Genetic tools and techniques for *Chlamydomonas reinhardtii*

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Abstract

The development of tools has always been a major driving force for the advancement of science. Optical microscopes were the first instruments that allowed discovery and descriptive studies of the subcellular features of microorganisms. Although optical and electron microscopes remained at the forefront of microbiological research tools since their inventions, the advent of molecular genetics brought about questions which had to be addressed with new "genetic tools". The unicellular green microalgal genus *Chlamydomonas*, especially the most prominent species *C. reinhardtii*, has become a frequently used model organism for many diverse fields of research and molecular genetic analyses of *C. reinhardtii*, as well as the available genetic tools and techniques, have become increasingly sophisticated throughout the last decades.

The aim of this review is to provide an overview of the molecular key features of *C. reinhardtii* and summarize the progress related to the development of tools and techniques for genetic engineering of this organism, from pioneering DNA transformation experiments to state-of-the-art techniques for targeted nuclear genome editing and high-throughput screening approaches.

Keywords

Chlamydomonas reinhardtii, genetic engineering, gene silencing, molecular toolkit, promoters, selectable markers

Introduction

Genetic tool development for *C. reinhardtii* has been a prospering field of research for more than 30 years and relevant review articles have been published periodically. *The Chlamydomonas Sourcebook* represents the most extensive collection of information on the organism with the most recent edition having been published in 2009 (Harris 2009). Although only few genetic tools are established for *C. reinhardtii* which would qualify as being "ready-to-use" without significant adaptation, a great number of individual tool elements, techniques and strategies have been published which can be applied separately, or in combination, to accomplish a specific genetic engineering task.

With this review article it is my intention to provide a comprehensive overview of the progress of genetic tool and technique development for *C. reinhardtii*, with a particular focus on options for transgene expression and techniques for endogenous gene expression inhibition. The historical context and important features of the organism are introduced briefly before important practical aspects for selection of appropriate engineering strategies are addressed, including tool delivery options, cell line characteristics, genetic elements for transgene expression, transformant selection and options for gene expression inhibition in chloroplast and nucleus.

Since promoters and transformation markers are important elements for virtually all genetic tools, an extended overview of the current options is provided as Supplementary Material for this article.

Historical background, general features and motivation to use *C. reinhardtii* as a research object

The invention of optical microscopes and their propagation by natural scientist like *Robert Hooke* and *Antoni van Leeuwenhoek* in the 17th century made it for the first time possible to gain insight into the morphology and physiology of microorganisms. Systematic investigations on aquatic microalgae belonging to the genus *Chlamydomonas* began in the early 19th century. In a series of lectures at the academy of sciences in Berlin between 1828 and 1835, *C. G. Ehrenberg* described and classified numerous microorganisms and in his third contribution in 1832, he introduced the novel genus *Chlamydomonas* (spelled *Chlamidomonas* in the respective article subsequently published (Ehrenberg 1834)). Since the first description of this new genus, scientific research with *Chlamydomonas* species has grown continuously because this photosynthetic green microalga represents an outstanding model organism for many physiological and genetic studies (Harris 2001). *Chlamydomonas* species are globally distributed and have been isolated from highly diverse habitats, ranging from polar to tropical climate zones and from soil to fresh or marine water (Harris 2009). *C. reinhardtii* is the best investigated microalgal species today and, as has been pointed out before (Merchant et al. 2007), represents a common ancestor of animals and plants. Therefore besides studying specific cellular features, key functions related to various distant research fields can be addressed with this species as a model organism. *C. reinhardtii* is a eukaryotic unicellular photosynthetic green microalga (order *Volvocales*, family *Chlamydomonadaceae*, see (Pröschold et al. 2001) for a detailed phylogenic description of the genus). Besides the typical eukaryotic compartmentation, wild type (wt) *C. reinhardtii* cells feature two apical flagella (anchored in basal bodies), a chloroplast, a pyrenoid, an eye-spot and are surrounded by a cell wall mainly composed of hydroxyproline-rich glycoproteins (Harris 2009). The mitochondrial, chloroplast and nuclear genomes have been fully sequenced, annotated and can be accessed via public databases (Blaby et al. 2014). The nuclear genome of vegetative cells is haploid and two mating types (*plus* and *minus*) are described, allowing sexual and asexual cell propagation. Axenic cultivation in the laboratory is simple and can be carried out from strict photolithoautotrophic (light, H_2O, CO_2) to strict chemoorganoheterotrophic (dark, acetate as the organic carbon source) conditions. Fast

vegetative growth rates with generation times of less than seven hours are common (Sager and Granick 1953), allowing fast generation of biomass.

Because of these reasons, a continuously growing number of researches have joined the "*Chlamy community*" to study such diverse research topics as (see citations for respective topical reviews) photosynthesis & light acclimation (Allahverdiyeva et al. 2014; Heinnickel and Grossman 2013; Minagawa 2011; Rochaix et al. 2012), respiration (Salinas et al. 2014), flagella & basal bodies organization and function (Dutcher 2014), life cycle and mating (Umen 2011), carbon metabolism (Johnson and Alric 2013), nutrient membrane transport (Blaby-Haas and Merchant 2012; Grossman 2000), circadian clock (Matsuo and Ishiura 2011; Schulze et al. 2010), photoreceptors (Kianianmomeni and Hallmann 2014), photosynthetic hydrogen (Grossman et al. 2011; Hemschemeier and Happe 2011; Melis et al. 2007) and high and low value compound production (Merchant et al. 2012; Rasala and Mayfield 2015; Rosales-Mendoza et al. 2012; Skjanes et al. 2013), and many more.

Genetic key features of *C. reinhardtii*

The genetic information of *C. reinhardtii* is compartmentalized into three types of organelles, mitochondria, chloroplast and nucleus. The three genomes differ significantly with respect to genome size, G+C content, gene number, DNA organization and copy number per cell. An exclusive advantage of *C. reinhardtii* is that DNA transformation is possible for all three genomes.

The mitochondrial genome

The size of the mitochondrial genome is ~15.8 kb (Vahrenholz et al. 1993) with a G+C content of ~45%, it encodes only 13 genes with no introns (Cardol and Remacle 2009). Early electron microscopic evidence indicated that ca. 99% of the mitochondrial DNA (mtDNA) molecules are linear and only up to 1% are circular (Ryan et al. 1978) and a recent work indicates that also these circular molecules can act as templates for transcription (Wobbe and Nixon 2013). It is clear that multiple copies of the mtDNA are present per cell, but the exact number is not certain. It was estimated that 46 mtDNA copies are present per cell from early biochemical analyses (Ryan et al. 1978). However work based on fluorochrome staining indicated that the mtDNA is organized as 30-40 small (~0.1 µm) nucleoids per vegetative cell (Hiramatsu et al. 2006) and it was estimated that each 0.1 µm nucleoid contains around 15 mitochondrial genome copies (Nishimura et al. 1998), which would represent a far higher copy number (450-600) per cell. Most recent evidence suggests that after mating, transmission of the mitochondrial DNA is uniparental by the mating type *minus* parent (see chapter 7 in Harris 2009), although conflicting data was reported before (Nishimura et al. 1998).

The chloroplast genome

One chloroplast is present per cell, the size of the circular chloroplast genome is ~203.8 kb with a G+C content of ~35%, encoding 99 genes with five genes containing introns (Higgs 2009; Maul et al. 2002). Typically ~80-90 chloroplast genome copies, organized in ~10-20 nucleoids of <0.2 µm in diameter (Harris 2009; Hiramatsu et al. 2006), are present per vegetative cell. The chloroplast DNA molecules seem to exist in multiple circular and linear conformations *in vivo* (Maul et al. 2002) and sexual transmission is uniparental from the mating type *plus* parent (see chapter 7 in Harris 2009).

The nuclear genome

By far the greatest extent of genetic information is stored in the nucleus. According to the most recent annotation, the haploid nuclear genome of ~111.1 Mb is arranged in 17 linkage groups with a G+C content of ~64% (Merchant et al. 2007) and around 19,500 protein-coding transcripts (including splice variants) are being predicted (Joint Genome Institute 2015). Since the nuclear genome is haploid in vegetative cells, recessive mutations do not occur and selection for mutations of specific genes can be straight forward. As mentioned before, the genomes of chloroplast and mitochondria are present in multiple copies per cell and heteroplasmy therefore can here be an issue (Remacle et al. 2006), however, isolation of homoplasmic cell lines is possible (Larosa et al. 2012).

Classical genetic techniques

Although molecular genetic tools and techniques are in the focus of this review, it is important to note that classical genetic techniques are well established for *C. reinhardtii*. Methods to induce gametogenesis, mating and tetrad analysis have been worked out many years ago and can be performed efficiently in the lab, although not every cell line mates equally well (Harris 2009).

Gametogenesis usually is induced by nitrogen deprivation (Sager and Granick 1954) and mating takes place when *plus* and *minus* gametes are mixed. Mediated by agglutinin glycoproteins, flagella of opposite mating type gametes adhere, cell wall lysis and cell fusion occurs and zygospore formation takes place. After a minimum of 2-3 (recommended are 4-6) days of zygospore maturation, germination of the diploid zygotes can be induced by transfer into fresh medium and illumination. After two meiotic divisions, four haploid tetrad cells arise within the zygospore wall. Tetrad cells can then be analyzed individually (see chapter 8 in Harris 2009 for a detailed protocol). Interestingly, occasionally zygotes undergo mitosis, instead of meiosis, resulting in stable vegetative diploid *Chlamydomonas* strains (Ebersold 1967; Shimogawara et al. 1999). Mating and tetrad analyses have been very important techniques in the past and will continue to be so in the future, e.g. in the context of forward genetics, where the number of unwanted random mutations can be reduced by crossing the mutant with the respective wt.

Molecular genetic tools and techniques

The first successful DNA transformation of *C. reinhardtii* was achieved by Rochaix & van Dillewijn more than 30 years ago (Rochaix and van Dillewijn 1982) and since then, many genetic tools and techniques have been developed. Due this progress, multiple strategies and/or tools are in some cases applicable to accomplish a certain engineering task. As an example, production of a given chemical compound within the chloroplast could be achieved via expression of a specific gene from the chloroplast genome, expression from the nucleus (and potentially directing the protein into the chloroplast via targeting signals), or application of a knockdown/knockout strategy to attenuate respective antagonistic pathways. Three aspects are of essential importance for selection of an appropriate genetic engineering strategy: (1) tool delivery; (2) the cell line; (3) the target effect of tool application. The currently available options regarding all three aspects will be addressed in detail in the following chapters.

(1) Techniques for genetic tool delivery

Genetic tools are macromolecules which must be introduced into the cell to unfold their effects, implicating that several protective layers (cell wall and one or more lipid bilayer membranes) have to be crossed without

deactivating the tool or irreversibly damaging the cell. Most often, DNA transformation is applied for tool delivery and three strategies are commonly used for *C. reinhardtii* to achieve this goal.

Application of mechanical force by biolistic particle bombardment

The first stable and highly efficient transformation of *C. reinhardtii* cells was reported in 1988 by restoring chloroplast *atpB* mutants via biolistic particle bombardment with appropriate DNA-coated tungsten microparticles (Boynton et al. 1988). Since then, gene gun DNA delivery was also applied successfully to transform the nuclear (Day et al. 1990; Debuchy et al. 1989; Kindle et al. 1989) and the mitochondrial (Randolph-Anderson et al. 1993; Remacle et al. 2006; Yamasaki et al. 2005) genomes. Homologous DNA recombination readily occurs in chloroplasts and mitochondria (Boynton et al. 1988; Larosa et al. 2012), however, DNA integration into the nuclear genome occurs preferably at apparently random loci. An early study reported a ratio of homologous vs. nonhomologous integration events of 1:24 after biolistic nuclear transformation (Sodeinde and Kindle 1993), however, this number could even be overestimating the homologous events (Zorin et al. 2005). The presence of a cell wall does not prevent biolistic transformation and since stable transformation of all three genomes is possible, particle bombardment can be regarded as the most versatile of the three delivery options. On the downside, the transformation protocols and the necessary equipment are quite sophisticated compared to the alternative methods.

Application of mechanical force by vortexing

This method to induce uptake and stable integration of exogenous DNA into the nuclear genome of *C. reinhardtii* was introduced shortly after establishment of the biolistic transformation and is based on simple vortexing of algal cell suspension in the presence of exogenous DNA and microparticles, e.g. glass beads (Kindle 1990) or silicon carbide whiskers (Dunahay 1993). Only little and cheap equipment is necessary to perform DNA transformation with this method and because of this advantage, the procedure is commonly applied for nuclear transformation. A disadvantage of the vortexing method is that the outer cell wall inhibits DNA uptake, therefore it can only be applied efficiently after autolysin treatment or with cell wall deficient strains (Kindle 1990). As described for biolistics, vortexing promotes random integration of the exogenous DNA into the nuclear DNA with a frequency of homologous vs. nonhomologous events of 1:1000 (Sodeinde and Kindle 1993) or even less (Gumpel et al. 1994; Nelson and Lefebvre 1995). Zorin et al. introduced a method based on the use of single-stranded, instead of double-stranded, DNA and showed a significant increase of the relative frequency of site-directed integration events (Zorin et al. 2005; Zorin et al. 2009). However, isolation of specific nuclear mutants resulting from homologous recombination with this method remained very challenging. Recently, a modified glass bead transformation protocol was introduced and used for simple generation of chloroplast mutants (Economou et al. 2014).

Application of electric fields

The short application of high intensity electrical fields during electroporation leads to reversible membrane breakdown and concomitant entry of exogenous DNA. This method has been applied successfully to introduce DNA into the nucleus of cells both with and without cell walls (Brown et al. 1991; Shimogawara et al. 1998). Zhang et al. demonstrated that the DNA insertion occurs at apparently random loci and subsequent insertion site sequence analyses indicated that endonucleolytic events during mutagenesis can occur and lead to cleavage of

the transforming DNA, as well as genomic DNA from lysed cells before integration (Zhang et al. 2014). Numbers on the relative frequencies of homologous vs. nonhomologous integration events after electroporation do not seem to be publicly available. It should be noted that electroporation was also successfully applied to introduce exogenous proteins into *C. reinhardtii* (Hayashi et al. 2001).

Besides the three main methods, two further strategies to introduce compounds into *C. reinhardtii* have been mentioned in the literature. Kumar et al. described that T-DNA transformation mediated by *Agrobacterium tumefaciens* was successful (Kumar et al. 2004), but hardly any further study applying this method has been published. Mechanical microinjection of KCl and $MgSO_4$ solutions into *C. reinhardtii* was performed even before the first successful DNA transformation (Nichols and Rikmenspoel 1978), but was apparently not yet applied for nucleic acid transfer. Very recently, the novel method of live-cell nanoinjection of fluorescent probes (Hennig et al. 2015) was successfully applied with cell-wall deficient *C. reinhardtii* cells (unpublished data in cooperation with Dr. S. Hennig and Prof. Dr. T. Huser, Bielefeld University, 2014). Although both, micro- and nanoinjection are not feasible for high-throughput macromolecular transfer, direct injection could become interesting alternatives to deliver molecular tools when generation of a genetically modified organism is not desired.

(2) Cell line selection for genetic engineering

Located at the *University of Minnesota*, the *Chlamydomonas Resource Center* houses a large collection of *Chlamydomonas* cell lines, which is of great value because it facilitates the selection of appropriate cell lines for specific scientific tasks. In the context of genetic engineering, three issues of general importance should be considered. First, it is important to decide if a cell wall or a cell wall deficient strain should be preferred. As mentioned before, transformation via vortexing methods is very simple in the case of cell wall deficient strains, however, the presence of a cell wall can be beneficial in terms of cell robustness, an important parameter for many biotechnological applications. Second, the strategy of transformant selection must be taken into account. Complementation of mutant cell lines or transformation of dominant selectable markers are the two most common ways for positive transformant identification. When complementation (e.g. transformation of an arginine auxotrophic strain like *CC1618* with an argininosuccinate lyase containing plasmid) is applied, it must be considered that in all subsequent experiments, growth of the parental strain has to be supplemented continuously with arginine, in contrast to the complemented strain. Since it is likely that the intracellular level of the supplemented substance will vary between wt and mutant, subsequent phenotype analyses can be misleading. For certain physiological experiments, it therefore seems more practical to use a non-auxotrophic parental stain and a marker conferring antibiotic resistance, since after initial selection, the antibiotic can be omitted from subsequent phenotype analyses, minimizing potential unwanted side effects. Third, the cell line dependent efficiency of transgene expression is often low (Fuhrmann et al. 1999; Schroda et al. 2000). Improved transgene expression was recently described for two cell lines, designated *UVM4* and *UVM11*, which were isolated after UV mutagenesis of the cell wall deficient parental strain *cw15-302* (Neupert et al. 2009). Alterations of the chromatin structure are suggested to be the reason for the improved transgene expression with these strains, but the exact molecular basis is still unknown (Neupert et al. 2009). Very recently, the strain *MET1*, an insertional mutant of a putative maintenance-type DNA cytosine methyltransferase, was identified to also show improved transgene expression characteristics (Kong et al. 2015).

(3) Target effect of genetic tool application

Molecular genetic tools are generally applied to achieve one of two apparently opposite goals, (a) the facilitation or enhancement of target gene expression or (b) the reduction of expression or editing of an endogenous gene target. Most genetic tools and techniques therefore fall into one of the two categories, although some tool elements can be adapted for dual use.

a) Tools and techniques for endogenous and exogenous gene expression

Stable and efficient transgene expression in *C. reinhardtii* has been notoriously difficult, especially from the nucleus (Fuhrmann et al. 1999; Neupert et al. 2009). Potential reasons are biased codon usage (Heitzer et al. 2007), epigenetic transgene silencing (Cerutti et al. 1997), positional effects and chromatin structure (Specht et al. 2014; Strenkert et al. 2011), aberrant processing, lack of suitable regulatory sequences or potentially further currently unknown mechanisms (Fuhrmann et al. 1999). Efficient transgene expression strongly relies on non-coding, cis-acting elements (Barnes et al. 2005; Lumbreras et al. 1998; Sizova et al. 2001) and several elements promoting stable transgene expression have been identified.

Promoters

Promoters drive gene expression and many promoter/5´ untranslated regions (UTRs) have successfully been used for recombinant transgene expression. At present, the hybrid HSP70A-RBCS2 promoter (Schroda et al. 2000), in combination with one or more enhancing introns, is most commonly used if high nuclear transgene expression is desired. For this reason, we selected this hybrid system to develop a versatile, modular vector tool for nuclear encoded recombinant protein production (Lauersen et al. 2013; Lauersen et al. 2015). A 1,425 bp promoter/5´-UTR region upstream of the endogenous ARG7 gene was recently demonstrated to convey promoter activity of similar strength as the hybrid HSP70A-RBCS2 promoter in the context of an ARS2 reporter assay (Specht et al. 2014), therefore representing an interesting alternative. Promoter trapping (Haring and Beck 1997) has also been performed and led to the identification of several efficient regulatory promoter sequences (Vila et al. 2012). As an example, the UBIRP (ubiquitine regulatory protein) promoter region, without further improvement, yielded 70% of the transcript level compared with the HSP70A/RBCS2 hybrid control construct (Vila et al. 2012). It can be expected that systematic analyses and introduction of selected modifications of these and other new promoter sequences will lead to the development of even better systems for high nuclear transgene expression in the near future. An extended overview of nuclear and chloroplast promoters is presented in Supplementary Table S1.

Introns

Most natural nuclear encoded *C. reinhardtii* genes contain one or more introns, suggesting that intron-exon gene organization is of great relevance for the organism. Indeed, the presence of introns has been shown to potentially exert a strong influence on the efficiency of transgene expression (Lumbreras et al. 1998). Eichler-Stahlberg et al. performed a semi-systematic analysis on the influence of RBCS2 introns on transgene reporter expression. A luciferase-based assay showed that incorporation of one or more RBCS2 introns can lead to increased levels of reporter activity and the highest activity was determined when introns 1, 2 and 3 were present in their physiological order, resulting in a more than fourfold increase of transgene expression (Eichler-Stahlberg et al.

2009). These data indicated that the selective integration of introns within the transgene construct can be of great importance for high-level transgene expression.

In most cases, the 3´-UTR seems less important in terms of gene expression regulation and several different, e.g. the RBCS2, the PSAD or the psbA 3´-UTRs, have been used successfully for nuclear or chloroplast gene expression, respectively (Bertalan et al. 2015; Fischer and Rochaix 2001; Fuhrmann et al. 2004).

Modulation of transgene expression

Many of the promoters listed in Supplementary Table S1 show varying activity depending on the prevailing conditions, allowing modulation of transgene expression to some degree. However, molecular tools for efficient and reversible "on/off" transgene expression are rare. The endogenous nuclear cytochrome c_6 promoter was successfully used by Quinn et al. for this purpose, since the promoter activity is repressed in the presence of copper but expression is strongly induced upon nickel addition or complete absence (below nanomolar levels) of copper (Quinn et al. 2003). Building on this nuclear promoter, an inducible and repressible chloroplast gene expression tool was developed by Surzycki and colleagues. Here, a nuclear NAC2 mutant was complemented with the NAC2 gene controlled by the cytochrome c_6 promoter. Presence of NAC2 is essential for photosynthesis, because the NAC2 protein targets to the chloroplast and stabilizes the 5′-UTR of the chloroplast psbD gene, allowing accumulation of functional photosystem II complexes (Nickelsen et al. 1994). Controlled induction or repression of NAC2 therefore allowed reversible induction or repression of photosynthetic competence, respectively, in the transformant cell lines (Surzycki et al. 2007). Importantly, this regulation principle can be implemented for any coding sequence in the chloroplast by fusing the transgene with the psbD 5′-untranslated region (Surzycki et al. 2007).

Another element for transgene expression regulation was introduced by Croft and colleagues. Thiamine pyrophosphate (TPP) dependent riboswitches were identified and it was shown that inclusion of the THI4 riboswitch leads to repression of recombinant protein production in the presence of TPP (Croft et al. 2007).

A combinatory strategy by Ramundo et al., where the NAC2 gene was placed under the control of the nuclear METE promoter (repression by vitamin B_{12}) and the THI4 riboswitch (repression by thiamine), led to the establishment of a vitamin-mediated, reversible chloroplast gene expression/repression tool (Ramundo et al. 2013).

An interesting option to increase nuclear promoter activity is the use of transcription activator-like effector (TALE) proteins and artificially designed TALE proteins were successfully used in *C. reinhardtii* to activate endogenous gene expression (Gao et al. 2015; Gao et al. 2014). The TALE-based technique represents a potential dual purpose tool, since coupling of TALEs with nuclease domains (TALEN) can be applied for specific gene knockout approaches, as will be discussed later.

Transformation markers and reporters

Early transgene expression trials relied on mutant complementation as a means to identify positive transformants but since then, a variety of dominant selectable markers/reporters for wt nuclear and chloroplast transformation has been established. It should be noted that in contrast to nuclear targets, marker-free and marker-removal strategies exist for chloroplast transformation approaches (Chen and Melis 2013; Day and Goldschmidt-Clermont 2011; Economou et al. 2014), which can be an important feature for applied biotechnological production processes.

Nuclear and chloroplast markers/reporters established for *C. reinhardtii* can be grouped into three main categories: (i) Markers complementing a specific (auxotrophic) phenotype; (iia) Markers mediating antibiotic/herbicide resistance by expression of a mutated, endogenous *C. reinhardtii* gene; (iib) Markers mediating antibiotic/herbicide resistance by expression of a foreign transgene; (iiia) Markers allowing color or (iiib) light emission detection. Supplementary Table S2 presents an extended overview of available options for each category. Amongst the nine markers belonging to category (iib), the ble marker is quite unusual. In contrast to the other eight, it does not represent an enzyme but instead a small, non-catalytic protein, which accumulates in the cell nucleus after expression (Fuhrmann et al. 1999) and directly binds and sequesters the respective DNA-cutting antibiotic (e.g. bleomycin) in a dose-dependent, linear stoichiometry (Dumas et al. 1994).

Simultaneous use of two separate plasmids for nuclear transformation can lead to co-transformation of both plasmids into a single cell and this feature was routinely applied in the past to co-transform genes present on plasmids which lacked a dominant selectable marker. However, due to the progress in plasmid construction strategies and the decline of costs for *de novo* DNA synthesis, expression vectors today incorporate distinct (in some cases easily exchangeable) dominant selectable markers which confer resistance to antibiotics like emetine, hygromycin B, paromomycin or zeo(my)cin, complement an auxotrophic phenotype or restore photosynthesis, alleviating the need for co-transformation (Berthold et al. 2002; Fischer and Rochaix 2001; Heitzer and Zschoernig 2007; Lauersen et al. 2015).

The establishment of several dominant positive selectable markers allows multiple transgene expression within a single cell by subsequent transformations. As an alternative, Rasala et al. employed viral 2A peptides to express multi-cistronic transgenes (Rasala et al. 2014; Rasala et al. 2012), representing an important step towards more complex metabolic engineering approaches.

In contrast to positive selectable markers, negative selectable markers represent factors that confer sensitivity towards a certain compound once expressed in the host cell and can be useful e.g. to identify trans-acting factors (Young and Purton 2014) or as markers for gene silencing approaches (Jiang et al. 2014; Rohr et al. 2004). Three established negative selectable marker in *C. reinhardtii* are the endogenous MAA7 gene (encoding the tryptophan synthase ß subunit; presence leads to sensitivity to 5-fluoroindole) (Rohr et al. 2004), the endogenous FKBP12 gene (encoding the 12-kD FK506-binding protein; presence leads to sensitivity to rapamycin) (Crespo et al. 2005) and the *E. coli* codA gene (encoding cytosine deaminase; presence leads to sensitivity to 5-fluorocytosine) (Young and Purton 2014).

b) Tools and techniques to reduce gene expression or edit an endogenous target

The prevalence of homologous DNA recombination within the organelles makes gene engineering and subsequent functional protein characterization comparably easy. Generation and isolation of specific knockdown or knockout mutants of nuclear encoded genes is substantially more difficult and many techniques were tested in forward or reverse genetics approaches but even today, targeted nuclear gene modification remains very challenging. Since an elaborate topical review was published very recently (Jinkerson and Jonikas 2015), a condensed summary in this respect will be presented here.

Random mutagenesis

Random mutagenesis of *C. reinhardtii* by DNA insertion or chemical/radiation mutagenesis is comparably easy and it is possible to generate large random mutant collections with several thousands of individual mutants. DNA

insertion has the advantage that knowledge of the transferred DNA sequence can be exploited for subsequent identification of the nuclear site of the mutation (Galvan et al. 2007) and a reverse genetics approach comprising two large transformant libraries (ca. 22,000 and 100,000 transformant lines, respectively) was published by Gonzalez-Ballester and colleagues. In their work, cells and DNA were systematically pooled to allow subsequent isolation of specific insertion mutants. Briefly, 96 individual transformant lines were pooled and genomic DNA was extracted from each pool. DNA superpools were then prepared by mixing equal volumes of genomic DNA from ten different pools and it was shown that these DNA superpools can be used as templates for PCR screenings, with one primer derived from the marker gene and the other from the nuclear target region. Once a positive signal was detected in a DNA superpool, it was possible to trace it back to the original transformant by sequential PCR and finally colony PCR reactions (Gonzalez-Ballester et al. 2011).

Dent et al. applied random DNA insertion mutagenesis in a forward genetics approach. Approximately 49,000 transformants were generated and phenotypically pre-screened, significantly reducing the number of cell lines that had to be maintained. Different PCR-based techniques were then applied for molecular analyses of the insertion sites and 459 flanking sequence tags could be identified for 439 mutants (Dent et al. 2015).

A general disadvantage of DNA insertion mutagenesis for generation of specific knockout lines is that multi-copy insertions, DNA truncations, rearrangements or loss of relatively large regions of the nuclear genome insertion site are not uncommon (Tam and Lefebvre 1993) and can make subsequent assignment of the mutant phenotype difficult. In this context, Zhang et al. recently used a PCR-based high-throughput genotyping method designated *ChlaMmeSeq* (*Chlamydomonas MmeI-based insertion site Sequencing*) to analyze 11,478 nuclear insertion sites and showed that endonucleolytic cleavage of the transforming DNA regularly occurs, potentially resulting in identification of misleading flanking sequences (Zhang et al. 2014).

Unambiguous identification of the locus responsible for a specific phenotype after mutation with chemicals or radiation, e.g. by traditional or PCR-based genetic mapping (Jinkerson and Jonikas 2015), is quite challenging because no obvious molecular tag is present and depending on the dose of the mutagen, many DNA mutations may occur in a single cell. *TILLING (targeting induced local lesions in genomes*) is a method to identify point mutations (Kurowska et al. 2011; McCallum et al. 2000) and one report describes successful application with *C. reinhardtii* to identify a LHCSR1 mutant (Truong 2011). However, this method has since then not been widely applied. Whole genome sequencing and bioinformatic data analyses were recently introduced as an alternative strategy to identify single nucleotide polymorphisms and specific point mutations in *C. reinhardtii* (Dutcher et al. 2012; Schierenbeck et al. 2015).

A noteworthy general obstacle when working with large random mutant populations is that maintenance of cell cultures requires regular, expensive and work intensive cell transfer and cryopreservation (Crutchfield et al. 1999) is not perfectly reliable for every *C. reinhardtii* cell line. Targeted gene silencing is an alternative approach and avoids such large mutant library maintenance.

Targeted gene silencing and gene editing

Several strategies exist to obtain cell lines with specifically reduced gene expression. RNA interference (RNAi) has been shown to be effective in *C. reinhardtii* (Schroda et al. 1999) and Rohr et al. have introduced the Maa7/X-IR (NE-537) plasmid tool, which allows easy application of the RNAi technology (Rohr et al. 2004). This procedure can also be used to effectively knockdown several homologous genes or even whole gene families (Mussgnug et al. 2007). In 2009, two artificial micro-RNA (amiRNA) gene knockdown tools were

introduced for *C. reinhardtii*, one based on the endogenous micro-RNA precursor MIR1162 (Zhao et al. 2009), the other based on the MIR1157 precursor (Molnar et al. 2009). The respective pChlamiRNA plasmid set created by Molnar et al., in combination with the web-based WMD software (Ossowski et al. 2008), allowed user-friendly application and was subsequently modified to generate an inducible amiRNA tool by replacing the original HSP70A-RBCS2 hybrid promoter with the NIT1 promoter (Schmollinger et al. 2010). Most recently, Hu et al. published an alternative amiRNA tool, which includes a luciferase reporter, and a one-step construction option for the amiRNA precursor (Hu et al. 2014).

A general downside of knockdown approaches is that RNAi efficiency and stability are transformant-specific and often complete silencing cannot be reached or maintained. Residual transcript expression can be advantageous for studies of essential genes, but on the other hand can make phenotype analyses difficult.

The generation of specific knockout mutants and/or targeted nuclear genome engineering can be achieved via directed endonucleolytic DNA cleavage. In case of DNA damage repair via error-prone non-homologous end joining, deletion or insertions commonly occur and often result in gene inactivation (Carroll 2014). If the DNA break is instead repaired via homologous recombination, addition of synthesized homologous DNA fragments, which contain a modified version of the target gene, can be utilized for targeted gene editing (Carroll 2014). To create such tools, highly DNA sequence specific nucleases have to be designed. The three most prominent strategies today are based on zinc-finger nucleases (ZFNs), TALEN or on the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system. The ZNF and the TALEN approaches both rely on engineering and fusion of specific DNA-binding protein domains (zinc-fingers or TALEs) to the FokI nuclease domain. In contrast, specificity of the Cas9 nuclease is guided by RNA in the CRISPR/Cas system. The latter is clearly superior to ZNFs and TALENs with regard to the simplicity of target design, allowing fast and easy adaption, however, off-target DNA cleavage can be quite problematic (Carroll 2014).

Advances to introduce nuclease-based tools to *C. reinhardtii* were, to some degree, made for all three approaches but today, the most developed option is the use of ZFNs and Sizova et al. successfully applied this method to target the COP3 gene (Sizova et al. 2013). As mentioned before, functional TALE proteins have been used in *Chlamydomonas* for gene expression activation (Gao et al. 2015; Gao et al. 2014) however, nuclease coupling and subsequent successful application for targeted gene knockout was not yet demonstrated.

The first trials to introduce the CRISPR/Cas tool to *C. reinhardtii* by Jiang et al. showed that the system is in principle functional, but lack of surviving transformants led the authors to conclude that Cas9 expression could be toxic to the cells (Jiang et al. 2014). This conclusion was recently challenged by work from the Hegemann lab (Humboldt University Berlin). Greiner provided evidence that *in vivo* expression of a Cas9-YFP fusion protein in *C. reinhardtii* was indeed possible and furthermore demonstrated specific Cas9 nuclease activity (Greiner 2014).

Summary

C. reinhardtii represents the most established eukaryotic microalgal model organism for fundamental and applied research. Many genetic tool elements and elaborate techniques have been developed and today, researchers often can select from several options to achieve a certain genetic engineering goal. Table 1 provides a bullet point style summary of the respective key topics addressed in this article and lists several considerations which are important for the selection of appropriate genetic engineering strategies for *C. reinhardtii*.

---------- **TABLE 1** ----------

Trends and prospects

As summarized in this review, substantial progress in the development of *C. reinhardtii* genetic engineering has been made in the past and numerous successful examples of genetic tool applications are published. However, not all of the tools and techniques can easily be implemented to new targets, because many were initially developed for a specific purpose, rather than for versatile use. The recent progress in the field of DNA synthesis technology has led to a dramatic reduction of the costs for gene synthesis, making the desirable rational re-design for optimal versatility of certain genetic tools feasible.

A major limitation still is the lack of tools for quick generation and isolation of distinct nuclear knockout mutants. Major progress can be expected from an indexed and mapped insertional mutant library prepared via the *ChlaMmeSeq* approach, which is currently in development (Jinkerson and Jonikas 2015).

The CRISPR/Cas9 system is the most valuable new tool for targeted nuclear genome editing in many species, but initial trials have indicated that the adaptation to *Chlamydomonas* could be comparatively difficult (Jiang et al. 2014). It can still be expected that a fully functional CRISPR/Cas tool will eventually be established and since the spectrum of applications for this tool is not limited to the generation of knockout mutants or gene editing (Mali et al. 2013), many more exciting applications can be foreseen once this has been achieved.

The ongoing progress of genetic tool development also paves the way for new approaches of metabolic pathway engineering, which often require multiple genetic engineering steps in plants (Bock 2013). It therefore seems likely that many ideas to change the metabolic composition or to produce novel compounds in *C. reinhardtii* will be realized in the near future.

Cloning and maintenance of large DNA fragments in yeast, including the complete *C. reinhardtii* chloroplast genome (O'Neill et al. 2012) and even entire chromosomes of *Phaeodactylum tricornutum* (Karas et al. 2013), have already been demonstrated and the further implementation of synthetic genomic approaches and modular functional device strategies, as illustrated by Scaife and colleagues with an example for improved triacylglycerol biosynthesis (Scaife et al. 2015), become realistic concepts.

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Conflict of interest

I declare no conflict of interest.

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Table 1 Summary of the key topics and respective considerations important for genetic engineering of *C. reinhardtii* addressed in this article.

Topic	**Considerations**
Genomes and genetic tool delivery	The genetic content present in mitochondria, chloroplast and nucleus differs significantly in many aspects and adaptation of transgenes can be advantageous. All three genomes can be accessed for genetic engineering and the most prominent methods are biolistic particle bombardment, vortexing and electroporation. Homologous recombination of the transferred DNA readily occurs in chloroplast and mitochondria but is a rare event in the nucleus.
Cell material	An extensive strain collection is maintained at the *Chlamydomonas Resource Center* at the *University of Minnesota*. Cell wall reduced cell lines allow easy transformation via vortexing methods, but can be less robust than cell lines with an intact cell wall. Existing mutant cell lines can be selected for strategies based on complementation. Cell lines with superior transgene expression characteristics have been isolated. Cells of opposite mating types (*plus* and *minus*) can be crossed, but not all cell lines mate well.
Regulatory elements	Many genetic elements promoting transgene expression were identified and promoters and introns have most extensively been investigated. Modulated or constitutive transgene expression can be achieved. An extended overview of available promoter options is presented in Supplementary Table S1.
Markers and reporters	The existing positive selectable markers are based on mediation of resistance to harmful chemicals, complementation of an auxotrophy or restoration of photosynthetic competence. Several reporters based on color detection or light emission are also established. An extended overview of available markers and reporters is presented in Supplementary Table S2. Multiple transgene expression can be achieved by multiple transformations or via multi-cistronic gene expression. Three available markers allow negative selection.
Random mutagenesis	DNA insertion and chemical/radiation mutagenesis allows generation of large scale nuclear mutant libraries. Existing libraries be screened for the availability of specific mutants, but maintenance of large mutant cell line libraries is work-intensive. Genotypic analysis of point mutants is more challenging compared to DNA insertion mutants, because the DNA tag can be exploited for insertion site identification. Undesired effects including DNA loss and/or rearrangements commonly occur during DNA insertional mutagenesis.
Targeted gene silencing and gene editing	Tools are available for generation of knockdown cell lines via RNAi and amiRNA approaches. ZFNs have successfully been used for specific target knockout. TALEN and CRISPER/Cas9 tools are under development.

Supplementary Tables

Supplementary Table S1 List of promoters described for recombinant transgene and/or reporter gene expression in *C. reinhardtii* from nuclear (n) or chloroplast (c) genomes, respectively. Alternative names are indicated in brackets.

Name	Organism, annotation	n/c	References
16S rRNA	*C. reinhardtii*, 16S rRNA gene	c	Klein et al. 1992; Rasala et al. 2011
ARG7	*C. reinhardtii*, argininosuccinate lyase	n	Specht et al. 2014
atpA	*C. reinhardtii*, atp synthase subunit	c	Blowers et al. 1990; Mayfield et al. 2003; Michelet et al. 2011
atpB	*C. reinhardtii*, atp synthase subunit	c	Blowers et al. 1990; Klein et al. 1992
ATX1	*C. reinhardtii*, copper chaperone *antioxidant*	n	Fei and Deng 2007
ß2TUB	*C. reinhardtii*, β2-tubulin	n	Brunke et al. 1984
C3	*C. reinhardtii*, C3	n	Voytsekh et al. 2008
CAH1	*C. reinhardtii*, carbonic Anhydrase	n	Ferrante et al. 2011; Kucho et al. 1999
CaMV 35S	*Cauliflower mosaic virus*, 35S	n	Guilley et al. 1982; Kumar et al. 2004; Ruecker et al. 2008
CHOP2	*C. reinhardtii*, channelopsin-2	n	Fuhrmann et al. 2004
CPX1	*C. reinhardtii*, coproporphyrinogen oxidase	n	Quinn et al. 2003
CYC6	*C. reinhardtii*, cytochrome c_6	n	Ferrante et al. 2011; Quinn et al. 2003
FDX5	*C. reinhardtii*, ferredoxin	n	Lambertz et al. 2010
FEA1 (H43)	*C. reinhardtii*, FEA1 (H43)	n	Baba et al. 2011; Fei et al. 2009
FOX1	*C. reinhardtii*, multi-copper oxidase	n	Deng et al. 2014
FTR1	*C. reinhardtii*, iron transporter	n	Fei et al. 2010
GPXH (GPX5)	*C. reinhardtii*, glutathione peroxidase	n	Leisinger et al. 2001
HSP70A	*C. reinhardtii*, heat shock factor 1	n	Schroda et al. 2000
HSP70A/ RBCS2	*C. reinhardtii*, hybrid promoter HSP70A/RBCS2	n	Lodha et al. 2008; Schroda et al. 2000; Strenkert et al. 2013
HSP70A/ RBCS3	*Volvox carteri*, hybrid promoter HSP70A/RBCS3	n	Hallmann and Wodniok 2006
HYDA1	*C. reinhardtii*, [FeFe]-hydrogenase isoform1	n	Pape et al. 2012
LHCBM6	*C. reinhardtii*, LHCBM6 (CABII-1, LHCB1)	n	Blankenship and Kindle 1992; Hahn and Kück 1999
LHCBM9	*C. reinhardtii*, LHCBM9	n	Grewe et al. 2014; Sawyer et al. 2015
LIP	*Dunaliella tertiolecta*, light-inducible protein	n	Park et al. 2013
METE	*C. reinhardtii*, B_{12}-independent methionine synthase	n	Croft et al. 2005; Helliwell et al. 2014
NAB1	*C. reinhardtii*, nucleic acid binding protein 1	n	Berger et al. 2014
NIT1	*C. reinhardtii*, nitrate reductase	n	Ohresser et al. 1997
nos	*Agrobacterium tumefaciens*, nopaline synthase	n	Diaz-Santos et al. 2013; Hall et al. 1993
petA	*C. reinhardtii*, cytochrome b_6f subunit	c	Young and Purton 2014
petD	*C. reinhardtii*, cytochrome b_6f subunit	c	Sakamoto et al. 1993
PSAD	*C. reinhardtii*, photosystem I subunit	n	Fischer and Rochaix 2001
psaA-exon1	*C. reinhardtii*, photosystem I subunit	c	Michelet et al. 2011; Young and Purton 2014
psbA	*C. reinhardtii*, photosystem II subunit	c	Michelet et al. 2011
psbA	Various species, photosystem II subunit	c	Gimpel and Mayfield 2013
psbD	*C. reinhardtii*, photosystem II subunit	c	Nickelsen et al. 1994
rbcL	*C. reinhardtii*, RUBISCO large subunit	c	Blowers et al. 1990; Franklin et al. 2002
RBCS2	*C. reinhardtii*, RUBISCO small subunit	n	Goldschmidt-Clermont and Rahire 1986; Kozminski et al. 1993
RBCS2	*Dunaliella tertiolecta*, RUBISCO small subunit	n	Walker et al. 2005
SQD2	*C. reinhardtii*, sulphoquinovosyltransferase	n	Iwai et al. 2014
SV 40	*Simian virus 40*, early promoter	n	Hasnain et al. 1985; Ladygin and Butanaev 2002

Supplementary Table S2 List of positive transformation markers and reporters developed for *C. reinhardtii*. The categories (cat.) are: (i) Auxotrophy/mutation complementation markers; (iia) Antibiotic/herbicide resistance mediated by a mutated, endogenous *C. reinhardtii* gene; (iib) Antibiotic/herbicide resistance mediated by a foreign transgene; (iiia) Markers for color or (iiib) light emission detection.
DCMU: 3-(3,4-dichlorophenyl)-l,l-dimethylurea;
$DiFMUX_2$: 6,8-difluoro-4-methylumbelliferyl beta-D-xylobioside; X: 5-Bromo-4-chloro-3-indolyl;
MUG: 4-methylumbelliferyl glucuronide. Alternative names are indicated in brackets.

Cat.	Name	Organism, annotation	Activity	References
(i)	AC29 (ALB3.1)	*C. reinhardtii*, ALBINO3.1	Complementation of yellow, acetate-requiring phenotype	Bellafiore et al. 2002; Ferris 1995
(i)	ARG7	*C. reinhardtii*, argininosuccinate lyase	Complementation of arginine auxotrophy	Debuchy et al. 1989
(i)	ARG9	*Arabidopsis thaliana/ C. reinhardtii*, N-acetyl ornithine aminotransferase	Complementation of arginine auxotrophy	Remacle et al. 2009
(i)	atpB	*C. reinhardtii*, ATP synthase subunit	Restoration of photosynthetic competence	Boynton et al. 1988
(i)	ATPC	*C. reinhardtii*, ATP synthase subunit	Restoration of photosynthetic competence	Smart and Selman 1993
(i)	NIC7	*C. reinhardtii*, quinolinate synthetase A	Complementation of nicotinamide auxotrophy	Ferris 1995; Ferris et al. 2002
(i)	NIT1 (NIA1)	*C. reinhardtii*, nitrate reductase	Restoration of growth on nitrate	Fernandez et al. 1989; Kindle et al. 1989
(i)	NIT2	*C. reinhardtii*, nitrogen metabolism transcription factor	Restoration of growth on nitrate	Camargo et al. 2007; Schnell and Lefebvre 1993
(i)	OEE1	*C. reinhardtii*, oxygen-evolving enhancer protein 1	Restoration of photosynthetic competence	Mayfield and Kindle 1990
(i)	petB	*C. reinhardtii*, cytochrome b_6f subunit	Restoration of photosynthetic competence	Cheng et al. 2005
(i)	psaA	*C. reinhardtii*, photosystem I subunit	Restoration of photosynthetic competence	Redding et al. 1998
(i)	psaB	*C. reinhardtii*, photosystem I subunit	Restoration of photosynthetic competence	Redding et al. 1998
(i)	psbA	*C. reinhardtii*, photosystem II subunit	Restoration of photosynthetic competence	Heiss and Johanningmeier 1992
(i)	psbA	Various species, photosystem II subunit	Restoration of photosynthetic competence	Gimpel and Mayfield 2013
(i)	PSY	*C. reinhardtii*, phytoene synthase	Restoration of carotenoid-less phenotype	McCarthy et al. 2004
(i)	rbcL	*C. reinhardtii*, RUBISCO large subunit	Restoration of photosynthetic competence	Chen and Melis 2013
(i)	THI10	*C. reinhardtii*, hydroxyethylthiazole kinase	Complementation of thiamine auxotrophy	Ferris 1995; Ferris et al. 2002
(i)	tscA	*C. reinhardtii*, small chloroplast RNA involved in psaA trans-splicing	Restoration of photosynthetic competence	Goldschmidt-Clermont 1991
(iia)	16S rRNA	*C. reinhardtii*, 16S rRNA gene	Resistance to spectinomycin and streptomycin	Newman et al. 1990
(iia)	23S rRNA	*C. reinhardtii*, 23S rRNA gene	Resistance to erythromycin	Newman et al. 1990
(iia)	ALS	*C. reinhardtii*, acetolactate synthase	Resistance to sulfometuron methyl and related sulfonylurea herbicides	Kovar et al. 2002

(iia)	CRY1	*C. reinhardtii*, ribosomal protein S14	Resistance to emetine and cryptopleurine	Nelson et al. 1994
(iia)	PDS	*C. reinhardtii*, phytoene desaturase	Resistance to norflurazon	Bruggeman et al. 2014
(iia)	PPX (PPO)	*C. reinhardtii*, protoporphyrinogen oxidase	Resistance to oxyfluorfen	Bruggeman et al. 2014; Randolph-Anderson et al. 1998
(iia)	psbA	*C. reinhardtii*, photosystem II subunit	Resistance to DCMU and metribuzin	Przibilla et al. 1991
(iib)	aadA	*Escherichia coli*, aminoglycoside adenyltransferase	Resistance to spectinomycin and streptomycin	Cerutti et al. 1997; Goldschmidt-Clermont 1991; Meslet-Cladiere and Vallon 2011
(iib)	aph 7″	*Streptomyces hygroscopicus*, aminoglycoside phosphotransferase	Resistance to hygromycin B	Berthold et al. 2002
(iib)	aphVIII	*Streptomyces rimosus*, aminoglycoside phosphotransferase	Resistance to paromomycin	Sizova et al. 2001; Sizova et al. 1996
(iib)	aphA6	*Acinetobacter baumannii*, aminoglycoside phosphotransferase	Resistance to kanamycin and amikacin	Bateman and Purton 2000
(iib)	ble	*Streptoalloteichus hindustanus*, BLE (BRP) protein	Resistance to bleomycin, phleomycin and zeo(my)cin	Lumbreras et al. 1998; Stevens et al. 1996
(iib)	cat	Transposon *Tn9*, chloramphenicol acetyltransferase	Resistance to chloramphenicol	Tang et al. 1995
(iib)	gat	Synthetic glyphosate acetyltransferase	Resistance to glyphosate	Bruggeman et al. 2014; Castle et al. 2004
(iib)	hpt	*E. coli*, hygromycin phosphotransferase	Resistance to hygromycin B	Butanaev 1994; Kumar et al. 2004
(iib)	nptII	Transposon *Tn5*, neomycin phosphotransferase	Resistance to kanamycin	An et al. 1985; Hall et al. 1993
(iiia)	ARS2	*C. reinhardtii*, arylsulfatase	Color reaction with e.g. X-Sulf	Davies et al. 1992; de Hostos et al. 1989; Specht et al. 2014
(iiia/b)	uidA	*Escherichia coli*, ß-glucuronidase	Color from e.g. X-Gluc or fluorescence from e.g. MUG substrates	Jefferson et al. 1987; Sakamoto et al. 1993
(iiib)	xyn1	*Trichoderma reesei*, xylanase 1	Fluorescence from e.g. $DiFMUX_2$ substrate	Rasala et al. 2012
(iiib)	BFP, CFP, GFP, OPF, RFP, YFP	*Entacmaea quadricolor (blue), Aequorea victoria (cyan, green, yellow), Discosoma sp. (orange, red)* fluorescent proteins	Blue, cyan, green, orange, red or yellow fluorescence	Franklin et al. 2002; Fuhrmann et al. 1999; Lauersen et al. 2015; Neupert et al. 2009; Rasala et al. 2013
(iiib)	GLUC RLUC LUCCP LUXCT	*Gaussia princeps*, luciferase *Renilla reniformis*, luciferase *Photinus pyralis*, luciferase *Vibrio harveyi*, luciferase	Chemiluminescence	Fuhrmann et al. 2004; Matsuo et al. 2006; Mayfield and Schultz 2004; Minko et al. 1999; Ruecker et al. 2008; Shao and Bock 2008

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