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

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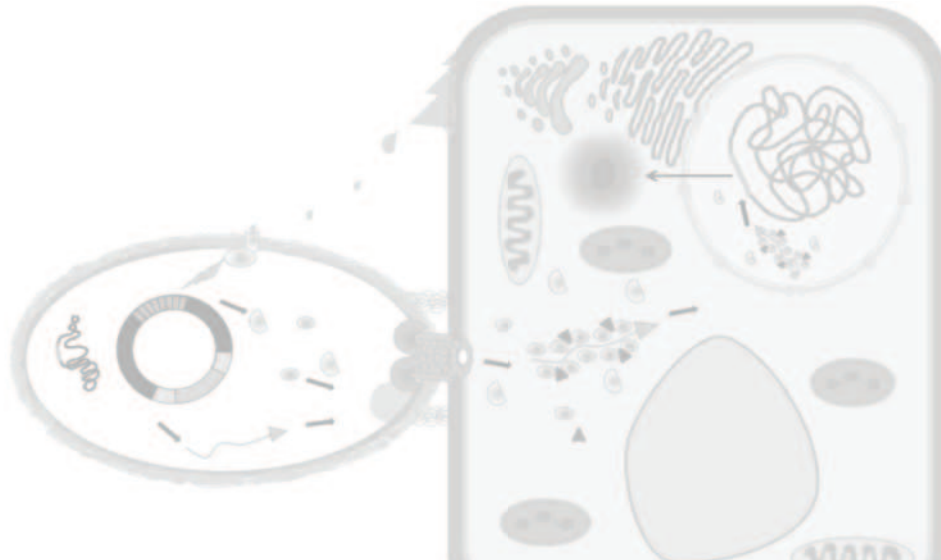


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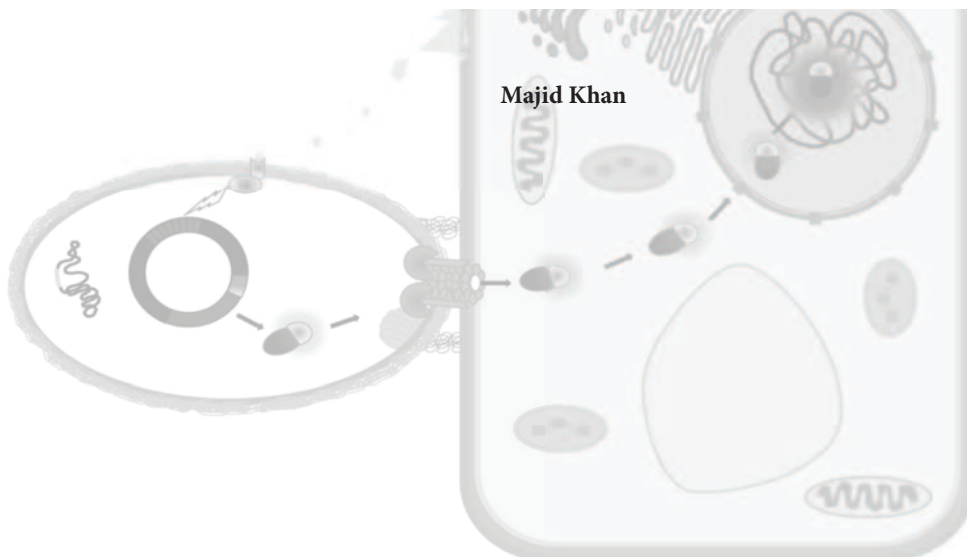
Title: Molecular engineering of plant development using Agrobacterium-mediated protein translocation

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GENERAL INTRODUCTION
CONTRIBUTION OF
THE AGROBACTERIUM
TRANSFORMATION
MACHINERY TO PLANT
DEVELOPMENTAL STUDIES

1



INTRODUCTION

The ancient book *Historia Plantarum* or *Enquiry into Plants* written by Aristotle'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).

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). Absciscic 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).

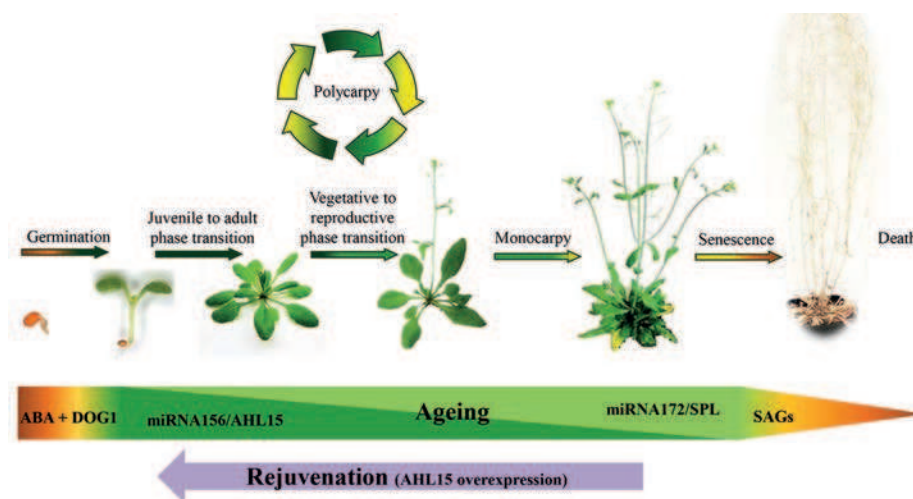


Figure 1. Schematic representation of plant ageing by transition from one to the next phase of development. The involvement of different key regulatory genes in ageing and rejuvenation is indicated.

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 photosynthetically 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-Seilanianz 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 plant's 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 (Hooymaas, 2010).

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 *VirA*, *VirB*, *VirC*, *VirD*, *VirE*, *VirF*, *VirG* and *VirH* (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

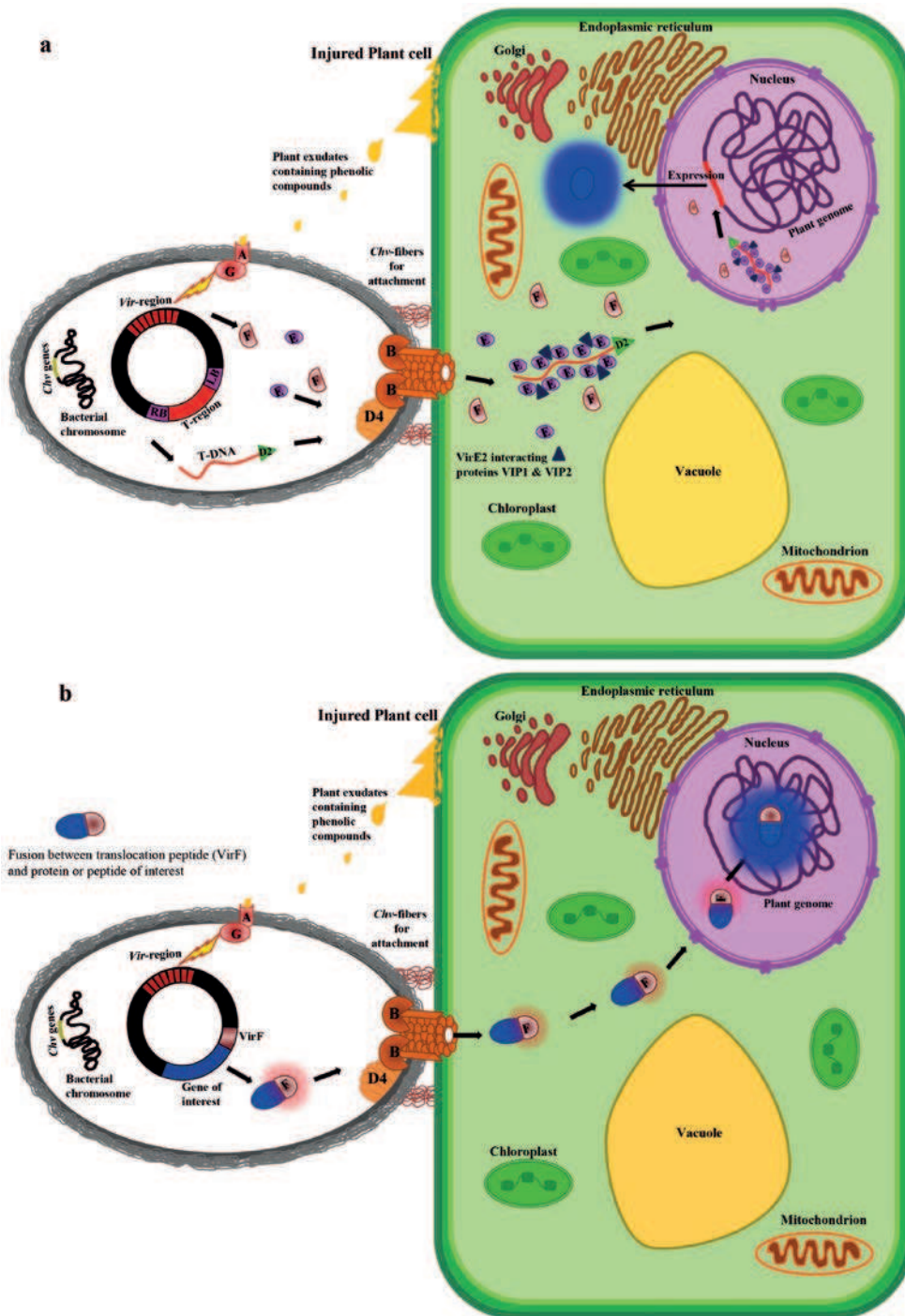


Figure 2. Schematic representation of (a) *Agrobacterium*-mediated transformation versus (b) protein translocation to a host plant cell.

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 *virB* and *virD* 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 peripheral 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 chaperone 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), cyclophilins and At KAP α) (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 IMP α -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 thuringiensis* (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 (1-5 days), *Agrobacterium* density ($1 \cdot 10^6$ to $1 \cdot 10^{10}$ 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 *Agrobacterium* 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 pleiotropic 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.

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