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Research review paper

Transient plant transformation mediated by *Agrobacterium tumefaciens*: Principles, methods and applications

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ABSTRACT

Agrobacterium tumefaciens is widely used as a versatile tool for development of stably transformed model plants and crops. However, the development of *Agrobacterium* based transient plant transformation methods attracted substantial attention in recent years. Transient transformation methods offer several applications advancing stable transformations such as rapid and scalable recombinant protein production and in planta functional genomics studies. Herein, we highlight *Agrobacterium* and plant genetics factors affecting transfer of T-DNA from *Agrobacterium* into the plant cell nucleus and subsequent transient transgene expression. We also review recent methods concerning *Agrobacterium* mediated transient transformation of model plants and crops and outline key physical, physiological and genetic factors leading to their successful establishment. Of interest are especially *Agrobacterium* based reverse genetics studies in economically important crops relying on use of RNA interference (RNAi) or virus-induced gene silencing (VIGS) technology. The applications of *Agrobacterium* based transient plant transformation technology in biotech industry are presented in thorough detail. These involve production of recombinant proteins (plantibodies, vaccines and therapeutics) and effectoromics-assisted breeding of late blight resistance in potato. In addition, we also discuss biotechnological potential of recombinant GFP technology and present own examples of successful *Agrobacterium* mediated transient plant transformations.

Contents

1. Introduction

The phytopathogenic gram negative bacterial species *Agrobacterium tumefaciens* is a causal agent of crown-gall disease in plants, which is accompanied by tumor formation on plant roots. *Agrobacterium*

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employs a unique virulence strategy to induce tumors; it delivers the virulent DNA molecule (Transferred DNA or T-DNA) into plant cells where it ultimately integrates into the host genome (Chilton et al, 1977). This *Agrobacterium* mediated genetic transformation of plants is one of the rare examples of naturally occurring trans-kingdom DNA transfer (Lacroix and Citovsky, 2013). The capability of *Agrobacterium* to integrate its own DNA into the host genome is predominantly determined by large Ti (tumor inducing) plasmid (Gelvin, 2003). Indeed, bacterial strains that are devoid of Ti plasmid are not virulent, i.e. they do not induce tumors. Moreover, virulence can be restored upon Ti plasmid acquisition. Two distinct regions harbored by Ti plasmid designated as T-DNA region and *vir* region are essential for tumor induction. T-DNA region is delineated by two around 25 bp imperfect repeats, designated as left and right borders (Gelvin, 2003). These regions contain genes, which encode for proteins involved in biosynthesis of plant-type hormones and opine (Zupan et al., 2000). In transformed plants, the expression of T-DNA genes induces hormone imbalance leading to cellular hyperproliferation and opine production. Opines are the exclusive source of nitrogen and energy to *Agrobacterium* providing a selective advantage over competing parasites (Chumakov, 2013). The *vir* region of Ti plasmid is not transferred to the host cell. It contains seven loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virF* and *virG*) encoding for most of the virulence proteins (Vir proteins) required for T-DNA transport and integration into host genome (Zupan and Zambryski, 1995). Immediately upon its discovery, the unique virulence strategy of *Agrobacterium* attracted attention of plant biotechnologists leading to the adaptation of *Agrobacterium* as an unprecedented tool for genetic transformation of plants. This adaptation involved the development of binary vector system consisting of a disarmed Ti plasmid eradicated of T-DNA region and a small-easily manageable plasmid (usually up to 20 kBa) to which T-DNA region devoid of *Agrobacterium* genes is allocated (Gelvin, 2003). Since the T-DNA region is determined only by delineating the left and right borders and not by any other DNA sequence, virtually any type of DNA can be placed between the borders and utilized for plant transformation.

It was originally shown that upon *Agrobacterium* infection of plant tissues, the expression of T-DNA harbored genes occurs in bimodal fashion: transient and stable (Janssen and Gardner, 1990). Transient expression usually peaks 2–4 days post infection of plant tissues and declines thereon in both the number of expressing cells and the expression level per single transformed cell (Lacroix and Citovsky, 2013). The stable expression requires T-DNA integration into host genome and when selection is applied it is characterized by increase in the expression level 10–14 days post infection of plant tissues (Janssen and Gardner, 1990). Although direct proof is lacking, indirect evidence indicates that transient expression predominantly occurs from T-DNA copies, which are not integrated into the host genome (reviewed in Lacroix and Citovsky, 2013). As the transient expression could approach high levels in infected tissues (Janssen and Gardner, 1990), it seems that *Agrobacterium* initially delivers much higher numbers of T-DNA copies into plant cells than the one(s) finally integrated into host genome. Following this scenario, the decrease in the transient expression peak could be explained by inherent instability of unintegrated T-DNA copies (Lacroix and Citovsky, 2013), while subsequent increase in expression level by growing number of selected cells containing stably integrated T-DNA. By convention, plants at transient expression peaks are said to be transiently transformed while tissues/plants displaying long term expression resulting from integration of T-DNA into genome are said to be stably transformed.

The stable transformation of plants mediated by *Agrobacterium* is inheritable in the case of germline transgene transmission, thus providing a basis for the development of fully transgenic plants, in which every single cell contains a T-DNA copy integrated into its genome. Such plants display uniform and long term expression of transgene and allow for the temporal and spatial control of the transgene expression level (e.g. Bartlett et al., 2008; L. Chen et al., 2014; Clough and Bent, 1998; Fan et al., 2008; Fillati et al., 1987; Harwood, 2012; Hiei et al., 1994, 2014; Hoekema et al., 1989; Ishida et al., 2007; Mayavan et al., 2013; Mrízová et al., 2014). Moreover, stably transformed plants can be used over many generations, although transgenes may become partially or fully inactivated by the silencing events (Fagard and Vaucheret, 2000). Within the last three decades much effort was paid to the development of *Agrobacterium* based stable transformation protocols for various plant species including crops. Indeed, reliable protocols are now available for the stable transformation of model plants and many crops including cereals that were originally thought to be recalcitrant to the *Agrobacterium* mediated transformation (e.g. Bartlett et al., 2008; Clough and Bent, 1998; Fillati et al., 1987; Hiei et al., 1994; Hoekema et al., 1989; Mrízová et al., 2014). Although other methods are suitable for plant transformation, such as protoplast or biolistic transformations, the *Agrobacterium* mediated transformation is preferred since plants bearing single transgene copy can be more easily obtained (e.g. Bartlett et al., 2008; Komari et al., 2004). Accordingly, vast majority of the approved genetically engineered agricultural crops have been developed using *Agrobacterium* (Hemmer, 2002). The importance of the green biotech sector for the global agriculture production can be demonstrated by the recent numbers: the global value of genetically modified (GM) seeds was worth of US$15.6 billion in 2013, which represents 35% of commercial seed market worth of US$45 billion (James, 2014). GM crops are widely grown in the USA and Asia, whereas in the European Union with most stringent GMO regulations, the MON 810 maize is the only GM crop approved for the commercial cultivation (Davison, 2010; James, 2014). A recent extensive global metadata analysis showed that the use of GM crops substantially reduced use of chemical pesticides while it increased crop yields and farmer profits (Klümper and Qaim, 2014). However, controversies about the health, social and environmental impact of GM crops are major reasons for the widespread negative public attitude towards this technology (Gilbert, 2013). One of the most criticized aspects is the genetic modification of commercial crops using genes imported from other species (Cressey, 2013). Nevertheless, the next-generation GM crops, which will be prepared using site specific nucleases, namely ZFNs, TALENs and CRISPR/Cas9, may harbor only precisely targeted modification of their own genomes (e.g. reviewed in Belhaj et al., 2014; K. Chen et al., 2014; Podevin et al., 2013; Voytas and Gao, 2014). Thus, such novel "transgene free" technology could possibly reduce the negative public concern on GM crops (Cressey, 2013).

Although *Agrobacterium* mediated development of stably transformed plants is indispensable for many aspects of modern plant science and GM crops prepared in this way are still of great promise for agriculture (Wang, 2015a,b), a substantial attention is also devoted to the *Agrobacterium* mediated transient plant transformation, especially in recent years (please, see Sections 3, 4 and 5 below). *Agrobacterium* mediated transient plant transformation methods allow for rapid and scalable recombinant protein production, rapid studies of protein subcellular localization and protein–protein interactions as well as for development of functional genomics assays. In this review, we focus on *Agrobacterium* and plant genetic factors affecting transfer of T-DNA from *Agrobacterium* into the plant cell nucleus and subsequent transient transgene expression. We also outline recent methods concerning *Agrobacterium* based transient transformation of model plant and crop species and stress out key factors leading to their successful establishment. In order to highlight the biotechnological potential of *Agrobacterium* based transient plant transformation methods, three state of the art applications are discussed in detail. These involve highly efficient production of pharmaceutical proteins, applications of recombinant GFP technology and effectoromics-assisted breeding of late blight resistance in potato.

2. *Agrobacterium* virulence proteins and interacting plant proteins

There are several steps which lead to the transport of T-DNA molecule from *Agrobacterium* into the host nucleus and its integration