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Research Focus

Agrobacterium is not alone: gene transfer to plants by viruses and other bacteria

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***Agrobacterium*-mediated genetic transformation is the most widely used technology for obtaining the overexpression of recombinant proteins in plants. However, complex patent issues related to the use of *Agrobacterium* as a tool for plant genetic engineering and the general requirement of establishing transgenic plants can create obstacles in using this technology for speedy research and development and for agricultural improvements in many plant species. Recent studies addressing these issues have shown that virus-based vectors can be efficiently used for high transient expression of foreign proteins in transfected plants and that non-*Agrobacterium* bacterial species can be used for the production of transgenic plants, laying the foundation for alternative tools for future plant biotechnology.**

Introduction

The delivery and expression of recombinant proteins in plant cells are essential for basic plant research and plant biotechnology, and usually require the stable integration of foreign genes into the plant genome as well as the production of transgenic plants. With its extensive host range, *Agrobacterium* is the most favored tool today for plant genetic engineering. This soil bacterium possesses the natural ability to transform its host by delivering a well-defined DNA fragment, the transferred (T) DNA, of its tumor-inducing (Ti) plasmid into the host cell (reviewed in [1,2]). T-DNA integration into the host genome and expression of its encoded-native bacterial genes cause a neoplastic growth and the production of tumors on infected plants [3], marking a successful genetic-transformation event. Replacing the bacterial genes with various genes of interest does not affect the transformation process and is the molecular basis for almost all *Agrobacterium*-mediated genetic-transformation protocols [2,4]. During more than three decades of *Agrobacterium* biotechnology research, numerous genetic-transformation protocols have been established for various plant species and a wide range of *Agrobacterium*-related patents have been claimed [5]. The complex landscape comprising patent and intellectual property (IP) rights for many biotechnological processes and materials, including *Agrobacterium*-mediated genetic transformation protocols [5], creates a real obstacle to the use of biotechnology in the private and public sectors. 'Golden Rice' [6], which was developed mainly in public institutions using public funding in an attempt to provide a free biotechnological product for developing countries [7], is one such example. It took strong determination on the side of the developers (to make their product free), their good public relations, and the good will of some of the companies holding IP rights, to resolve the issue of 70 technical and intellectual property rights belonging to 32 different universities and companies [7,8]. Thus, potential obstacles to the use of *Agrobacterium*-based technologies by the general public and nonprofit institutions should not be simply overlooked, and good will from holders of IP rights should not be used as a pattern for resolving licensing issues. The ruling of The Court of Appeals for the Federal Circuit in the Madey versus Duke case [9] in favor of the plaintiff, who sued the university for violation of his patented IP rights, is an example of an academic institution allegedly having mistakenly overlooked these rights in the name of academic freedom. Interestingly, the court rejected the university argument that its nonprofit status allows it to use patented items for experimental or educational purposes. Even with the extensive range of *Agrobacterium*-related patent claims [5], most research studies working towards developing transformation protocols for new plant species, cultivars, variants [10] and even non-plant species [11] still focus on *Agrobacterium*, mostly because of the lack of alternative organisms capable of genetically transforming their hosts.

Agrobacterium can also technically be used for the transient production of recombinant proteins in plant cells, usually following the infection of host tissues. This technology, often referred to as 'agroinfiltration', has a major advantage over transgenic plants in that it does not require stable integration of the T-DNA into the host genome, and protein expression can be achieved within several days of infection. However, *Agrobacterium*-infected leaves usually express low levels of protein compared with transgenic plants because only a small fraction of the entire plant is actually transfected. Virus-based vectors offer a reasonable alternative to *Agrobacterium* as a tool for transient protein expression in plant cells because of their ability to replicate in host cells autonomously, move locally from cell to cell, and sometimes even move systematically from leaf to leaf. This can potentially result in the high yield of recombinant proteins. Interestingly, the method of choice for infecting plant cells with recombinant plus sense RNA viral vectors

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[e.g. tobacco mosaic virus (TMV)] is through recombinant cDNA molecules encoded by *Agrobacterium* T-DNAs, which are delivered to plant cells by agroinfiltration. Although simple to apply and shown to be successful in the delivery of recombinant TMV-based vectors, cloning the TMV genome on a T-DNA molecule destined to be delivered to the host cell nucleus usually results in a low infection rate. This is probably because the first viral infectious genome is produced in the host cell nucleus. This artificial step in the TMV life cycle, which in nature resides exclusively in the host cell cytoplasm, probably leads to the inefficient formation of active replicons from the primary nuclear transcript (Figure 1). Although this inefficiency can be masked by the ability of the virus to move from cell to cell, technical improvements in the viral genome, making it more resistant to unwanted nuclear processing, will further improve virus-mediated gene-expression technology.

'Non-*Agrobacterium*'-mediated genetic transformation of plant species

Inspired by the complex patent landscape of *Agrobacterium* technology, and with a vision for a utopian 'open source' platform for plant biotechnology, Wim Broothaerts *et al.* [12] took it upon themselves to take the first step in harnessing non-*Agrobacterium* species for the task of plant genetic engineering. They showed that three non-*Agrobacterium* species – *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* – are capable of genetically transforming different plant tissues and plant species. They provided these bacterial species with the machinery needed for the transformation process: a set of virulence genes encoded by an *Agrobacterium* Ti-plasmid and the T-DNA segment residing on a separate, small binary plasmid. The T-DNA segment itself carried genes encoding a hygromycin selection marker and *uidA* reporter. Broothaerts *et al.* then used these bacterial species to

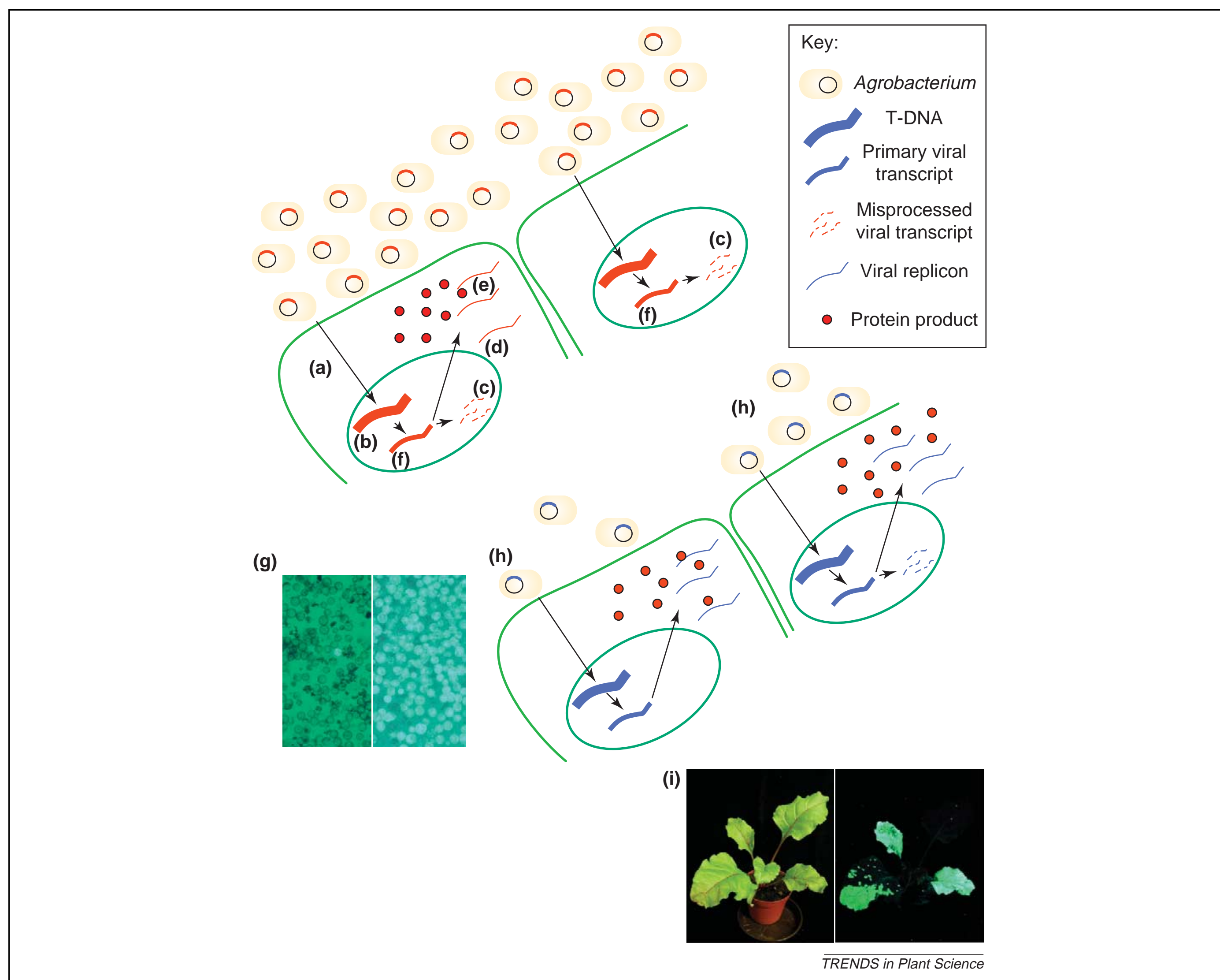

Figure 1. Genetic improvements in viral vectors and their systemic delivery. *Agrobacterium*-mediated delivery of **(a)** TMV-based vectors by **(b)** T-DNA molecules is an inefficient process owing to **(c)** improper processing or **(d)** export of **(e)** active viral replicons from **(f)** the primary nuclear transcript. Genetic improvements of the TMV vectors have led to their improved delivery as DNA precursors as well as to **(g)** higher local transfection efficiency: infection of tobacco protoplasts with original (left) and genetically improved (right) TMV vectors carrying the GFP reporter gene. These improvements have resulted in a significant decrease in the *Agrobacterium* inoculum concentration needed for local infection **(h)** and have enabled the establishment of the 'magnification' process, a systemic transfection method for *Agrobacterium*-mediated delivery of viral vectors to all mature leaves of the target plant **(i)**: *Beta vulgaris* plants systemically infected with fully optimized viral vector carrying the GFP reporter gene; photographed under normal (left) or UV (right) light, 10 days post-inoculation. Images were kindly provided by Marillonnet *et al.* [14] and reproduced, with permission, from Ref. [14].

Box 1. How can non-*Agrobacterium* species affect plant biotechnology?

Three key elements are essential for nearly every genetic-transformation protocol: the susceptibility of the infected tissue to *Agrobacterium*; the ability of the target tissue to regenerate; and an efficient selection scheme for the recovery of transformed plants.

The establishment of new transformation protocols and the improvement of existing ones rely extensively on matching *Agrobacterium* strains to existing regeneration protocols, developing new regeneration and selection methods for transformable tissues and modifying culture conditions. Most of the effort invested in establishing such protocols for commercially important plant species has resulted in numerous patent claims for the *Agrobacterium*-mediated genetic transformation of various plant species and tissues. Non-*Agrobacterium* species can potentially be used to achieve the following goals.

Improve plant–bacteria interactions

Many crops are highly resistant to *Agrobacterium* infection, or unable to regenerate from susceptible tissues. Exchanging *Agrobacterium* with other bacterial species that can interact better with the target tissues could potentially help in overcoming *Agrobacterium*-tissue incompatibility and facilitate the establishment of new transformation protocols.

Avoid plant defense response

It is likely that infection by *Agrobacterium*, a plant pathogen, stimulates a plant defense response, which could explain the low transformation and regeneration ability of various plant species. The use of, for example, a plant-associated bacterium is likely to evoke different plant responses, and improve transformation efficiency.

Provide an open-source alternative to patent claims

Most of the biological genetic-transformation patent claims explicitly refer to *Agrobacterium*. *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti*, and perhaps other bacterial species in the future, are available under an open-source-modeled license with no major commercial restrictions. This new paradigm could potentially facilitate the use of plant genetic-engineering technologies by private and public sectors worldwide.

transiently and stably transform various plant species, targeting various different tissues. Co-cultivation of tobacco leaf disks with all three strains resulted in T-DNA transfer into the host cells, as evidenced by the observed β-glucuronidase (GUS) activity encoded by the *uid*A gene. Furthermore, recovery of putative transgenic tobacco plants following selection on hygromycin and Southern blot analysis of these plants indicated that the T-DNA had stably integrated into the genome of the plant. Similarly, co-cultivation of rice calli with *S. meliloti* resulted in transient GUS activity and recovery of a single transgenic GUS-expressing and hygromycin-resistant rice plant. Finally, floral-dip transformation of *Arabidopsis* plants by *Rhizobium* sp. and *S. meliloti* gave rise to eight hygromycin-resistant transgenic plants. These results clearly demonstrate that non-*Agrobacterium* species can genetically transform not only dicots but also a monocot species, even though monocots are traditionally considered less susceptible to *Agrobacterium* infection. Similar to the *Agrobacterium*-based protocols, various plant tissues (e.g. leaf disks, calli and oval tissue) were used as the target tissues, demonstrating that existing protocols can potentially be modified for use with non-*Agrobacterium* bacterial species. Although it is still too early to determine the viability of these new '*Agrobacterium*-like' species for the transformation of commercially and scientifically important plant species, these findings indicate the way forwards in plant biotechnology (Box 1) by providing species with technological and legal advantages over *Agrobacterium* technology.

Improved virus-based vector for highly efficient transient expression in plants

The relatively high yield of viral-vector-based expression systems compared with *Agrobacterium*-mediated transient expression, and the speed of the viral expression technology relative to that of producing transgenic plants, hold great promise for plant biotechnology. Nevertheless, until recently, this technology was rather limited by transgene size and the low infectivity of viruses [13]. Sylvestre Marillonnet *et al.* [14] addressed the issue of low levels of infectivity by genetically modifying a TMV-based vector and introducing a new protocol for delivering the improved viral vector to multiple areas of the plant targeted for infection. Because the delivery of viral vectors by *Agrobacterium* T-DNA results in the production of the first viral transcript in the nucleus, it is exposed to RNA processing, which can affect its ability to propagate efficiently in the cytoplasm. The authors identified several intron-like and thymine-rich sequences in TMV vector sequences that could induce such unwanted RNA processing. Removing some of the putative cryptic splice sites and increasing the GC content of the TMV viral-vector sequence by silent mutations resulted in a significant increase in the percentage of infected cells, without affecting the replication and the ability of the viral vector to move from cell to cell. Next, Marillonnet *et al.* explored the possibility that the large RNA transcript of the viral vector might also affect the efficiency of its export to the cytoplasm by the host RNA export machinery, which is more accustomed to relatively shorter transcripts. To allow the host RNA processing and export system improved access to the viral primary transcript, Marillonnet *et al.* added several introns into the viral and target gene sequences and showed that these modifications do improve the overall transfection efficiency of plant cells by agroinfiltration (Figure 1). These genetic modifications resulted in optimized viral vectors that not only replicated well in infected cells but also were delivered by the T-DNA of *Agrobacterium* more efficiently, thus potentially allowing the use of a lower concentration of *Agrobacterium* inoculum for the initial infection. Because these vectors lacked viral coat protein and were thus unable to move systemically in infected plants, the authors introduced a system for *Agrobacterium* inoculation of the entire plant by infiltrating whole mature plants with a dilute *Agrobacterium* suspension (Figure 1). The efficiency and simplicity of the inoculation process, termed 'magnification' [13,14], is a direct result of having an improved and optimized viral vector with virus replication and cell-to-cell movement capabilities.

The efficient inoculation of all the aerial parts of a plant with primary RNA transcript using *Agrobacterium* could provide the basis for a new process to produce recombinant proteins in plants on an industrial scale [14].

Concluding remarks

During the past decade, we have witnessed remarkable progress in plant genetic-transformation technology. The production of transgenic model plants is considered a necessary routine in nearly every plant research laboratory, and genetically modified plants are already a viable part of modern agriculture. This rapid progress has resulted in the constant flow of new and improved transformation protocols for many plant species, but has also given rise to restricted patent claims and IP rights that could hinder the future use of *Agrobacterium* technology in both the private and public nonprofit sectors. The development of new technologies for the stable and transient expression of recombinant proteins in plants could potentially provide more versatile biotechnological tools for the scientific and commercial communities in their constant pursuit of basic plant research and new commercial products.

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