doi:10.1093/plphys/kiab081 PLANT PHYSIOLOGY 2021: 186: 215–220

Plant Physiology

Prospects for Reengineering *Agrobacterium tumefaciens* for T-DNA Delivery to Chloroplasts

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This work was supported by research grants National Science Foundation MCB 1716102 and National Science Foundation IOS 2037155.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Pal Maliga (maliga@waksman.rutgers.edu).

Letter

Dear Editor,

The current bottleneck of plastid transformation in *Arabidopsis thaliana* is the difficulty of obtaining fertile plants from transplastomic tissue culture cells. Inherent polyploidy of leaf cells and the tendency for somaclonal variation in tissue culture make plastid transformation in Arabidopsis a challenging task (Yu et al., 2017; Ruf et al., 2019; Yu et al., 2019). Tissue culture limitation in Arabidopsis nuclear gene transformation has been overcome by direct transformation of the female gametocyte by floral dip, and identification of nuclear transgenic events by germinating the seed on a selective medium (Clough and Bent, 1998). Our goal is to re-engineer *Agrobacterium* for T-DNA delivery to chloroplasts to directly transform the plastids in the female gametocyte. Success of the project depends on reengineering the *Agrobacterium* virulence proteins for T-DNA delivery to chloroplasts. We provide proof of concept that proteins can be directly exported from *Agrobacterium* to chloroplasts. The protein of our choice is the phiC31 phage site-specific integrase (Int) because visitation of the recombinase to chloroplasts creates a permanent footprint. To target Int from *Agrobacterium* to chloroplasts, we translationally fused Int with the VirF Type IV secretion system (T4SS) signal at its C-terminus to facilitate its movement through the T4SS to the plant cytoplasm (Vergunst et al., 2000). To target the fusion protein to chloroplasts, we fused the Int N-terminus with the tobacco (*Nicotiana tabacum*) Rubisco small subunit transit peptide (Gnanasambandam et al., 2007). The protein sequence of the chimeric gene is shown in Supplemental Figure S1. Int engineered for direct transfer from *Agrobacterium* to chloroplasts (Agro-Int) is carried on a binary plasmid pJCD6B. Plasmid pJCD6B is a pPZP200 binary vector derivative (Hajdukiewicz et al., 1994), which is lacking T-DNA border sequences (Figure 1A). In the absence of borders, there is no T-DNA transfer to the plant nucleus (Peralta and Ream, 1985; Jen and Chilton, 1986); therefore, the only source of Int activity is the enzyme pumped over from *Agrobacterium* carrying plasmid pJCD6B via the T4SS. Agro-Int is expressed in *Agrobacterium* in the *virE* promoter/virF terminator cassette. Target excision by Agro-Int is schematically shown in Figure 1A.

We used as a control *Agrobacterium* binary vector pKO117, encoding a chloroplast-targeted Int on its T-DNA that is expressed in a cassette designed for expression in the plant nucleus (Lutz et al., 2004; Figure 1B). In cells transformed with vector pKO117, the T-DNA integrates in the plant nuclear genome where *int* is constitutively expressed. Chloroplast targeting of pKO117 Int is ensured by the translational fusion with the Rubisco small subunit transit peptide. To distinguish the two Int enzymes, we refer to Int expressed in the plant nucleus from pKO117 T-DNA as Nuclear-Int.

We tested the export of Agro-Int into chloroplasts by the excision of a target sequence in the chloroplast genome of Nt-pSS33 plants (Figure 2A). The Nt-pSS33 transplastomic tobacco plants carry an aurea bar^{au} gene between 54- and 215-bp *attB*/*attP* target sites to facilitate integrase-mediated excision of the target gene (Tungsuchat-Huang et al., 2010). The virulence genes were activated in *Agrobacterium* by

Received May 29, 2020. Accepted February 2, 2021. Advance access publication February 23, 2021

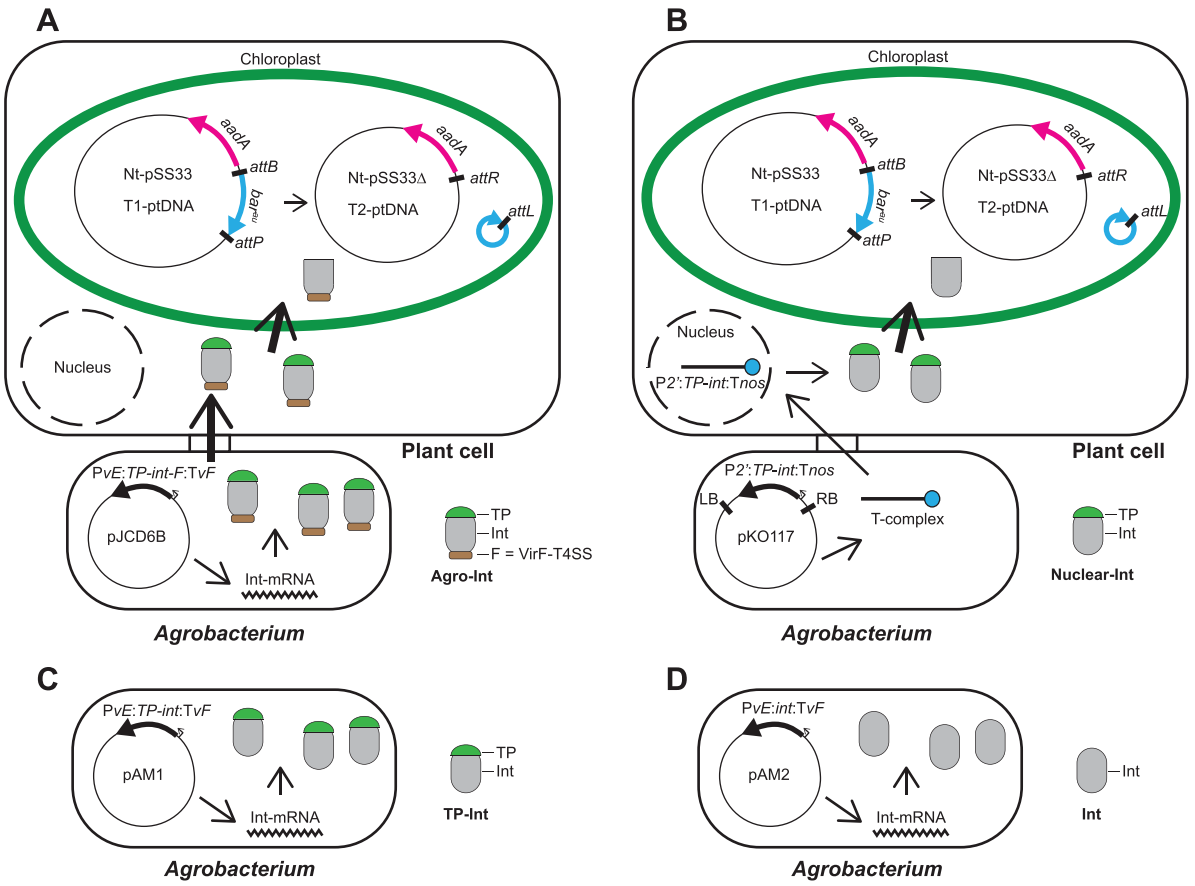

Figure 1 Schematic diagram of *bar*^{au} gene excision by Agro-Int or Nuclear-Int yielding plastid genome Nt-pSS33Δ. A, Int engineered for direct transfer from *Agrobacterium* to chloroplasts (Agro-Int) is expressed in *Agrobacterium* from plasmid pJCD6B and exported to chloroplasts. The tobacco Rubisco small subunit transit peptide (TP) and the VirF (F) T4SS signal were fused at the Int N- and C-termini, respectively. PvE, Promoter *virE*; TvF, terminator *virF*. B, Co-cultivation of *Agrobacterium* carrying a binary plastid pKO117 (Lutz et al., 2004) is followed by the transfer of the T-complex to the nucleus, symbolized with a blue circle (VirD2) and a black line (T-strand). Int is expressed in a nuclear P2′/Tnos cassette and is fused with the pea Rubisco small subunit transit peptide for plastid targeting. LB and RB are the T-DNA left- and right-borders. C, TP-Int in binary vector pAM1. D, Int in binary vector pAM2. T1-ptDNA and T2-ptDNA refer to transformed plastid genomes before and after *bar*^{au} excision.

acetosyringone treatment, and the *Agrobacterium* suspension was injected into the leaves of greenhouse-grown Nt-pSS33 plants (Figure 2C).

Excision of the *bar*^{au} gene in the leaves of greenhouse plants was tracked by polymerase chain reaction (PCR) in leaf sectors injected with *Agrobacterium* suspension using total cellular DNA as template (Figure 2D). In the Day 1 Agro-Int sample, the PCR product was 1.9 kb, the unexcised fragment size. The Days 2–4 samples contained increasing amounts of 0.9-kb fragments indicating that the chloroplast *bar*^{au} gene was excised in the chloroplast genomes. In Nuclear-Int samples, from Day 1 only, the 0.9 kb excised fragment was detectable (Figure 2D). The 0.9-kb fragment was isolated from the gel and sequenced. The sequence revealed a perfect *attR* junction in both the Agro-Int and Nuclear-Int samples, formed by recombination of the *attB* and *attP* sites mediated by the phiC31 site-specific integrase (Figure 2B), as predicted (Groth et al., 2000). Because proteins may be exported from *Agrobacterium* by more than one mechanism (Costa et al., 2015), we also tested an *Agro-Int* gene variant lacking the T4SS signal at its C-terminus (plasmid pAM1; Figure 1C). If Agro-Int is exported via the Type IV protein secretion system, we expected that the *bar*^{au} gene is not excised in leaf sectors injected with *Agrobacterium* carrying plasmid pAM1, encoding an integrase lacking the T4SS signal at its C-terminus (Figure 1C). Indeed, pAM1 binary plasmid samples failed to yield any excision products (Figure 2D), indicating that Int export is dependent on the *Agrobacterium* Type IV protein secretion machinery. When Int was expressed in *Agrobacterium* without an N-terminal TP and a C-terminal T4SS signal (plasmid pAM2; Figure 1D), a second control protein, no excision of the *bar*^{au} gene was detected either (Figure 2D).

To detect excision in greenhouse leaves after injection with Agro-Int, we used relatively high DNA template concentrations for PCR. This condition favored amplification of the smaller excision product, showing complete excision of the target sequence after injection with Nuclear-Int. When the Nuclear-Int-injected DNA sample was subjected to DNA gel blot analyses, an unbiased method to detect excised and nonexcised plastid DNA (ptDNA) copies, the target *bar*^{au} gene was excised in most ptDNA copies, while

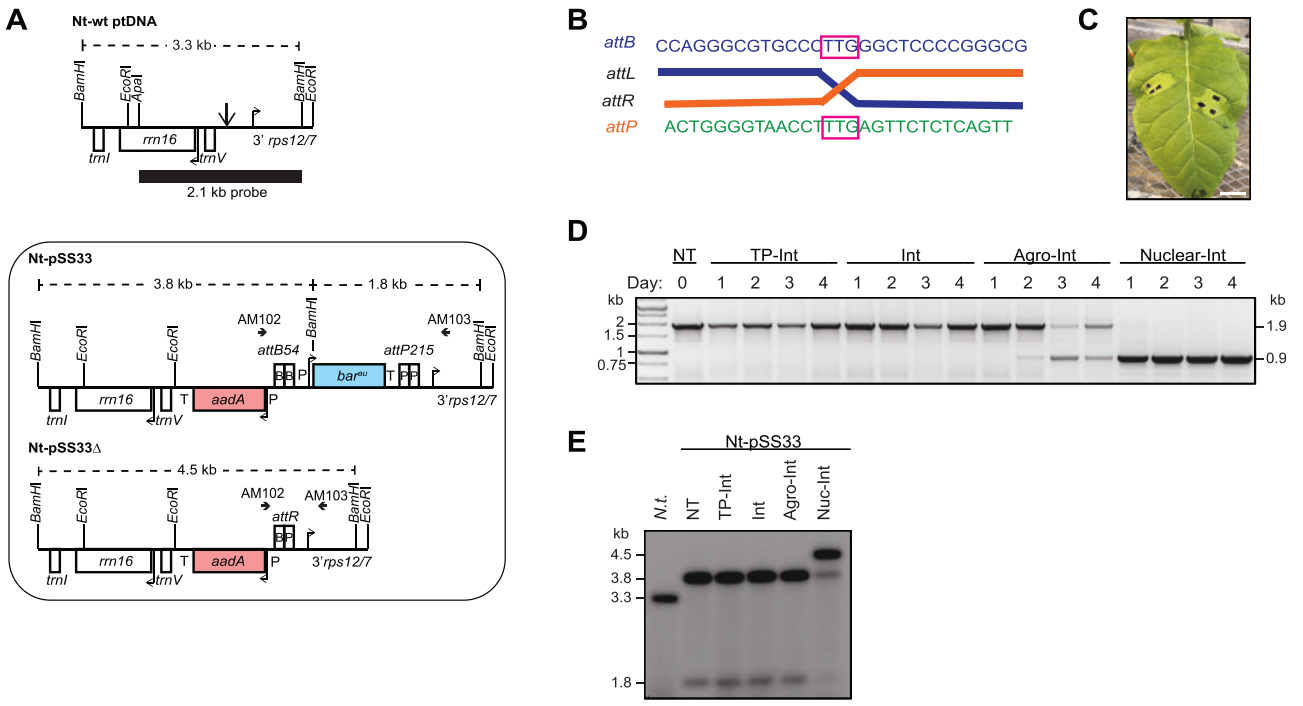

Figure 2 Integrase exported from *Agrobacterium* excises *bar*^{au} gene in the Nt-pSS33 chloroplast genome. A, Partial map of the Nt-pSS33 plastid genome before and after the excision of *bar*^{au} gene. The DNA fragment used to probe the DNA gel blots and the size of hybridizing fragments are shown. Shown are: the *trnI*, *rrn16* and *trnV* plastid genes and the 3′-*rps12/7* operon promoter; the *attB*, *attP* target sites, and the *attR* recombination junction; the AM102 and AM103 primer sites; and the *Bam*HI, *Eco*RI, and *Apa*I restriction sites. B, The minimal *attB*/*attP* sequence and the *attL* and *attR* recombination junctions (Groth et al., 2000). C, Tobacco leaf 3 d after Agroinfiltration. D, PCR amplification confirms target excision in syringe-injected leaves. The AM102 and AM103 primers amplify a 1.9-kb fragment before and a 0.9-kb fragment after excision of the *bar*^{au} gene. E, DNA gel blot confirms excision of the target sequence in Agroinfiltrated leaf sections. Hybridization of *Bam*HI-digested total cellular DNA yielded 3.8- and 1.8-kb fragments before excision and 4.5-kb fragment after excision. For fragment positions, see Figure 2A.

no excision was detected on the DNA gel blots in the Agro-Int-infiltrated leaves (Figure 2E). Although the plastid DNA had the footprint of Int-mediated excision in the form of an *attR* sequence junction (Figure 2B), the excision took place in only a small fraction of ptDNA copies that were undetectable by DNA gel blot analyses. A factor in low-level Agro-Int export could be that *Agrobacterium* quickly shuts down the energy-costly infection machinery once successful infection is established (Wang et al., 2019).

Routine *Agrobacterium*-mediated transformation chloroplast genome engineering has not yet been achieved. De Block et al. (1985) reported chloramphenicol-resistant tobacco plants after cocultivation with a vector carrying a chloramphenicol acetyltransferase (*cat*) gene on its T-DNA. Chloramphenicol resistance was conferred by a nopaline synthase promoter (Pnos) fused to the coding sequence of a *cat* gene. Chloramphenicol acetyltransferase activity and the *cat* DNA were associated with chloroplasts, but no stable transgenic lines were obtained. This observation is compatible with delivery of the *cat* gene to chloroplasts, but lack of maintenance of the *cat* gene due to lack of flanking chloroplast targeting sequences that are necessary for the integration of foreign DNA in an intergenic region. Such chloroplast targeting sequences are universal features of current routine chloroplast transformation vectors (Maliga, 2004; Bock, 2015). A recent report on selection of transplastomic clones by chloramphenicol resistance provides support for this claim (Li et al., 2011). In a different study, a segment of chloroplast DNA comprising 5S rRNA and a tRNA was inserted in the T-region of a binary vector, which also carried a *Pnos-NPTII* gene that enabled selection of kanamycin-resistant clones (Venkateswarlu and Nazar, 1991). Transformed chloroplast genomes apparently formed by a single crossover event, yielding an inherently unstable cointegrate structure. Today's vectors carry a marker gene (and linked genes of interest) that inserts in the chloroplast genome at intergenic locations by two recombination via flanking homologous sequences (Maliga, 2004; Bock, 2015). Aside from obvious deficiencies of vector design, in none of the two cases was an attempt made to re-target the VirD2 and VirE2 proteins known to be responsible for escorting the T-complex to the nucleus. Thus, even if the marker genes were serendipitously expressed in the chloroplast, these experiments did not provide a reproducible methodology for chloroplast engineering.

Five *Agrobacterium* proteins play roles in the nuclear gene transformation (Table 1). When considering re-designing *Agrobacterium* for T-DNA delivery to chloroplasts, VirD2

Table 1 The role of *Agrobacterium* virulence (Vir) proteins in plant cells

Nuclear transformation	Chloroplast transformation
In plant cell VirD2—Nuclear targeting of T-DNA by NLSs VirD5—cell division (Zhang et al., 2017) VirE2—Protection of T-DNA, NLSs for nuclear targeting VirE3—chaperone to VirE2 VirF—E3 ligase; integration in nucleus	In plant cell PT-VirD2—Plastid targeting by removing NLSs, adding plastid targeting sequences PT-VirE2—Protection of T-DNA, plastid targeting allowed by removal of NLSs
In *Agrobacterium* VirD1—Excision of T-DNA VirD2—Excision of T-DNA, export of T-DNA to plant cell	In *Agrobacterium* PT-VirD1—Excision of T-DNA PT-VirD2—Excision of T-DNA, export of T-DNA to plant cell

Unless specifically cited, for references on Vir proteins in nuclear transformation see recent reviews (Gelvin, 2010; Lacroix and Citovsky, 2019). Proposed re-engineering of VirD2 and VirE2 are discussed in the text.

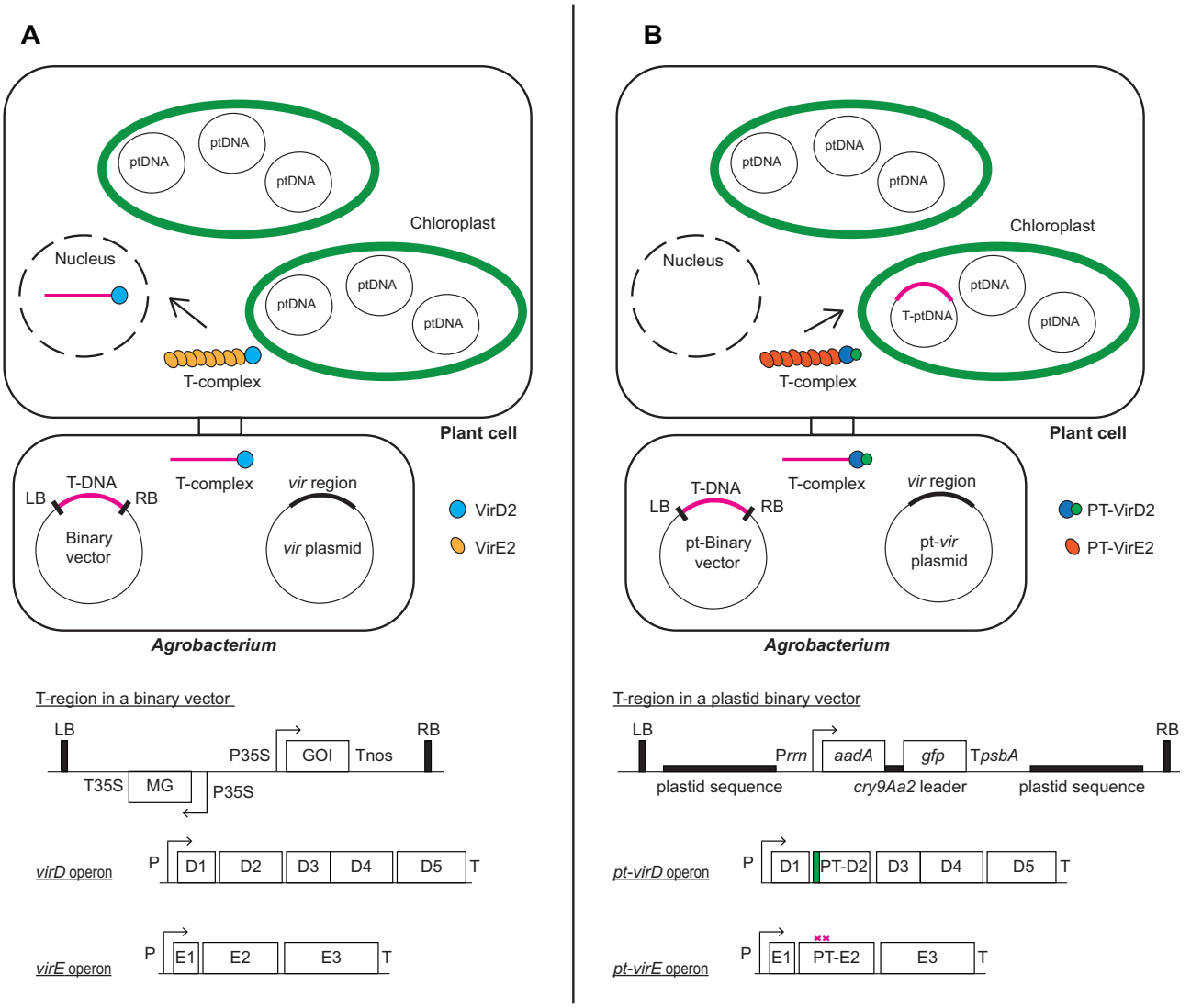

Figure 3 Chloroplast transformation by *Agrobacterium*: the steps involved and hurdles to overcome. A, Major steps of nuclear transformation by *Agrobacterium*. B, The engineering steps to enable chloroplast transformation by *Agrobacterium*. Shown are a plant cell with a nucleus and two chloroplasts, the plastid type in leaf cells, and an *Agrobacterium*, cell with a virulence (*vir*) plasmid (not to scale) encoding the operons required for T-DNA transfer to the plant cell, and a binary vector encoding a T-DNA, with the 25-bp direct repeat required for T-DNA excision. ptDNA and T-ptDNA mark wild-type and transplastomic plastid genome copies, respectively. LB and RB are the T-DNA left- and right-borders; MG, marker gene; GOI, gene of interest.

and VirE2 stand out as targets. VirD2 plays important roles in both *Agrobacterium* and the plant cell. Chloroplast targeting of VirD2 requires removal of nuclear localization signals (NLSs) and fusion of the N-terminus with a chloroplast-targeting transit peptide. Removal of NLSs requires replacement of the VirD2 T4SS signals because the VirD2 T4SS signals and C-terminal NLSs are co-localized (van Kregten et al., 2009). A suitable replacement would be the VirF T4SS that does not contain NLS (Vergunst et al., 2005). PT-VirD2, the VirD2 engineered for plastid (chloroplast) delivery is depicted in Figure 3B. The changes required for PT-VirD2 to be transferred to and function in the plant cell should not impact its functions in the *Agrobacterium* cell, including T-strand excision, covalent linkage of the T-DNA to Tyr29 of VirD2 and guiding it through the T4SS into the plant cell. The role of VirE2 in nuclear gene transformation is to protect the T-DNA from degradation and guide it to the nucleus (Table 1). Mutagenesis of VirE2 NLSs may be sufficient to tailor it for chloroplast engineering (red crosses in Figure 3B); or it will have to be replaced with a single-stranded DNA binding protein to protect the T-DNA on its journey to the chloroplast. To avoid competition between chloroplast and nuclear targeting, the wild-type VirD2 and VirE2 protein should be replaced with PT-VirD2 and PT-VirE2 in the *Agrobacterium virD* and *VirE* operons (Figure 3B).

Fortuitous expression of antibiotic resistance markers in chloroplasts carried on the *Agrobacterium* T-DNA suggests that the import of the T-complex into chloroplasts may be less of a problem than anticipated. Successful chloroplast transformation following polyethylene glycol-induced uptake of transforming DNA suggests that it may be sufficient to introduce the transforming DNA into the cytoplasm (Golds et al., 1993; O'Neill et al., 1993). Exploring the use of alternative cleavable and non-cleavable transit peptides for T-DNA targeting (Armbruster et al., 2009; Sjuts et al., 2017) should follow demonstration that the engineered PT-VirD2 protein itself has been re-targeted to chloroplasts and is able to mobilize the T-DNA to plant cells, as discussed here for the integrase protein. A convenient marker system will be dicistronic operons encoding spectinomycin resistance and the green fluorescence protein (Figure 3B), which will express GFP only, when the operon is introduced in chloroplasts (Yu et al., 2020).

Engineering of the VirD2 and VirE2 proteins are just two of the major hurdles that need to be overcome to create a system for T-DNA delivery to chloroplasts and employing it in a floral dip protocol. A major benefit of side-stepping the tissue culture process will be the elimination of need for tissue culture expertise and the relatively small effort required to obtain fertile transplastomic plants. A simplified process of chloroplast transformation will lead to widespread applications of Arabidopsis plastid genome engineering which, combined with the available extensive genomic resources, will have a major impact on basic science and biotechnological applications.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Amino acid sequence of the phiC31 integrase engineered for protein export from *Agrobacterium* to chloroplasts in plasmid pJCD6.

Supplemental Figure S2. DNA sequence of integrase genes in binary vectors pJCD6B, pAM1, and pAM2.

Supplemental Methods S1. Detailed information on experimental procedures.

Acknowledgments

Authors thank undergraduate student Juliana DiGiacomo for contributing to the construction of plasmid pJCD6B.

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