capsicum annuum en Tulipa gesneriana*.

ACKNOWLEDGEMENTS
CURRICULUM VITAE

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ACKNOWLEDGMENTS

A successful scientific career requires good health, the potential to work hard, a scientific mind and patience. So first of all I am very thankful to my God who blessed me with all these blessings. Beside these blessings you also need support, encouragement, and guidance which you can get only from your family, teachers, friends and colleagues. Among my family I am very thankful to my parents, who are not only responsible for my existence but they also have supported, encouraged and educated me both socially and morally. My PhD is actually the result of my parents' prayers and wishes. Next, I would like to acknowledge my master supervisor **Raham Sher Khan** for making my scientific research base and the **Institute of Biotechnology and Genetic Engineering (IBGE) The University of Agriculture Peshawar (Pakistan)** for awarding me with a faculty development overseas PhD scholarship and **Institute of Biology Leiden (IBL)**, for giving me the opportunity to study in the Netherlands at Leiden University.

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The successful completion of PhD in science is not possible without good supervision and guidance, but my supervisor **Remko Offringa** not only supervised me in understanding, working with and writing science but also taught me how to supervise the science students. Although I did not have many interactions with my promotor **Paul Hooykaas**, I am very thankful to him for his guidance and suggestions during work discussions and coffee breaks and also for his treating not only me but everyone with a smiling face. I am also thankful to senior staff members of Molecular and Developmental Genetics department: **Paul van Heusden, Bert van der Zaal** and **Sylvia de Pater** for their feedback and suggestions during discussions.

Finding my PhD position at the IBL in the Sylvius Laboratory of Leiden University and settling down in Leiden was made possible because of the help of my friends, and now I will defend my thesis also with the help of my friends in the form of my paranymphs. So in acknowledging my friends and colleagues first I would like to say thanks to **Ghulam Hasnain** for contacting me with my supervisor and then to **Muhammad Khurshid, Muhammad Faraz, and Muhammad Tayyab Akhtar**, who not only helped me to settle here in Leiden but also treated me like my brothers. I would also like to say thanks to my other Pakistani friends especially to **Muhammad Ibrahim** for accompanying me during prayer times in the lab, and for nice gatherings, food and jokes to **Muhammad Zaman, Muhammad Fawad Khan, Mujeeb ur Rahman, Irshad ul Haq, Muhammad Belkarme, Muhammad Tariq, Muhammad Waseem Akhtar, Muhammad Shahbaz Anwar, Nasir Bilal, Muzammil Iqbal, Amir Aslam, Amir Aziz, Muhammad Yahya, Muhammad Kamran, Farooq Ahmad, Samrah Masud, Muzafar Ali khan, Sohaib Aslam, Zeshan Hassan** and **Imran Haider.**

Nobody is perfect in this world. You need help, support and encouragement and also most of the time during a PhD in science you have to be in the lab. So if you don't have good and helpful friends and colleagues there, not only your working efficiency decreases but your life in the workplace can become boring and miserable. So I am very thankful to all my friends and colleagues at my lab: **Myckel, Elco, Kasper, Eike, Philippe, Omid, Cheryl, Yuanwei, Arezoo,**

Niels, Reza, Kiki, Gary, Yao, Xiao, Xiaoyu, Xiao-Rong, Anne, Niharika, Felix, Daan, Shwai, Suyun, Haxi, Amke, Ward, Marianne, Gerda, Jan and **Johan.**

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CURRICULUM VITAE

Majid Khan was born on August 20th 1981 in Charsadda, Pakistan. In 2001, he obtained his two year bachelor degree from the Government Post Graduate College Charsadda with majors in Zoology and Chemistry. In 2006, he obtained his four year bachelor degree in Biotechnology from the Center of Biotechnology and Microbiology at the University of Peshawar, Pakistan. From 2006 to 2007 he served as a lecturer in Government Post Graduate College Charsadda. In 2007, he was appointed as a lecturer at the Agriculture University Public School and College, where he also started his master studies at the Institute of Biotechnology and Genetic Engineering (IBGE) of the University of Agriculture Peshawar, Pakistan. In the last year of his master studies he did his master's thesis entitled "*Agrobacterium*-mediated transformation of tomato for enhanced resistance to fungal phytopathogens" under the supervision of Dr. Raham Sher Khan. After earning his MSc degree in Biotechnology in 2010, he passed the GRE test and was selected as a lecturer in IBGE under the faculty development overseas PhD scholarship for the Strengthening Institute of Biotechnology and Genetic Engineering (SIBGE) project of the University of Agriculture Peshawar with financial support by the Higher Education Commission (HEC) of Pakistan. This PhD scholarship allowed him to move to the Netherlands in 2012, where he started his PhD research on *Agrobacterium*-mediated translocation of developmental regulators to plant cells under the daily supervision of Dr. Remko Offringa and promotor Paul Hooykaas in the Molecular and Developmental Genetics department at the Sylvius Laboratory, Institute of Biology Leiden (IBL) at Leiden University, the Netherlands.