Building a multipurpose insertional mutant library for forward and reverse genetics in *Chlamydomonas*

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**Abstract**

**Background:** The unicellular green alga, *Chlamydomonas reinhardtii*, is a classic model for studying flagella and biofuel. However, precise gene editing, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas9) system, is not widely used in this organism. Screening of random insertional mutant libraries by polymerase chain reaction provides an alternate strategy to obtain null mutants of individual gene. But building, screening, and maintaining such a library was time-consuming and expensive.

**Results:** By selecting a suitable parental strain, keeping individual mutants using the agar plate, and designing an insertion cassette-specific primer for library screening, we successfully generated and maintained ~150,000 insertional mutants of *Chlamydomonas*, which was used for both reverse and forward genetics analysis. We obtained 26 individual mutants corresponding to 20 genes and identified 967 motility-defect mutants including 10 mutants with defective accumulation of intraflagellar transport complex at the basal body. We also obtained 929 mutants defective in oil droplet assembly after nitrogen deprivation. Furthermore, a new insertion cassette with splicing donor sequences at both ends was also constructed, which increased the efficiency of gene interruption.

**Conclusion:** In summary, this library provides a multifunctional platform both for obtaining mutants of interested genes and for screening of mutants with specific phenotype.

**Keywords:** *Chlamydomonas*, Insertional mutants, Mutant library, Flagella, Intraflagellar transport, Oil droplet

**Background**

*Chlamydomonas reinhardtii* is a unicellular green alga that shares organelles with both higher plants and animals [1]. This organism performs photosynthesis via cup-shaped chloroplasts, moves toward nutrients and light using a pair of flagella/cilia, and accumulates oil droplets in response to nitrogen starvation [2]. So *Chlamydomonas* is recognized as an important model for studying photosynthesis, flagella/cilia, and stress response [3, 4]. Recently, It has been acknowledged as “green yeast” for the study of biofuels such as biodiesel and hydrogen, and has been used as a biofactory for the production of antibodies and biopharmaceuticals [5, 6].

Classic and modern genetic manipulation techniques have been established in the last 60 years since Gilbert Morgan Smith isolated and provided the first *C. reinhardtii* strain to the scientific community [7]. Ultraviolet (UV) light [8], ethyl methanesulfonate (EMS) [9] and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [10, 11] was widely used for mutagenesis and generated many useful mutants. For example, the genes involved in photosystem-II and photosynthetic electron transport were first characterized in Paul Levine’s laboratory by screening UV-induced acetate auxotrophy mutants [8, 12]. Cell wall-deficient mutants have been generated by MNNG mutagenesis and isolated under stereomicroscopy. These mutants have been widely used for gene transformation...
using glass beads [13]. Remarkably, temperature-sensitive flagellar mutants generated by MNNG mutagenesis are motile at permissive temperatures, but become immotile at restrictive temperatures, and have been used to identify the components of intraflagellar transport (IFT) [11, 14].

Transformation or introducing a foreign gene in the genome is a prerequisite of modern genetics. Several methods have been developed to transform DNA fragments into the nuclear genome of *Chlamydomonas*. In addition to glass beads mentioned above, other techniques include electroporation, biolistic transformation and biotransformation by *Agrobacterium tumefaciens* are also used in *Chlamydomonas* [15–17]. Biolistic transformation can also be used to transform DNA into the chloroplast and mitochondria of *Chlamydomonas* [18, 19]. Meanwhile, various transformation markers have been developed. These include auxotrophic markers such as arginosuccinate lyase (*ARG7*) and nitrate reductase (*NITI*), which encode enzymes that complement metabolic deficiencies [15, 20], as well as antibiotic markers such as *aphVII*, *aphVIII*, and *ble*, which provide resistance to hygromycin, paromomycin and zeocin respectively [21–23]. Several novel markers have also been developed recently, such as herbicide resistance genes glyphosate acetyltransferase (*GAT*), protoporphyrinogen oxidase (*PPO*), and phytoene desaturase (*PDS*) and codon-optimized kanamycin-resistance gene *nptII* and tetracycline resistance gene *tetX* [24–26].

Similar to other classical organism models, both forward and reverse genetics strategies are widely employed in the research of *Chlamydomonas*. Forward genetics study generally composed of four major steps: mutagenesis, phenotypic screening, mutation loci (gene) mapping, and phenotype rescue. Much progress has been made to optimize these four steps. At present, mutagenesis introduced by insertion of a DNA cassette into the genome is preferred [27–29]. The mutated gene can be identified using PCR map-based cloning, and whole genome sequencing [29–31]. The valuable *Chlamydomonas* mutant library screened by forward genetics could also be cryopreserved for further analysis [32]. Specific mutated phenotypes can be screened using high throughput methods such as fluorescence-activated cell sorting [33]. Phenotype rescue is relatively difficult, because the expressions of foreign genes are relatively low in *Chlamydomonas* [34]. However, this problem may be solved partially by modification of transgene sequences, such as codon optimization, incorporation of native promoters and introns, co-expression with antibiotic gene or luciferase, and use of a host strain defective in transgene silencing [34–36].

Several methods are also available for reverse genetic studies in *Chlamydomonas*. Target genes can be knocked down by using small interfering RNAs [37] and artificial miRNAs [38, 39]. Conditional knockdown of *Chlamydomonas* genes can be achieved by using inducible promoters such as nitrate-induced promoter *NITI*, nickel-induced promoter *CYC6*, and CO2-induced promoter *CAH1* [40, 41]. However, the knockdown effects of RNAi [37] and artificial miRNAs [38] varies depending on genes and may disappear after several months. Gene knockout based on homologous recombination (HR) provides a great opportunity to study the function of non-lethal genes. Of note, is that the natural HR rate in *Chlamydomonas* is extremely low [42]. Several strategies have been described to increase the HR rate, such as use of single-stranded DNA, use of HR constructs coding an antibiotic marker in frame with the target gene, and induction of DNA breaks in the target gene via zinc-finger nucleases [42, 43]. However, these methods are either limited to special genes or the procedures are tedious. Recently, gene editing using CRISPR and Cas9 has been reported in *Chlamydomonas*, but the efficiency is too low to be applied to any genes, especially when the phenotypes of mutated genes are unknown [44–46]. Thus, constructing a random insertional mutant library provides an alternative method to obtain the mutants of specific genes. This strategy has been successfully applied to construct a single-use insertion mutant library and obtained 45 individual mutants representing 37 different genes by screening the approximately 100,000 insertional mutants with PCR [29]. Most recently, an indexed, mapped mutant library was developed based on the high-throughput approach, in which the mutants were transferred by highly automatic robots and cryopreserved in liquid nitrogen. The insertion sites were determined by deep sequencing methods, *Chlamydomonas Mmel* based insertion site Sequencing (ChlaMmeSeq) and Linear and Exponential Amplification of insertion site sequence coupled with Paired-end Sequencing (LEAP-Seq) [27, 47]. As a result, 37,000 mutants covering 73% of the *Chlamydomonas* genome were identified based on a deconvolution method [47]. However, construction and maintenance of such a library requires automatic robot technology, which is expensive for a small group. The library is mostly used for reverse genetics and more than half of the insertions lie in the untranslated region (UTR) [47]. Therefore, the need for improvement in construction, maintenance, and screening the library still remains.

In this study, a library of approximately 150,000 *Chlamydomonas* transformants was constructed and maintained with optimized procedures. Both reverse and forward genetics were performed to identify mutants...
of specific genes or mutants with specific phenotypes. We obtained 967 mutants with defective motility, 929 mutants with fewer oil droplets in response to nitrogen deprivation, and 26 individual mutants representing 20 different genes related to flagella, glycosylation, and starch metabolism. This library can also be used to screen mutants for any *Chlamydomonas* gene of interest or mutants with specific phenotype defects.

**Results**

**Optimizing methods for construction of the insertional library**

The genomic size of *Chlamydomonas* is about 111 Mb and the genome encodes approximately 17,000 genes according to the prediction in Joint Genome Institute (JGI) version 5.5 of *Chlamydomonas* genome [1]. Since the integration of DNA fragment in the genome of *Chlamydomonas* is random, the minimum number of insertional mutants is 111,000 if one insertional mutation locating within any 1 kb of genome is required. Taking into account that the average size of *Chlamydomonas* genes is approximately 6 kb, each gene can be hit about five times. However, to generate and maintain this quantity of mutants is challenging for an individual group. To optimize the use of this resource, we intended this library to be used not only for reverse genetics, but also forward genetics, such as screening mutants for flagella assembly and formation of oil droplets.

1. **Selection of the parental strain**

Intraflagellar transport (IFT) plays a key role in flagellar assembly and disassembly [4]. In order to obtain the mutants defective in IFT protein accumulation at the basal body, we generated an IFT46::YFP fusion construct using an endogenous promoter of *IFT46* and transformed it into the flagella-less mutant *ift46-1* (Fig. 1a). IFT46::YFP rescued the motility defect of *ift46-1* and
the distribution of flagellar length in the rescued strain was similar to the wild type, with an average length of 11.1 μm (Fig. 1b). The rescued strain was designated as HS211. Western blot results demonstrated that IFT46 was expressed in CC-125 cells only and not in the ift46-I mutant, while the fusion protein IFT46::YFP was detected in HS211 (Fig. 1c). These data suggest that the IFT46::YFP fusion protein functions as endogenous IFT46. Thus, HS211 could serve as a parental strain for screening of flagella assembly mutants and IFT basal body localization-defect mutants.

Next, we compared the transformation efficiency of HS211 with wild type strain. Since the aphVII gene conferring to hygromycin resistance was used to generate the HS211 strain, transformation efficiency of HS211 was tested using the aminoglycoside 3′-phosphotransferase (aphVIII) cassette as a selection marker. Using 5 × 10⁷ cells for one transformation, an average of 1342 transformants were obtained in HS211, whereas only 236 were obtained in CC-125 cells. The transformation efficiency of HS211 was sixfold higher than that of the wild type, CC-125 (Fig. 1d). This indicates that one transformation using 30 ng aphVIII in an electroporation cuvette generated almost 1400 transformants after 3 days, which suggests that only ~100 times transformations can generate enough mutants for construction of the library.

2. Insertion cassette and screening primer design

Besides serving as a selection marker for transformation, an ideal cassette for insertional mutagenesis can terminates transcription of the interrupted gene completely. Therefore, the 2.6 kb MlyI digested fragment from plasmid pMJ013b was used, which contains the promoter and terminator of PSAD gene, the aphVIII gene, an intron from CYTc6, and an RBL12 terminator [27] (Fig. 1e). The intron was intended to enhance the expression of the aphVIII gene and two inverted terminators at the 3′UTR were designed to terminate gene transcription of interrupted gene, irrespective of the direction of insertion. The fragment was therefore, selected as the insertion cassette.

The principle behind PCR-based screening was that when an insertion occurred in a target gene, a PCR product can be obtained using an insertion cassette-specific primer and a target gene-specific primer [29]. Since GC content of the Chlamydomonas genome is approximately 60%, the PCR product should be <1 kb in order to be considered reliable. Therefore, the target gene-specific primers were designed in two directions, forward and reverse, at an interval of approximately 1.0 kb. In order to cover every possible insertion events, the target gene-specific primers were designed from 500 bp upstream of 5′UTR to the end of 3′UTR of any interested genes (Fig. 1e).

The insertion cassette-specific primer requires to be within the 200- to 500-bp region from the insertion cassette border [29]. This region not only ensures amplification specificity and efficiency, but also reduces the possibility of corruption of the annealing sites caused by recombination or endonucleolytic cleavage during transformation [27]. The primer previously used has been designed as per the aphVIII gene sequence or the endogenous sequence of the PSAD promoter and RBL12 terminator. The aphVIII gene in this 2.6-kb cassette was approximately 700 bp from the border, while the primers annealing to PSAD promoter and RBL12 terminator tended to generate non-specific amplification since there is another copy of these two genes in the genome. Therefore, we designed the cassette-specific primer LGR06, annealing to the junction between PSAD and RBL12 terminator, which was 273 bp near the border of insertion cassette. Compared with the cassette-specific primer LGL03, which anneals to the PSAD promoter, the sequence of LGR06 does not exist within the Chlamydomonas genome. PCR amplifications with LGR06 generate fewer non-specific products and a cleaner background using the library and HS211 genomic DNA as templates. This primer increased efficiency of library screening (Additional file 1: Fig. S1).

3. Determination of the super pool size

A super pool size up to approximately 1000 was adopted for mutants screening in the previously generated library [29]. Larger pool size can reduce labor cost significantly if PCR amplification sensitivity can be guaranteed. We identified the maximal size of the super pool for library screening by mixing three characterized insertional mutants with varying numbers of uncharacterized insertional mutants, to generate a series of super pools ranging from 480 to 2400 in size (Fig. 1f). PCR amplification of three different insertion loci indicated that two loci could be detected in super pool reached to 2, 400, another one only could be identified by PCR in super pool 1920, so we choose 1440 as the optimal size for super pool (Fig. 1f).

4. Maintenance and replication of transformants

Maintenance and replication of large-scale Chlamydomonas mutants is a time-consuming task. To overcome this problem, a single-use library of up to 100,000 transformants was generated [29]. Alternatively, a library consisting of 18,334 transformants was cultured in 96- or 384-ul plates and cryopreserved with automatic robots operated under highly sterile conditions [47]. We intended to save all transformants on an agar plate and therefore devised an optimal economic
strategy. After electroporation, the transformants grew on solid tris-acetate-phosphate (TAP) plates under low light for 1 week (Fig. 2a), then each clone was picked up with a toothpick and applied along a line across the agar surface, where the clone propagated to the entire length of the line within a week. Thus, 48 lines were obtained (Fig. 2b1). This method not only guaranteed enough cell count for further analysis and reduced the duration for replication of the library, but also reduced loss of transformants due to contamination, since only 48 colonies would be lost in case a plate was contaminated. After a year, the livability of our library was approximately 97.8%, and the contamination rate was lower than 3%.

5. Isolation of the super pool DNA

Successful PCR amplification of insertion loci depends on quality of DNA templates from each super library. The library should contain sufficient DNA for thousands of PCR runs and also contain similar amount of genomic DNA of each mutant. To achieve this goal, we inoculated 1440 transformants from the basic pool onto 15-cm sized plates by drawing a shorter colony line (Fig. 2b2), such that the similar amount of cells from each clone could be guaranteed. All the transformants grown into visible clones of similar size within several days, then the clones were scraped and resuspended in liquid TAP medium, forming a cell culture with concentration >5 × 10^6 cells/ml. After the cell reached stationary phase under constant light, genomic DNA was isolated using the phenol/chloroform method [48]. Generally, about 250 μg genomic DNA can be obtained from a single super pool library preparation, which is adequate to screen 2500 pairs of primers.

Screening the mutants based on reverse genetics strategy

We planned to screen insertional mutants of 61 genes of interest, which requires more than 600 target gene-specific primers to screen 104 super pool libraries (Additional file 2: Table S1 and Additional file 3: Table S2). This means that at least 62,000 PCR reactions and 630 PCR runs need to be performed. To save time and cost, we optimized several aspects of the PCR screening strategy. First, the super pool libraries were divided into two groups, each including 48 libraries. If a reliable mutant was identified in one group, the other group was skipped. Second, DNA templates were arrayed into a matrix consisting of 4 rows and 12 columns. All PCR components were premixed and the reaction buffers were prepared only with addition of different target-specific primers. Thus, templates and buffers could be transferred into PCR microplates with a multichannel pipette (Fig. 2b4). Third, since it is generally accepted that insertional mutagenesis near the start codon tends to induce a complete knock-out effect, the screening process for each target gene was initiated with the first six target primers either forward or reverse from the 5′UTR end. If a correct insertion was identified by sequencing the PCR product, the other primers and libraries were not screened (Fig. 2b5). As a result, the total number of PCR runs was largely reduced. Finally, the volume of each PCR was reduced to 20 μl and the amount of Taq polymerase used per reaction was reduced from 2 units (supplier recommended) to 0.5 units, which improved amplification specificity per contra.

Once a correct insertion was identified from a super pool, the basic pools from this positive super pool were recovered for DNA isolation (Fig. 2b6). All 96 colonies from each basic pool were replicated onto a fresh 9-cm solid agar plate, allowing all transformants to recover to similar extents (Fig. 2b6). Then, cells from these plates were washed with 20 ml TAP medium and genomic DNA from each basic pool was isolated using the phenol/chloroform method, but on a small scale (Fig. 2b6). Approximately 10 μg basic library DNA was harvested, which was adequate for identification of positive transformants from the basic pool (Fig. 2b7). Once the positive basic pool was identified, all 96 transformants from the positive basic pool were inoculated separately, into liquid TAP medium contained in 96 microtiter plates. The cells were mixed together by line and row respectively, and colony PCR was performed. Positive results were obtained from both, the row and line mixtures as a positive transformant (Fig. 2b8). Finally, the positive transformant was duplicated from the original 9-cm solid agar plates for further analysis (Fig. 2b9).

Screening flagellar assembly-defect mutants

In addition to screening mutants for any gene of interest by reverse genetics, the library was also utilized to screen flagellar-defect mutants. Since the flagella of gametes are longer than those of vegetative cells [49], an aliquot of each clone was transferred into liquid nitrogen-deprived TAP (TAP-N) medium in 96-well microtiter plates for gametogenesis and screened for mutants with motility defects under a stereomicroscope (Fig. 2c1). We identified 967 motility-defect mutants (Fig. 2c2). Since motility defects may result from flagella loss or from the flagella being short or paralyzed, the flagellar length of these mutants was measured. We found that 66% of these mutants failed to assemble flagella, 11% showed shorter flagella, while the rest exhibited flagella with normal length, but were paralyzed. This suggests that most motility defects are caused by aberrations in flagella assembly (Fig. 3a).
Because the IFT marker IFT46::YFP was introduced into the parental strain HS211, all motility-defect mutants with aberrant IFT distribution at the basal body were screened. In the wild type HS211, the puncta of IFT46::YFP were distributed along the flagella and IFT46::YFP accumulation was observed at the basal body.
(Fig. 3c). In most of 638 flagella-less mutants, IFT46::YFP was concentrated at the basal body, while the fluorescence signal from some of these mutants was a little weaker. However, ten mutants showed similar phenotype with the ift46-1 mutant, lacking IFT46 accumulation at the basal body (Fig. 3c). The loss of IFT46::YFP signal was probably caused by knockout of IFT46::YFP in the parental strain, interference of IFT46 transcription, or regulation of IFT46 anchoring to basal body. To investigate this, IFT46::YFP expression in ten mutants was analyzed by western blot (Fig. 3b). IFT46::YFP proteins were absent in seven mutants, but were detected in the remaining three mutants, at reduced quantities compared to the parental strain HS211. This indicates that certain key elements regulating IFT46 anchorage to the basal body may have been knocked out in these three mutants. The flanking sequences of the insertion cassette in these mutants were amplified using restriction enzyme site-directed amplification (RESDA)-PCR in order to map the insertion loci (Table 1). In the mutant DB23-09, the cassette was inserted into the ninth intron of IFT88, which is required for the localization of IFT-B complex to the basal body [50]. The characterization of ift88 mutant as exhibiting IFT46 distribution defect suggests that the screening strategy we used was reasonable. The other two insertion loci in mutants AV27-20 and BF29-31 were localized to genes Cre14.g633700, which is an uncharacterized protein with transmembrane domain, and Cre11.g467653, which is annotated as threonine-specific protein kinase. Nevertheless, further analyses are required to confirm their role in regulation of IFT46 accumulation at the basal body.

**Screening mutants with defective in oil droplet assembly**

*Chlamydomonas* accumulates oil droplets in response to nitrogen deprivation [51]. Therefore, we used this library to screen oil droplet assembly mutants. The parental strains and mutants were cultured in TAP-N medium for 48 h, and stained with Nile red. Oil droplets accumulated in the cell body were observed under a fluorescence microscope (Fig. 2d1). A total of 929 mutants with fewer oil droplets were characterized (Fig. 2d2); of these, 11 mutants were selected and observed under a confocal microscope (Fig. 2d3). The fluorescence intensities of Nile red in 5 mutants were decreased significantly as compared to the parental strain, representing a reduced number and size of oil droplets (Fig. 4a). Thin layer chromatography (TLC) indicated that 4 mutants synthesized less triacylglycerol (TAG) after nitrogen deprivation (Fig. 4b, c). The mutant of AP01-30 and DA06-10 only have 60% TAG compared the parental strains HS211. These data suggest that a large amount of mutants
Table 1  The mutants lacking IFT46::YFP accumulation at the basal body

| Strain     | Accession of inserted genea | Annotation                                      | Insertion loci |
|------------|-----------------------------|------------------------------------------------|----------------|
| AP03-22    | Cre11.g467717               | Nitriase-related; carbon–nitrogen hydrolase     | R, 5th intron  |
| AQ01-40    | Not identified              | –                                              | –              |
| AV18-35    | Between Cre14.g612250 and Cre14.g612226 | No annotation                                   | Intergenic region |
| AV27-20    | Cre14.g633700               | Uncharacterized conserved protein                | R, 4th intron  |
| BE13-33    | Not identified              | –                                              | –              |
| BF29-31    | Cre11.g467653               | Threonine-specific protein kinase                | R, 2nd intron  |
| DB08-33    | Not identified              | –                                              | –              |
| DB23-09    | Cre07.g335750               | Intraflagellar transport protein 88             | F, 9th intron  |
| CZ18-45    | Not identified              | –                                              | –              |
| CY19-39    | Cre12.g554400               | No annotation                                   | F, 1st exon    |

a Gene accession numbers were from Phytozome v10.3 C. reinhardtii website

b F and R indicate that the insertion cassettes have the same or opposite orientation as the targeted genes, respectively

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Fig. 4  Mutants with defects in oil droplet formation and TAG synthesis. a Nile red staining of oil droplets in the mutants and HS211 after 3-day nitrogen starvation. Scale bars 10 μm. CHL chlorophyll fluorescence. b Analysis of TAG biosynthesis in mutants with defective oil droplet formation. c The relative amount of TAG in the mutants as compared to that in WT. *p < 0.05; **p < 0.01
possessing defects in oil droplet formation have been identified in this study, which is a great resource to study the molecular mechanism for assembly and disassembly of oil droplets.

**Characterization of mutants for targeted genes**

Initially, we planned to screen 61 mutants for genes involved in flagellar assembly, biogenesis of basal body, and vesicles trafficking (Additional file 2: Table S1). We identified 65 correct insertion loci corresponding to 37 genes from the super pool library during 6 months. Due to contamination and low recovery rate of certain transformants, only 48 insertion loci could be amplified in the basic pool. Finally, 26 mutants corresponding to 20 target genes were identified, including transition zone mutant POC2, flagella mutant PKD2, glycosylation mutant MAN1, and starch metabolism mutant SSS2 (Fig. 5a; Table 2). Among them, 31% insertion cassettes were integrated in the UTR, 23% were integrated in exons, and approximately 46% of insertions occurred in the introns (Fig. 5b). To determine whether mutagenesis in introns could induce complete knockout of target genes in *Chlamydomonas*, we compared the mRNA level of target genes in three individual mutants with parental strain HS211 by quantitative PCR, with probes locating at upstream, downstream, or across the insertion loci. The transcriptions of the three targeted genes were still detectable in the mutants. However, the transcript fragments upstream of the insertion loci (inserted in 13th intron) in mutant *fap215* were up-regulated. In contrast, the transcript across the insertion loci in mutant *fap215* and *poc2* (inserted in 2nd intron) was undetectable. The transcription downstream of the insertion loci was variable, which was down-regulated in mutant *fap164* (inserted in 2nd intron), but up-regulated by 20-fold in mutant *poc2* (Fig. 5c). Partial transcription was still detectable in the three mutants, which suggests that the 2.6-kb *MlyI*-digested expression cassette did not always terminate gene transcription completely when it was inserted into the intron. Therefore, another optimized insertion cassette was designed.

**Optimizing the insert insertion cassette**

Although the 2.6-kb *MlyI*-digested fragment contains two inverted terminators at its right border, it lacks a terminator on the left side. Additionally, terminators may not be functional for the target gene if the insertion cassette was located within an intron. To overcome this, we designed a new insertion cassette with a splicing donor sequence from the RubisCO gene (TCCATTT-GCAG/GATGTTCGA) at both sides of the insert fragment. Thus, the insertion cassette became a part of the mRNA even if it was integrated into an intron. We also added three tandem overlapping stop codons and a transcription termination signal at each side (Fig. 6a). As a result, transcription of a target gene would terminate at
the artificial transcription termination signal and polyadenylated at the new terminal. Thus, transcription and translation would stop simultaneously. We transformed the modified \textit{aphVIIr} fragment into the strain HS211, and found that more than twofold mutants with motility defects were generated under the same conditions as compared to the original \textit{aphVII} fragment without splicing donor segments (Fig. 6b). We also simulated gene interruption by inserting different cassettes into the \textit{RbcS2} intron3 which was integrated to luciferase, and analyzed the luciferase activity of \textit{Chlamydomonas} transformants in order to compare the termination efficiency of the new insertion cassette with or without splicing donor sequences (Additional file 4: Fig. S2). Luciferase interrupted by insertion cassettes possessing splicing donor sequence displayed relatively lower activity, with an average intensity of ~3000–7300 in pHK601-5, pHK603-5 and pHK603-10 compared to ~13,000 in cassettes pHK602-2 which has no splicing donor sequence (Fig. 6c). Furthermore, insertion cassettes with splicing donor sequence generated less positive luciferase transformants. Only 2.5 and 3.9% positive transformants could be detected in pHK601-5 and pHK603-10, compared to 7.2% in pHK602-2. Transformant displaying high luciferase activity (>10^4) could be hardly detected in pHK601-5, pHK603-5 and pHK603-10 (Fig. 6d).

These results suggest that the new insertion cassette increases the efficiency of transcription termination, subsequently increasing the possibility of generating the null mutant.

**Discussion**

Due to a lack of reliable targeted gene editing methods such as TALEN or CRISPR-Cas9, large-scale insertion mutagenesis provides an alternative strategy to screen mutants of specific genes in \textit{Chlamydomonas}. In this study, we generated and maintained an insertional mutagenesis library including approximately 150,000 clones, which can be utilized for both, reverse and forward genetics. A total of 26 mutants corresponding to 20 interested genes, related to glycosylation, starch metabolism, lipid metabolism, and flagellar proteins were identified by PCR-based screening. Meanwhile, two independent libraries related to flagellar assembly

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**Table 2 List of identified mutants**

| Targetedgenes | Accessiona | Description | Function category | Insertion locb |
|---------------|------------|-------------|------------------|---------------|
| POC2          | Cre10.g436000 | Polycystin-related | Transition zone | R, 2nd intron; F, 1st exon |
| CRX           | Cre03.g202950 | EF-hand calcium-binding domain containing protein | Caleodoxin | R, 2nd intron |
| MAN1          | Cre07.g336600 | Alpha-1,2-mannosidase | Glycosylation | R, 7th exon |
| CUL3          | Cre17.g734400 | Ubiquitin ligase SCF complex subunit Cullin | Ubiquitin ligase | R, 3'UTR |
| XYL1T         | Cre09.g391282 | Glycoprotein 2-beta-1-xylosyltransferase | Glycosylation | F, 6th intron; F, 7th intron |
| PKD2          | Cre17.g715300 | Polycystin cation channel protein 2 | Flagellar protein | F, 11th exon; F, 3rd exon |
| AMYB1         | Cre06.g307150 | Beta-amylase | Starch metabolism | R, 5'UTR; R, 3'UTR (two different mutants) |
| SSS2          | Cre03.g185250 | Soluble starch synthase II | Starch metabolism | R, 1st intron |
| FAP215        | Cre03.g180450 | 5'-nucleotidase and flagellar associated protein | Flagellar protein | R, 13th intron |
| FAP208        | Cre11.g482001 | Ankyrin repeat-containing protein | Flagellar protein | F, 22nd exon |
| FAP249        | Cre17.g746697 | Flagellar associated protein | Flagellar protein | R, 5'UTR (ligated with 480 bp unknown sequence) |
| FAP164        | Cre17.g735350 | Flagellar associated protein | Flagellar protein | F, 2nd intron |
| CDK13         | Cre01.g009500 | Protein kinase domain (PKinase) | Flagellar protein | F, 2nd intron (ligated with partial left border of insertion fragments) |
| PDCD6         | Cre03.g200050 | Ca2+-binding protein, EF-hand protein superfamily | ESCRT | R, 2nd intron; R, 4th intron |
| VPS4          | Cre02.g079300 | AAA-ATPase of VPS4/SKD1 family | ESCRT | R, 3'UTR |
| VPS37         | Cre08.g362550 | Subunit of the ESCRT-I complex | ESCRT | F, 2nd intron |
| PEPC2         | Cre03.g171950 | Phosphoenolpyruvate carboxylase | Lipid metabolism | R, 19th exon |
| IFT139        | Cre06.g268800 | Intraflagellar transport protein 139 | IFT | R, 22nd intron |
| UBA1          | Cre09.g386400 | Ubiquitin-activating enzyme E1 | Ubiquitin | R, 3'UTR |
| FRE2          | Cre05.g241400 | Ferric-chelate reductase (NADH) | Ferric protein | R, 5'UTR |

*a* Gene accession numbers were from Phytozome v10.3 \textit{C. reinhardtii} website

*b* F and R indicate that the insertion cassettes have the same or opposite orientation as the targeted genes, respectively
and oil droplet formation were obtained from this insertional library based on phenotype screening. The flagellar assembly mutant library included 967 mutants, which was expected to cover most of the ~1000 flagellar proteins [52, 53].

One advantage of this insertional library is that we transformed a fluorescence-labeled marker IFT46::YFP into the parental strain, which enabled us to screen specific mutants with defects in IFT. We obtained three mutants with reduced IFT46 expression in whole cells but lacking the accumulation at the basal body. The insertion loci of these mutants were identified and the interrupted genes may be involved in transporting or anchoring the IFT complex to the basal body. We also optimized several procedures for library construction and screening. First, the parental strain HS211 not only contains fluorescence IFT marker but also displays higher transformation efficiency. We also noticed that a strain expressing the aph-VIII gene showed 4.5-fold higher efficiency than wild type if another selection marker such as aphVII was used. This indicates that *Chlamydomonas* tends to acquire a second antibiotic resistance more easily if an antibiotic resistance gene is already expressed. The molecular mechanism of this phenomena still need to be identified, but it speeded library construction by reducing the number of transformation. Second, we optimized the insertion cassette specific primer, which complement to the junction between two inverted terminator, and increased the specificity for the PCR screening. Third, we maintained all the transformants on the 9-cm sized agar plate with a fixed 48-clones format. This format ensures much more cells to be maintained for replication and further analyses. Because

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**Fig. 6 Generating a novel insertion cassette.**

a) Diagram of insertion cassette AphVIIr which contains the 1.7-kb AphVII gene, forward splicing donor sequence, and reversed splicing donor sequence. Splicing donor sequence is indicated below. Intron/exon splicing sites, stop codons, and terminator are marked.  

b) The AphVIIr insertion cassette generated more than 2 times motility-defect mutants as compared to insertion cassette AphVIII.  

The luciferase activity of *Chlamydomonas* cells transformed with different insertion cassettes. Luciferase interrupted by insertion cassettes possessing splicing donor sequence displayed relatively lower activity. The pattern of splicing donor sequence (red bar) is indicated by the side of AphVII (gray bar). The cutoff of positive luciferase activity is set as 100.  

d) The distribution of luciferase expression level in *Chlamydomonas* transformants. Insertion cassettes possessing splicing donor sequence generated less transformants displaying positive luciferase expression.
there is more space between each colony, the cross-contamination rates were reduced. However, the procedure of picking fresh transformants into 48-clones format is labor-intensive and cryopreservation of the whole library in liquid nitrogen is recommended to prevent mutant loss. Finally, we normalized the amount of genomic DNA of each transformant by picking the same amount of each individual clones from solid agar plates to make the DNA mixture, which increase the chance to identify the insertion junction of mutants with similar PCR efficiency.

Interestingly, we found that the insertion cassette was inserted in UTR region among 30% mutants. The expression level of target gene was significantly reduced, which may be useful to screen the mutant of lethal genes. In addition, 46% of the insertions were localized in an intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied. Hochmal et al., reported mutant IMcrx, in intron. Their effect on the expression of the targeted gene varied.

Algal strains and growth conditions
The flagella-less C. reinhardtii mutant and the wild type CC125 were kindly provided by the Chlamydomonas Resource Center. HS211 was used as parental strain for library construction. HS211 is a rescue strain of mutant ift46-1 obtained by transforming a linearized plasmid pHK266, which contains the IFT46 full length fused to the 5’ end of YFP (see “DNA manipulation” for details). Unless otherwise indicated, algal cells were grown mixtrophically in liquid TAP medium or on solid TAP agar (1.5%) plate under continuous illumination (30 μE m−2 s−1) at 22 °C. For maintenance of the library, algal colonies were grown under weak illumination (5 μE m−2 s−1) at 20 °C. For motility defect and oil droplet defect screening, algal colonies were inoculated into liquid TAP with omission of NH4Cl.

DNA manipulation
The universal plasmid pHK86 includes fused GFP::YFP sequences driven by the PSAD promoter, and the selectable marker gene AphVII driven by HSP70A promoter [55]. For mutant ift46 rescue, the genomic DNA of IFT46 gene was amplified by PCR from the plasmid pGEM T Easy-IFT46 (provided by Joel Rosenbaum) using primers IFT46-F and IFT46-R (all primers listed in Additional file 3: Table S2). After digestion with NdeI and EcoRV, the IFT46 fragment was ligated to the 5’ side of YFP in plasmid pHK86, yielding the plasmid pHK214. The AphVIII in pHK214 were exchanged with the AphVII fragment amplified from the plasmid pHyg3 (provided by Wolfgang Mages), which yielded the plasmid pHK266. The plasmid pHK266 was linearized by Kpnl and used to rescue the mutant ift46.

For library transformation, the 2.6-kb insertion cassette of AphVIII was digested from the plasmid pMJ013b with Mfy1 [27].

For optimization of the insertion cassette, the forward-splicing donor (FSD) sequence was synthesized by annealing the oligonucleotides F-Spe-F, F-Spe-R, F-Hind-F, and F-Hind-R, and the reversed one (RSD) was synthesized by annealing the oligonucleotides R-Nco-F, R-Nco-R, R-Cla-F, and R-Cla-R. The annealing process
followed was as previously described by Hu et al. [38]. After digestion with SpeI/HindIII or NcoI/ClaI, the forward and reverse splicing donor sequences were ligated to the 5′- and 3′-ends of AphVII, respectively, using pBluescript II KS as the backbone, which yielded the plasmid pHK415. The 1.9-kb fragment FSD::AphVII::RSD digested from pHK415 by SpeI/ClaI was used for transformation.

The RbcS2 intron3 was ligated to the CTGGAG/GTGCTG of luciferase using In-Fusion kit (Clontech). Different version of insertion cassettes were amplified from pHK415 and ligated to the restriction site of Apal in RbcS2 intron3.

**Nuclear transformation of *Chlamydomonas reinhardtii***

Nuclear transformations of *C. reinhardtii* were performed by electroporation as previously described by Huang et al. [56]. In brief, algae cells were grown in 200 ml TAP medium until the cell density reached 6 × 10^6 cells/ml. The cells were concentrated to 2 × 10^8 cells/ml with pre-chilled TAP, supplemented with 60 mM Sorbitol. Then, 250 µl concentrated cells were mixed with 30 ng DNA fragment in a 4-mm electroporation gap cuvette at 4 °C. Electroporation was performed using ECM630 electro cell manipulator (BTX Harvard Apparatus) with following parameters: voltage, 800 V; resistor, 1575 Ω; and capacitor, 50 µF. Transformants were recovered by incubation in 10 ml liquid TAP (60 mM Sorbitol) for 14 h under low illumination (5 μE m⁻² s⁻¹). The cells were sedimented by centrifugation at 2500 rpm for 3 min and the pellet was mixed with 1 ml 20% starch and positive colonies were screened on solid TAP agar plates containing 10 µg/ml paromomycin or 12.5 µg/ml hygromycin B.

**DNA and RNA extraction**

Total genomic DNA of *Chlamydomonas* was extracted using standard CTAB/phenol/chloroform method as previously described by Huang et al. [48]. The DNA concentration of each super library was determined using *HindIII* digested lambda DNA (ThermoFisher Scientific, USA) as control and diluted to 100 ng/µl for PCR. Total RNA of *Chlamydomonas* was isolated using TRIzol (ThermoFisher Scientific, USA) according to the manufacturer protocol. RNA concentration was determined by Quawell 5000 (Quawell Technology, USA). Residual genomic DNA in total RNA was removed by DNase I (RNase-free) (ThermoFisher Scientific, USA) before reverse transcription.

**PCR procedures**

For PCR-based screening, the target gene specific primers were designed using Primer Premier 5.0 software with following parameters: length, 20–26 bp; Tm, 58–62 °C; GC%, 40–60% (all primers listed in Additional file 3: Table S2). PCR was performed in a 20-µl solution including 0.25 µM target gene specific primer, 0.125 µM insertion cassette specific primer LGR06-F, 0.2 mM dNTP, 0.5 M betaine (Sigma), 3% DMSO (Amresco), 0.5 unit Trans Taq DNA polymerase (HiFi) (Transgen, China) and 100 ng genomic DNA templates. The PCR program for super library screening was as follows, pre-incubation at 95 °C for 5 min, 34 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and amplification at 72 °C for 2 min. The colony PCR was performed according to the method previously described by Cao et al. [57].

For mRNA expression quantification, reverse transcription of RNA was performed with oligo-dT primers using First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) according to the manufacturer instructions. Quantitative real-time PCR was performed by ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) with SYBR Green Realtime PCR Master Mix (TOYOBO, Japan) according to the manufacturer instructions. Using housekeeping gene *Chlamydomonas* β subunit-like polypeptide (CBLP, Cre06.g278222) as an internal control [58], the expression of other genes was quantified using standard curve assay in SDS 2.4 software (Applied Biosystems). All primers used in real-time PCR are listed in Additional file 3: Table S2.

For flanking sequence identification, RESDA-PCR was employed [59]. Degenerated primers (DegAlul, DegPstI, DegSacII, and DegTaqI) and primer Q0 were the same as the original method. Primers For1, For2, Rev1, and Rev2 were used as specific primers.

**Protein extraction and western blot analyses**

Whole-cell protein extraction and concentration were performed as previously described by Hu et al. [38]. Whole cell proteins (5 µg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to 0.45-μm nitrocellulose membranes (BioRad, USA) at 300 mA for 3 h. After blocking with 5% non-fat milk, the nitrocellulose membranes were probed with rabbit anti-IFT46 antibody (1:5000, immunized with the N-terminal 20 amino-acid peptide by Genscript) or mouse anti-α-tubulin antibody (1:200,000, #T9026, Sigma). Immunodetection of IFT46 was detected by ECL (Millipore) with goat anti-rabbit secondary antibody (1:5000, #6154, Sigma), and α-tubulin was detected with goat anti-mouse secondary antibody (1:5000, #A4416, Sigma).

**Extraction of total cellular lipids and thin-layer chromatography**

Extraction of total cellular lipids was performed as previously described by Bligh and Dyer [60] with minor
modifications. In brief, cells (approximately $3 \times 10^7$ cells) were harvested by centrifugation at 6000g at room temperature for 5 min. The pellets were mixed with 400 µl methanol:chloroform (1:1, v/v) and vortexed for 5 min. The suspension was mixed with 120 µl PAK buffer (1 M KCl, 0.2 M phosphate acid). The mixture was centrifuged at 12,000g for 5 min and the lower chloroform phase was transferred to a new glass tube. Finally, additional chloroform was added into the organic phase until the constant volume was 20 µl.

For thin-layer chromatography, equal volume of total cellular lipid extract was loaded as a spot on 10 × 20 cm silica gel GF254 TLC plates (Haiyang, China). Neutral lipids in the samples were separated using a hexane/diethylether (3/1, v/v) solvent mixture. Then, the silica gel plates were dried under the hood and stained by diethylether (3/1, v/v) solvent mixture. Then, the silica lipids in the samples were separated using a hexane/CHL solution and examined with differential interference contrast was 650–700 nm.

**Microscopy**

Motility-deficient mutants were identified using stereo-microscopy (Nikon SMZ1500, Japan). Mutants with changes in IFT46::YFP basal body localization were screened using LSM710 confocal microscope (Carl Zeiss, Germany) (excitation: 514 nm; emission: 519–560 nm). To screen oil droplet accumulation-deficient mutants, cells were stained in 0.1 µg/ml Nile red (Sigma, USA) for 15 min in the dark at 37 °C [61]. Cells with fewer oil droplets were identified using ECLIPSE Ti-U fluorescence microscope (Nikon, Japan) (excitation: 465–495 nm; dichroic mirror: 505 nm; emission: 515–555 nm).

Live cell images were captured using Leica SP8 confocal microscope. For Nile red, the excitation was 488 nm and the emission was 560–590 nm. For chlorophyll fluorescence (CHL), the excitation was 488 nm and the emission was 650–700 nm.

**Measurement of flagellar length**

*Chlamydomonas* cells were fixed in 0.5% Lugol’s iodine solution and examined with differential interference contrast (DIC) microscopy using ECLIPSE Ti-U fluorescence microscope (Nikon, Japan) equipped with a 40× objective. Lengths of flagella were measured with NIS-Elements BR software. For each sample, at least 300 flagella were measured.

**Luciferase activity assay**

The luciferase activity was analyzed using the Renilla Luciferase Assay System (Promega, TM055) in 96 well microtiter plates according to the manual, with the modification that the algae cells were ruptured with glass-beads by vortex for 15 min.

**Additional files**

**Additional file 1: Figure S1.** Comparison of the specificity of primer LGLO3 and LGR06. More than sixteen non-specific PCR products were obtained with primer LGLO3, whereas only four non-specific bands were obtained using primer LGR06.

**Additional file 2: Table S1.** List of genes for insertional mutants screening and PCR identified insertion loci from super libraries.

**Additional file 3: Table S2.** List of primers used in plasmid construction, RESDA-PCR, Realtime-PCR, and library screening.

**Additional file 4: Figure S2.** Different version of insertion cassettes. The 238 bp intron3 of *RbcS2* was fused to the luciferase at the site of CTGGAG/GTGCCTG. The insertion cassette with or without splicing donor sequence at either side of AphIVI gene was inserted to the restriction site of Apal in *RbcS2* intron3.

**Abbreviations**

- CRISPR-Cas9: clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9
- IFT: intraflagellar transport
- EMS: ethyl methane-sulfonate
- MNNG: N-methyl-N′-nitro-N-nitrosoguanidine
- HR: homologous recombination
- JGI: joint genome institute
- RESDA-PCR: restriction enzyme site-directed amplification
- TAP-N: nitrogen-deprived TAP medium
- TLC: thin layer chromatography
- TAG: triacylglycerol
- FSD: forward-splicing donor
- RSD: reverse-splicing donor

**Authors’ contributions**

KH organized the project. XC, GL, WK, LZ, BL, XM, NX, XX, XD performed the experiments, and analyzed the data. KH, GL, XC wrote the paper. All authors read and approved the final manuscript.

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**Acknowledgements**

We would like to thank Dr. Jue Ning (University of Texas Southwestern) for suggestion on PCR solution for super library screening.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files, including supplemental Additional file 1: Figure S1, Additional file 2: Table S1 and Additional file 3: Table S2. The experiment materials generated during the current study are available from the corresponding author on reasonable request.

**Funding**

This project was supported by the National Natural Science Foundation of China (31371354 to Dr. Kaiyao Huang and 31400654 to Dr. Gai Liu).

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received:** 29 September 2016 **Accepted:** 2 May 2017 **Published online:** 15 May 2017
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