Cell Synchronization Enhances Nuclear Transformation and Genome Editing via Cas9 Enabling Homologous Recombination in Chlamydomonas reinhardtii

Max Angstenberger,* Francesco de Signori, Valeria Vecchi, Luca Dall’Osto, and Roberto Bassi*

ABSTRACT: In Chlamydomonas reinhardtii, the model organism for eukaryotic green algae and plants, the processes of nuclear transformation and genome editing in particular are still marked by a low level of efficiency, and so intensive work is required in order to create and identify mutants for the investigation of basic physiological processes, as well as the implementation of biotechnological applications. In this work, we show that cell synchronization during the stages of the cell cycle, obtained from long-term cultivation under specific growth conditions, greatly enhances the efficiency of transformation and allows the identification of DNA repair mechanisms that occur preferentially at different stages of the cell cycle. We demonstrate that the transformation of synchronized cells at different times was differentially associated with nonhomologous end joining (NHEJ) and/or homologous recombination (HR), and makes it possible to knock-in specific foreign DNA at the genomic nuclear location desired by exploiting HR. This optimization greatly reduces the overall complexity of the genome editing procedure and creates new opportunities for altering genes and their products.

KEYWORDS: Chlamydomonas reinhardtii, genome editing, CRISPR/Cas9, nonhomologous end joining, homologous recombination, cpftsy

As the model organism for eukaryotic green algae and plants, Chlamydomonas reinhardtii has been widely studied over recent decades, as summarized in ref 1. Its fully sequenced^2 haploid genome offers great benefits in establishing techniques for nuclear transformation^3 and electroporation,^4 as well as genome editing techniques such as the CRISPR/Cas9 system. Special strains that are even easier to transform are also available, for example, the CW15 strain with a reduced cell wall. Nevertheless, transformation, and specific gene targeting techniques in particular, still need to be further optimized in order to obtain an efficient platform for creating mutants that can be applied to other algae and plant species.

The recent confirmation of the transformation of C. reinhardtii using ribonucleoproteins^6 of Cas9 and coupled single-guide RNA (sgRNA) opened the way for the production of gene knockout mutants and, in some cases, the knock-in of foreign DNA delivered in specific sequences (compare Figure 1).

The latter was recently confirmed^9 as enabling un specific knock-ins via nonhomologous end-joining (NHEJ), which displayed the disadvantage of sequence changes often observed during DNA integration (Figure 1C,E) which, together with low transformation efficiency, makes identification of specific genotypes problematic and requiring extensive sequencing procedures. Therefore, specific knock-ins caused by HR (Figure 1F−I), with a high probability of complete and sequence-specific DNA integration, display great potential in altering gene structures and therefore altering protein sequences, to implement protein tagging systems,^10 for example, or to replace genes with copies containing single point mutations. Significantly, such specific knock-ins created through the HR pathway (Figure 1F), that is, replacing a target sequence using homologous flanking regions, only appear naturally at very low frequencies in C. reinhardtii;^11−14 instead, random DNA integration is dominant and mediated by NHEJ. Inducing DNA double-strand breaks (DSB) using the Cas9 nuclease at specific sequences and providing homologous repair templates were shown to increase the probability of specific knock-ins in various species. HR activity is often suppressed by the NHEJ pathway in
The introduction of DNA repair templates in eukaryotic cells can result in various outcomes depending on the repair mechanisms involved. In the context of genome editing, two primary pathways are considered: homologous recombination (HR) and non-homologous end joining (NHEJ).

**Homologous Recombination (HR):** HR is a precise and efficient way to repair DNA by utilizing a homologous template. This pathway is particularly useful for gene knock-ins, where the precise integration of DNA into the genome is desired. HR is a complex process that requires the presence of a homologous sequence to act as a template for repair.

**Non-Homologous End Joining (NHEJ):** NHEJ is a less precise but also very efficient way to repair DNA by joining broken ends without a homologous template. This pathway is activated in response to DSBs in the genome. NHEJ can lead to a variety of outcomes, including insertion of random sequences, which can be advantageous for genome editing applications.

In C. reinhardtii, eukaryotic cell cycle stage-specific integration of DNA has been observed. During the late DNA synthesis phase (S phase) and early mitotic phase (M phase), HR may be more prevalent, allowing for the precise integration of DNA. In contrast, NHEJ is dominant in the G1 phase, allowing for the repair of DNA breaks and potentially leading to the insertion of random sequences.

**Cell Synchronization:** Cell synchronization can be achieved through various methods, including light and temperature treatments, which affect the cell cycle stages. In C. reinhardtii, synchronized cells are used to access HR pathways during specific cell cycle stages. For instance, cells can be synchronized to enter the mitotic prophase with associated condensation of chromatin, which is conducive to HR.

**Transformation Efficiency:** The transformation efficiency of C. reinhardtii has been studied extensively, and methods to optimize this efficiency have been developed. For example, the use of a zinc-finger nuclease to introduce a DSB at a specific site in the genome can enhance the efficiency of genome editing.

**Conclusion:** The use of HR and NHEJ in the context of genome editing in C. reinhardtii has shown promise in targeted gene knock-ins and precise DNA integration. Further research is needed to improve the efficiency and specificity of genome editing in this microalgal model system.

---

**Figure 1:** Schematic overview of single guide RNA (sgRNA), guided Cas9 introduced DNA double-strand breaks (DSB), and possible DNA repair pathways in eukaryotes. Repair mechanisms include non-homologous end joining (NHEJ) and homologous recombination (HR), the possible outcomes of which depend on the provision of DNA repair and the respective active repair mechanism. In the uppermost part, the selected exemplary nuclear (gDNA) target sequence (t) and 5′ and 3′ flanking regions (5F, 3F) are depicted. Using sgRNA-guided Cas9, a DSB can be introduced in the target region, but the process is not entirely predictable. As long as a nonhomologous repair template confers antibiotic resistance (resA cassette), an active NHEJ pathway can lead to several results: (A) target sequence repair with error-prone sequence modifications, (B) insertion of random (r) sequences, (C) insertion of DNA clockwise, and anticlockwise in (D), and (E) insertion of truncated repair DNA. As a consequence, there are likely to be remnants of the chosen target sequence. In contrast, the provision of homologous repair DNA containing the flanking regions can lead to different results in an active HR pathway: the most desirable outcome is shown in (F), that is, the sequence-specific replacement of the target sequence with resA. Other options are multiple insertions of resA (G), inverted insertions (H) and deletions (I).
This approach made it possible for the knockout frequency to be quickly determined by counting the pale green colonies as compared to the native green ones, created by random mutations. Interestingly, the knockout frequency of C. r. cpfts was very low in earlier experiments (0.5−1%) compared to knockouts of other target genes, for example, the chlorophyllide-a oxygenase (CAO, ∼20%, data not shown), which also leads to a pale phenotype, though less pronounced. Nevertheless, analyzing the low knockout frequency of the cpfts gene offers the advantage of showing the full effect of an optimized GE frequency, avoiding saturation. To enable specific recombination events by HR at the cpfts locus, a construct with 2 kb flanking regions surrounding the sgRNA guided target sequence was created. Large flanking regions were previously shown to enhance HR events in C. reinhardtii and in other microalgae, increasing the probability of such events.

### RESULTS AND DISCUSSION

**C. r. CW15 Cell Synchronization under Long-Term Cultivation.** As reported by ref 23, cell synchronization of C. r. can be achieved by applying a temperature of 28 °C during the light phase (resulting in cell growth) and 18 °C in the dark phase (leading to an exact doubling of cell numbers). We observed the growth behavior of CW15 in similar light-dark and warm-cool cycles, as carried out by ref 23, in a multicuvitator system that measured the optical density over several days (not shown). Interestingly, under these growth conditions (12 h light at 28 °C and 12 h darkness at 18 °C), we observed a change in growth behavior during prolonged cultivation, leading to a higher daily cell number than expected, if two daughter cells were being generated by each mother cell. In order to further characterize such growth behavior, a batch culture of CW15 was maintained under these conditions and after 3 to 4 days of growth, cells were used to inoculate a new culture, in order to achieve a long-term adaptation (2 weeks of preculturing with ongoing cultivation). Following a subsequent
TE data is displayed in comparison with the corresponding cell number (shown as log2, gray circles) on the third day of growth under 10^6 cells/mL to 6.97. In the following 4.5 h, the cell number doubled, from 3.57 to 7.14 × 10^6 cells/mL. The cell number after 3 h of light, cell division stopped and the cell number showed a pronounced minimum at +8 h, but increased again at the end of illumination period to 1.2 × 10^7 cells/mL. The following minimum of TE at +8 h could be explained by cells entering the S phase, in which DNA repair pathway, recombination efficiency, and the occurrence of DNA DSBs in the target sequence significantly decreased in the maximal TE using pHyg3. No pale colonies could be found after all those transformations, confirming the aforementioned inefficiency of the HR pathway in C. r. Nevertheless, the different maxima of TE using pHyg3 and HCP point to the recognition by the cell of homologous foreign DNA, which cannot be used for HR-mediated recombination in this physiological state, probably due to missing DNA DSBs in the target sequence cpf35y and/or an inactive HR pathway caused by a lack of necessary proteins.

First, we defined an optimized transformation protocol aimed at minimizing false positive clones and simplifying the procedure based on. The final optimized protocol is described in detail in the Methods section (see also Table S1) and includes the reduction of materials, working time and a more stringent selection using a higher hygromycin concentration. Once optimized conditions on synchronized CW15 had been established, the analysis of transformations at different time points on the third day of cultivation with respective constructs (Figure 4A) displayed a strong enhancement of the transformation efficiency (TE).

Compared to the TE of unsynchronized cells, the TE using synchronized cells and pHyg3 remained rather low during the first hours of illumination (around 10^-7 clones/ng DNA/cells), but was drastically enhanced to a maximum at +4 h of illumination (+1800%). In the following hours, TE decreased to a pronounced minimum at +8 h, but increased again at the end of illumination period to 5 × 10^-7 clones/ng DNA/cells. TE using HCP displayed a similar pattern, but interestingly reached a maximum at +6 h (~10^-4 clones/ng DNA/cells), although less pronounced compared to that for pHyg3. No pale colonies could be found after all these transformations, confirming the aforementioned inefficiency of the HR pathway in C. r. Nevertheless, the different maxima of TE using pHyg3 and HCP point to the recognition by the cell of homologous foreign DNA, which cannot be used for HR-mediated recombination in this physiological state, probably due to missing DNA DSBs in the target sequence cpf35y and/or an inactive HR pathway caused by a lack of necessary proteins.

By combining the TE values of synchronized CW15 cells with the cell number on day 3 after inoculation (Figure 4B), it was possible to associate the defined cell cycle stages based on TE tendency. As with the G1 phase, NHEJ is the dominant DNA repair pathway, reflected in the maximal TE using pHyg3 at +4 h of illumination. The following minimum of TE at +8 h can be explained by cells entering the S phase, in which DNA integration must be avoided in order to protect the karyome from the random rearrangement of the newly synthesized DNA. Nevertheless, the possibility that HR could be active at this stage was further investigated. Before entering the M phase, the expected synchronization of the culture could be observed. After 3 h of light, cell division stopped and the cell number remained constant for about 6 h before cell division resumed. In the following 4.5 h, the cell number doubled, from 3.57 × 10^6 cells/mL to 7.14 × 10^6 cells/mL, implying synchronized cell division was underway in the culture. A second cell division occurred during this dark phase, resulting in 1.2 × 10^7 cells/mL the next day (a factor of 1.78). On the basis of general knowledge of the eukaryotic cell cycle, we could assume that the two major phases (interphase and M phase) were underway in the vast majority of the CW15 cells at different points of time during this cultivation procedure. Due to the occurrence of two subsequent cell divisions in the dark phase, a second interphase (though shorter) occurred, as described by ref 31. This shorter interphase was not examined further and so is not displayed in the M phase in Figure 2B.

**Determination of Transformation Efficiency in Synchronized C. r. CW15 Cultures.** Using CW15 synchronized cultures made it possible to investigate the effect on transformation efficiency and the occurrence of different DNA repair mechanisms (NHEJ/HR) by using a non-homologous (pHyg3, Figure 3) or homologous linearized transformation construct (HCP, containing homologous flanking regions of cpf35y, Figure 3) at different time points during cultivation. Random DNA integration by NHEJ was therefore expected to lead to hygromycin-resistant, normal green colonies, whereas HR-mediated recombination events at cpf35y should generate pale green clones.
phase, eukaryotic cells undergo a shorter G2 phase, although not described by the literature for C. r., which we associated with around +9 h after illumination. Cells then re-enter the M phase at around +10 h of light and begin to divide.

HR activity was therefore expected to be present during the last hours of illumination in order to enable sequence-specific DNA repair to take place before cell division. These results strongly suggest the need for the Cas9 nuclease to introduce DNA DSBs enabling recombination events.

**Genome Editing of Synchronized CW15 Using the Cas9 Nuclease.** The Cas9 nuclease was expressed in E. coli and purified using Ni-affinity chromatography. The presence of recombinant enzyme was evaluated by SDS-PAGE in the eluted fractions (Figure S1A), which showed a high degree of purity. Another critical feature for a high activity was the removal of bacterial nucleic acids, achieved by EDTA incubation and subsequent washing to remove the chelating agent. The concentrated Cas9 preparation (Figure S1B) was further evaluated by immunodecoration analysis using an α-His tag antibody (Figure S1C). An in vitro assay proved the capability of restricting the defined target DNA sequence of cpf3sy (Figure S1D) in the presence of the respective sgRNA (T1, T2). Since both target sequences are close to the end of the DNA molecule (Figure 3), little change in size occurred, but it nevertheless proved to be a functional Cas9 nuclease.

Interestingly, while testing different target sequences as sgRNA for cpf3sy in transformations (not shown), we saw that not all events led to the creation of a pale phenotype, suggesting the inefficiency of cpf3sy related sequences in genome editing. The defined target sequence 1 (T1, Figure 3) was therefore maintained and an additional target sequence (T2, Figure 3) was chosen. Both sgRNAs led to the creation of pale mutants and so were used together for genome editing experiments. Although T2 is also present in HCP (Figure 3), a restriction of the homologous template was not thought to interfere with putative HR activity, since about half of the homologous flanking region would still be present and because there was no incubation of Cas9 ribonucleoproteins together with HCP at activating temperatures prior to transformation.

When transforming synchronized CW15 at different time points using Cas9, pale green mutants were obtained in all cases at higher percentages than for unsynchronized cultures (Figure 5A, C−E), underlining once again the advantage of synchronization. Interestingly, TE increased only at +4 h when using HCP together with Cas9 ribonucleoproteins (HCP-GE) compared to the use of HCP alone, underscoring the dominance of the NHEJ pathway in leading to random
Figure 6. Target sequence analysis of selected pale mutants of C. r. CW15 from different transformation approaches. (A) PCR analysis carried out by the flanking region surrounding primers (C5_fw/C3_rv) for locus cpfts. Four pale mutants for each construct (pHyg3, HCP) at two different time points (+1 h, +12 h) were analyzed together with the wild type; the amplicons expected after HR-mediated knock-in of about 5.8 kb are surrounded by black boxes. (B) Similar PCR analysis were carried out with optimized primers (C5_fw2, C3_rv2) for 10 pale clones from transformation E (see Figure S8). As positive controls for each cell line, additional PCRs were performed using primers Cas9_fw and Cas9_rv and are shown in the lower part of the A–B panels. (C) Selected pale mutants from transformations C, F at +12 h (see Figure S8) were used for the amplification of sufficient amounts of recombinant knock-in fragments of cpfts for purification and further analysis, as shown in (D), that is, Nested-PCRs were performed using the purified fragments as template and primers Aph7_fw/Aph7_rv for detection of the inserted aph7 gene, in addition to primers CP_fw2/Cp_rv for detection of the target sequence T2 of cpfts as a control. (E) Summary of results of additional PCR analysis performed on pale clones from corresponding transformations of C. r. CW15 at +12 h after the start of illumination (see also Figure S8), showing the numbers of analyzed pale clones, the larger DNA insertions in cpfts detected, the desired HR-mediated knock-ins (flanking-based target sequence replacement) and its occurrence rate. (F) Schematic representation of knock-ins of the aph7 cassette into cpfts detected, based on HR-mediated recombination, which was confirmed by sequencing the corresponding XhoI and SpeI restricted amplicons (cloned into bluescript vector KS-) from clones 1F and 4F (see Figure S2). The disrupted genomic sequence of cpfts is depicted at the top, showing the occurrence of the gene disruption that leads to the pale phenotype. For the numbering, compare with Figure S8, except for transformation F, which is not shown in detail.

mutants. In all other cases, TE decreased or was unchanged when additionally using Cas9, pointing to more specific DNA repair, that is, HR.

The maximal efficiencies for creating pale mutants (around 5%) occurred at +8 h and +12 h of illumination, which were associated with the S phase and the G2/M phase respectively, since HR activity has a higher efficiency for functional knockouts and was assumed to be present at these time points. This hypothesis was further supported by the rather low percentage of pale mutants at time point +4 h, which was attributed to the G1 phase with high NHEJ activity. Since TE at +8 h (S phase) was very low (Figure 4B), time point +12 h was chosen for further optimization experiments to test different amounts of the components used (Figure S8) due to quite a high TE and a high percentage of pale mutants accompanied by the expected HR activity.

Interestingly, the provision of more plasmid DNA (150 to 400 ng HCP) decreased the TE but resulted in a similar percentage of pale mutants, confirming the recognition of the homologous template and, moreover, that the introduction of the DSB was the limiting step. This conclusion was further confirmed when using double the amount of sgRNA (5.4 μg to 10.8 μg) because the TE increased again (+100%) and the percentage of pale mutants was more than doubled (factor 2.1). A further increase of sgRNA (21.6 μg) and Cas9 (6 μg) resulted in the highest percentage of pale mutants observed (14.3% and 13.8%), with an average of 14%. A further increase of sgRNA to 40 μg decreased the yield of pale green clones, which confirms the optimal ratio previously determined. Overall, the efficiency of creating pale mutants could be increased by about 1300% by switching from unsynchronized cells (1.1% of pale green clones) to synchronized cells at +12 h (14%). In principle, this could be optimized even further by using different combinations of components.

Determination of HR-Mediated Knock-Ins in Pale Mutants. A major advantage of knock-ins caused by HR is the easy detection of functional knockout mutants by means of PCR screening. Indeed, large insertions can be easily detected and a functional knockout is the highly probable consequence. Since NHEJ-mediated gene disruption in most cases results in a knock-out has to be determined by other means, for example, by the analysis of the protein content. In order to analyze pale mutants obtained from different time points (+1 h and +12 h of illumination, Figure 6A), PCR was performed using primers for cpfts that bind to the exterior of the flanking regions (Figure 3) in order to avoid false positive PCR artifacts.53 Interestingly, the native PCR amplicon could not be detected in most clones from synchronized cultures, indicating a relatively high knock-in frequency (either unspecific, by NHEJ, or specific, by HR) of larger DNA molecules (for example, plasmid) into the cpfts target itself. Clones derived from transformation at +1 h were devoid of the expected HR-
mediated recombination product (target sequence replacement) of about 5.8 kb, as had been expected due to the inactive HR pathway. On the other hand, two out of four clones from transformation at +12 h did show the 5.8 kb recombination product, so supporting the presence of HR in the assumed G2/M phase. The total occurrence of HR-mediated recombination events could be even higher at this time point or at others, since there are different outcomes (Figure 1F–I) from recombination-like events, for example, multiple insertions and deletions. Interestingly, the sought-after knock-ins of HCP into cpftsy could not be identified at any time point other than +12 h (tested for +1, +2, +4, +6, +8, and +10 h with about 40 pale clones in total, data not shown).

Pale clones derived from the transformations shown in Figure 5B (as indicated for PCR analysis) were analyzed with optimized primers. In all 10 pale clones from transformation E (Figure 6B), the native sequence of cpftsy could not be amplified, indicating larger DNA insertions in all of them. The first four clones displayed the expected HR-mediated knock-in product of about 5.8 kb. When several clones carrying the same mutation had been identified, four of them were chosen for further analysis, as shown in Figure 6C (clone number and respective transformation number as indicated, compare with Figure 5B).

All clones displayed the recombinant PCR product expected after HR-mediated knock-in of HCP into cpftsy, and were amplified in triplets and a doublet for further analysis. After gel extraction and purification of the respective fragments, Nested-PCR was performed (Figure 6D), which showed the abundance of the inserted aph7 gene, as well as the T2 sequence of cpftsy used as a control. In Figure 6E, the results of all tested clones are displayed, showing the maximal yield of specific knock-ins into cpftsy from transformations D and E (compare with Figure 5B), on average 35%. To further verify the specific knock-ins and provide smaller molecules for easy cloning and subsequent sequencing, PCR was again performed, as described above, using cDNA from clones 1(F) and 4(F) and partially digested thereafter with Xhol and SpeI (see Figure S2). This revealed the expected products of about 3.5 kb and 2.3 kb (Xhol), the latter digested further to 1.1 kb and 1.2 kb (SpeI) respectively. Final sequencing confirmed the HR-mediated sequence-specific knock-in in those clones, which is shown schematically in Figure 6E.

**CONCLUSION**

Overall, the demonstrated synchronization of *C. r.* CW15 cultures is a powerful option for enhancing the efficiency of nuclear transformation, also confirmed in mammalian cells and, in particular for precise genome editing via HR, as demonstrated, for example, in different yeast species. Furthermore, this type of cell synchronization makes future in-depth research possible into the cell cycle of *C. r.* and could be very useful for further research into DNA damage responses during the cell cycle. The phases of the latter could be more precisely defined by using antibodies for cell cycle relevant proteins, for example, cyclins or by determining cyclin-dependent protein kinase activity. We demonstrated that access to certain cell cycle stages makes possible the genetic manipulation of the nuclear genome in different ways, depending on the desired outcome. When simple random DNA integration is sufficient, usage of the dominance of NHEJ at +4 h after illumination is the best option, since it enables the highest transformation efficiency, as also described by ref 38 for the eukaryotic parasite *Trypanosoma cruzi*.

With regard to genome editing strategies, avoiding NHEJ as much as possible and favoring HR-mediated DNA integration offers multiple benefits, including the reduction of unspecified DNA integration and enhanced gene targeting. A time point in the cell cycle of *C. r.* was identified (+12 h of illumination, Figure 4B, 6) offering a higher HR/NHEJ ratio and used to create mutants with an HR-based knock-in. Further research is required to evaluate the additional possibility of inactivating the NHEJ pathway in *C. r.*, which could reduce or even eliminate unspecified DNA integration accompanied by specific knock-ins using cell synchronization. Furthermore, the greatest efficiency of gene targeting in producing knockouts could be observed at this time point (+12 h), which confirms the advantage of HR-based gene disruption in leading to a higher probability of functional knockouts of genes. Such knockout mutants also offer the benefit of being easily identified by PCR, due to the high frequency of knock-in events using larger DNA molecules. Additional PCR analysis of targeted loci also enables the identification of the desired knock-in events (target sequence replacement by HR) that were shown to happen at acceptable rates. The actual efficiency of HR as identified in this work might be even higher, given the different outcomes of HR, as described for the fungus *Ashbya gossypii*.

Specific knock-ins make advanced genome editing possible, for example, eliminating native genes and replacing them with altered sequences for specific purposes, such as point mutated or extended gene copies leading to tagged proteins. Thus, 5% of colonies growing on selective medium will display a desired mutant knock-in sequence even in the absence of a visible phenotype. Since cpftsy displays a rather inefficient target for genome editing and was mainly chosen as the optimal phenotype of a knockout, our improved strategy should increase the efficiency of genome editing for more easy accessible targets and so provide high frequencies of the creation of knockouts and specific knock-ins. Finally, valuable information concerning the nuclear HR pathway in *C. r.* could be obtained, that is, its identification in special phases (G2/M) of the cell cycle, which is consistent with other eukaryotes.

**OUTLOOK**

Further optimization of HR usage could be achieved by identifying the optimal length of the homologous region and the additional determination of optimal Cas9, sgRNA, and plasmid amounts as well as other types of DNA nucleases like Cas12a. Moreover, the strategy presented should be applicable to other algae species, when conditions of synchronization have been identified and nuclear transformation is possible, especially if the genetic handling of species is problematic, enabling more efficient nuclear transformation and genome editing strategies, necessary for basic research and biotechnological applications.

**METHODS**

**Chemicals, Reagents, and Enzymes.** If not otherwise stated, all the chemicals and reagents used were provided by Sigma-Aldrich and AppliChem and all the enzymes used were supplied by New England Biolabs, Promega and Thermo Fisher Scientific.
**Gene Identity.** The phytozome database (https://phytozome.jgi.doe.gov) was used for *C. r. relevant data.* Database entry for *cpfts	us* is Cre05.g241450.

**Strains, Cell Culture, and Transformation.** The *Chlamydomonas reinhardtii* strain CW15 was used in all the experiments described and was cultivated in 20 mL flasks containing TAP-medium, supplemented with 100 μg/mL of ampicillin. Normal growth conditions were set to 25 °C with 200 μmol photons m⁻² s⁻¹ of white light for 16 and 8 h of darkness. Synchronizing growth conditions were adjusted to ref 23 with 200 photons m⁻² s⁻¹ of white light for 12 h at 28 °C, followed by 12 h of darkness at 18 °C. Cell number was determined using a Countess II FL cell counter (Life Technologies) and the results were divided by a calibration factor of 2.

**Transformation.** For optimized transformation of *C. r. CW15 based on ref 4, the respective number of cells (unsynchronized: 5 × 10⁵; synchronized: 10⁶) were harvested at 8000 g for 7 min and resuspended in 50 μL of TOS-Medium (80% v/v TAP, 40 mM Sucrose). The suspension was mixed with different amounts of linearized plasmid DNA (pHyg3: Ndel; HCP: Xbal; for unsynchronized cells: 250 ng; for synchronized cells: in general 150 ng, 60 ng for time point +4 HCP-GE (see Figure 5A) and 400 ng for time points +8 h as well as +12 h (see Figure 5A,B)). For genome editing experiments, a further 3 μg Cas9 (deviating amounts see Figure 5B) and 5.4 μg sgRNA, each consisting of 50% sgRNA for *cpfts	us* target1 and target2 (deviating amounts see Figure 5B), were added and the mixture incubated on ice for 5 min. Transformation was carried out in 0.4 cm spaced cuvettes by electroporation using a Gene Pulser II (Bio-Rad) set to 200 Ω, 50 μF and 0.6 kV. Recovery was achieved in 1.5 mL TOS-Medium containing reaction tubes kept in darkness overnight on a mixing rotator. After subsequent centrifugation at 8000 g for 7 min, cells were resuspended in 1 mL 30% starch containing TAP-Medium, supplemented with 60 μg/mL hygromycin and 100 μg/mL ampicillin. This solution was distributed on two 60 μg/mL hygromycin and 100 μg/mL ampicillin containing 1.5% Agar-TAP-plates to obtain selection.

**E. coli Wall.** Taq polymerase was expressed in *E. coli* BL21 and isolated according to the literature.60 As a general buffer, 10 mM Tris-HCl pH 8.3, 50 mM KCl and 4 mM MgCl₂ were chosen, while special amplifications based on GC rich templates like those present in *cpfts	us* (S’ Flanking region, see Figure 3) were performed using 75 mM Tris-HCl pH 8.3, 20 mM (NH₄)₂SO₄ and 4 mM MgCl₂. Reactions also contained 10 pmol of each primer, 0.53 mM dNTPs (each) and 0.26 M Betain. Annealing temperatures 2–5 degrees below the supplier’s reported melting temperature of primers were chosen, with elongation to 1 min/kb at 72 °C. All amplifications for cloning were achieved by using Hybrid Polymerase (EURX) in accordance with the supplier’s instructions. In addition, target sequence analysis of *cpfts	us* using flanking regions surrounding primers (see Figure 3) was carried out using the same buffer, containing (NH₄)₂SO₄, as that for Taq amplifications of GC rich templates.

**Amplification, In Vitro Transcription, Restriction, and Cloning.** Taq polymerase was expressed in *E. coli* BL21 and isolated according to the literature.60 As a general buffer, 10 mM Tris-HCl pH 8.3, 50 mM KCl and 4 mM MgCl₂ were chosen, while special amplifications based on GC rich templates like those present in *cpfts	us* (S’ Flanking region, see Figure 3) were performed using 75 mM Tris-HCl pH 8.3, 20 mM (NH₄)₂SO₄ and 4 mM MgCl₂. Reactions also contained 10 pmol of each primer, 0.53 mM dNTPs (each) and 0.26 M Betain. Annealing temperatures 2–5 degrees below the supplier’s reported melting temperature of primers were chosen, with elongation to 1 min/kb at 72 °C. All amplifications for cloning were achieved by using Hybrid Polymerase (EURX) in accordance with the supplier’s instructions. In addition, target sequence analysis of *cpfts	us* using flanking regions surrounding primers (see Figure 3) was carried out using the same buffer, containing (NH₄)₂SO₄, as that for Taq amplifications of GC rich templates.

**Amplification of template DNA for *in vitro* transcription of sgRNA was obtained using Taq polymerase with the NH₄ buffer, as mentioned above, using primers (see Table S2) sg_CP-fw1 and T7_CP-fw1 (for target T1, see Figure 3) as well as sg_CP-fw2 and T7_CP-fw2 (for target T2, designed using www.e-crisp.org, see Figure 3). As a first step, target sequence was amplified primers binding the Cas9 required RNA sequence (sg_CP-fw1, sg_CP-fw2) on plasmid DGE277 were used. After dilution of the PCR products obtained to about 0.1–1 ng/μL, a second amplification was performed with primers targeting the target sequence and containing a 5′ addition of the T7 polymerase motif (T7_CP-fw1, T7_CP-fw2). After determining the concatenation of the PCR products obtained, 1 μg of template DNA was used for *in vitro* transcription, using the Hi Scribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) at 37 °C overnight, with subsequent purification of sgRNA as mentioned above. Recombinant Cas9 activity was tested at 37 °C for 1 h using 100 ng of PCR product created with Primers Cp-fw and Cp-rv (see Figure 3), 400 ng sgRNA for *cpfts	us* target 1 and 2 (see Figure 3), and 750 ng of recombinant Cas9 (see chapter below) in cleavage buffer (20 mM Tris-HCl pH 7.5, 20 mM KCl, 5 mM MgCl₂).

**Isolation of Nucleic Acids.** Plasmid isolation from *E. coli* was carried out using the GenJet Miniprep Kit (Thermo Scientific). Contrary to the supplier’s instructions, the elution buffer was prewarmed to 55 °C and elution was repeated once using the eluto.

**Isolation of genomic DNA from *C. reinhardtii* CW15 was carried out after ref 49. Harvested cells (12 000g for 30 s) from 1–2 weeks old cultures grown in plastic multiwells of 2–3 mL TAP-Medium each were resuspended in 700 μL 2X CTAB buffer, supplemented with 100 μg Proteinase K and 50 μg RNaseA. After incubation for 2–5 h at 60 °C, genomic DNA was extracted using 1 unit of chloroform/isoamyl alcohol (24:1) after centrifugation at 12 000g for 5 min. This step was repeated with 1 unit of phenol (10 mM Tris-HCl buffered at pH 8)/chloroform/isoamyl alcohol (25:25:1) and 1 unit of chloroform. Precipitation was achieved using 0.3 M sodium acetate pH 5 and the addition of 1 unit of isopropanol at –20 °C for 1 h. After centrifugation at 12 000g for 15 min, the supernatant was removed and the sediment was washed twice with 70% ethanol at 12 000g for 5 min. Sediments were dried at 42 °C for 1 h and resuspended in 20–50 μL 10 mM Tris-HCL pH 8.

**Isolation of in vitro transcribed sgRNA was carried out in the same way as described for genomic DNA, starting by increasing the final volume after the reaction to 1 mL with DEPC-H₂O and continuing with phenol (Tris-HCl buffered pH 5)/chloroform/isoamyl alcohol (25:25:1) extraction.**

**Concentration of nucleic acids was determined using a Nanodrop One (Thermo Scientific). In the case of plasmid DNA for transformation of *C. reinhardtii*, concentration was precisely determined on 1% agarose gels compared to the marker GeneRuler 1kb Plus (Thermo Scientific) using the ImageJ software (https://imagej.nih.gov/ij/).**

**Amplification, In Vitro Transcription, Restriction, and Cloning.** Taq polymerase was expressed in *E. coli* BL21 and isolated according to the literature.60 As a general buffer, 10 mM Tris-HCl pH 8.3, 50 mM KCl and 4 mM MgCl₂ were chosen, while special amplifications based on GC rich templates like those present in *cpfts	us* (S’ Flanking region, see Figure 3) were performed using 75 mM Tris-HCl pH 8.3, 20 mM (NH₄)₂SO₄ and 4 mM MgCl₂. Reactions also contained 10 pmol of each primer, 0.53 mM dNTPs (each) and 0.26 M Betain. Annealing temperatures 2–5 degrees below the supplier’s reported melting temperature of primers were chosen, with elongation to 1 min/kb at 72 °C. All amplifications for cloning were achieved by using Hybrid Polymerase (EURX) in accordance with the supplier’s instructions. In addition, target sequence analysis of *cpfts	us* using flanking regions surrounding primers (see Figure 3) was carried out using the same buffer, containing (NH₄)₂SO₄, as that for Taq amplifications of GC rich templates.

**Amplification of template DNA for *in vitro* transcription of sgRNA was obtained using Taq polymerase with the NH₄ buffer, as mentioned above, using primers (see Table S2) sg_CP-fw1 and T7_CP-fw1 (for target T1, see Figure 3) as well as sg_CP-fw2 and T7_CP-fw2 (for target T2, designed using www.e-crisp.org, see Figure 3). As a first step, target sequence was amplified primers binding the Cas9 required RNA sequence (sg_CP-fw1, sg_CP-fw2) on plasmid DGE277 were used. After dilution of the PCR products obtained to about 0.1–1 ng/μL, a second amplification was performed with primers targeting the target sequence and containing a 5′ addition of the T7 polymerase motif (T7_CP-fw1, T7_CP-fw2). After determining the concatenation of the PCR products obtained, 1 μg of template DNA was used for *in vitro* transcription, using the Hi Scribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) at 37 °C overnight, with subsequent purification of sgRNA as mentioned above. Recombinant Cas9 activity was tested at 37 °C for 1 h using 100 ng of PCR product created with Primers Cp-fw and Cp-rv (see Figure 3), 400 ng sgRNA for *cpfts	us* target 1 and 2 (see Figure 3), and 750 ng of recombinant Cas9 (see chapter below) in cleavage buffer (20 mM Tris-HCl pH 7.5, 20 mM KCl, 5 mM MgCl₂).
subsequent restriction and ligation using T4 Ligase into final constructs.

**Expression and Purification of Recombinant Cas9.**

*E. coli* strain BL21 was transformed with the Cas9 nuclease (160 kDa) encoding plasmid pET- NLS-Cas9-6xHis (Addgene). After expression, the recombinant Cas9 contained a nuclear localization signal and a C-terminal his-tag for purification using nickel affinity chromatography. After overnight cultivation of a 5 mL culture containing 25 µg/mL chloramphenicol and 100 µg/mL ampicillin, 1 L LB-Medium containing the same antibiotics was inoculated and the culture grown to an OD₆₀₀ of 0.5. Expression of recombinant Cas9 was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, and cultivation continued overnight at 20 °C. Cells were harvested at 5000g for 10 min and purified in accordance with ref S2 using Nickel NTA affinity chromatography. In order to remove nucleic acids from *E. coli* from the protein solution, the latter was supplemented with 10 mM EDTA and incubated for 1 h. Subsequent concentration using centricons (10 kDa cutoff) was carried out twice to a small volume and again diluted in order to remove nucleic acids and EDTA, achieving a final volume of 750 µL. Thereafter, the protein solution was analyzed on SDS-page and immunoblotting was carried out. Protein concentration was determined using a Nanodrop One with an extinction coefficient at 280 nm of 120.45 M⁻¹ cm⁻¹.

**Construction of Knock-In Construct HCP.**

All the elements necessary for creating HCP (5′ flanking (SF): chromosome_S:3459480Â—3461402, aph7 cassette, 3′ flanking (3F): chromosome_S:3461425Â—3463347; see Figure 3) were amplified as described above using SF_fw/rv (SF), pBT-fw/rbsc2_rv (aph7 cassette) and 3F-fw/rv (sequences see Table S2), cloned into bluescript KS- and sequenced. The 5′ flanking region (SF) was amplified using a reverse primer (SF_rv) that also carried nucleotides for an in-frame stop codon after sequence-specific knock-in into cpofts. The aph7 cassette from the transformation vector pHyg3, conferring resistance to hygromycin, consisted of the promoter from the RubisCO small subunit (5F) was amplified using a reverse primer (5F_rv) and a C-terminal his-tag for purification using nickel affinity chromatography. Further sequence extension of the promoter after sequence-specific knock-in was carried out.
(12) Gumpel, N. J., Rochaix, J. D., and Purton, S. (1994) Studies on Homologous Recombination in the Green Alga Chlamydomonas Reinhardtii. Curr. Genet. 26, 438–442.
(13) Nelson, J. A., and Lefebvre, P. A. (1995) Targeted Disruption of the NIT7 Gene in Chlamydomonas Reinhardtii. Mol. Cell. Biol. 15, 5762–5769.
(14) Zorin, B., Lu, Y., Szizova, I., and Hegemann, P. (2009) Nuclear Gene Targeting in Chlamydomonas as Exemplified by Disruption of the PHOT Gene. Gene 432, 91–96.
(15) Zhang, R., Patena, W., Armburster, U., Gang, S. S., Blum, S. R., and Jonikas, M. C. (2014) High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. Plant Cell 26, 1398–1409.
(16) Szizova, I., Greiner, A., Awasthi, M., Kateriya, S., and Hegemann, P. (2013) Nuclear Gene Targeting in Chlamydomonas Using Engineered Zinc-Finger Nucleases. Plant J. 73, 873–882.
(17) Irion, U., Krauss, J., and Niüsslein-Volhard, C. (2014) Precise and Efficient Genome Editing in Zebrafish Using the CRISPR/Cas9 System. Development 141 (24), 4827–4830.
(18) Frit, P., Barboule, N., Yuan, Y., Gomez, D., and Calsou, P. (2014) Alternative End-Joining Pathway(s): Bricolage at DNA Breaks. DNA Repair 17, 81–97.
(19) Lin, S., Stahl, B. T., Alla, R. K., and Doudna, J. A. (2014) Enhanced Homology-Directed Human Genome Engineering by Controlled Timing of CRISPR/Cas9 Delivery. eLife 3, e04766.
(20) Schorsch, C., Köhler, T., and Boles, E. (2009) Knockout of the DNA Ligase IV Homolog Gene in the Sphingoid Base Producing Yeast Pichia Cerevisiae Significantly Increases Gene Targeting Efficiency. Curr. Genet. 55 (4), 381–389.
(21) Angstenberger, M., Kirsch, J., Aktas, O., and Büchel, C. (2019) Knock-Down of a LigiV Homolog Enables DNA Integration via Homologous Recombination in the Marine Diatom Phaeodactylum Tricornutum. ACS Synth. Biol. 8 (1), 57–69.
(22) Buffalo, N. D. (1958) A Comparative Cytological Study of Four Species of Chlamydomonas. Bull. Torrey Bot. Club 85, 157–178.
(23) Strenkert, D., Schmollinger, S., Gallaher, S. D., Salome, P. A., Purvine, S. O., Nicora, C. D., Mettler-Altmann, T., Soubeyrand, E., Jonikas, M. C. (2014) High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. Plant Cell 26, 1398–1409.
Expression Systems for Highly Efficient Arabidopsis Genome Editing Facilitate Isolation of Complex Alleles in a Single Generation. *Funct. Integr. Genomics* 20, 151–162.

(52) Zuris, J. A., Thompson, D. B., Shu, Y., Guilinger, J. P., Bessen, J. L., Hu, J. H., Maeder, M. L., Joung, J. K., Chen, Z. Y., and Liu, D. R. (2015) Cationic Lipid-Mediated Delivery of Proteins Enables Efficient Protein-Based Genome Editing in Vitro and in Vivo. *Nat. Biotechnol.* 33 (1), 73–80.

(53) Laemmli, U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227 (5259), 680–685.

(54) Berthold, P., Schmitt, R., and Mages, W. (2002) An Engineered Streptomyces Hygroscopicus Aph 7" Gene Mediates Dominant Resistance against Hygromycin B in Chlamydomonas Reinhardtii. *Protist* 153 (4), 401–412.