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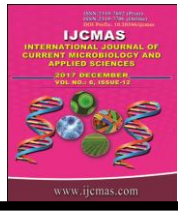

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A Review on Molecular Tools of Microalgal Genetic Transformation and their Application for Overexpression of Different Genes

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A B S T R A C T

Microalgae are in focus because of its tremendous potential to produce different value added compounds such as pigments, polysaccharides, cosmetics, fatty acids, proteins, vitamins and so on. Genetic engineering of microalgae promises broader applications in the field of molecular farming for the production of above mentioned industrially valuable compounds. For transformation of any of these gene widely used molecular tools such as particle bombardment, *Agrobacterium* mediated transformation, electroporation, and less commonly used Conjugation, Agitation with glass beads, Agitation with silicon carbon whiskers, Protoplast transformation with polyethylene glycol and dimethyl sulfoxide are used. The basic mechanism of these methods is to somehow disturb the cell wall and cell membrane of the algal cell and allow the gene to reach the nucleus via nuclear pore to integrate into the nucleus. Using above mentioned methods genes responsible for lipids, terpenoids, carotenoids and other different therapeutic recombinant proteins have been overexpressed. There are different challenges and existing problems for transformation and integration of genes in microalgae which needs to be overcome to fully utilize the potential of this microbe for molecular cloning and overproduction of different value added compounds.

Introduction

One of the main challenges for commercial viability of algal technology is low biomass production of microalgae which is utilized for the different value added products. Besides medium engineering and bioreactor designing, genetic engineering is an emerging alternative strategy of combating the problem of low biomass productivity in this field. Genetic engineering of microalgae is a fairly new approach and has been explored in a limited capacity to produce fatty acids enzymes, and vaccines in a limited number of microalgae mainly *Chlamydomonas reinhardtii*, *Dunaliella salina*, and *Phaeodactylum tricornutum* (Rasala *et al.*, 2012; Geng *et al.*, 2003; Hamilton *et al.*, 2014; Vira *et al.*, 2016). The advantages of using these photosynthetic cell factories include biosafety and low cost of substrates in the form of carbon dioxide, light and water

(Rasala *et al.*, 2014). Different transformation techniques have been developed for the transformation of this very important microorganism which has been mentioned in this review. Besides this, genetic engineering of microalgae has also been employed to modify the metabolism of microalgae, the targets being the genes involved in lipid biosynthetic pathway, photosynthetic activity, carbon assimilation mechanisms, and nutrient metabolism (Gimpel *et al.*, 2015). Overexpression of different genes in microalgae responsible for lipids, terpenoids, carotenoids and other different therapeutic recombinant proteins have been mentioned in this review.

In spite of greater ability and different approaches to produce variety of bioproducts from microalgae, there are technical difficulties and challenges which need to be addressed for commercial exploitation of algal biotechnology.

Molecular tools for algal transformation

The basis of algal genetic transformation methods is to cause temporal permeabilization of the cell membrane/cell wall enabling DNA molecules to enter the cell, by various physical/ chemical means. Thus, entry of the DNA into the nucleus through nuclear membrane and integration into the genome occurs with or without any external assistance.

The incorporation of DNA into host cell mainly occurs by random recombination, resulting in integration of introduced DNA and, thus culminates in stable/partial genetic transformation (Hallmann, 2007). In reality, permeabilization of a cell membrane in order to introduce the DNA is not difficult; however, the affected algal cell must survive this severe damage *i.e.* the DNA invasion and recommence cell division at the earliest.

Particle bombardment

Various transformation methods are available for algal systems that permit the recovery of viable transformants. Amongst these, the most popular method is gene gun transformation also referred to as micro-projectile bombardment, micro-particle bombardment, particle gun transformation, or simply biolistics (Gangl *et al.*, 2015).

In this method, DNA-coated heavy-metal (tungsten/gold) micro-projectiles are used, which allows transformation of nearly any type of cell or cell organelles despite the rigidity or thickness of the cell wall (Fig. 1). This method has been successfully applied in different algal species (Table 1) for basic studies as well for production of different compounds (Gangl *et al.*, 2015). This method is also useful for transformation in chloroplasts (Boynton *et al.*, 1988) and mitochondrial genome of *C. reinhardtii* (Randolph-Anderson *et al.*, 1998).

Electroporation

Electroporation is yet another method used for genetic transformation in microalgae. Cell-wall reduced mutants, protoplasts, and cells with thin cell walls can be transformed by this method *i.e.* electroporation.

Electroporation is carried out in specially designed electroporation cuvettes and electroporation chamber where voltage across the plasma membrane exceeds its dielectric strength (Fig. 2). Large electronic pulse allows molecules like DNA to pass the phospholipid bilayer of the cell membrane.

This method was employed in cells of *C. merolae* (Minoda *et al.*, 2004), *D. salina* (Geng *et al.*, 2003), *C. vulgaris* (Chow and Tung, 1999) and *C. reinhardtii* (Brown *et al.*, 1991) successfully (Table 1).

Agrobacterium-mediated transformation

Recently, the *Agrobacterium*-mediated transformation has been demonstrated successfully both in fresh and marine water algal species (Table 1). This gram-negative plant pathogen *Agrobacterium tumefaciens* has been widely used in genetic engineering of plants (Gangl *et al.,* 2015). The *Agrobacterium* infection causes tumors mostly in dicots and few monocots, but now demonstrated in certain microalgae are being infected but they do not develop tumors. Thus, *Agrobacterium*-mediated transformation offers high transformation efficiency with low cost compared to others. The transformation efficiency of *Agrobacterium*-mediated transformation is reported to enhance in the presence of acetosyringone by activating the *vir* genes of *Agrobacterium* (Stachel *et al.,* 1985; Men *et al.,* 2003; Kumar *et al.,* 2004). The gene of interest gets transferred to the algal nucleolus with the help of *vir* gene and randomly integrates into the nuclear DNA (Fig. 3). However, there is a report of *Agrobacterium*-mediated transformation of *H. pluvialis* cells without the use of acetosyringone (Kathiresan *et al.,* 2009). This method is preferred over other methods mostly because of high chances of integration of gene into the algal genome at a specific site.

Glass bead/inert particle mediated transformation

Alternatively, another less complex and less expensive transformation method involves agitation of microalgae with small inert particles and DNA. It involves preparation of homogenous suspension of microalgae that is agitated with DNA in the presence of macro- or micro-particles, and polyethylene glycol. A number of investigators have also used silicon carbide whiskers (~0.3-0.6 µm thick and ~5-15 µm long) as micro-particles which is a ceramic compound composed of silicon and carbon. These hard and rigid micro-particles are efficient in genetically transforming cells with intact cell walls. The examples include *Amphidinium sp.* (Te Lohuis and Miller, 1998) *Symbiodinium microadriaticum* (Te Lohuis and Miller, 1998), and *C. reinhardtii* (Dunahay, 1993). Although microalgae with cell wall get transformed by this method, but cell wall reduced microalgae provide more advantage in terms of higher transformation efficiency. The cell wall reduced mutants of *C. reinhardtii* have been transformed through agitation in the presence of glass beads (0.4-0.5 mm in diameter) along with DNA and polyethylene glycol (Kindle, 1990). Most investigators prefer to work with cell wall less strains, thus this easy and cheap method is routinely used for transforming *C. reinhardtii*. In case of *C. ellipsoidea*, cell-wall free protoplasts were transformed without any micro- or macro-particles by only agitation of the protoplast in the presence of DNA and polyethylene glycol (Jarvis and Brown, 1991).

Thus, there are considerable ways of introducing DNA into algal cells. The most widely used method for genetic transformation is particle bombardment as depicted in Table 1 because of its ability to transform all kinds of cells irrespective of the cell wall composition. The only hurdle in this method is the cost of the instrument and remarkable running expenses. The second preferred method is electroporation which has same advantages as particle bombardment of being simple and quick method for transformation. *Agrobacterium*-mediated transformation has been used for a couple of algal species and is gaining momentum because of less cost requirement during cell transformation. Other methods such as conjugation, agitation and protoplast transformation methods have also been applied for different algal species though they are not routine methods. The glass bead

method is classified as frequently used method for model organism *C. reinhardtii* which is quick and easy method of transformation. In conclusion, the method of transformation can be defined based on species and cell wall physiology of the microalgae.

Potential applications of genetic transformation in microalgae

The potential of microalgae have been demonstrated by their ability to produce lipids, antioxidants, high-value bioactive compounds, and proteins such as vaccines, antibodies, and hormones by using genetic engineering.

Microalgae have been targeted for biofuel production owing to its suitable features as mentioned earlier. Genetic manipulation is a promising strategy for the over-production of lipids among algal species and different metabolic engineering strategies were employed for increasing lipid production as listed in Table 2.

Studies have also been carried out to reveal the role of genes in the regulation of algal lipid accumulation (Guckert and Cooksey, 1990). The algal cells accumulate lipid under different stress conditions which inhibits cell cycle progression (Guckert and Cooksey, 1990).

Under nitrogen stress, transcriptomic and proteomic analysis showed up-regulation of mRNAs and proteins associated with fatty acid and lipid biosynthesis (Miller *et al.*, 2010; Guarnieri *et al.*, 2011). A number of potential gene targets which are involved in lipid biosynthesis and regulation were targeted and higher lipid productivity was achieved in different microalgal species (Li *et al.*, 2010). Reports suggest that elimination of starch synthesis pathway would switch photosynthetic carbon flux toward triacylglyceride (TAG) synthesis (Li *et al.*, 2010).

Along with lipids, other high-value added compounds will be produced from the microalgae using algal transgenics such as terpenoids and carotenoids. The examples for different terpenoids and carotenoids production in microalgae are listed in Table 3.

Microalgae have also been established as useful bioreactors for the expression of valuable proteins such as vaccines, antibodies, enzymes and hormones (Gan *et al.*, 2016).

Specifically *C. reinhardtii* has been targeted for the production of recombinant protein production owing to its well characterized genome, functional mutants and genetic transformation tools available for transformation (Lauersen *et al.*, 2012).

The examples of different recombinant proteins produced in microalgae are mentioned in Table 4.

In addition to the above products, genetic engineering of microalgae has also been used in carbon capturing mechanism, aquaculture and in production of essential oils (Gangl *et al.*, 2015).

Several attempts have been made to improve the photosynthetic efficiency of microalgae; most of these are focused on reducing the size of the chlorophyll antenna by using different techniques including the most efficient RNAi-based strategy to knock down both LHCI and LHCII in *C. reinhardtii* (Zeng *et al.*, 2011).

In *P. tricornutum*, overexpression of Δ5-elongase gene from *O. tauri* helped in accumulation of high value omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) (Hamilton *et al.*, 2014).

Fig.1 Particle bombardment system and mechanism

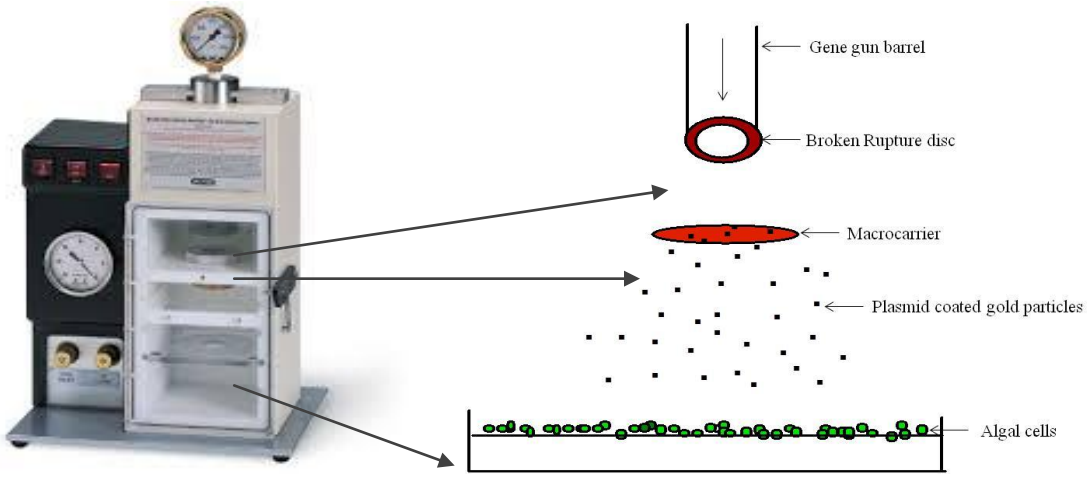

Fig.2 Electroporation apparatus and mechanism

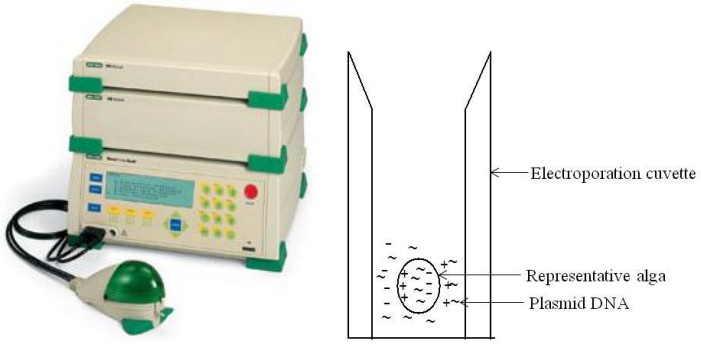

Fig.3 Mechanism of *Agrobacterium*-mediated transformation

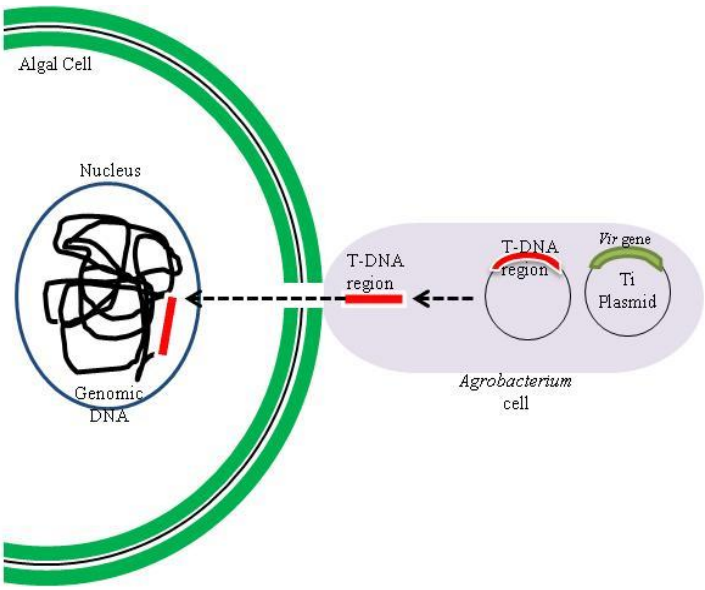

Table.1 Methods of algal transformation

Transformation method	Transformed algal species	Reference
Particle bombardment	*C. reinhardtii*	Mayfield *et al.*, 2004
		Sun *et al.*, 2003
		Bateman and Purton, 2000
		Debuchy *et al.*, 1989
		Kindle *et al.*, 1989
	P. tricornutum	Miyagawa *et al.*, 2009
		Zaslavskaia *et al.*, 2001
		Falciatore *et al.*, 1999
		Apt *et al.*, 1996
	Chlorella zofingiensis	Liu *et al.*, 2014
	Parachlorella kessleri	Rathod *et al.*, 2016
	H. pluvialis	Steinbrenner and Sandmann, 2006
	D. salina	Tan *et al.*, 2005
	Gracilaria changii	Gan *et al.*, 2003
	Porphyridium sp.	Lapidot *et al.*, 2002
	Euglena gracilis	Doetsch *et al.*, 2001
	L. japonica	Qin *et al.*, 1999
	C. kessleri	El-Sheekh, 1999
	Chlorella sorokiniana	Dawson *et al.*, 1997
	Navicula saprophila	Dunahay *et al.*, 1995
	Cyclotella cryptica	Dunahay *et al.*, 1995
Electroporation	*C. zofingiensis*	Liu *et al.*, 2014
	Scenedesmus obliquus	Guo *et al.*, 2013
	C. vulgaris	Niu *et al.*, 2011
	C. vulgaris	Chow and Tung, 1999
	C. merolae	Minoda *et al.*, 2004
	D. salina	Geng *et al.*, 2003
	U. lactuca	Huang *et al.*, 1996
	C. reinhardtii	*Brown et al.*, 1991
***Agrobacterium*-mediated transformation**	*C. vulgaris*	Sharif *et al.*, 2015
	Scenedesmus almeriensis	Dautor *et al.*, 2014
	Parachlorella kessleri	Rathod *et al.*, 2013
	Schizochytrium sp.	Cheng *et al.*, 2012
	C. vulgaris	Cha *et al.*, 2012
	D. bardawil	Anila *et al.*, 2011
	Nannochloropsis sp.	Cha *et al.*, 2011
	Chaetoceros sp.	Miyagawa *et al.*, 2011
	H. pluvialis	Kathiresan *et al.*, 2009
Conjugation	*Prochlorococcus*	*(Tolonen et al.*, 2006*)*
	Pseudanabaena	*(Sode et al.*, 1992*)*
	Synechococcus	*(Sode et al.*, 1992*; Brahamsha*, 1996*)*
	Synechocystis	*(Sode et al.*, 1992*)*
Agitation with glass beads	*C. reinhardtii*	León *et al.*, 2007; Ohresser *et al.*, 1997; Purton and Rochaix, 1995; Kindle, 1990;
	D. salina	Feng *et al.*, 2009
Agitation with silicon carbon whiskers	*Amphidinium sp.*	Te Lohuis and Miller, 1998
	Symbiodinium microadriaticum	Te Lohuis and Miller, 1998
	C. reinhardtii	Dunahay, 1993
Protoplast transformation with polyethylene glycol and dimethyl sulfoxide	*Pleurochrysis carterae*	Endo *et al.*, 2016
	C. ellipsoidea	Kim *et al.*, 2002
	Chlorella	Hawkins and Nakamura, 1999

Table.2 Metabolic engineering of microalgae for lipid production

Algal strain	Target protein	Outcome of transformation	Reference
C. reinhardtii	*Diacylglycerol acyltransferase*	Polyunsaturated fatty acids, especially α-linolenic acid, an essential omega-3 fatty acid, were enhanced up to 12%	Ahmad *et al.*, 2015
	Fatty acid-ACP thioesterase	Shorter chain FA with the endogenous gene and no increase in lipids for all genes	Blatti *et al.*, 2012
	Acyl-Coa: diacylglycerol acyltransferase	No increase in TAG accumulation and TAG profiles	La Russa *et al.*, 2012
	Acyl-CoA: diacylglycerol acyltransferase	34% increase in TAGs with one gene; 24% and 37% decrease in TAGs with two genes	Deng *et al.*, 2012
	Lipid trigger transcription factor	Total extractable lipids increased by 11%	Yohn *et al.*, 2012
	Δ4 desaturase	Increased accumulation of FA 16:4	Zäuner *et al.*, 2012
P. tricornutum	*Pyruvate dehydroganase kinase*	Neutral lipids increase by 82%	Ma *et al.*, 2014
	Malic enzyme	Total lipids increased by 2.5 times	Xue *et al.*, 2014
	Δ5 desaturase	65% more neutral FA with 58% increased accumulation of EPA	Peng *et al.*, 2014
	Fatty acid (FA)-ACP thioesterase	Increased production of C12 and C14 FA	Radakovits *et al.*, 2011
	Fatty acid-ACP thioesterase (Endogenous)	82% increase in total FAs	Gong *et al.*, 2011a
Thalassiosira pseudonana	*Multifunctional lipase*	Total lipids increased by 3.3 fold.	Trentacoste *et al.*, 2013
Chlorella minutissima	*Five TAG biosynthetic enzymes*	No change with individual genes but increase in TAGS with five genes	Hsieh *et al.*, 2012
C. cryptic	*Acetyl-CoA carboxylase*	No increase in total lipid accumulation	Dunahay *et al.*, 1996

Table.3 Production of terpenoids and carotenoids in microalgae through genetic engineering

Algal strain	Target protein/enzyme	Outcome of transformation	Reference
C. reinhardtii	*Squalene synthase*	Squalene was not detected	Kajikawa *et al.*, 2015
	Squalene epoxidase	Detection upto 1.1 (μg mg^{-1}) dry weight of squalene	Kajikawa *et al.*, 2015
	Phytoene desaturase	Norflurazon resistance and Increase of different carotenoids	Liu *et al.*, 2013
	Phytoene synthase	Increase in lutein content by 2.6 times	Couso *et al.*, 2011
	Phytoene synthase	Increase in lutein content by 2.2 times	Cordero *et al.*, 2011
	Phytoene desaturase	No significant changes in carotenoid profile	Vila *et al.*, 2008
	β-caroteneketolase	Detection of 4-keto-lutein but no astaxanthin detected	Leon *et al.*, 2007
	β-caroteneketolase	keto-carotenoids not detected	Wong, 2006
	Geranylgeranyl-pyrophosphate synthase	No changes in carotenoids/isoprenoid profile	Fukusaki *et al.*, 2003
C. zofingiensis	*Phytoene desaturase*	54.1% increase in astaxanthin and 32.1% increase in total carotenoids	Liu *et al.*, 2014
D. salina	*Phytoene desaturase*	Carotenoid profiles not reported	Sun *et al.*, 2008
H. pluvialis	*Phytoene desaturase*	Norflurazon resistance and 26% increase in astaxanthin	Steinbrenner and Sandmann, 2006

Table.4 Production of recombinant proteins in microalgae by using genetic engineering approach

Gene overexpressed	Function	Reference
10NF3,14FN3	Domains 10 and 14 of human fibronectin, potential antibody mimics14FN3	Rasala *et al.*, 2010
83K7C	Full-length IgG1 human monoclonal antibody against anthrax protective antigen 83	Tran *et al.*, 2009
ARS2-crEpo-his6	Human erythropoietin fused to ARS2 export sequence w/6xhis tag	Eichler-Stahlberg *et al.*, 2009
CSFV-E2	Swine fever virus E2 viral protein	He *et al.*, 2007
CTB-D2	D2 fibronectin-binding domain of Staphylococcus aureus fused with the cholera toxin B subunit	Dreesen *et al.*, 2010
CTB-VP1	Cholera toxin B subunit fused to foot and mouth disease VP1	Sun *et al.*, 2003
hGAD65	Diabetes-associated anutoantigen human glutamic acid decarboxylase 65	Wang *et al.*, 2008
hGH	Human growth hormone	Geng *et al.*, 2013
HMGB1	High mobility group protein B1	Rasala *et al.*, 2010
hMT-2	Human metallothionine-2	Zhang *et al.*, 2006
HSV8-lsc	First mammalian protein expressed	Mayfield *et al.*, 2003
HSV8-scFv	Classic single-chain antibody	Mayfield *et al.*, 2005
hTRAIL	Human tumor necrosis factor-related apoptosis-inducing ligan d (TRAIL)	Yang *et al.*, 2006
IgG1	Murine and human antibodies	Tran *et al.*, 2009
M-SAA	Bovine mammary-associated serum amyloid	Manuell *et al.*, 2007
M-SAA- Interferon β1	Multiple sclerosis treatment fused to M-SAA	Rasala *et al.*, 2010
Proinsulin	Blood sugar level-regulating hormone, type I diabetes treatment	Rasala *et al.*, 2010
VEGF	Human vascular endothelial growth factor isoform 121	Rasala *et al.*, 2010
VP28	White spot syndrome virus protein 28	Surzycki *et al.*, 2009
Xylanase1	Commercial enzyme	Rasala *et al.*, 2012

Existing problems

Genetic engineering in microalgae is a challenging science to understand and cater the needs of eukaryotic system due to its complexity of nuclear genome and tougher cell wall, which pose hints for establishing the transformation protocols in species of interest. Even after establishment of transformation methodologies, selection of positive transformants among false positives will be a rate limiting challenge. The frequency of transgene expression in nuclear genome of microalgae is extremely low due to presence of gene silencing. Few of the indicated reasons for gene silencing are DNA methylation, positional effects and epigenetic mechanisms (Hallman, 2007). It is often related to several factors such as the control of developmental stages and the response of a cell to transposable elements, or other foreign DNA or unnaturally placed DNA (Wu-Scharf *et al.*, 2000; Cerutti *et al.*, 1997). The possible solution to this problem is screening of a larger number of transformants which are expressing higher amounts of protein.

The transgene expression of heterologous origin in microalgae is further hampered on account of codon bias, which is typical for almost all the species. When commonly used codons of the DNA donor species are infrequently found in the genes of the target organism, the corresponding tRNA abundance becomes very low and this led to less or no translation and expression of the desired protein. The situation can be worst when the codon is not present at all in the target species. One strategy to circumvent this problem is to look for heterologous gene source from different organisms that have codon usage similar to genes of the target organism. Thus, the *Streptoalloteichus hindustanus (ble)* gene was recognized as efficient selectable marker for transformation of both *V. carteri* (Hallmann and Rappel, 1999) and *C. reinhardtii* (Stevens *et al.*, 1996). Another approach is to completely re-synthesize the heterologous gene by using codon optimization for the target species.

Another difficulty in transgenesis of microalgae is the presence of introns in heterologous gene. Heterologous genes with introns will likely not be spliced correctly, thus cDNAs should be used. However, the problem in this case is poor expression of transgene. This problem can be solved by inserting homologous introns into the heterologous coding region. The usefulness of such chimeric genes has been already demonstrated in *V. carteri* (Hallmann and Rappel, 1999) and *C. reinhardtii* species (Lumbreras *et al.*, 1998).

Another reason for lower expression is due to choice of promoter component. The heterologous promoters, like CaMV35S and SV40 promoters have been demonstrated to work in some species, but not in all algal species (Hallmann, 2007). One of the possible reason behind this maybe inadequate recognition of the heterologous promoter region and lack of adequate regulation; thus heterologous 3′ untranslated regions may cause incorrect polyadenylation and can also inauspiciously influence regulation (Hallman, 2007). Therefore, use of endogenous promoter for specific species of interest is highly recommended. Major troubleshooting maybe due to insufficient DNA delivery, failure to integrate into the host organism's genome, and false positive results can be because of adaptability of microalgae to the antibiotics, or improper transport through the plasma membrane into the extracellular compartment or into the chloroplast. Till date, studies on genetic engineering have been demonstrated in model microalga such as *C. reinhardtii* and *P. tricornutum* due to the availability of molecular tools. Profound optimized protocols are required to develop

and understand algal transgenics, which will aim to provide molecular tools that are compatible and promising future for genetic engineering in microalgae.

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