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Chapter

Agrobacterium-Mediated Transformation

Risha Amilia Pratiwi and Muhammad Imam Surya

Abstract

Agrobacterium-mediated transformation (AMT) heavily relies on the capability of bacterial pathogen *Agrobacterium tumefaciens* in transferring foreign genes into a wide variety of host plants. Currently, AMT is the most commonly used method for generating transgenic plants. On the other hand, *A. tumefaciens* was very useful for plant breeding. It also accelerated the technology of plant breeding to obtain specific characters. Gene transfer from bacteria to plants is a complex mechanism that involves several functional steps. This chapter will give brief information related to AMT mechanism, including the history of crown gall disease, the natural pathogenesis of *A. tumefaciens*, and the general protocol of AMT.

Keywords: *Agrobacterium tumefaciens*, crown gall disease, natural pathogenesis, plant transformation, plant breeding technology

1. Introduction

Plants are essential natural resource for the survival and welfare of human being. The many uses of plants can be summarized in "TREES" word, an abbreviation for timber, restoration, ecological, educational and recreation, and source of sustenance [1]. Given the importance of plants, people have been upgrading both productivity and quality of cultivated plants. Plant breeding technique comes as an art, science, and business of manipulating the genetic pattern of plants by humans to develop superior cultivars, related to improving humankind, ranging from unintentional changes that are resulted by conventional selection to precision breeding by molecular tools [2, 3].

Plant breeding activities are considered to have been going on for at least 10,000 years, as long as the age of human civilization from nomadic hunter-gatherer to sedentary lifestyle [2]. The earlier farmers collected their best seeds to be replanted. This conscious human selection activity to get the best performing plant, although relatively simple, is the fundamental principle of phenotype-selection based. Later people incorporated some superior properties of different closely related parents through artificial mating or sexual hybridization, generating new genetic recombination which dramatically led to increased crop yields, easy cultivation, tolerant to environmental stresses, and resistant against pest [2–4].

The advance of molecular biology and genetic engineering provides new opportunities in plant breeding technology. Moreover, the application of molecular markers developed from QTL analysis enhances breeding efficiency by enabling marker-assisted selection for particular agronomic traits [5]. On the other hand, next generation sequencing (NGS) has revolutionized genomic and transcriptomic

approaches to biology. These new sequencing tools are also valuable for discovery, validation, and assessment of genetic marker in populations. NGS technologies have conferred new opportunities for high-throughput genotyping in various plant species. Recent improvements in high-throughput sequencing have enable sequences to be used to detect and score single nucleotide polymorphisms (SNPs) by bypassing time-consuming process of marker development. However, genotype-by-sequencing (GBS), a series of genetic analyses that includes molecular marker discovery and genotyping using NGS technologies, has opened new possibilities in plant breeding and plant genetics studies, including linkage maps, genome-wide association studies, genomic selection, and genomic diversity studies [6]. Furthermore, horizontal gene transfer (HGT), also known as lateral gene transfer, refers to the movement of genetic information across normal mating barriers, between more or less distantly related organisms, and thus stands in distinction to the standard vertical transmission of genes from parent to offspring [7]. HGT from bacteria to plants has been restricted to *Agrobacterium rhizogenes*, and the related bacterium *A. tumefaciens* transforms a wide variety of host plants by transferring a segment of the large tumor-inducing plasmid, called T-DNA into host cells [8]. Most genetic engineering of plants uses AMT to introduce novel gene content.

The development of AMT technology is supported by research on RNA interference technology for functional analyses of genes involved in transformation mechanism or gene(s) of interest and gene editing technology that allows precise manipulation of targeted genome sequences [9]. Although there are several species of *Agrobacterium* that have the capability to cause tumors—such as *A. rhizogenes* that cause hairy roots disease, the discussion of *Agrobacterium*-mediated transformation in this chapter specifically refers to *A. tumefaciens*. The *Agrobacterium*-plant interaction is a complex process that its effectiveness and efficiency are affected by many factors. This review focuses on the mechanism of AMT and updates technology to increase the successfulness of plant-gene transformation mediated by *A. tumefaciens*.

2. Agrobacterium-mediated transformation mechanism

2.1 History of crown gall disease

The generation of transgenic plant mediated by *A. tumefaciens* basically mimics the event of naturally plant transformation. The dawn of natural plant transformation study began when fleshy rough roundish surface morphology on the roots' crown (region joining root and shoot) of over 20 different fruit trees was observed [10]. To investigate the causative agent of these tumor-like outgrowths later named "crown gall disease," infected root crown tissues were isolated. The bacteria described as *Agrobacterium tumefaciens* then were presented [11]. While these Gram-negative soil bacteria were inoculated to wounded young tissues of healthy plants, secondary tumors that cannot be distinguished from the crown gall were produced. On the other hand, the old tissues were not very susceptible [10].

The development of in vitro cultivation technique supports the study of secondary tumor. Explant derived from the interior of secondary tumor continued to unlimited proliferation in auxin in auxin and cytokinin lacking medium and synthesized unusual amino acid derivative; guanido amino acids octopine N2-(D-1-carboxyethyl)-L-arginine and nopaline N2-(1,3-dicarboxypropyl)-L-arginine [9, 12]. Both properties distinguished tumor cells than normal cells.

Bacterial isolation from secondary tumor cultures revealed that no one of these cultures has yielded any growth of *A. tumefaciens*. Injection of paste from these

bacteria-free cultures to healthy plants exhibited no evidence of tumor initiation as it is observed due to paste injection from young primary tumors. The fact that volume of secondary tumor still increased—although bacteria were absent, indicated that bacteria only trigger tumorigenesis, not involved in the whole process. Somehow, the host cells were permanently transformed so that they were able to convert normal cells into neoplastic cells [13].

Further investigation to confirm the involvement of *A. tumefaciens* in early step of tumorigenesis was conducted through temperature challenge. Bacterial inoculation on periwinkle plant (*Vinca rosea* L.) at temperature of 32°C did not inducted swelling growth, even bacterial and plant cells grew well at that temperature. The bacterial inability to forming tumorous property was not influenced by host plant physiological disturbances as a whole but was solely dependent upon environmental change of bacterial inoculation site, in this case an elevated temperature.

Plants inoculated with bacteria at a temperature of 26°C for 5 days, before being held at 32°C, retained tumorigenic state. These periods provided sufficient opportunity for bacteria to interact with host plants, and then they drove the cellular alteration of the plants. Once the cellular alteration was fully complete, temperature at 32°C would not matter; plants were entirely independent in converting normal cells into neoplastic cell. The nature of plant oncogenic transformation as bacterial influence was known as "tumor-inducing principle" [13, 14].

The development of molecular technique made a breakthrough in the investigation of the origin of tumor-inducing principle. On the examination of the pathogenic strains of *A. tumefaciens*, one or several homolog large (±140–235 kbp in size) supercoiled circular plasmids were isolated [15]. None of the avirulent *Agrobacterium* strains tested belonged to such a plasmid. Based on its association in inducing tumors, the plasmid was called "tumor-inducing(Ti)-plasmid" [15, 16].

Ti-plasmid borne harbored by four apparently distinct genetic loci (**Figure 1**). Three loci that consist of genes regulating auxin, cytokinin, and opine synthase are located on T-DNA, a highly conserved DNA fragment defined by 25 bp repeat sequence borders on each end. The genes regulating auxin and cytokinin accumulation determine the oncogenicity of the plasmid. The gene regulating opine synthesis is necessary for expression in host plants as exclusive nutrition for *A. tumefaciens* [19]. The fourth locus, apart from T-DNA, controls the catabolism of opine compound [9, 20]. All genes carry the signal necessary for the expression in host plant cell. During bacterial infection to host cell, only T-DNA was cleaved out of the Ti-plasmid borne, transferred, and ultimately incorporated to DNA nuclear of host plant cells [17, 18]. Therefore, the complete Ti-plasmid was not found in tumor cells, given their relatively large size could not infiltrate host nuclear ore complex passively [15]. Genes regulating auxin and cytokinin synthesis modifying the phytohormone ratio in the host cell resulting uncontrolled cell proliferation that leading to tumor growth.

2.2 The natural pathogenesis of *A. tumefaciens*

AMT is a complicated mechanism, which includes (1) signal recognition from plant host to *A. tumefaciens*, (2) T-DNA processing, (3) T-DNA traveling in plant host cell, (4) T-DNA integrating to plant host genome, and (5) expression of T-DNA in the plant host cell. The mechanism of T-DNA transfer is facilitated by a set of virulent genes located on Ti-plasmid borne [approximately 35 virulent genes grouped in at least 8 operons, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G, and *vir*H, encoding VirA, VirB, VirC, VirD, VirE, VirF, VirG, and VirH protein, respectively (**Figure 1**)], apart from T-DNA, whereas others are on chromosome (chromosomal virulent genes—*chv*) [22, 23].

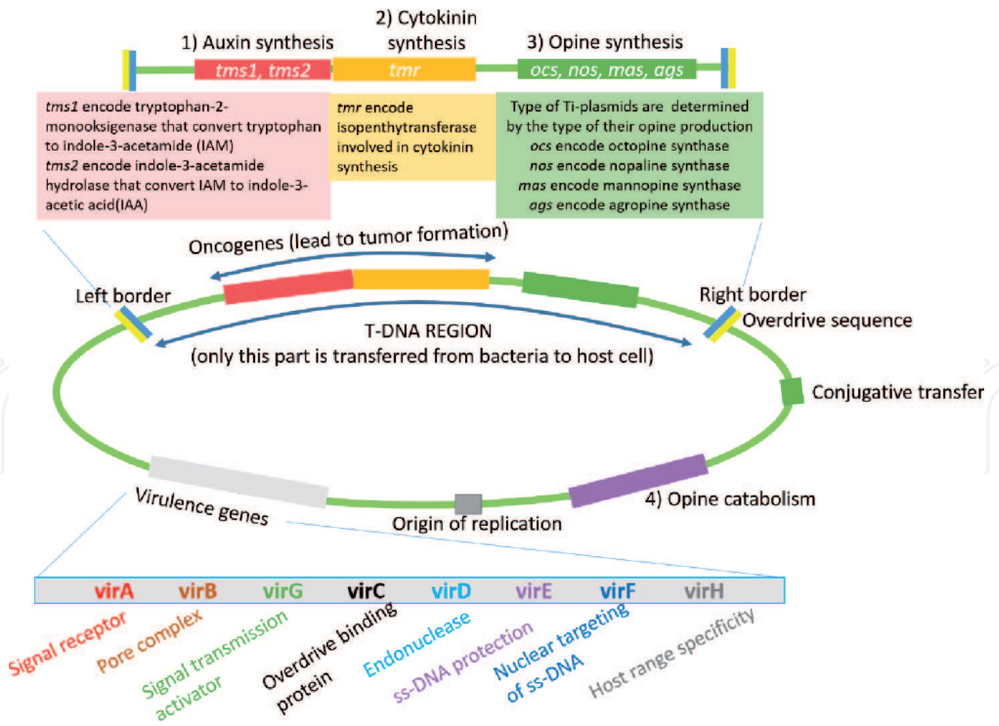

Figure 1.
Schematic representation of a Ti-plasmid borne (not to scale, modified from [20, 21]).

2.2.1 Signal recognition

Agrobacterium-plant interaction occurs when a large plant-derived chemicals, which include organic acid compounds (pH 5.0–5.8) as routine secreted chemical and phenolic compounds as the wound-releasing chemical, are exposed to the bacteria. Signal recognition of *A. tumefaciens* to plant cell involves three systems (**Figure 2**) [24]. First, bacterial chemotaxis attracted by phenolic compounds that are exuded from fresh wound site of plants, such as acetosyringone and α-hydroxy acetosyringone [25]. Initially, bacteria engage with plant cell surface reversibly, stimulating adhesion production that causes adhesion through unipolar polysaccharide (UPP)-dependent polar attachment and UPP-nondependent attachment. This irreversible surface attachment establishes a site for multicellular biofilms formation, matrix elaboration, cell division, biofilm maturation, and "buddy daughter" cell dispersal [26]. Secondly, host signal compounds are also recognized by transmembrane protein receptors (VirA) on periplasmic space of bacterial cell that trigger phosphorylation of positive regulatory protein VirG [25–27]. Thirdly, protein ChvG/ChvI encoded by chromosomal virulent genes perceive acidity from sugar that activate basal expression of *vir*G too [22]. The phosphorylated VirG leads to the activation of another virulence gene [25]. Monosaccharides enhance signaling process by binding with ChvE then synergize with VirA [28].

2.2.2 T-DNA processing

The process of excising T-DNA from Ti-plasmids depends on VirD1 and VirD2 as endonucleases. The 25 bp border sequences on the bottom strand of T-DNA act as nicking site for VirD1 and VirD2. VirD1, a site-specific helicase, unwinds double-stranded T-DNA. A nuclease, VirD2, cuts the bottom strand of T-DNA from the right and left border, becoming single-stranded linear DNA termed T-strand [21, 29]. VirD2 then covalently caps the 5′ end of T-strand at the right border, forming the VirD2/T-strand complex [28]. The 3′ end of the nicked right border acts as a priming site for the bottom strand of T-DNA regeneration [29]. VirC1 binding

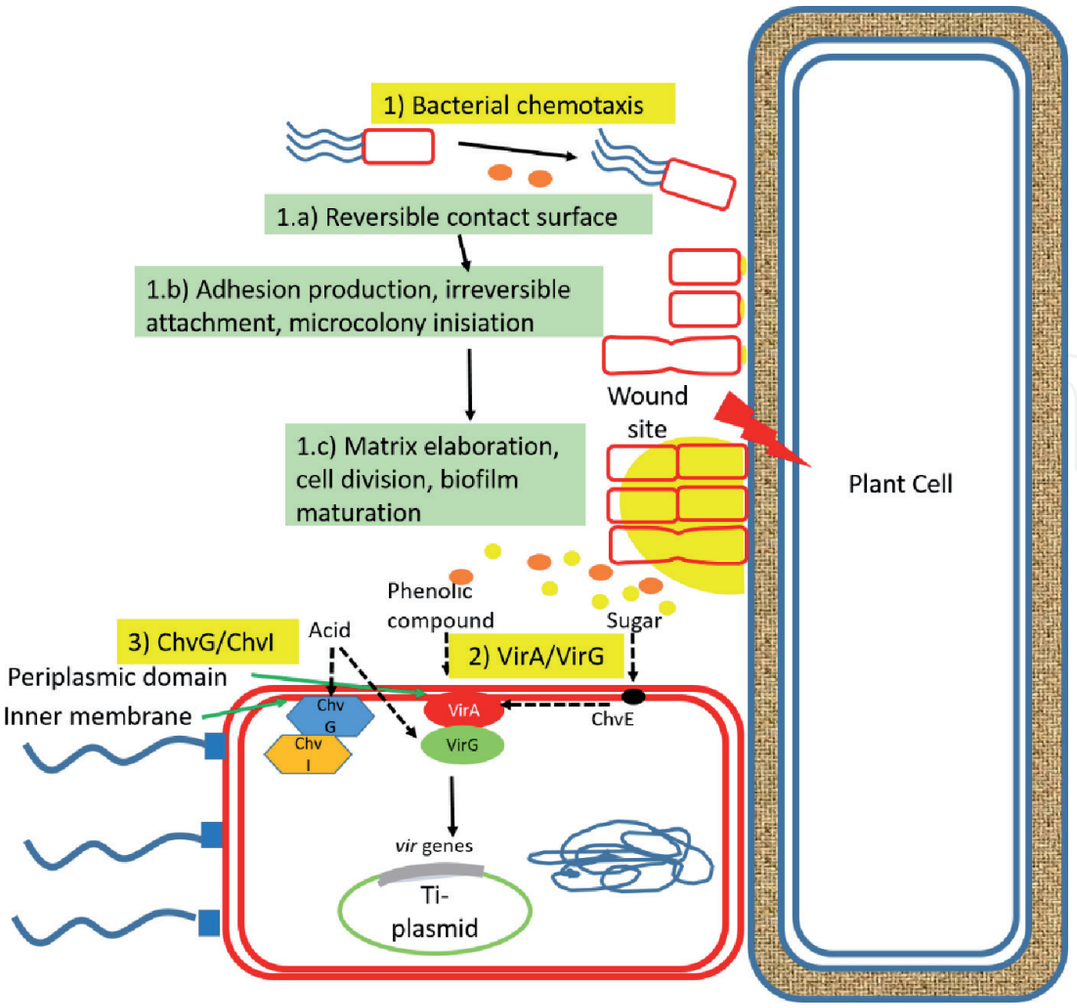

Figure 2.
Schematic representation of A. tumefaciens *signal recognition mechanism, modified from [26].*

the "overdrive" sequence near the right border of T-DNA (**Figure 1**) through its C-terminal ribbon-helix-helix DNA binding fold enhances the number of T-strand molecules [30, 31]. The right fraction of the 25 kb terminus sequence of T-DNA determines the director of DNA transfer [32] (**Figure 3**).

2.2.3 T-DNA traveling

The T-DNA traveling to the host cannot separate from the role of VirD2 and VirE2. Both VirD2 and VirE2 have the C-terminal nuclear localization signal (NLS) sequence that piloted VirD2/T-strand to the host nucleus [33]. VirD2/T-strand, a rodlike structure, exits bacterial cells through Ti-pilus, type IV secretion system (T4SS), which is assembled by 11 VirB and VirD4 proteins [34]. The hydrophilic protein VirE2 is accumulated in the bacterial cytoplasm and translocated into the host cell through clathrin-mediated endocytosis. Besides helping transport the T-strand, VirE2 in the host cytoplasm coats along the T-strand noncovalently and form VirD2/T-strand/VirE2 (termed Ti-complex) to protect it from any nuclease digestive activity [35–37]. Ti-complex in the plant cytoplasm is trafficked to the plant nucleus via the endoplasmic reticulum network inside the plant cytoplasm (**Figure 4**) [24].

2.2.4 T-DNA integration

T-DNA integration followed by transgene expression is the final and crucial stage in the genetic transformation mediated by *Agrobacterium*. The molecular

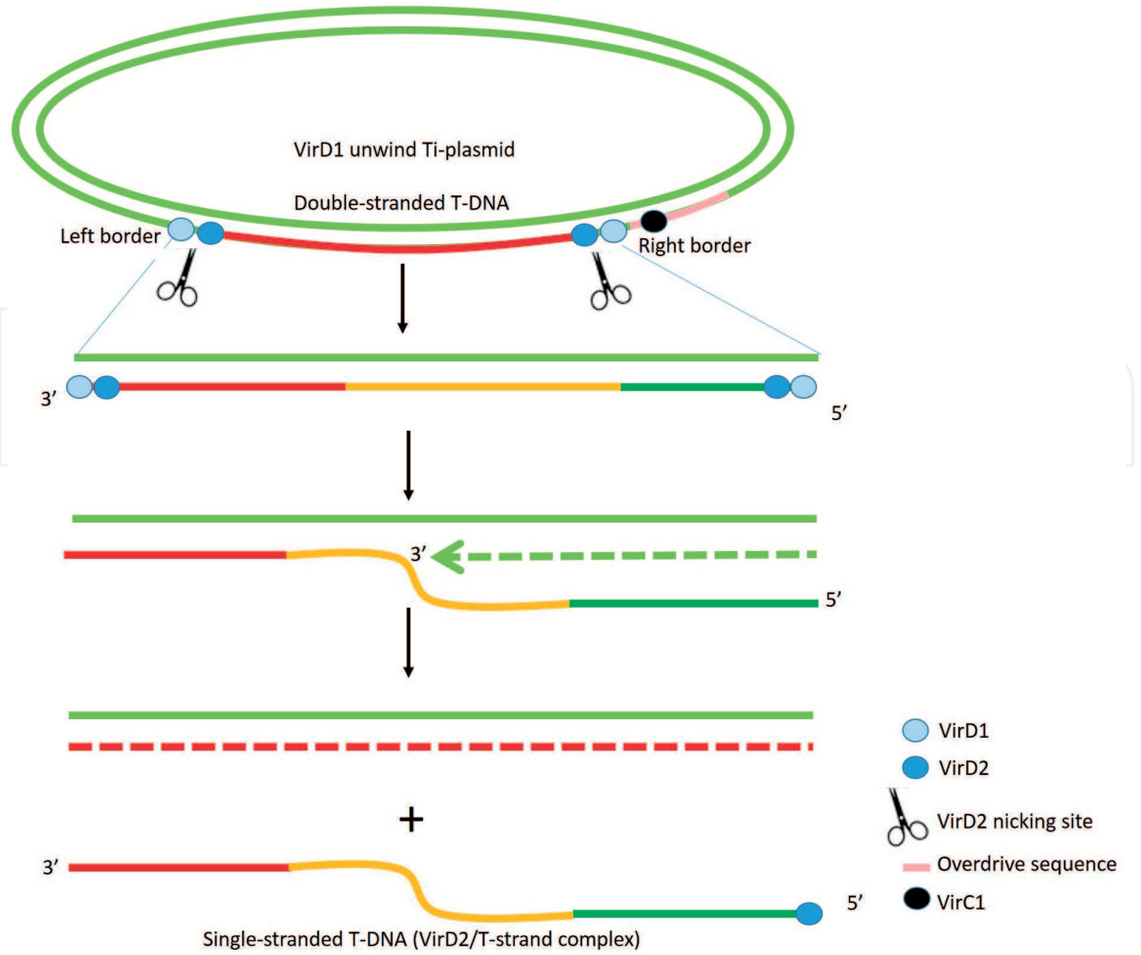

Figure 3.
T-strand generation from T-DNA, modified from [29].

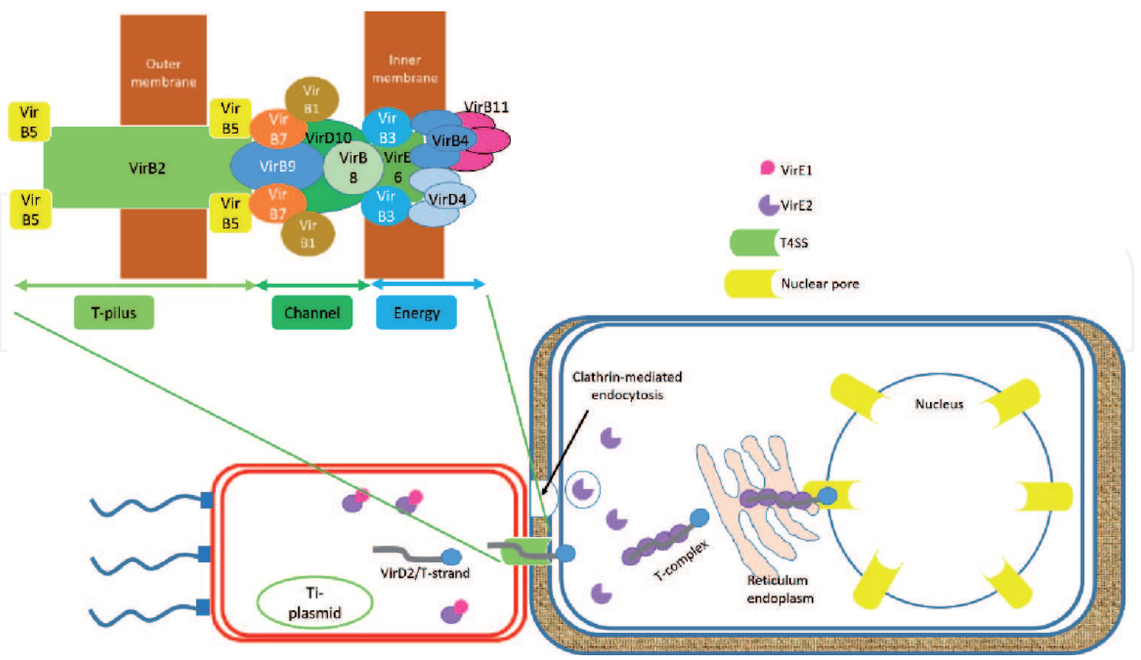

Figure 4.
T-complex traveling, modified from [38].

mechanism of T-DNA integration into host plant genome actually is still not fully understood. T-DNA integration occurs at random sites, not preferentially in transcriptional active or hypomethylated regions of the plant genome. Some important

genes in the T-DNA integration process are limited to genes related to chromatin formation and histone modification [39].

VirE2 may play a role in T-strand targeting to chromatin by binding to the bZIP transcription factor VirE2 interacting protein 1 (VIP1). VIP1 mediates the association of VirE2/single-strand DNA with mononucleosomes, a unit of chromatin in the nucleus [39]. When T-complex arrived in the plant nucleus, its protein component should be disassembled by the ubiquitin-proteasome system so that the T-strand can be exposed. T-complex disassembling process and VirE2 degradation are assisted by VirF [40, 41].

VirD2 has no ligation activity, so T-strands are not likely to join directly with the host genome. Possibly, the host DNA polymerase copies the T-strand to form a double-stranded T-DNA, and then it joins with the site breaks of DNA host plant that it is caused by environmental stress due to *Agrobacteria* incubation or normal metabolic processes. Non-homologous end joining (NHEJ) as a way of repairing broken double-stranded DNA is proposed as the main pathway of bacterial plant DNA integration. NHEJ only requires little or no sequence homology on the damaged part, although in fact there are microhomology between the T-DNA and the integration points on the host chromosome [42] (**Figure 5**).

2.2.5 T-DNA expression

Bacterial T-DNA that integrates with plant genome cells faces two possible fates. First, the T-DNA is expressed, in various levels. Second, the T-DNA is only

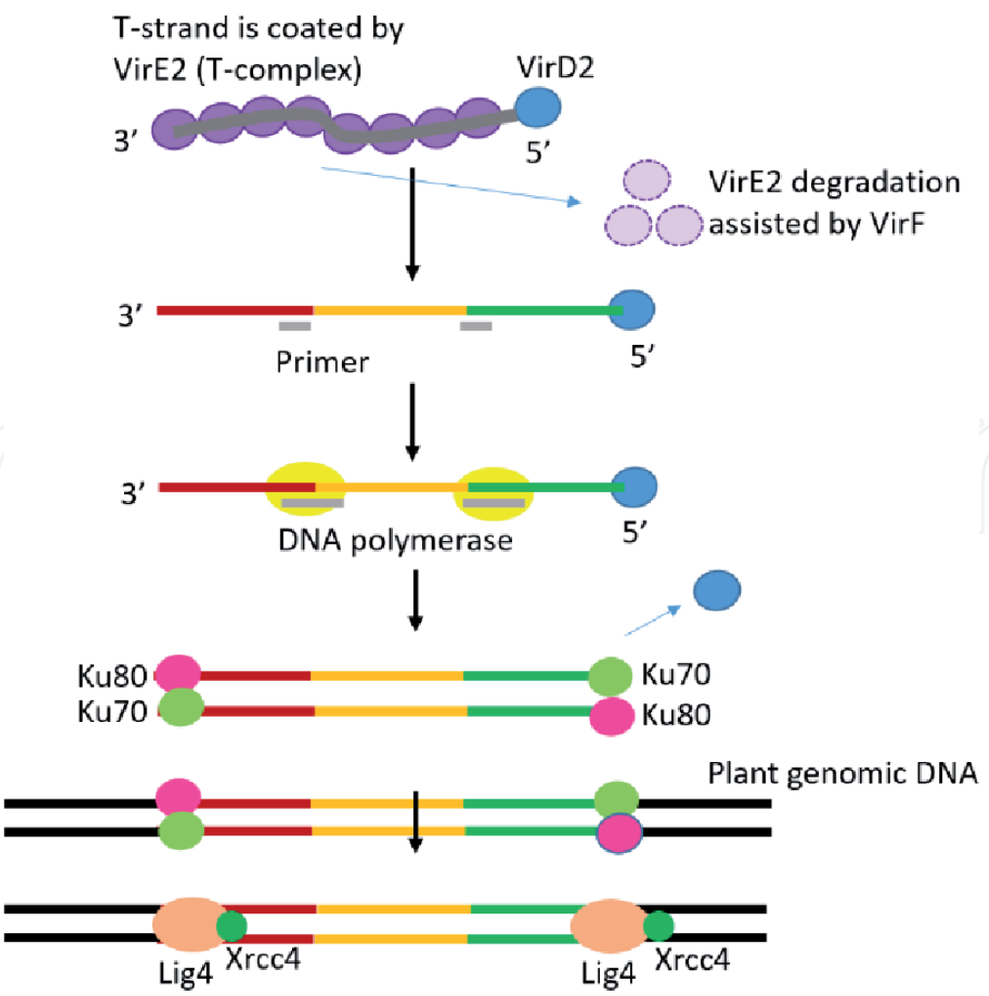

Figure 5.
T-DNA integration, modified from [42].

integrated but cannot be expressed. A broad range of transgene expression, from very high to totally silent, depends on species [12].

The expression on auxin and cytokinin coding genes in T-DNA causes the accumulation of both phytohormones. Phytohormone ratio abnormalities bring plant cell to uncontrolled cell proliferation, leading to tumor growth. The expression of opine synthesis coding genes produces opine—the type depends on bacterial strain, an exclusive nutrition for *Agrobacterium*. In young tissue, swelling is observed from the fourth or fifth day after bacterial inoculation, well-developed in a month, and growing rapidly until it reaches an inch or two in diameter for several months [10]. The overall mechanism of AMT is summarized in **Figure 6**.

2.3 *Agrobacterium*-mediated transformation

2.3.1 The engineered A. tumefaciens *Ti-plasmid*

The wild-type Ti-plasmids are not suitable for being gene vectors because the T-DNA has oncogenes that cause tumor growth in host cells. Construction disarmed Ti-plasmid by deletion of oncogenes, and opine biosynthetic coding gene makes the plasmid non-oncogenic, the 25 bp of each repeat border sequence remaining. The promise of AMT relies on the substitution of T-DNA by any foreign DNA sequence so that *A. tumefaciens* can be a "vehicle" in insertion gene(s) of interest that are transmissible to the progeny [18, 43]. Selectable marker genes are inserted into the T-DNA in order to distinguish the transformed cells from normal cells, tandem with experimental transgenes. Some herbicide resistance markers that are commonly used are phosphinothricin, chlorsulfuron, sulfonamide, and glyphosate. The insertion of bacterial selectable marker, such as trimethoprim, streptomycin, spectinomycin, sulfonamides, bleomycin, hygromycin, kanamycin, neomycin, or gentamicin, evaluates the uptake engineered plasmid to bacterial cell [8].

Disarmed Ti-plasmid is difficult to be manipulated in vitro due to its large size. Since the virulence genes may act in *trans* on the T-DNA sequences in the same cell, it was transferred to small independently plasmid that has origin of replication for *Agrobacterium*, called "helper vector." In addition, the elimination of virulence gene causes the Ti-plasmid to accommodate longer transgene. Furthermore, scientist constructs the binary vector, so called because it is designed to be replicate in multiple host (*E. coli* and *A. tumefaciens*). The binary vector consist of left and right borders, origin of replication for multiple host, selectable marker genes, and gene(s) of interest. This engineered plasmid is now used in plant genetic transformation.

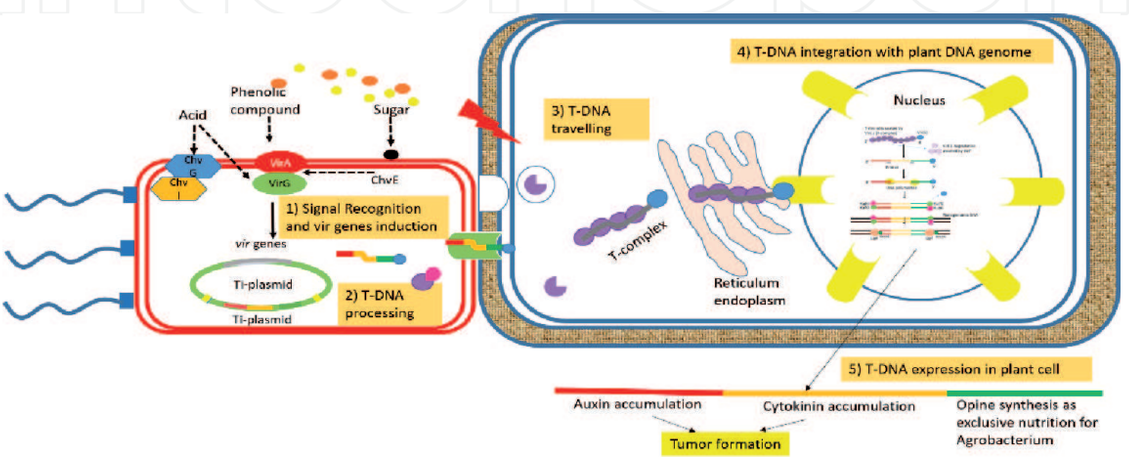

Figure 6.
Overall mechanism schematic of AMT.

2.3.2 Agrobacterium*-mediated transformation protocol*

AMT is a general method for genetic modification in many plant species. It is because it allows efficient insertion of stable, un-rearranged, single-copy sequences into plant genome. Two critical points for successful transformation were indicated: the use of actively dividing embryonic callus cells derived from the scutella of mature seeds as the starting material and the addition of a phenolic compound, acetosyringone, in the cocultivation steps [44, 45]. Moreover, Cheng et al. reported that there is no significant difference in the transformation efficiencies between immature embryos, pre-cultured ones, and embryogenic callus [46].

Several protocols of AMT have been reported either in Monocotyledoneae or Dicotyledoneae plants. In general, *Agrobacterium*-based method was used for transgenic plant. The protocol consists of seven steps, which can be briefly summarized as follows: stage (I) preparation of sterilize seed or samples and inoculum; stage (II) explant preparation, infection, and cocultivation with *A. tumefaciens*; stage (III) selection; stage (IV) regeneration; stage (V) acclimatization and molecular identification of T_0; stage (VI) cultivation and self-crossing of T_0; and stage (VII) T_1 plant analysis (**Figure 7**).

2.3.2.1 Preparation of sterilize seed or samples and inoculum

Immature embryo was a common sample that is used for transformation. Some experience reported that transformation efficiencies depend on the genotype or variety [47, 48]. To obtain the immature embryo, seed is planted in sterile media (such as husk, compost, mixed soil, etc.) and grown in environmentally controlled growth rooms. Immature embryo is harvested after pollination, but it depends on the species. On the other hand, callus is also produce from hypocotyl or cotyledon explants.

Inoculum is prepared by culture *A. tumefaciens* strain that contains appropriate antibiotics. The bacteria are grown with a loop and suspend in specific media such as LB, LS-inf-AS medium. Inoculum should be prepared fresh. In some

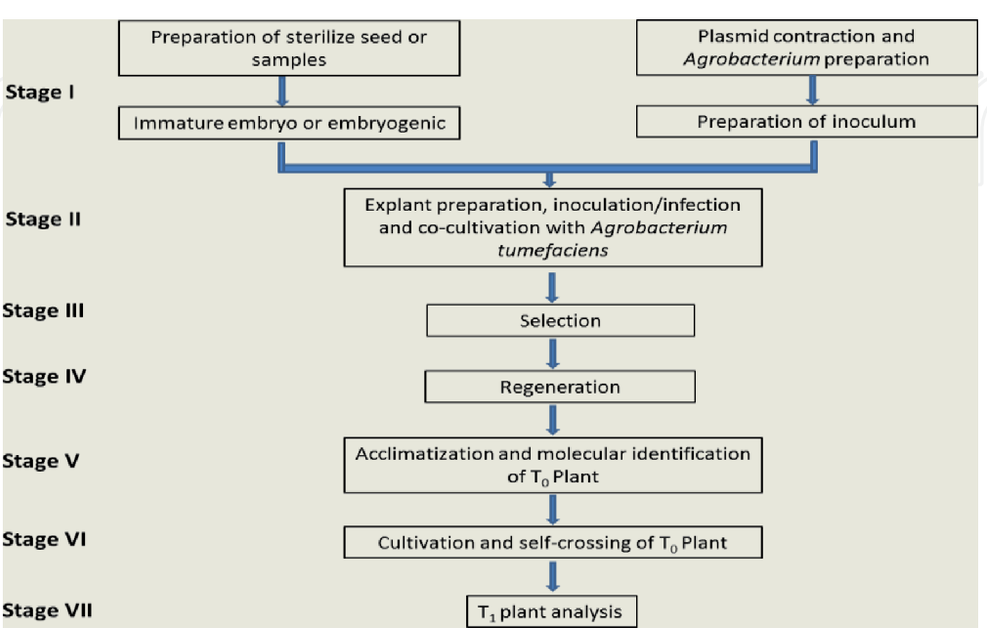

Figure 7.
The protocol of Agrobacterium*-mediated transformation.*

cases, the growth of *A. tumefaciens* in liquid culture before transformation is not necessary. On the other hand, the *A. tumefaciens* preparation and plasmid construction are able to follow the commercial plasmid and *A. tumefaciens* preparation.

2.3.2.2 Explant preparation, infection, and cocultivation with A. tumefaciens

The embryonic, immature embryo or callus is able to be used as the explants. The explant should be sterilized before the infection or transformation process. Both of the suspension of the embryos and bacteria are transferred to the new plate or empty petri dish. After wrapping the petri dish, the cocultivation step follows by incubating in the dark at 24–29°C for 2–7 days, depending on the species. The *A. tumefaciens* concentration and the infection time were found to be important factors in preventing explant turning necrosis and improving transformation efficiency. We can follow some recommendation from several protocols for specific species.

2.3.2.3 Selection

Selection is one of the critical factors in the success of transformation. The process of selection can be occurred after the stage of transformation, regeneration, or on T_0 and T_1 plant. Moreover, antibiotic selection is one of the methods to check the successful transformation. In addition to antibiotic selection, PCR should be used to confirm the presence of the targeted transgene in each transformant at each generation.

2.3.2.4 Regeneration

Regeneration of transformed plants occurred after the proliferation. The shoots grown out from the proliferation explants is pulled out and placed in a new medium. Generally, the regeneration stage is following the in vitro propagation methods which are divided into shoot regeneration and selection, cut and recut shoot regeneration, and root regeneration.

2.3.2.5 Acclimatization and molecular identification of T_0

The acclimatization of T_0 can occur after the roots grow strongly. The transgenic T_0 plant can be grown directly in a soil or mixed media under the environmental controlled or green house.

2.3.2.6 Cultivation and self-crossing of T_0

The primary transformant (T_0) was obtained by *A. tumefaciens* transformation. After the study of transgene inheritance in successive generation, T_1 seeds are produced by self-crossing pollination of the primary transformant (T_0). The process of cultivation occurred in the greenhouse. The seed of primary transformant (T_0) is harvested from T_0 plant.

2.3.2.7 T_1 plant analysis

T_1 plant is the plant that obtained from the harvested seed of T_0 plant. The analysis of T_1 plant are referring to the morphological or physiological expression of the specific gene which is inserted.

3. Conclusions

Currently, AMT become a common tool for genetic engineering. The mechanism of AMT was affected by several factors and also depends on the species. On the other hand, several *Agrobacterium* and plasmid have been commercialized, and it was accelerating plant breeding technology. This chapter gave brief information related to AMT mechanism, including the history of crown gall disease caused by *A. tumefaciens*, the natural pathogenesis of *A. tumefaciens,* and the general protocol of *Agrobacterium*-mediated transformation in plants.

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