Chapter 15 Exploitation of *Agrobacterium tumefaciens*

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15.1 Introduction[1]

During invasion of wounded plants, soil agrobacteria transfer a defined segment of their Ti and Ri plasmids into the plants. The transferred DNA, termed T-DNA, is integrated into the plant nuclear genome. Genes encoded by Ti and Ri plasmid T-DNAs are expressed in plants and confer the synthesis of plant growth factors as well as sugar and amino acid derivatives, called opines. Expression of T-DNA genes *iaa*M, *iaa*H, and *ipt* (see Hohn, Chap. 15, this Vol.) leads to production of phytohormones, auxin and cytokinin that induce proliferation of transformed cells to form undifferentiated tumors, crown galls. In contrast, cells transformed by *rol* A, B and C genes of Ri plasmid T-DNAs differentiate to hairy roots. While genetic analysis of the function and expression of these T-DNA genes provided a key for better understanding of various aspects of hormonal regulation and cell differentiation, studies of the T-DNA transfer and integration contributed directly to the development of T-DNA-based transformation vectors and transgenic plant technology. How T-DNA gene vectors are exploited to gain more insight to molecular biology of plants is the focus of this chapter.

15.2 T-DNA, a Universal Tool of Plant Molecular Biology[2]

Development of modern plant gene vectors derived from the T-DNA was based on the observations that:

- Foreign DNAs inserted in the T-DNA are accurately transferred from *Agrobacterium* to plants.
- T-DNA-encoded genes are not required for the transfer of the T-DNA.
- *vir*ABCDE and G operons of Ti and Ri plasmids encode an inducible DNA processing system that mediates the recognition of direct 25 bp repeats located at the borders of T-DNA segments of Ti and Ri plasmids.
- The function of these 25 bp repeats is analogous to that of conjugational transfer origins of bacterial plasmids, thus:
- Any DNA sequence flanked by these 25 bp end-repeats can be transferred from *Agrobacterium* into plants and:
- Separation of the T-DNA and virulence genes does not influence the transformation process.

Certain requirements are essential for T-DNA vector designs. Since *Escherichia coli* is used as a host for construction of vectors with transferable T-DNA segments, T-DNA vectors should contain selectable markers for both *E. coli* and *Agrobacterium* as well as a replicative or integrative maintainance function. Vectors made in *E. coli* can be transferred to *Agrobacterium* by transformation, electroporation, or conjugation. In the latter case, DNA sequences recognized by a suitable plasmid mobilization system have to be added to the constructs. Finally, for selection of transformed plant cells, a marker gene selectable in plants should also be inserted in the T-DNA. Notwithstanding its relatively short history, a great number of different T-DNA-based vectors have already been constructed along the following two lines.

15.2.1 Recombination-Based Ti Plasmid Vectors[3]

Recombination-based vectors are Ti or Ri plasmid derivatives from which some or all T-DNA on-

cogenes have been removed. Foreign DNAs are inserted into the T-DNA by homologous recombination using a target DNA sequence that is homologous to commonly used bacterial antibiotic resistance genes or to diverse *E. coli* cloning vectors and located within the T-DNA borders of Ti or Ri plasmids. The same target DNA is also part of a second plasmid, referred to as "intermediate vector." Foreign genes are subcloned into the target DNA of intermediate vectors and transferred from *E. coli* to *Agrobacterium* by plasmid conjugation or mobilization. A variety of genetic methods have been developed to select or screen for the integration of foreign DNA into the T-DNA using single or double cross-over recombination and diverse replicative or nonreplicative vectors in agrobacteria. To identify T-DNA-transformed cells, selectable and/or screenable marker genes are provided either within the T-DNA or in the intermediate vector carrying the cloned foreign DNA. Recombination-based vectors are commonly referred to as "armed" or "disarmed" plasmids depending whether or not their T-DNAs contain oncogenes. Apart from special applications, the "armed" Ti plasmids are no longer used as vectors. In contrast, "armed" Ri plasmid are frequently employed to obtain transgenic plants from hairy roots. Disarmed Ti plasmid vectors, such as pGV3850 or the SEV system, for example, are still in use today. These vectors are based on single cross-over recombination within a target site located inside the T-DNA borders that results in an intermediate vector-Ti plasmid cointegrate (Fig. 1a).

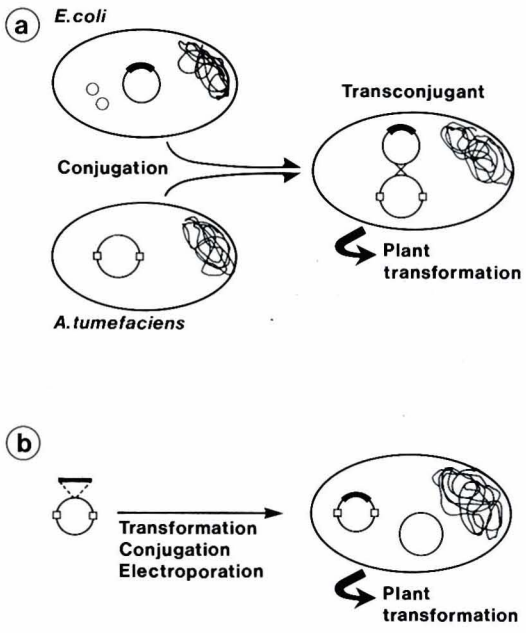

Fig. 1a, b. T-DNA plant gene vectors. **a** Insertion of cloned DNA into recombination-based vectors by single crossover. Foreign DNA is cloned into an intermediate vector and transferred into *E. coli* strain containing helper plasmids that promote conjugational transfer into *Agrobacterium*. The intermediate vector is unable to replicate in *Agrobacterium* but maintained by recombination with the T-DNA of Ti plasmid vector. *Open boxes* refer the T-DNA borders and the *thick line* for foreign DNA. **b** Use of binary vectors. Foreign DNA is cloned into the T-DNA of binary vectors and transferred into *Agrobacterium* that contains a T-DNA-less virulence helper Ti or Ri plasmid

15.2.2 Binary Vectors[4]

Binary vector systems consist of two elements: a helper Ti or Ri plasmid providing virulence functions, and a cloning vector containing bacterial and plant selectable marker genes and cloning sites flanked by T-DNA end-repeats (Fig. 1b). Most binary cloning vectors were derived from the well-characterized wide-host-range plasmid RK2 that can efficiently be mobilized between *E. coli* and *Agrobacterium*. An advanced binary system, referred to as PCV (*P*lant *C*loning *V*ector) system, is based on a plant vector cassette (Fig. 2a) that carries only the ori_V (replication origin) and ori_T (origin of conjugational plasmid transfer) regions of plasmid RK2 in combination with diverse T-DNAs. ori_V and ori_T are active only when *trans*-acting RK2 functions *trf*a (replication function) and *tra* (plasmid transfer) are expressed in the same cell. To provide helper functions for replication and conjugation of PCV vectors, defective RK2 plasmid derivatives were inserted into the chromosome of *E. coli* and *Agrobacterium* hosts or into a T-DNA-less Ti plasmid pMP90RK. In *Agrobacterium* hosts carrying chromosomal insertions of *trf*a and *tra* RK2 genes, any Ti or Ri plasmids can be used as virulence helper. The presence of RK2 *tra* func-

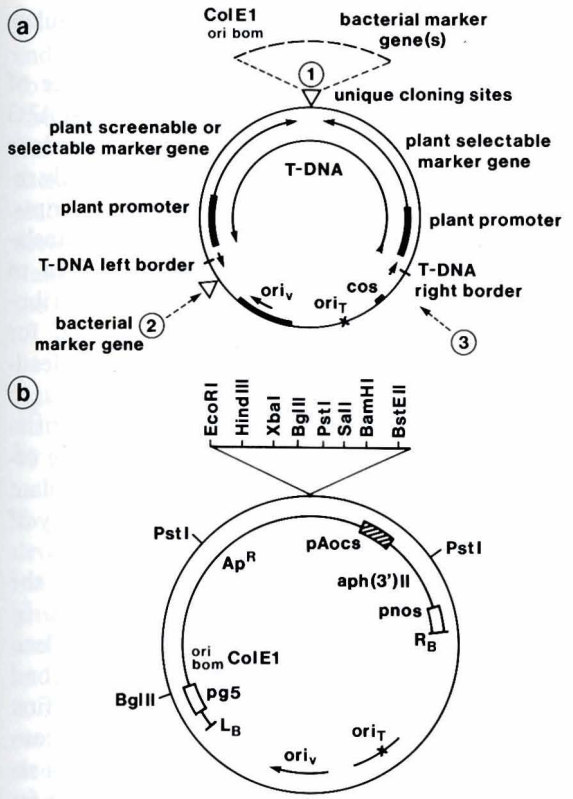

Fig. 2a, b. Plant vector cassette for construction of binary vectors. **a** Schematic design of a plant vector cassette that contains cloning sites, plant selectable and screenable marker genes within the T-DNA carried by an $ori_V - ori_T$ basic RK2 replicon. *Arrows 1, 2,* and *3* are unique cleavage sites for introduction of additional elements into the cassette. **b** Plasmid pPCV002, a prototype of simple binary plant gene vectors. *pnos* nopaline synthase promoter; *aph(3')II* coding sequence of kanamycin resistance gene of transposon Tn*5*; *pAocs* polyadenylation signal of octopine synthase gene; Ap^R ampicillin resistance gene; *ori* and *bom* of *ColE1* replication and conjugational transfer origins of plasmid ColE1; *pg5* promoter of T-DNA gene 5; L_B and R_B left and right ends of the T-DNA

tions in both hosts results in a ping-pong conjugation of binary vectors that helps to test the stability of T-DNA constructs before plant transformation. Since ori_V plasmids are maintained at a low copy number, most PCV vectors contain also a multicopy ColE1 replicon to facilitate the cloning in *E. coli* (Fig. 2b).

15.3 Gene Transfer and Transgenic Plant Technology[5]

One of the first observations during the pioneering experiments with wild-type T-DNAs showed that although T-DNA genes of agrobacteria were active in plants, other bacterial, yeast and animal genes inserted via T-DNA into the plant genome were not transcribed. It thus became evident that T-DNA-encoded genes must contain all signals necessary for transcription in plants. To achieve the expression of bacterial genes in plants, transcription promoter and terminator sequences of the nopaline synthase (*nos*) gene of the T-DNA were used first to construct chimeric genes with the coding sequence of neomycin phosphotransferase [*aph*(3')II] and chloramphenicol acetyltransferase (*cat*) genes of transposons Tn*5* and Tn*9*. Expression of these chimeric antibiotic resistance genes could be followed by simple enzyme assays and permitted the selection of transformed plant cells. This opened the way for the development of transformation techniques using co-cultivation of agrobacteria with plant protoplasts, leaf-disks, stem and root explants; direct DNA uptake, protoplast fusion with charged liposomes, macro- and microinjection or bombardement with microprojectile-bound DNA. From cells transformed with disarmed T-DNA vectors fertile transgenic plants were regenerated that transmitted the introduced genes to their offsprings in a Mendelian fashion. Since little was known about gene expression in plants, most studies focused initially on the regulation of the expression of plant genes in foreign genetic background. Exploration of maize alcohol dehydrogenase and sucrose synthase, pea Rubisco and Cab, soybean lectin, and leghemoglobin genes, etc. in tobacco indicated that regulation of the transcription in response to environmental-, hormonal-, tissue-specific and developmental stimuli, is similar in diverse plant species. These studies, together with the analysis of the expression of plant virus genomes, contributed basic information and useful plant promoters to achieve regulated expression of foreign genes in plants.

15.4 Gene Expression in Plants[6]

Expression cassettes consisting of characterized plant promoter and polyadenylation signal sequences separated by suitable cloning sites were constructed using transcriptional regulatory elements of T-DNA genes (octopine, nopaline, mannopine synthase genes, etc.), 35S and 19S RNA genes of Cauliflower Mosaic Virus (CaMV) and plant genes, such as the light-regulated SSU or Cab. These cassettes were inserted into the T-DNA of binary vectors in linkage with diverse chimeric antibiotic resistance genes, to clone and express various foreign genes in plants. Certain empirical rules established during these studies indicated that:

- The active core of plant promoters contains a TATA-box followed by a transcription initiation site at 40 to 100 bp 3′-downstream.
- 5′-Upstream of the core ("minimal") promoter region are *cis*-regulatory elements located that modulate the level of transcription in a quantitative (i.e., SV 40 type positive enhancers) or qualitative (i.e., tissue-specific enhancers and silencers) fashion by interaction with *trans*-acting regulatory proteins (transcription factors).
- Increasing the copy number of certain enhancers results in a proportional increase in the level of transcription.
- Many promoters contain AT-rich DNA sequences (e.g., AT-boxes in heat shock, lectin, leghemoglobin, etc. promoters) in the 5′-upstream region that probably mediate interaction with common nuclear matrix proteins (e.g., HMG class) regulating conformational changes of active chromatin.
- From genes transcribed by RNA polymerase II polyadenylated transcripts are synthesized. Signals for polyadenylation (i.e., AATTAA/T) are located 3′-downstream of the translational stop codon. The distance between the stop codon and the polyadenylation site influences the steady-state level of transcripts.
- Most plant genes contain introns, therefore the derived primary transcripts undergo splicing. It is apparent that viroids that cause serious plant diseases affect splicing. The length of introns (especially that of the first intron) may regulate the level of gene expression.
- Ribosomes bind to the 5′ leader sequence of mRNAs and initiate translation at the first ATG codon. The presence of nonframe ATG codons in chimeric gene constructs therefore greatly reduces the translation of foreign transcripts. Kozak's rule for efficient initiation of translation at ATG/G starts can also be applied to plant genes. A consensus sequence for ribosome-binding sites is not yet established for plant genes. However, it was observed that leader sequences of certain viral RNAs (i.e., tobacco or alfalfa mosaic virus) can be used as translational enhancers. Alteration of the coding sequence of foreign genes according to plant codon usage may also increase the efficiency of translation.
- Ribosomes do not necessarily dissociate at the stop codon during scanning the transcript. In the case of a dicistronic transcript this may lead to initiation of the translation of the second coding region. Since the translation of the first coding region is more efficient, chimeric constructs carrying a foreign gene as first, and a selectable marker gene as a second cistron can facilitate an increased production of foreign proteins in plants.
- N- and C-terminal sequences of plant proteins may be recognized by diverse processing mechanisms that mediate the targeting of the proteins to cellular compartments, such as chloroplast, mitochondrium, peroxisome, or endoplasmic reticulum. A fusion of corresponding DNA sequences to foreign genes can successfully be applied for targeting foreign proteins to plant organelles.

The use of T-DNA-based expression vectors resulted in a burst of applications. New selectable markers, such as hygromycin, bleomycin, gentamycin, streptomycin, and methotrexate resistance genes were constructed by expression of diverse bacterial genes and a dihydrofolate reductase gene from mouse. Reporter enzyme systems providing sensitive assays for monitoring gene expression in vitro, in vivo, and by histological methods were developed by expression of β-glucuronidase (*gus*), β-galac-

tosidase (*lacZ*) and light-producing luciferase (*luc* and *lux*) enzymes from fireflies and *Vibrio harveyi*. To engineer plants resistant to or tolerant of insects, viruses, or herbicides, the crystal toxin protein of *Bacillus thüringiensis*, virus coat proteins, antisense virus transcripts and herbicide-resistant or inactivating enzymes, such as EPSP-synthase, acetolactate synthase, and phosphinotricine acetyltransferase, were expressed in transgenic plants with the help of T-DNA vectors.

15.5 Studying Gene Regulation by Chimeric Plant Promoters[6,7]

To understand how environmental, hormonal, or developmental signals are transmitted through various signal transduction pathways to individual genes, the interaction of *cis*-regulatory elements and transcription factors is studied using promoter and enhancer test T-DNA vectors. These vectors contain a reporter gene, the activity of which can easily be followed in transformed protoplasts (transient expression assays), or in diverse tissues of transgenic plants (transformation assays). Promoter derivatives carrying successive deletions in the 5'-upstream region are linked to a promoterless reporter gene. Alternatively, the reporter gene is linked to core promoter region of a known gene (i.e., minimal promoter region of *nos* or CaMV 35S RNA promoters), upstream of which suitable cloning sites are provided for insertion of putative *cis*-regulatory DNA sequences. This approach is used for functional analysis of individual promoter elements, as well as for studying possible interactions between promoter elements and transcription factors. Promoter analysis of light-regulated, heat shock, hormonally or anaerobically regulated genes revealed various DNA sequences that are targets for binding of transcription factors and/or for methylation in vivo. A complex picture emerging from these studies suggests that similar regulatory DNA sequences may be part of different promoters, thus an interaction between transcriptional factors and diverse promoter elements may mediate various and specific responses in transcription. Since promoters of all T-DNA genes are recognized by plant transcription factors that modulate their activity in a hormone-regulated and tissue-specific fashion, the T-DNA itself provides an excellent tool to gain more insight into hormone signal transduction and transcriptional regulation of plants.

15.6 Insertional Mutagenesis: a Link Between Classical and Molecular Plant Genetics[8]

From a genetic point of view, T-DNA is a unique insertion element that is integrated into the plant nuclear genome after transfer from agrobacteria, and therefore may cause insertional inactivation of plant genes. To identify T-DNA insertions in functional plant genes, a gene fusion approach was developed. A promoterless reporter gene was linked to the right border of a T-DNA, which also carried a bacterial plasmid replicon and a plant selectable marker gene. The ATG start codon of the reporter gene was either retained or deleted in order to generate either transcriptional or translational gene fusions (Fig. 3). Following selection of transformants using the selectable marker, the frequency of T-DNA-induced gene fusions was determined. In tobacco and *Arabidopsis* about 40% of all insertions resulted in transcriptional fusions, while 15 to 20% of T-DNA inserts induced translational gene fusions. Differences in the complexity and distribution of transcribed DNA sequences between tobacco and *Arabidopsis* excluded the possibility that a similar frequency of gene fusions in both plant species resulted from random T-DNA insertions. The data rather indicated that T-DNA is preferentially integrated in plant chromosomal loci that are potentially transcribed.

The gene fusion technique has the advantage that the expression of T-DNA-tagged plant genes can be followed in vitro and in vivo throughout the life cycle of plants or under influence of various external stimuli. Both transcriptional regulatory elements and coding sequences of T-DNA-tagged genes can be rescued from the nuclear DNA of transgenic plants with the help of a bacterial plasmid replicon carried by the T-DNA. Plant DNAs are digested with a restriction endonuclease that

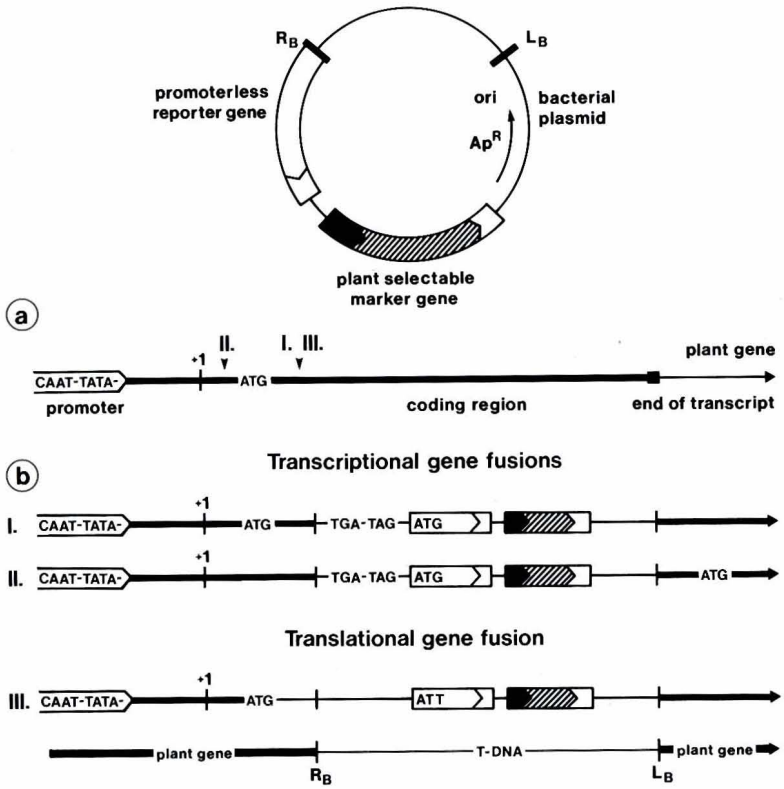

Fig. 3a, b. Insertional mutagenesis with T-DNA gene fusion vectors. **a** Schematic structure of T-DNA gene fusion vectors. A promoterless reporter gene is linked to the right border (R_B) of the T-DNA that also contains a bacterial plasmid replicon (Ap^R and *ori*) and a plant-selectable marker gene joined to the left T-DNA border (L_B). Underneath the T-DNA vector a hypothetical plant gene is depicted with promoter region (CAAT-TATA), transcription start (+1), translation start (ATG) and transcription termination site. *Arrows I, II,* and *III* indicate T-DNA integration sites. **b** Principle of T-DNA-induced transcriptional and translational fusions. *Line I* shows a T-DNA insert in the coding region of a plant gene, while in *line II* a T-DNA insert is depicted after integration in the transcribed but untranslated leader region of a plant gene (located between +1 and ATG). Due to the presence of stop codons in all reading frames upstream of the ATG codon of the reporter gene, *T-DNA insert in I* results in a dicistronic transcript. The *first coding region* of this transcript encodes a truncated plant protein, while the *second one* encodes the reporter enzyme. *T-DNA insert in II* leads to a monocistronic transcript starting at +1 position of the plant gene and terminating at the polyadenylation site of the reporter gene within the T-DNA. From both *I* and *II* transcriptional gene fusions an intact reporter protein is synthesized. *III* shows a T-DNA insert with a promoterless reporter gene that does not contain ATG translational start codon. T-DNA insertion in the coding region of a plant gene may lead to in-frame fusion between plant and reporter genes. This results in the translation of a fusion protein that consists of an N-terminal plant protein domain and a C-terminal reporter enzyme domain

has no recognition site within the T-DNA, circularized by self-ligation and transformed into *E. coli*, where the T-DNA and flanking plant DNA is recovered as a plasmid. Plant DNA sequences rescued in linkage with the promoterless reporter gene of the T-DNA are dissected and inserted into promoter and enhancer test vectors for further studies of the regulation of the identified plant promoters, or used as probes to isolate wild-type alleles of the tagged genes from genomic and cDNA libraries.

Arabidopsis thaliana, a plant with excellent genetics, became a model for plant molecular biology in general and for insertional mutagenesis studies in particular. A search for T-DNA-induced mutations in *Arabidopsis* showed that while insertions in diverse genes can be obtained at a fairly high fre-

quency by gene fusions, only a portion of these mutations result in morphological alterations or other visible mutant phenotypes. Selectable antibiotic resistance genes carried by T-DNA gene-tagging vectors provide suitable markers for mapping of the induced mutations even in the absence of visible mutant phenotypes. A large number of mutations induced by irradiation or by chemical mutagenesis (e.g., EMS) is also available to test allelism with the T-DNA-induced mutations. Wild-type alleles cloned on T-DNA plant gene vectors (or corresponding cDNAs cloned in T-DNA-based expression vectors) are used for complementation of the induced mutations. Insertional mutagenesis in plants thus offers nearly as much flexibility as similar approaches in bacteria or in yeast. Recent isolation and characterization of *chlorata* (*ch*-42), *agamous* (*ag*), *apetala* (*ap*-2), and *glabrous* (*gl*-1) genes of *Arabidopsis* demonstrated that T-DNA-tagging is an efficient approach to identify genes regulating basic processes, such as photosynthesis or differentiation in plants.

15.7 Outlook

Application of T-DNA gene vectors in basic and applied plant science is virtually unlimited. As in the past, studies of the mechanism of T-DNA transfer are expected to facilitate further improvement of T-DNA vectors and plant transformation systems. Some rules established for *Agrobacterium*-plant interaction may lead to the discovery of new forms of interspecies gene transfer. Exploiting the growing knowledge on regulation of plant gene expression may help to achieve cell type-, tissue-, developmental stage-specific; hormone-, light-, heat-, gravity- or drought-induced; osmotically or chemically regulated expression of foreign or modified plant genes in a great variety of plants. Studies of transcription factors and corresponding genes will give more insight to the regulation of gene expression during plant development. T-DNA genes, exploited to alter plant morphology and development genetically, may also be applied as experimental tools in studies of hormone signal transduction, cell division, or organ differentiation. Further development of insertional mutagenesis techniques involving the use of plant transposable elements may facilitate simple identification of new plant genes. T-DNA-induced gene fusions to suicide marker genes, that cause cell lethality, might help the isolation of genes that are expressed only in certain cell types or during certain stages of development. Alternatively, T-DNA-mediated insertion of strong promoters into the plant genome may be used for dominant activation of life defense genes involved in tolerance to drought, heat, salinity, or toxic chemicals. Transformation with T-DNAs carrying segments of the plant genome may result in recombination with homologous chromosomal loci that can be exploited in development of site-specific mutagenesis techniques for plant. Last but not least, combination of T-DNAs with telomeric elements of plant chromosomes might facilitate the identification of centromeric and autonomously replicating (ARS) DNA sequences to achieve chromosome engineering in plants.

15.8 Summary

The properties of T-DNA transfer and integration into the plant genome make it the system of choice for engineering of stably transformed plants. The versatility of T-DNA vectors that exploit the natural gene transfer process between agrobacteria and plants is such that T-DNA can be used for a variety of purposes other than simple gene transfer into plants. Over the years the unique ability of *Agrobacterium* to transfer the T-DNA to the plant cell has provided us with means to study plant-bacterial interaction, gene transfer and control of gene expression, differentiation, and development of plants. With the T-DNA vectors and marker genes currently available it is predictable that the collection of various approaches using T-DNA as a tool to investigate plant biology is far from being exhausted.

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