PLANT TRANSFORMATION TECHNOLOGIES

Neal Stewart, Alisher Touraev, Vitaly Citovsky and Tzvi Tzfira

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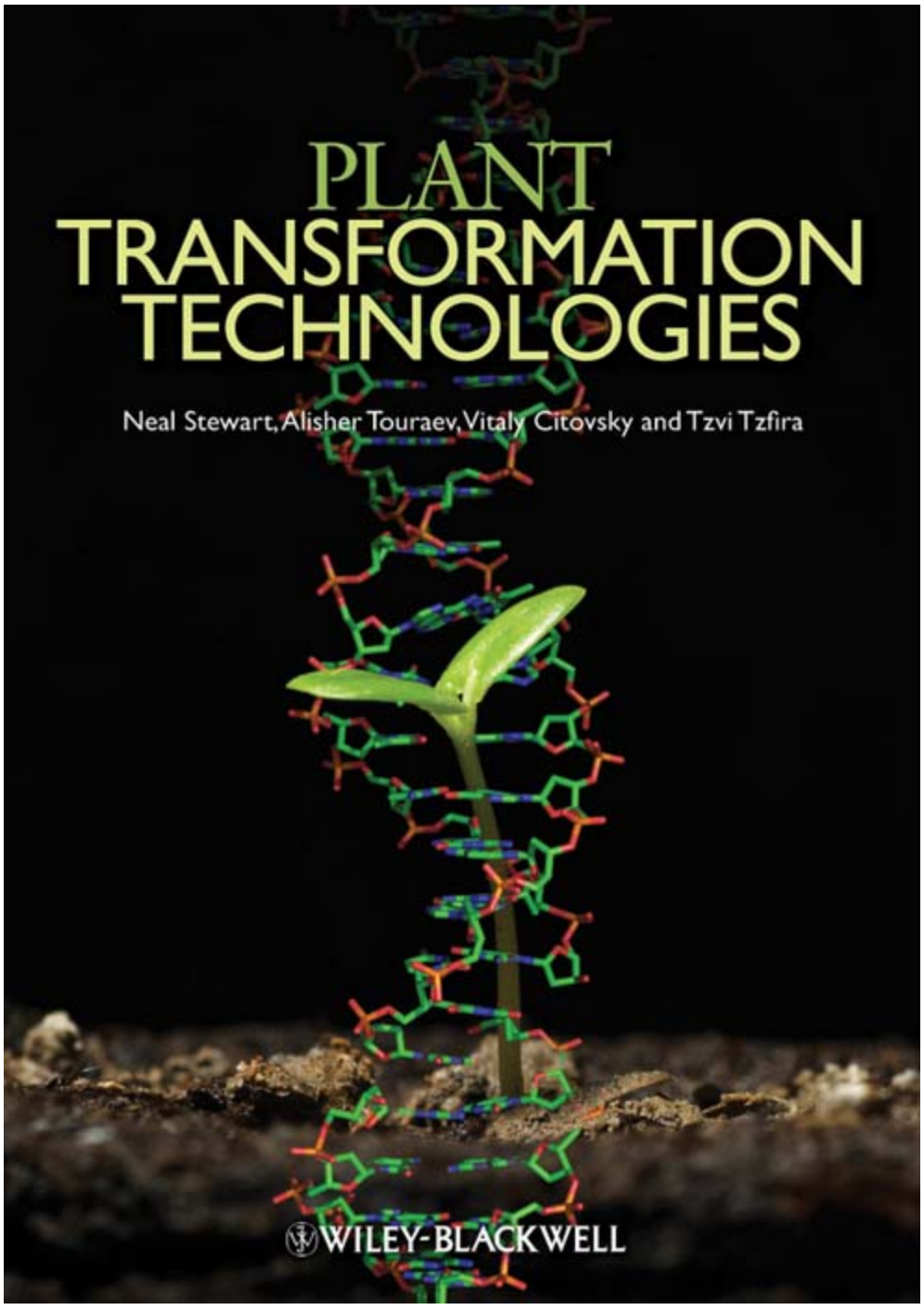
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Preface

From the early 1980s to the present, biotechnologies have yielded, with a great degree of success, the ability to genetically transform a wide variety of plant species. These plant transformation technologies have literally changed the face of agriculture and plant biology. Invariably, whenever any discussion ensues about developments in plant biotechnology with one of the pioneers of the field, especially in industry, we often hear stories of life on the frontier and excitement of breaking new ground in the 1980s. However, “all the really exciting research has already been done,” we are told.

In February 2007, the International Conference on Plant Transformation Technologies was held in the beautiful city of Vienna, Austria. Over 300 participants from 47 countries learned that there was plenty of exciting plant biotechnology research in progress. In the course of the conference, research was presented by groundbreaking researchers, such as Dr. Mary-Dell Chilton, who demonstrated that there was still plenty left to do in plant transformation. Indeed, the editors of this volume were so convinced of this fact that we decided to invite many of the presenters and others to contribute to this volume, which gives a taste of the excitement we felt at the conference.

Thus, borrowing from the title of the Vienna conference, this book covers many topics on the cutting edge of transgenic plants. Of course, we are just really beginning to understand how the original methods of plant transformation, those using *Agrobacterium tumefaciens* and particle bombardment, truly work. Several chapters are reviews and updates on these technologies, and pertinent genomic interactions among organisms. Several other chapters present new vector systems and describe plasmids

that we think will make a huge impact on making plant transformation more accessible for a wider variety of species. Indeed, this theme of accessibility and efficiency is prominent in chapters written by experts in their respective fields. It seems that plant biotechnology is becoming more egalitarian to more types of scientists—from molecular biologists, of course, but now accessible to ecologists and environmental scientists. Transformation technologies have played a huge role in this movement.

The upcoming frontier of challenges for biotechnology deals with issues of transgene precision and regulations. There is now a premium on minimizing the amount of transgenic DNA in plants while maximizing stability of gene expression and trait performance. For regulatory and commercial purposes, characterized integration of transgenes in known locations and precise expression patterns are viewed as helpful to target traits in predictable ways. Indeed, genetic engineering is better than genetic tinkering and transgenic technologies that maximize precision have increasing value in the world marketplace. Chapters were written on methods to gain higher precision of transgene integration and marker-free transformation. However, transgene silencing, either on purpose or by accident, which is one of the key research breakthroughs in recent years, is also important.

As we look to the future of plant biotechnology, we can envision new methods of transformation that could be more efficient and widen the breadth of species and genotypes that can be manipulated. Methods such as those using calcium alginate micro-beads and those targeting alternative cells, such as pollen grains, could be game-changers. In the future, nanotechnologies alone or coupled with established methods such as *Agrobacterium*-mediated transformation will likely be important contributors to biotechnology as well. The ever-expanding color pallet of

fluorescent proteins and pigmented proteins will also likely be very useful as transformation tools and indicators of expression from the subcellular to ecological levels. Looming ahead are potentially greater regulatory hurdles and demands for safety beyond that required of traditional technologies. Thus, plant transformation technologies will be invented to serve dual purposes of increasing trait and crop value as well as biosafety. We believe that the most exciting times lie ahead in plant biotechnology as we plan for the second Plant Transformation Technologies conference held in Vienna February 2011.

We thank all the authors who contributed to the conference and the book by freely sharing their knowledge and expertise. We appreciate the work of Justin Jeffryes and his team at Blackwell for commissioning and working with us during our unpredictable schedules. We also thank Ronald D'Souza for his work at the proofing stage. The editors express special thanks to Ms. Julia Szederkenyi for her great assistance in organizing chapters, formatting and contributing to the final form as well as Jennifer Young Hinds for her help. A volume such as this could never be produced without the work of so many people that we have not acknowledged by name, but we wish to express our heartfelt thanks to each one.

Section 1

Agrobacterium-Mediated Transformation

1

Host Factors Involved in Genetic Transformation of Plant Cells by Agrobacterium

Benoît Lacroix, Adi Zaltsman, and Vitaly Citovsky

Introduction

Agrobacterium tumefaciens and several other species of the *Agrobacterium* genus possess the unique ability to transfer a DNA segment from a specialized plasmid (tumor inducing or Ti plasmid in the case of *A. tumefaciens* and hairy root inducing or Ri plasmid for *Agrobacterium rhizogenes*, the two main species of pathogenic *Agrobacterium*) into a host plant cell. This feature is widely used in plant biotechnology, and *Agrobacterium* is, by far, the most important tool employed to produce transgenic plants (Newell 2000). Not surprisingly, the biology of *Agrobacterium* and its interactions with host plant have been the subject of numerous studies in the past three decades (for recent reviews, see Gelvin 2003; Citovsky *et al*. 2007; Dafny-Yelin *et al*. 2008).

In brief, the main steps of host genetic transformation mediated by *A. tumefaciens* are the following. The induction of *Agrobacterium*'s virulence machinery results in expression and activation of the virulence genes (*vir* genes) (Stachel *et al*. 1985b, 1986; McLean *et al*. 1994; Turk *et al*.

1994; Lee *et al*. 1996). This first step mobilizes a single-stranded DNA segment from the Ti or Ri plasmid. This segment of transferred DNA (T-DNA), delimited by two 25-bp direct repeat sequences known as left and right borders (LB and RB) (Peralta and Ream 1985; Wang *et al*. 1987), is termed the T-strand, and it represents the substrate of DNA transfer to the host cell. VirD2, associated with VirD1, forms a nuclease able to excise the T-strand by a strand-replacement mechanism, at the completion of which VirD2 remains covalently linked to the 5′-end (RB) of the T-strand (Ward and Barnes 1988; Young and Nester 1988; Durrenberger *et al*. 1989; Pansegrau *et al*. 1993; Jasper *et al*. 1994; Scheiffele *et al*. 1995; Relic *et al*. 1998). This VirD2–T-DNA complex is then translocated into the host cell cytoplasm by a mechanism relying on the VirB/VirD4 secretion system (Zupan *et al*. 1998; Vergunst *et al*. 2000; Christie 2004). The 11 proteins encoded by the VirB operon together with the VirD4 protein form a type IV secretion system, similar to the system allowing plasmid exchange by conjugation between bacteria. The type IV secretion system consists of a protein complex, spanning *Agrobacterium* internal membrane, periplasm and external membrane, and of an extracellular appendage, termed the T-pilus, composed mostly of VirB2 molecules forming a hollow channel (Christie *et al*. 2005). The VirB/VirD4 secretion system mediates the export of the VirD2–T-DNA complex out of the bacterial cytoplasm, and likely plays a role in its entry in the host cell. This secretion system is also required for the export of several *Agrobacterium* virulence proteins, that is, VirD5, VirE2, VirE3, and VirF, via their C-terminal secretion signals (Vergunst *et al*. 2000; Schrammeijer *et al*. 2003; Vergunst *et al*. 2003; 2005; Lacroix *et al*. 2005). There, the T-DNA–VirD2 complex is packaged by the single-stranded DNA-binding protein VirE2 (Christie *et al*. 1988; Citovsky *et al*. 1989; Sen *et al*. 1989). The resulting helical structure, called the T-complex, with the help of several

bacterial and host proteins, is then imported into the host cell nucleus, targeted to the host chromatin, and ultimately integrated into the host genome (reviewed in Gelvin 2003; Lacroix *et al*. 2006a; Citovsky *et al*. 2007). The native T-DNA contains genes encoding enzymes that modify growth regulators and induce uncontrolled cell proliferation, which results in neoplastic cell growths (crown galls), and proteins mediating production and secretion of opines, amino acid, and sugar phosphate derivatives, secreted by the transformed cells and utilized almost exclusively by the *Agrobacterium* as carbon and nitrogen source (Escobar and Dandekar 2003).

The transfer of T-DNA is not sequence-specific, and any sequence of interest can be inserted between the T-DNA borders. The ability to engineer *Agrobacterium* to introduce genes of interest for plant genetic transformation is the basis of *Agrobacterium*'s use in biotechnology. The natural host range of *Agrobacterium* is very large, including most of the dicotyledonous and gymnosperm families (De Cleene and De Ley 1976). However, although the number of plant species transformable by *Agrobacterium* under laboratory conditions is always increasing (Newell 2000), in practice, producing transgenic plants efficiently is still a challenge for many plant species. Moreover, even nonplant species can be transformed by *Agrobacterium* under laboratory conditions (Lacroix *et al*. 2006b), including yeast (Bundock *et al*. 1995; Piers *et al*. 1996), various fungi (de Groot *et al*. 1998; Michielse *et al*. 2005), and cultured human cells (Kunik *et al*. 2001). This chapter focuses on numerous host plant factors that play important roles in the transformation process, from the initial interactions between *Agrobacterium* and plant cells and the activation of *Agrobacterium*'s virulence, to the integration of T-DNA into the host genome.

Plant Signals Affecting *Agrobacterium*'s Virulence Machinery

The rhizosphere is a complex and dynamic environment, where plant-associated bacteria such as *Agrobacterium* need subtle regulation systems to efficiently induce their virulence machinery (Brencic and Winans 2005). *Agrobacterium*'s virulence depends mostly on transcriptional activation of a set of virulence (*vir*) genes; this regulatory system allows the integration of environmental signals to ensure a timely expression of these genes. Moreover, the induction of virulence system obviously represents a high cost in energy for the bacterial cell, and its activation must be tightly regulated to ensure that it occurs only at the proximity of a susceptible host tissue. To this end, *Agrobacterium* harbors sensors able to recognize signals emitted by its host plants, and to activate the virulence machinery in response to these signals. The induction of *vir* gene expression in *Agrobacterium* relies on a two-component regulatory system encoded by the *virA* and *virG* genes that respond, directly or indirectly, to different plant and environmental cues (Klee *et al*. 1983; Stachel and Nester 1986). *virA* and *virG* have low basal expression, but their expression is highly inducible by a self-regulated system (Winans *et al*. 1988). The expression of other *vir* genes is virtually nonexistent in absence of induction, and it is strongly enhanced when the VirA–VirG system is activated. VirA–VirG represents a two-component regulatory system, in which VirA is the membrane-spanning sensor kinase that responds to external signals and activates the response regulator VirG by phosphorylation. Phosphorylated VirG recognizes and binds to a 12-bp long specific sequence, the *vir* box, which is present in all *vir* gene

promoters, and serves to activate transcription (Brencic and Winans 2005).

Several signals, from both host plants and the environment, can modulate *vir* gene expression (Table 1.1); these include phenolic compounds, monosaccharides, low pH, and low phosphate (McCullen and Binns 2006). Among these signals, only phenolics are absolutely required for virulence induction, whereas the other signals render *Agrobacterium* cells more sensitive to phenolics and/or enhance virulence induction levels.

Table 1.1 Plant and environmental signals that influence *Agrobacterium* virulence

Phenotype	Signal	Bacterial receptors	References
Virulence activation	Phenolics (i.e., acetosyringone and related molecules)	VirA	Stachel *et al.* (1985a) and Lee *et al.* (1992, 1995)
	Monosaccharides	ChvE	Cangelosi *et al.* (1990) and Shimoda *et al.* (1990)
	Low pH	ChvG/ChvI	Melchers *et al.* (1989b) and Gao and Lynn (2005)
Virulence inhibition	DIMBOA, MDIBOA	VirA	Sahi *et al.* (1990) and Zhang *et al.* (2000)
	IAA	VirA	Liu and Nester (2006)
	Salicylic acid	VirA	Yuan *et al.* (2007) and Anand *et al.* (2008)
	Ethylene	Unknown	Nonaka *et al.* (2008b)

Phenolic Compounds Activating Agrobacterium's Virulence

Initially, during the analyses of plant cell exudates, a single phenolic compound, acetosyringone (3,5-dimethoxyacetophenone) was identified. It was present at elevated concentrations and able to induce *vir* gene expression even in the absence of the plant cells (Stachel *et al*. 1985a, 1986; Bolton *et al*. 1986). Since then, more than 80 related phenolics, including glycoside derivatives (Joubert *et al*. 2004), have been shown to act as *vir* inducers with variable efficiency (Melchers *et al*. 1989a; Palmer *et al*.

2004). These studies revealed that all *vir*-inducing molecules share common structural features that enable this family of chemicals to interact with bacterial receptors and to act as virulence inducers, suggesting that these molecules are recognized by a unique bacterial receptor (Lee *et al*. 1992). Whereas direct interaction between radioactively labeled acetosyringone and VirA has not been detected (Lee *et al*. 1992), genetic studies have demonstrated that phenolic inducers most likely interact directly with the linker domain of VirA, thereby activating VirA's kinase activity (Lee *et al*. 1995). Indeed, the specific range of phenolic compounds recognized by different *Agrobacterium* strains was dependent on the *virA* locus, and could be transferred from one strain to another via the transfer of *virA*.

Reducing Monosaccharides

Sugar monomers are involved in *vir* gene activation in two ways: by enhancing VirA–VirG system sensitivity to phenols and by elevating the saturating concentration of phenols for virulence activation (Cangelosi *et al*. 1990; Shimoda *et al*. 1990). In addition, the range of phenolics recognized by the *Agrobacterium vir* gene induction system increases when monosaccharides are present as they act as coinducers (Peng *et al*. 1998). Several monosaccharides, such as D-glucose and D-galactose, are coinducers (Ankenbauer and Nester 1990; Shimoda *et al*. 1990), which share minimal structural features (i.e., the presence of a pyranose ring and acidic groups), also suggesting that they are recognized by a specific receptor. The virulence response to monosaccharides indeed relies on a chromosome-encoded factor, ChvE. This periplasmic sugar-binding protein is believed first to bind monosaccharides, then to interact with the periplasmic domain of VirA, and to enhance the VirA

ability to activate *vir* gene expression (Cangelosi *et al*. 1990; Lee *et al*. 1992; Shimoda *et al*. 1993; Banta *et al*. 1994).

Low pH and Low Phosphate

Low pH (i.e., ~5.7) enhances virulence activation, and this effect is mediated by VirA (Melchers *et al*. 1989b; Chang *et al*. 1996) as well as ChvE (Gao and Lynn 2005). Low pH and low concentration of phosphate (both are frequently observed in a variety of soils) activate the *virG* expression (Winans 1990), likely by inducing another two-component regulatory system—also required for *vir* gene induction—composed of ChvG and ChvI (Charles and Nester 1993).

Production of Virulence Inducers by Plant Tissues

The presence of the *vir* gene inducers mentioned above can be associated with some characteristics of the plant cell or tissues susceptible to *Agrobacterium* DNA transfer. It is well known that wounded sites of the plant tissue are particularly susceptible to *Agrobacterium* infection (Smith and Townsend 1907), and wounding of plant tissue is thus classically used in many *Agrobacterium*-mediated plant genetic transformation protocols. Consistently, wound repair is usually associated with low pH, high activity of the phenylpropanoid pathway, and presence of monosaccharides involved in cell wall modification and synthesis (Baron and Zambryski 1995), showing that the most vulnerable sites for infection are usually associated with the presence of virulence-inducing signals. Moreover, phenolic compounds are classically secreted by plant roots in the rhizosphere, along with sugars, organic acids, amino acids, and other secondary metabolites (Walker *et al*. 2003).

Wounding is not absolutely required for infection (Escudero *et al*. 1995; Brencic *et al*. 2005); thus, alternative pathways