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Truyen N. Quach

Shirley J. Sato

Mark R. Behrens

Paul N. Black

Concetta C. DiRusso

See next page for additional authors

Authors

Truyen N. Quach, Shirley J. Sato, Mark R. Behrens, Paul N. Black, Concetta C. DiRusso, Heriberto D. Cerutti, and Tom Elmo Clemente

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GENETIC TRANSFORMATION

A facile *Agrobacterium*-mediated transformation method for the model unicellular green algae *Chlamydomonas reinhardtii*

Truyen N. Quach[1] · Shirley J. Sato[2] · Mark R. Behrens[3] · Paul N. Black[3] · Concetta C. DiRusso[3] · Heriberto D. Cerutti[1,4] · Tom Elmo Clemente[5]

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Abstract

A reliable and simple *Agrobacterium*-mediated transformation system for the unicellular green algae model organism *Chlamydomonas reinhardtii* has been developed. The protocol has been successfully employed with both neomycin phosphotransferase II (*nptII*) and the phleomycin resistance (*bleI*) genes coupled with the selective agents paromomycin and zeocin, respectively. A set of binary vectors were assembled that carry the selectable marker cassettes under control either of the Rbcs2 alone or fused to the HSP270A leader sequence, PsaD, or ß-tubulin2 promoters. The corresponding T-DNA elements also harbored a cassette with a codon-optimized version of yellow fluorescence protein (YFP) under control of the Rbcs2 promoter in which the YFP open reading frame was interrupted with the first intron of Rbcs2 to prevent expression in *Agrobacterium tumefaciens*. The resultant binary vectors were introduced into *A. tumefaciens* strain C58C1/pMP90, and the derived transconjugants were used for transformation studies with the walled *C. reinhardtii* strain CC124. Estimated transformation frequencies ranged from 0.09 to 2.86 colonies per 10^6 cells inoculated. Molecular characterizations on a subset of the transgenic lineages revealed that most of the transgenic events harbored single locus insertions. Moreover, sequencing of captured junction fragments about the T-DNA insertion site showed that minimal disruption of the *C. reinhardtii* genome occurred. However, the transgenic lineages often harbored truncated T-DNA regions within the non-selectable marker gene cassettes.

Keywords *Chlamydomonas* · *Agrobacterium* · Genetic engineering · Yellow fluorescence protein

Introduction

The unicellular alga *Chlamydomonas reinhardtii* has a long history as a model system for study of the fundamental underpinnings of core biological processes. These include photosynthesis, flagellar assembly and mechanics (Harris 2001), heavy metal transport and compartmentalization (Hanikenne 2003), epigenetic regulation of gene expression (Jeong *et al.* 2002; van Dijk *et al.* 2005; Casas-Mollano *et al.* 2008), and a platform to understand intracellular carbon flux changes under nutrient sufficient and depleted environments (Miller *et al.* 2010; Msanne *et al.* 2012). Genetic engineering of cells is a powerful tool for both functional genomics programs and translation of foundational discoveries into application. In this regard, *C. reinhardtii* is rather unique given that all three of its genetic compartments are competent for genetic manipulation (Kindle 1990; Kindle *et al.* 1991; Sizova *et al.* 1996; Bateman and Purton 2000; Remacle *et al.* 2006). These genetic engineering tools, coupled with the wealth of other genomic resources and databases (Vallon and Dutcher 2008; Dal'Molin *et al.* 2011), including a draft of the *C. reinhardtii* genome (Merchant *et al.* 2007), have greatly enhanced the attractiveness of this model algal system.

The introduction of transgenes into the nuclear compartment of *C. reinhardtii*, while rather robust, has been historically hampered by poor expression of transgene(s) (Fischer

✉ Truyen N. Quach
tquach2@unl.edu

1 Center for Plant Science Innovation, University of Nebraska-Lincoln, Lincoln, NE, USA

2 Center for Biotechnology, University of Nebraska-Lincoln, Lincoln, NE, USA

3 Department of Biochemistry, University of Nebraska-Lincoln, Lincoln, NE, USA

4 School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA

5 Department of Agronomy & Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA

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and Rochaix 2001; Shao and Bock 2008) and by a tendency of the resultant transformants to carry large genetic deletions about the junction of the inserted foreign allele (Dent *et al.* 2005). The former limits the potential of *C. reinhardtii* as a feedstock for biological products (Franklin and Mayfield 2004), and the latter can complicate hypothesis testing in functional genomics programs when phenotyping insertional mutant populations. Most protocols communicated for nuclear transformation of *C. reinhardtii* implement a direct DNA delivery system, employing electroporation, glass beads, silicon carbide whiskers, or microprojectile bombardment (Dunahay 1993; Neupert *et al.* 2012). In higher plants' nuclear transformation, implementing direct DNA delivery techniques often leads to complex, multicopy transgenic alleles, which can be mitigated by utilizing *Agrobacterium*-based protocols (Cheng *et al.* 2001; Dai *et al.* 2001; Shou *et al.* 2004). To this end, the objectives of the studies communicated herein were to develop a dependable *Agrobacterium*-mediated transformation protocol for *C. reinhardtii* and genotype a subset of the derived transgenic lineages to gauge the utility for both biotechnology applications and use in functional genomics efforts with respect to direct DNA methods.

Materials and methods

Vector Constructions A *C. reinhardtii* codon-optimized version of the selectable marker gene neomycin phosphotransferase II (*nptII*) coupled with the 5′ leader sequence derived from ribosomal protein L13 (rpl13) along with a codon-optimized version of the visual marker yellow fluorescence protein (YFP) fused to the 5′ untranslated region of RbcS2 was used in the transformation studies. The YFP open reading frame (ORF) was interrupted by the first intron of the *C. reinhardtii* RbcS2 gene to block translation within *A. tumefaciens* cells. Both *nptII* and YFP genes were commercially synthesized (GenScript Co. Piscataway, NJ). In some experiments, a second selectable marker gene, *ble* (Stevens *et al.* 1996), was tested. The synthetic *nptII* gene was assembled into three expression cassettes regulated by either the *C. reinhardtii* RbcS2 promoter alone or fused with the HSP70A element (Sizova *et al.* 2001), the PsaD promoter (Fischer and Rochaix 2001), or the ß2-tubulin promoter (Davies and Grossman 1994) while the single *ble* selectable marker was placed under control of the RbcS2 promoter.

A total of seven binary vectors were assembled (Fig. 1). The respective *nptII*, *ble*, and YFP cassettes were subcloned into the base binary plasmid pPZP202 (Hajdukiewicz *et al.* 1994), and the resultant vectors were designated as pPTN1127, pPTN1129, pPTN1130, pPTN1133, pPTN1170, and pPTN1176 (Fig. 1). The first three harbored just the *nptII* cassette under control of one of the three regulatory elements RbcS2 (pPTN1127), PsaD (pPTN1129), or ß2-tubulin (pPTN1130). The plasmid pPTN1133 carried an *nptII* cassette under control of the RbcS2-HSP70A regulatory element and a YFP cassette regulated by the ß2-tubulin promoter. The plasmid pPTN1170 carried the same YFP and *nptII* cassettes as pPTN1133 plus the *ble* selectable marker while vector pPTN1176 harbored the two selectable marker cassettes, *nptII* and *ble* (Fig. 1). The binary plasmids were mobilized into *A. tumefaciens* nopaline strain C58C1/pMP90 (Koncz and Schell 1986) *via* tri-parental mating, and the derived transconjugants were used in the transformation studies for *C. reinhardtii* (Fig. 2).

***Agrobacterium*-Mediated Transformation** The *A. tumefaciens* transconjugants were grown in YEP medium with the appropriate antibiotics to an OD_{650} of 0.8 to 1.0. The cultures were centrifuged in 50-mL conical tubes, and the bacterial pellet was suspended to the same OD_{650} in co-cultivation medium composed of low salt, supplemented with 1% glucose, buffered with 20 mM MES (pH 5.4), plus 200 µM acetosyringone. The bacterial cultures were placed on a shaker for 1 h and subsequently placed on ice in the dark until use.

C. reinhardtii cells, strain CC124, were cultured in TAP medium under photosynthetic active radiance (PAR) of 40 to 50 µmol m^{-2} s^{-1} at room temperature until cell densities reached OD_{750} of approximately 0.5 at which time the cells were harvested by centrifugation and re-suspended to a concentration of 2×10^8 cells mL^{-1}. An aliquot of 200 µL of the algae culture was spread on co-culture medium solidified with 0.8% agar (Sigma Cas No. 9002–18-0), and the plates were subsequently air-dried in a laminar flow hood for 30 min. Following the dry-down step, the algal lawn was overlaid with 200 µL of the *A. tumefaciens* inoculum, and plates were co-cultured in the dark at 24 °C for 48 h. At the completion of the co-cultivation step, the cells were scraped off the plate and washed 2 times in TAP medium supplemented with 100 mg L^{-1} each of cefotaxime and carbenicillin. The cells were then plated onto delay medium (TAP supplemented with the cefotaxime and carbenicillin) to counter-select against *A. tumefaciens* for 15 h under PAR of 40 to 50 µmol m^{-2} s^{-1}, after which the cells were collected and re-plated onto TAP selection plates supplemented with cefotaxime and carbenicillin and with paromomycin at 15 mg L^{-1} or zeocin at 7.5 mg L^{-1} for *nptII* and *ble* selectable marker cassettes, respectively. The selection plates were maintained at room temperature under light (40 to 50 µmol m^{-2} s^{-1} PAR) for 7 d prior to counting resistant colonies.

Microprojectile-Mediated Transformation Microprojectile bombardments were conducted using the PDS-1000He Particle Delivery System (Bi-Rad Lab., Hercules, CA). Plasmid DNA (1.0 mg mL^{-1}) was precipitated onto 25.0 µL of 0.6 µm gold particles suspended in 50% glycerol (60.0 mg mL^{-1}) by

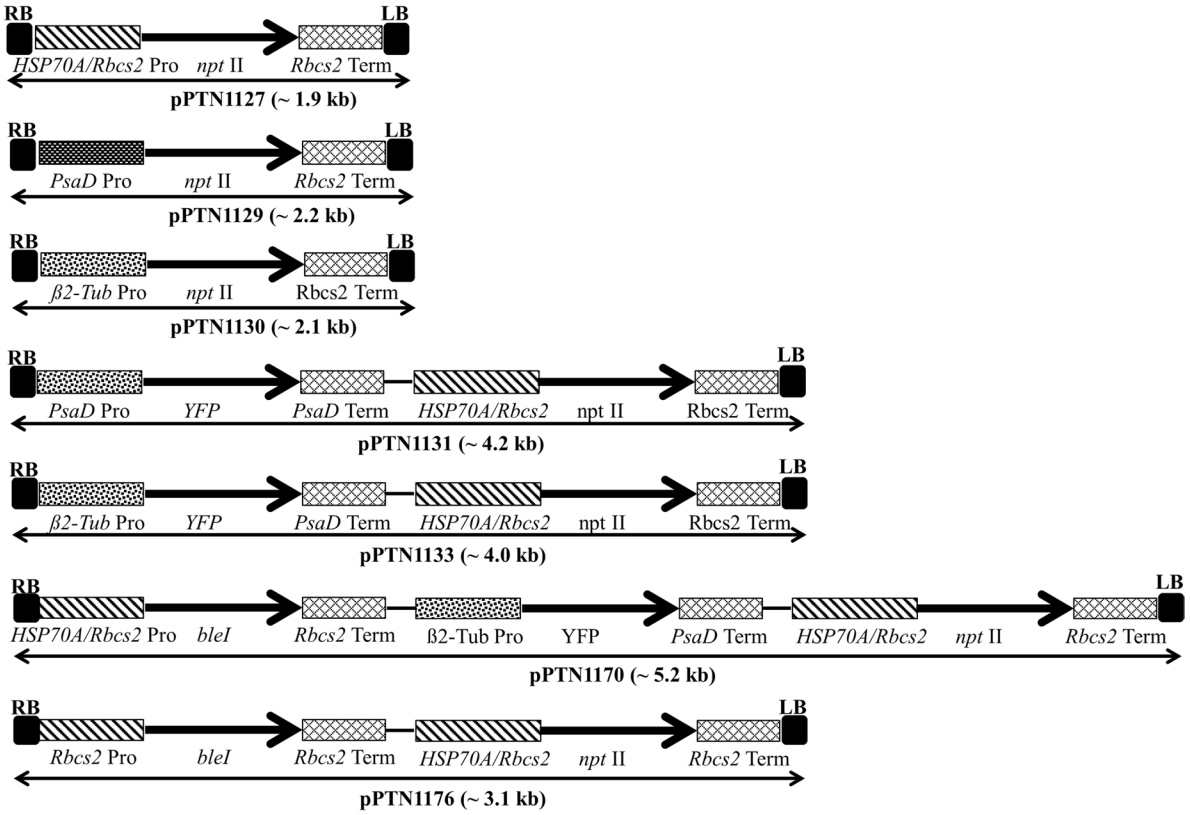

Figure 1. Diagrammatic representation of T-DNA elements used in the study. Binary vector names along with approximate size, in parentheses, of the corresponding T-DNA are listed underneath the *bidirectional arrow*. RB and LB indicate right border and left border elements, respectively. Rbcs2 Pro and Rbcs2 terms indicate Rbcs2 promoter and terminator, respectively. PsaD and ß2-Tub Pro refer to PsaD and ß2-tubulin promoters. HSP70A indicates 5′UTR of HSP70A gene. Note: Sizes of the respective elements are not drawn to scale.

the sequential addition of 2.5 µL plasmid prep, 25 µL of 2.5 M $CaCl_2$, and 10 µL 0.1 M spermidine with continuous vortexing for 3 min. The coated particles were subsequently washed with 140 µL of 70% ethanol followed by a second wash with 100% ethanol and finally suspended in 24.0 µL of 100% ethanol. Each particle preparation was partitioned onto two macrocarriers. The algal plates were prepared as described above with the exception that the medium used was full-strength TAP supplemented with the antibiotic regime of carbenicillin and cefotaxime to limit contamination potential. The plates were positioned 9 cm from the launch assembly. Following bombardment, cells were collected in 5 mL TAP plus carbenicillin and cefotaxime, centrifuged and re-suspended in 15 mL of TAP plus antibiotics, and placed in a 50-mL Erlenmeyer flask under dim light (40 to 50 µmol m^{-2} s^{-1} PAR) with no shaking for 15 h. The cultures were subsequently collected and plated onto selection plates as described above.

Characterization of Transgenic *C. reinhardtii* Events Putative resistant colonies were re-cultured onto fresh selection plates for an additional 10 d and subsequently bulked-up in 250 mL cultures for down-stream characterization. Monitoring for expression of the selectable marker gene *nptII* was carried out using a commercially available ELISA kit (Agdia® Corp. Elkhart, IN, Cat# 73000/0480). Imaging for expression of the YFP visual marker was conducted using a Nikon 1A confocal laser microscope system (Nikon Instruments, Melville, NY) with dual scanning at 488 nm laser excitation and 500 to 550 nm emission. Fluorescence from chloroplast was monitored with 641 nm laser excitation and 662 to 737 nm emission. Images were captured at 100 × magnification.

Total genomic DNA was isolated from *C. reinhardtii* cells using either a modified CTAB (Murray and Thompson 1980) protocol or a method adapted from Dellaporta *et al.* (1983). Southern blot analyses were carried out on approximately 10 µg of genomic DNA digested with *Bam* HI (Fig. 3) or *Not* I (Fig. 4), and the resultant fragments were separated on a 0.8% agarose gel. The resultant gel was blotted onto a nylon membrane (Bio-Rad, Hercules, CA, Cat#162–0196). Probes used in the hybridization step were prepared by random prime labeling incorporating ^{32}P-radiolabelled dCTP using Prime-It II kit (Stratagene Corp., La Jolla, CA, Cat#

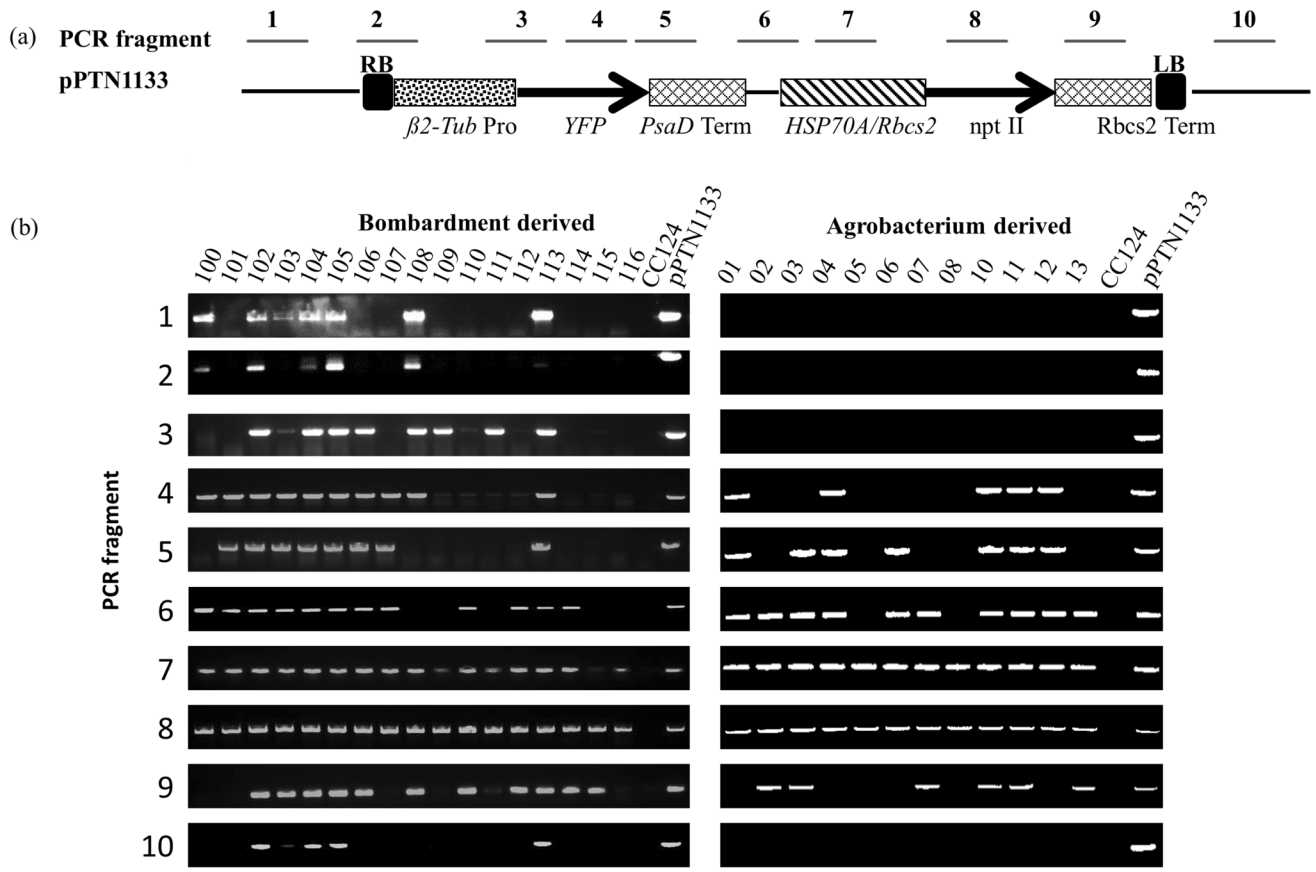

Figure 2. PCR analyses for integration of plasmid DNA into *Chlamydomonas* genome in the microprojectile-derived and *Agrobacterium*-mediated transgenic events derived from binary vector pPTN1133. (*a*) Diagram of PCR amplification along the T-DNA and a segment of pPTN1133 plasmid backbone. (*b*) PCR results of using corresponding primer sets.

300385). Hybridizations and washing steps were carried out as previously described (Eckert *et al.* 2006). For analyses of DNA integration in the transgenic events of both *Agrobacterium* and microprojectile derivatives, a series of primer sets (Supplementary Table S1) were used to amplify various segments of the T-DNA and the binary plasmid backbone sequence (Fig. 2).

DNA junction fragments about the insertion sites of the introduced transgene(s) were captured using either inverse PCR (Ochman *et al.* 1988) or thermal asymmetric interlaced (TAIL) PCR (Liu *et al.* 1995) methods. Amplified DNA fragments were cloned into pCR2.1-TOPO (Invitrogen Cat# 45–0641) and sequenced (Eurofins MWG Operon). The sequence junction fragments were blast searched (www.phytozome.net). As a confirmatory step and to analyze the integrity of the transgenic allele insertion, the junction fragments were subsequently re-amplified directly from the transgenic events with primer set designs based on the DNA sequence captured from the initial TAIL or inverse PCR reactions.

Results and Discussion

Evaluation of Synthetic *nptII* and YFP Transgenes in *C. reinhardtii* To confirm functionality of the synthetic codon-optimized versions of the selectable and visual marker genes, microprojectile bombardment of intermediate pUC-based plasmids, designated pPTN1111 and pPTN1106, which harbor *nptII* and YFP cassettes, respectively, and are present in the binary vector pPTN1133 (Fig. 1) as a transgene stack, was introduced into *C. reinhardtii*. Based on recovery of paromomycin-resistant colonies under a selection pressure of 15 mg L^{-1} that were also positive by ELISA assay, the estimated transformation frequency was 3.4 CFU per million cells. Co-bombardment transformations using pPTN1111 and pPTN1106 resulted in an estimated transformation frequency of 2.5 CFU per million cells, translating to a co-expression rate of 3.5% with expression of YFP observed in 0.09 CFU per million cells. The co-expression frequency was significantly lower than what was observed in higher

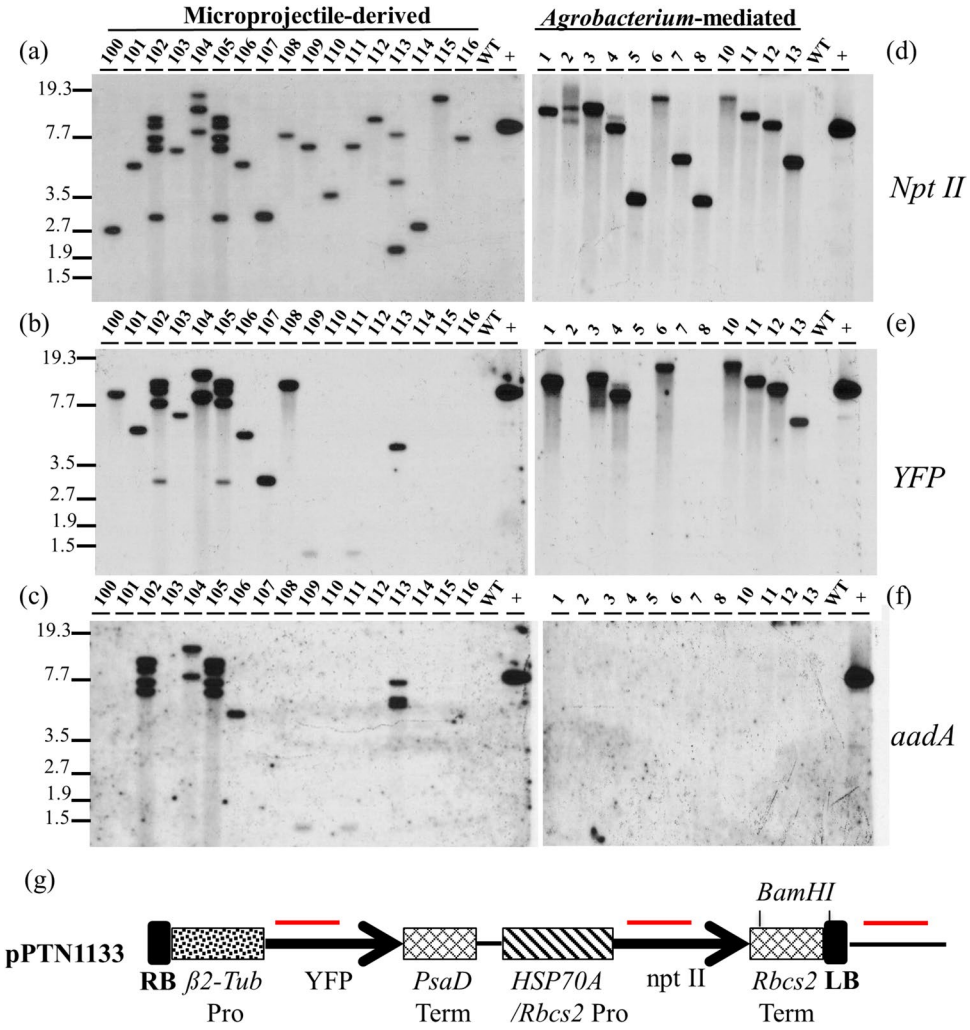

Figure 3. Southern blot analyses on transgenic *Chlamydomonas reinhardtii* events. (*a*) Microprojectile-derived transgenic events obtained from delivery of binary vector pPTN1133 hybridized with *nptII* ORF. (*b*) Blot in *a* stripped and re-hybridized with YFP ORF. (*c*) Blot in *b* stripped and re-hybridized with *aadA* ORF. (*d*) *Agrobacterium*-mediated transgenic events derived from binary vector pPTN1133 probed with *nptII* ORF. (*e*) Blot in (*d*) stripped and re-hybridized with YFP ORF. (*f*) Blot in *e* stripped and re-hybridized with aadA ORF. *Lane* + indicates 50 pg of pPTN1133. Molecular marker is in kb. Genomic DNA was digested with *BamHI*. (*g*) Diagram of pPTN1133 showing *BamHI* sites and relative probe binding locations (*red bars* corresponding to *nptII*, *YFP*, and *aadA*).

plants when delivering multiple transgenic cassettes into cells *via* biolistics (Gordon-Kamm *et al.* 1990; Chen *et al.* 1998, 2006; Tang *et al.* 1999).

Several possibilities, including genetic and epigenetic, can account for the poor co-expression frequencies often encountered with *Chlamydomonas* transformation (Cerutti *et al.* 1997; Fischer and Rochaix 2001). Molecular analyses employing 12 PCR primer sets that spanned the T-DNA element of pPTN1133, including regions just outside the respective border elements, were conducted on 17 of the transgenic lines not co-expressing YFP derived from these microprojectile-mediated transformations. The results revealed that within these selected lines eight of the 17 appeared to have an intact YFP cassette with 13 missing some portion of the binary vector backbone (Fig. 2); the latter rarely occurred with direct DNA delivery into the nuclear compartment of higher plants. These data suggested that approximately 50% of the transgenic lineages not co-expressing YFP were due to genetic truncations in the transgenic allele.

Agrobacterium*-Mediated Transformation of *C. reinhardtii To assess the ability of *A. tumefaciens* to transform and subsequently characterize the complexity of the integration sites of transgenic alleles introduced into the *C. reinhardtii* genome *via Agrobacterium*-mediated transformation, a facile protocol was designed based on known parameters critical for the bacterium to transfer T-DNA elements through the type IV secretion system of the organism into a host genome. These parameters include low pH and reduced phosphorus coupled with the presence of a phenolic inducer of the two-component regulatory system of the secretion apparatus, acetosyringone, during the co-cultivation of the *A. tumefaciens* with the algae cells (Stachel *et al.* 1985; Fullner and Nester 1996; McCullen and Binns 2006). Secondly, the protocol implements an antibiotic counter selection scheme to impede *A. tumefaciens* multiplication while maintaining growth rates of the *C. reinhardtii.* Hence, the protocol provided a microenvironment conducive for *A. tumefaciens* during the co-cultivation step without compromising growth

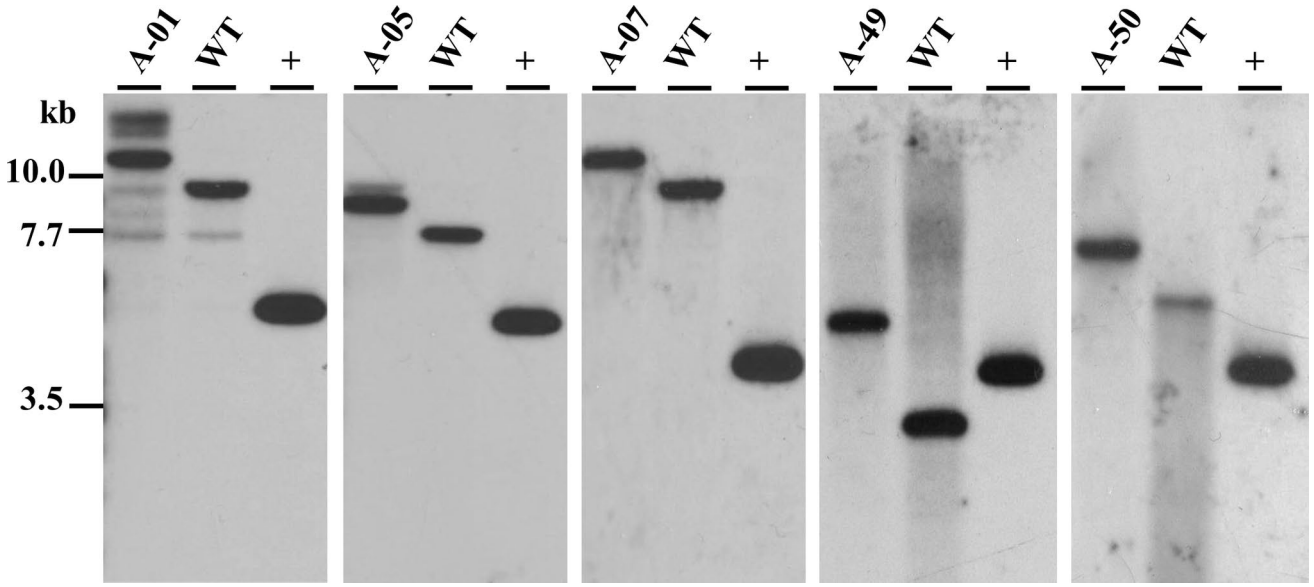

Figure 4. Southern blot analysis using junction fragment probes. Total genomic DNA isolated from five selected *Agrobacterium*-mediated transgenic *Chlamydomonas reinhardtii* events (pPTN1133) designated A-01, A-05, A-07, A-49, and A-50. *WT lanes* refer to wild-type CC124 total genomic DNA. *Lanes* designated with + refer to 50 pg of plasmid DNA harboring the corresponding cloned junction fragment. Genomic DNA was digested with *NotI*. Probes used in the respective hybridization of blots correspond to junction fragments obtained from DNA proximal to the LB region of the selected transgenic events.

of *C. reinhardtii* during the selection phase of the transformation protocol.

A set of binary vectors were assembled that carry a synthetic *nptII* cassette regulated by either the Rbcs2, PsaD, or ß2-tubulin promoter alone or stacked with a YFP and/or *ble* cassettes (Fig. 1). The base binary vector backbone, pPZP202 (Hajdukiewicz *et al.* 1994), that was used, along with the disarmed nopaline strain of *A. tumefaciens*, C58C1/pMP90 (Koncz and Schell 1986), is a standard component in transformation systems often used for higher plants. The transformation protocol was evaluated on the walled strain, *C. reinhardtii* CC-124. To mitigate confounding results due to bacterial expression of YFP, the ORF was synthesized with the Rbcs intron from *C. reinhardtii*.

A summary of the *Agrobacterium*-mediated transformations carried out is listed in Table 1. The results indicated comparable transformation frequencies between the *Agrobacterium*-mediated delivery and microprojectile bombardment introduction of the respective plasmids, pPTN1133 and pPTN1131, with the latter vector identical to the former except that the YFP cassette was under control of the PsaD rather than the ß2-tubulin promoter (Fig. 1). Observed mean transformation frequencies, calculated based on antibiotic resistant colonies per million cells, ranged from 0.09 to 2.86 (Table 1). Importantly, the respective transformations tabulated in Table 1 were conducted at different times by individuals from different laboratories thereby reflecting the reliability, repeatability, and critically the translatability of this *Agrobacterium*-mediated transformation protocol for *C. reinhardtii*.

Molecular Characterization of Transgenic Cell Lineages of *C. reinhardtii* Southern blot analyses on a subset of the transgenic cell lineages derived from both microprojectile bombardment and *Agrobacterium*-mediated transformation methods with the binary vector pPTN1133 (Fig. 1) are shown in Fig. 3. With respect to the former transformation method, the whole circular plasmid was delivered into the cells. The data collected from the hybridization analyses revealed a higher number of multi-locus insertions with the direct DNA approach as compared to *Agrobacterium*-mediated events (Fig. 3). Surprisingly, though, a relatively high percentage of single locus events (13/17 = 76%) was also observed with microprojectile bombardment, which was atypical in nuclear transformation in higher plants with direct DNA methods.

Co-transformation-based genotyping *via* PCR of the non-selectable YFP cassette was detected in approximately 59% (10/17) of the microprojectile bombardment events and 42% (5/12) of the *Agrobacterium*-mediated events (Fig. 2*b*). Presence of the plasmid backbone outside of the T-DNA was monitored by re-probing the blots with the bacterial selectable marker gene, *aadA* (Fig. 2*c*). The data revealed that the *aadA* marker was integrated in seven of the 17 microprojectile events and absent in all the *Agrobacterium*-mediated events (Fig. 2*c*). Hence, unlike what is typically observed in

Table 1. Estimated transformation frequencies of *Chlamydomonas reinhardtii*

Transformation method[a]	Plasmid[b]	Promoter[b]	Selection (mg/L^{-1})[c]	Frequency[c]
Agrobacterium-mediated	pPTN1127	Rbcs2-HSP70	Paromomycin (15)	0.70 ± 0.02
Agrobacterium-mediated	pPTN1127	Rbcs2-Hsp70	Paromomycin (15)	0.31 ± 0.04
Agrobacterium-mediated	pPTN1127	Rbcs2-HSP70	Paromomycin (15)	0.79 ± 0.05
Agrobacterium-mediated	pPTN1129	PsaD	Paromomycin (15)	2.86 ± 0.51
Agrobacterium-mediated	pPTN1130	ß-tub2	Paromomycin (15)	1.17 ± 0.12
Agrobacterium-mediated	pPTN1133	Rbcs2-HSP70	Paromomycin (15)	0.58 ± 0.08
Agrobacterium-mediated	pPTN1133	Rbcs2-HSP70	Paromomycin (15)	0.28 ± 0.07
Agrobacterium-mediated	pPTN1133	Rbcs2-HSP70	Paromomycin (15)	0.19 ± 0.03
Agrobacterium-mediated	pPTN1133	Rbcs2-HSP70	Paromomycin (15)	0.24 ± 0.05
Agrobacterium-mediated	pPTN1170	Rbcs2-HSP70	Paromomycin (15)	0.29 ± 0.05
Agrobacterium-mediated	pPTN1170	Rbcs2	Zeocin (7.5)	0.18 ± 0.04
Agrobacterium-mediated	pPTN1176	Rbcs2-HSP70	Paromomycin (15)	0.20 ± 0.04
Agrobacterium-mediated	pPTN1176	Rbcs2	Zeocin (7.5)	0.09 ± 0.02
Microprojectile-derived	pPTN1133	Rbcs2-Hsp70	Paromomycin (15)	0.10 ± 0.04
Microprojectile-derived	pPTN1133	Rbcs2-HSP70	Paromomycin (15)	0.18 ± 0.07

[a]*Transformation method column* refers to gene transfer system used for *C. reinhardtii* transformation. [b]*Plasmid* and *promoter columns* indicate the respective plasmid name and promoter element controlling the *nptII* selectable marker cassette, respectively. [c]*Selection* and *frequency columns* refer to the selection agent and level used in parenthesis, and colony counts per 1 million cells inoculated (*Agrobacterium*-mediated) or per µg DNA delivered (microprojectile-derived), respectively

nuclear higher plant transgenic events derived from direct DNA methods (Cheng *et al*. 2001; Svitashev *et al.* 2002; Makarevitch *et al.* 2003), a significant number of transgenic events lack integration of the backbone regions of delivered plasmids. Genotyping by PCR (Fig. 2) of 12 selected *Agrobacterium*-mediated transgenic lineages, not co-expressing YFP, suggested that a lack of co-expression in eight of the lines was due to truncations in the YFP cassette (Fig. 2).

Junction fragments about the foreign alleles introduced through *Agrobacterium*-mediated transformation were captured using either TAIL-PCR (Liu *et al.* 1995) or inverse PCR (Ochman *et al.* 1988) methodology. The integrity of the junctions, map positions, and gene models in which the T-DNA elements reside from the genotyped events are summarized in Table 2. The junction fragment sequences blast searched against the *C. reinhardtii* genome (www. Phytozome.net) suggested that, analogous to T-DNA integration in higher plant nuclear compartments, integration was essentially a random event. Among the junction fragments analyzed, five were predicted to interrupt genes (Table 2). Importantly, the analyses indicated that the junction fragments about the site of T-DNA integration are essentially

Table 2. Map position of T-DNA alleles in selected *Chlamydomonas reinhardtii* events

Clone designation[a]	Junction integrity[b]	Location[c]	Annotation[c]
1133-(1)	5 bp deletion	Cre13.g571050	AFRID/BRIGHT DNA binding domain
1133-(5)	22 bp deletion	Cre01.g068400	Unknown
1133-(6)	4 bp insertion	Cre01.g065900	MAPKK-related Ser/Thre Kinase
1133-(7)	5 bp insertion	Cre07.g315800	MAPEG family
1133-(42)	26 bp insertion	Cre09.g389650	Tyrosine protein kinase
1133-(43)	14 bp deletion	Non-genic Ch7	NA
1133-(48)	0 bp	Cre09.g404900	Unknown
1133-(49)	11 bp deletion	Cre12.g484650	Unknown
1133-(50)	2 bp deletion	Non-genic Ch2	NA
1170-(Z2)	2 bp insertion	Cre03.g148650	Unknown
1170-(Z4)	6 bp insertion	Cre06.g306250	Unknown
1170-(Z9)	11 bp deletion	Cre13.g567550	Tyrosine protein kinase

[a]*Clone designation column* indicates binary plasmid and clone number in parenthesis. [b]*Junction integrity column* indicates number of base pair deletions/insertions adjacent to the T-DNA sequence observed. [c]*Location* and *annotation columns* indicate map position and gene call of insertion site of the corresponding T-DNA element, respectively

intact (Table 2). This was unlike other reports studying direct DNA integration in the *C. reinhardtii* nuclear genome where large deletions near the site of integration are often observed (Dent *et al.* 2005). This attribute was important to consider when implementing transformation approaches for insertional- and/or activation-tagging for functional genomics studies with *C. reinhardtii*.

The junction fragments isolated from five events were used as probes in Southern blot analysis to visualize molecular weight shifts in the hybridization signals between wild-type CC124 and transgenic lineages as an additional confirmation that the junctions captured were linked to the T-DNA. As can be seen in Fig. 4, in each case the expected upshift in molecular weight of the hybridization signal is observed, supporting the linkage of the captured DNA junction element with the T-DNA.

The junction fragment sequence information about the T-DNA alleles allowed the present study to gather deeper genotypic data on the integrity of the T-DNA element integrated (Fig. 5) in the genome of a subset of the transgenic events produced *via Agrobacterium*-mediated transformation. A visual representation of the T-DNA elements integrated in nine of the transgenic events derived from *Agrobacterium*-mediated transformation (pPTN1133; Fig. 1) is shown in Fig. 5. Each of the integrated T-DNAs has significant truncations proximal to the right border element where the visual marker YFP cassette resides, which supports the PCR genotyping results (Fig. 2). This lack of foreign locus integrity in turn can negatively impact co-expression of transgenes in *C. reinhardtii*. For example, *Agrobacterium*-mediated transformations conducted with *A. tumefaciens* transconjugants carrying pPTN1133 and pPTN1170 were monitored for co-expression of YFP. The results revealed that among 54 transgenic pPTN1113 lines selected on paromomycin and monitored for co-expression of YFP, only one was expressing YFP (Fig. 6), translating to a co-expression frequency of 1.9%. Among 51 transgenic pPTN1170 lines selected on paromomycin, one was identified as co-expression YFP (2.0% co-expression); and across 54 transgenic pPTN1170 lines selected on zeocin, none was positive for YFP expression. Employing a dual selection approach, supplementing selection plates with both paromomycin and zeocin, transformation frequencies were drastically reduced; but among three transgenic pPTN1170 dual selected transgenic events, one was positive for YFP expression (Fig. 6).

Using a co-bombardment strategy to simultaneously introduce YFP and *nptII* cassettes into *C. reinhardtii*, co-expression of YFP in *nptII* positive cell lineages ranged from 0.7 up to 3.9% across nine independent co-transformation set-ups. Therefore, unlike in higher plants, co-expression of non-selected transgene(s) was relatively low. Hence, while co-transformation occurred at a rate of over 40% using either microprojectile bombardment or *Agrobacterium*-mediated gene transfer, integrity of the transgenic allele appeared to

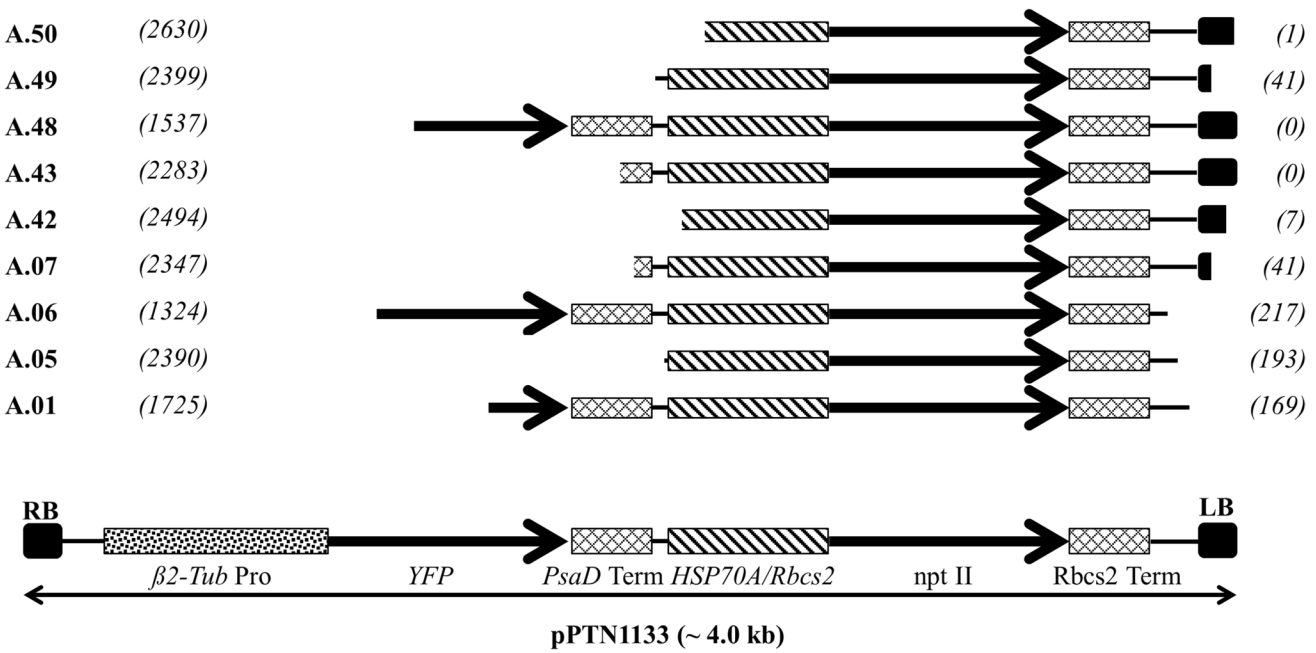

Figure 5. Integrity of T-DNA elements present in selected *Agrobacterium*-mediated transgenic events. A diagrammatic view of the T-DNA element of the binary vector pPTN1133 is shown at the *bottom*. Transgenic event designations are listed along the *left side* of the figure. The *number* in parenthesis next to the event designation refers to the base pair deletion within the T-DNA proximal to the RB element. The *number* in parenthesis along the right side corresponds to the base pair deletion proximal to the LB element.

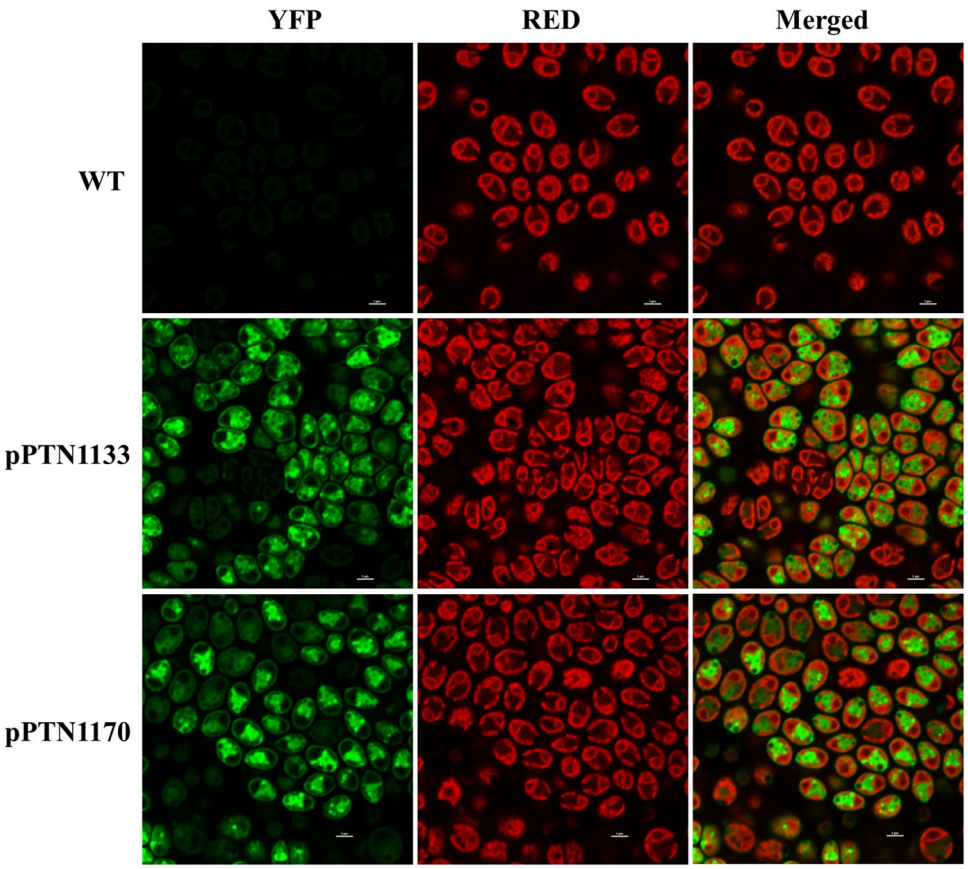

Figure 6. Confocal images of *Agrobacterium*-mediated transgenic *Chlamydomonas reinhardtii* events expressing YFP. *Panels* labeled WT, pPTN1133, and pPTN1170 refer to wild-type CC124 cells, an *Agrobacterium*-mediated transgenic event carrying T-DNA element of binary vector pPTN1133 selected on paromomycin, and a dual selected, paromomycin and zeocin, event carrying the T-DNA of binary vector pPTN1170, respectively. *YFP* column* of images is false colored green. *Red panel* images display autofluorescence emanating from chlorophyll, while *merged panel* images are a stacked image of corresponding YFP* and red images.

be a significant factor impacting co-expression of the non-selectable transgenic cassette (Figs. 2 and 3).

A previous report communicating an *Agrobacterium*-mediated transformation system for *C. reinhardtii* using the octopine strain of *A. tumefaciens* LBA4404 (Hoekema *et al.* 1983) revealed a 50-fold increase in transformation frequency as compared to the direct DNA delivery using glass beads (Kumar *et al.* 2004). This protocol utilized the binary vector pCAMBIA1304 which harbors a hygromycin selectable marker along with a fusion visual marker cassette coupling ß-glucuronidase (GUS) and green florescent protein (GFP). Both cassettes were under control of the viral 35S CaMV promoter element (Benfey and Chua 1990), and the GC content of the transgenes was not optimized for *C. reinhardtii.* These two features of pCAMBIA1304 were not conducive for a reliable transformation system for *C. reinhardtii.* The 35S CaMV promoter, although reportedly functional in *C. reinhardtii* (Tang *et al.* 1995), has relatively poor activity in this alga (Day *et al.* 1990) and other algae cells (Walker *et al.* 2005). Codon optimization of transgenes has been shown to be a very important parameter for translational efficiency in *C. reinhardtii* (Fuhrmann *et al.* 1999; Shao and Bock 2008). Hence, this may account for the apparent non-repeatability of this earlier *Agrobacterium*-mediated transformation protocol in the *C. reinhardtii* scientific community, which is reflected by the minimal number of subsequent communications further exploiting an *Agrobacterium*-mediated transformation system to manipulate the *C. reinhardtii* nuclear genome (Pratheesh *et al.* 2012, 2014).

To indirectly evaluate the effectiveness of the 35S CaMV promoter in *C. reinhardtii*, a comparative transformation study was conducted using a binary vector harboring standard higher plant *nptII* cassette under control of the 35S CaMV promoter and pPTN1133 in which *nptII* is under control of the Rbcs2-HSP70 promoter. Across three independent microprojectile bombardment experiments, no paromomycin-resistant colonies were recovered with the *35S-CaMV::nptII* cassette while a mean paromomycin-resistant CFU per million cells of 0.13 ± 0.3 was obtained with the *Rbcs2-HSP70::nptII* cassette (pPTN1133). This outcome supported the importance of proper designs of transgenic alleles for successful recovery of *C. reinhardtii* transformants.

More recently, Mini *et al.* (2018) did a comparative study between a direct DNA-based and *Agrobacterium*-mediated transformation of *C. reinhardtii*. In this report, the researchers utilized an *nptII* allele from *Streptomyces rimosus* (Sizova *et al.* 1996, 2001) as the selectable marker rather than the *E. coli* version typically used in transformation systems of higher plants (Fraley *et al.* 1983). They also

evaluated both walled and wall-less strains of *C. reinhardtii*, and two *A. tumefaciens* strains, the octopine strain LBA4404 (Hoekema *et al.* 1983) and nopaline strain C58C1 (Koncz and Schell 1986). The outcomes reported showed that both *Agrobacterium* strains could transform the wall-less strain (cw15) with equal efficiency; however, only the nopaline strain was able to transform the walled strain (CW15). These results also confirmed the importance of proper designs of the genetic elements for successful and reliable *Agrobacterium*-mediated transformation of this model alga, including proper selection of promoter elements, codon optimization of the open reading frames, and a proper co-culturing microenvironment (Mini *et al.* 2018).

Key attributes of the *Agrobacterium*-mediated transformation protocol described herein include (1) comparable transformation efficiencies with direct DNA methods when using a walled cell strain; (2) no autolysin pretreatment required; (3) robust application, as it has been successfully conducted in independent laboratories; and (4) junction fragments about the transgenic allele are essentially intact. This latter attribute is of paramount importance for insertional- and/or activation-tagging approaches to gain insight on the underlying biology governing this model alga and for the delivery and expression of transgenic alleles. The presence of large deletions around the foreign allele, which is common when using direct DNA methods for nuclear transformation of *C. reinhardtii* (Dent *et al.* 2005), makes it difficult to link genotype to phenotype in a mutant collection and to create cell lines expressing a transgenic allele without collateral damage about the locus in which the transgenic allele resides. The only direct DNA protocol communicated for *C. reinhardtii* that possesses the attribute of intact junction fragments required the combination of electroporation with linearized DNA containing no vector backbone (Gonzalez-Ballester *et al.* 2011). This method, while reliable, adds labor and cost in the development of the mutagenized population of *C. reinhardtii.*

The drawback of the *Agrobacterium*-mediated transformation protocol outlined herein and by others (Mini *et al.* 2018) is the observation that the non-selectable transgenic allele is often truncated (Fig. 5). Historically, lack of co-expression of non-selected transgenic cassettes in the *C. reinhardtii* nuclear compartment has been attributed to epigenetic phenomena (Cerutti *et al.* 1997), improper regulatory elements controlling gene(s) of interest (Fischer and Rochaix 2001), or GC content issues requiring codon optimization for efficient translation (Shao and Bock 2008). While each of these parameters clearly can have an impact on co-expression frequencies and levels, genotyping of the transgenic *C. reinhardtii* events generated *via* microprojectile bombardment and *Agrobacterium*-mediated transformation methods suggests deletions of the non-selected transgenic cassette are the predominate underlying cause for lack of co-expression. Indeed, taking a mutant screening approach, *C. reinhardtii* isolates were identified in which the presence of intact non-selected transgenic cassettes was improved, which translated to higher co-expression frequencies (Neupert *et al.* 2009). Hence, research investments in DNA repair mechanisms of *C. reinhardtii* (Vlček *et al.* 2008) may provide insights into steps to improve upon this parameter along with informing future genetic designs that permit for more precision in genetic variation at a locus introduced by genome editing reagents (Sizova *et al*. 2021).

Successful genome editing in *C. reinhardtii* has been achieved through deployment of CRISPR-Cas-mediated approaches (Ghribi *et al.* 2020) with reports communicating CRISPR reagents that incorporate endonucleases from organisms *Lachnospiraceae* bacterium ND2006 (Ferenczi *et al.* 2017) and *Streptococcus pyogenes* (Akella *et al*. 2021), CRISPR/Cas12a and CRISPR/Cas9 systems, respectively, being effective in creating targeted INDELs in the genome (Ghribi *et al.* 2020). The editing reagents have been introduced as DNA elements, both transiently (Guzmán-Zapata *et al.* 2019) and as a stable transgenic allele (Park *et al.* 2020), or as *in vitro* assembled ribonucleoproteins (Ferenczi *et al.* 2017).

Interestingly, frequencies of precise knock-ins were rather high (> 10%) when the assembled ribonucleoprotein complex was co-delivered with either small single-stranded DNA (Ferenczi *et al.* 2017; Akella *et al.* 2021) or double-stranded expression cassette templates (Kim *et al.* 2020). These reports opened the possibility that an improved facile knock-in method for *C. reinhardtii* can be developed by coupling an *Agrobacterium*-mediated reagent delivery approach with a retron-based *in vivo* synthesis of single-stranded multi-copy DNA templates by employing Cas9 Retron precise Parallel Editing *via* homologY (CRISPEY) editing reagents (Sharon *et al.* 2018; Zhao *et al.* 2022). The CRISPEY editing reagents can be assembled into a binary vector and the T-DNA cargo introduced either as a stable expressing transgenic allele, using a common *A. tumefaciens* strain like C58C1/pMP90 (Koncz and Schell 1986), or transiently expressed using a novel mutant strain (Gelvin and Lee 2021) that makes an integrative deficient T-strand complex that is translocated to the algal cell, but fails to form a stable transgenic allele.

Conclusion

A reliable and simple *Agrobacterium*-mediated delivery was described that provides workable transformation frequencies for the algae model organism *C. reinhardtii*. The major attribute of the *Agrobacterium*-mediated method for *C. reinhardtii* was the integrity of the genomic junction fragments.

The drawback of the approach for transformation of this algae, however, was the low co-expression frequency of the non-selectable transgenic allele, which is primarily due to truncations in the T-DNA.

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