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Algal Transgenics and Biotechnology

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ABSTRACT

Transgenesis in algae is a complex and fast-growing technology. Selectable marker genes, promoters, reporter genes, transformation techniques, and other genetic tools and methods are already available for various species and currently ~25 species are accessible to genetic transformation. Fortunately, large-scale sequencing projects are also planned, in progress, or completed for several of these species; the most advanced genome projects are those for the red alga *Cyanidioschyzon merolae*, the diatom *Thalassiosira pseudonana*, and the three green algae *Chlamydomonas reinhardtii*, *Volvox carteri* and *Ostreococcus tauri*. The vast amount of genomic and EST data coming from these and a number of other algae has the potential to dramatically enlarge not only the algae's molecular toolbox. A powerful driving force in algal transgenics is the prospect of using genetically modified algae as bioreactors. In general, today's non-transgenic, commercial algal biotechnology produces food additives, cosmetics, animal feed additives, pigments, polysaccharides, fatty acids, and biomass. But recent progress in algal transgenics promises a much broader field of application: molecular farming, the production of proteins or metabolites that are valuable to medicine or industry, seems to be feasible with transgenic algal systems. Indeed, the ability of transgenic algae to produce recombinant antibodies, vaccines, insecticidal proteins, or bio-hydrogen has already been demonstrated. Genetic modifications that enhance physiological properties of algal strains and optimization of algal production systems should further improve the potential of this auspicious technology in the future.

Keywords: algae, *Chlamydomonas*, *Chlorella*, *Dunaliella*, genetic engineering, microalgae, molecular farming, transformation, *Volvox*
Abbreviations: ***aphVIII***, aminoglycoside phosphotransferase gene; ***ARS***, arylsulfatase gene; **CaMV 35S promoter**, cauliflower mosaic virus 35S promoter; **EST**, expressed sequence tag; GFP, green fluorescent protein; ***GUS***, β-glucuronidase gene; ***HUP1***, *Chlorella kessleri* hexose/H^+ symporter gene; ***lacZ***, β-galactosidase gene; **SV40 promoter**, simian virus 40 promoter

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Invited Review

INTRODUCTION

Algae are accountable for the net primary production of ~52,000,000,000 tons of organic carbon per year, which is ~50% of the total organic carbon produced on earth each year (Field *et al*. 1998), but this is not the only reason why they are of enormous biological importance. They constitute a group of ~40,000 species, a heterogeneous group that describes a life-form, not a systematic unit; this is one reason why a broad spectrum of phenotypes exists in this group. Algae are mostly eukaryotes, which typically, but not necessarily, live in aquatic biotopes. They are described as "lower" plants that never have true stems, roots and leaves, and they are normally capable of photosynthesis. The nontaxonomic term "algae" groups several eukaryotic phyla, including the Rhodophyta (red algae), Chlorophyta (green algae), Phaeophyta (brown algae), Bacillariophyta (diatoms), and dinoflagellates, as well as the prokaryotic phylum Cyanobacteria (blue-green algae). There are coccoid, capsoid, amoeboid, palmelloid, colonial, plasmodial, filamentous, parenchymatous (tissue-like), and thalloid organizational levels; some algae at the last-mentioned level developed complex structures that resemble the leaves, roots, and stems of vascular plants. The size of algae ranges from tiny single-celled species to gigantic multi-cellular organisms. Remarkably, algal species span eight orders of magnitude in size (**Fig. 1**): The smallest eukaryotic alga, *Ostreococcus tauri* (Prasinophyceae) (Courties *et al*. 1994), has a cell diameter of less than 1 µm which makes it the smallest known free-living eukaryote having the smallest eukaryotic genome; in contrast, the brown alga *Macrocystis pyrifera* (Phaeophyceae), also known as the giant kelp, grows up to 60 m and is often the dominant organism in kelp forests.

Algae of different sizes and shapes not only occupy all aquatic ecosystems but also occur in almost all other habitats. Some of these habitats are really extreme: There is an outstanding salt tolerance of halophilic algae like *Dunaliella salina* (Chlorophyceae), which is capable of growing in environments that are nearly saturated with NaCl. Cryophilic green algae like *Chlamydomonas nivalis* (Chlorophyceae) are adapted to low temperature, poor nutrition, permanent freeze-thaw cycles and high irradiation, and by the way, they color snow fields orange or red. A principal alga of hot acidic waters is the red alga *Cyanidium caldarium* (Bangiophyceae), which can grow, albeit slowly, at a pH of zero and at temperatures up to ~56°C. Aerial, sub-aerial and aeroterrestrial algae like *Apatococcus lobatus* (Chlorophyta) are normally spread by airborne spores and grow in the form of biofilms in aerophytic biotopes (bark of trees, rocks, soils, and other natural or man-made surfaces). Hypolithic algae, like *Microcoleus vaginatus* (Cyanobacteria), can live in very arid environments like the Death Valley or the Negev desert.

Other species of algae prefer to live in symbiotic relationships with animals or fungi. Unicellular yellow-brown algae, so-called zooxanthellae, like *Symbiodinium microadriaticum* (Dinophyceae, dinoflagellates), live symbiotically in the gastrodermis of reef-building corals. The jellyfish-related hydra *Chlorohydra viridissima* (Hydrozoa) is a bright green species owing to the presence of algae called zoochlorellae (*Chlorella* sp., Chlorophyta). Symbioses between sponges and algae are abundant in nutrient-poor waters of tropical reefs. Lichens, like the common yellow-colored *Xanthoria parietina*, are "composite organisms" made of a fungus (mostly Ascomycota) and a photosynthetic alga; they prosper in some of the most inhospitable habitats.

The diversified traits and living conditions of algae make them extremely attractive for commercial utilization particularly if the desired candidate alga is accessible to genetic manipulation. Because algal transgenics and bio-

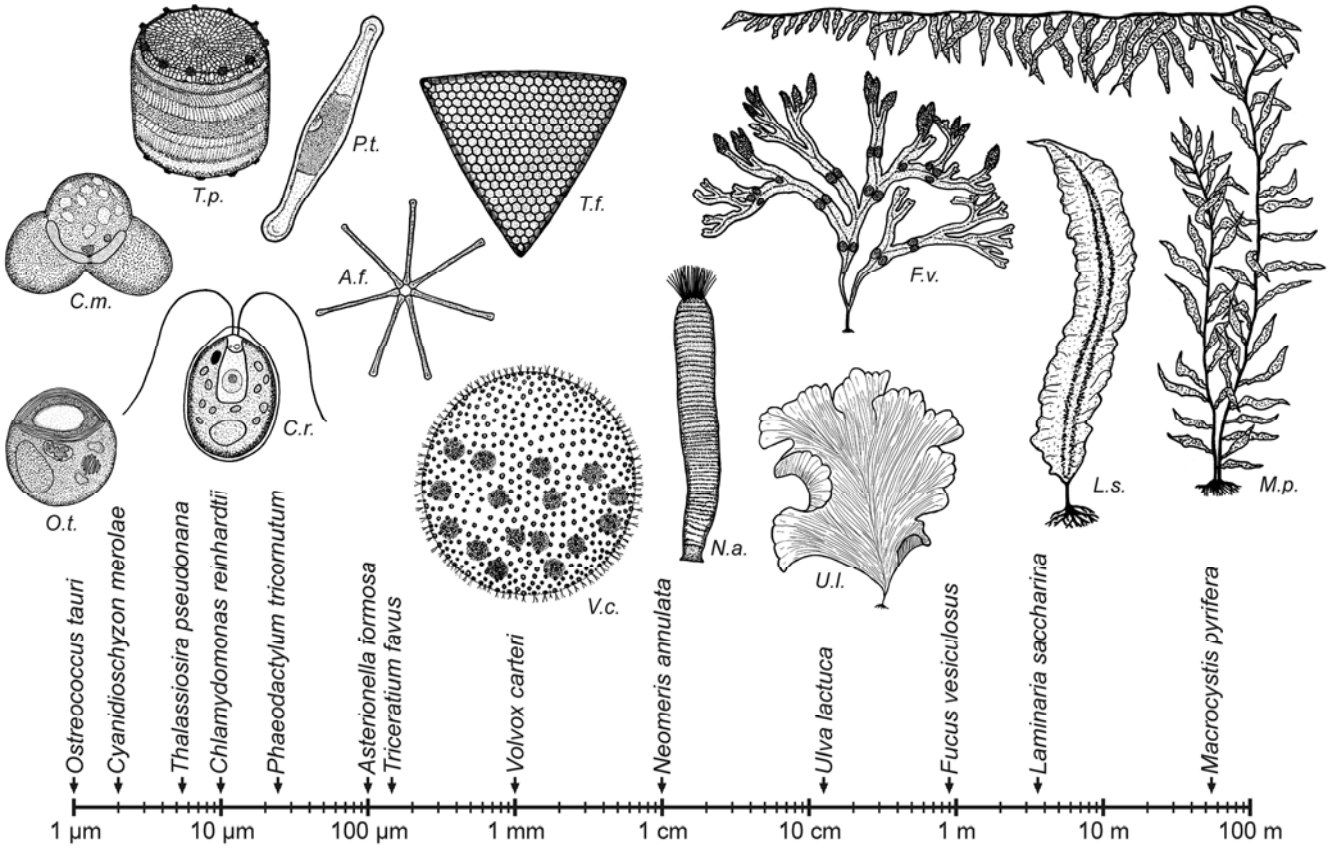

Fig. 1 Spectrum of phenotypes and sizes of algal species. Algae vary greatly in shape and the sizes of the smallest and the largest algae differ by a factor of ~10^8. Some examples: *Ostreococcus tauri* (Chlorophyta), *Cyanidioschyzon merolae* (Rhodophyta), *Thalassiosira pseudonana* (Bacillariophyta), *Chlamydomonas reinhardtii* (Chlorophyta), *Phaeodactylum tricornutum* (Bacillariophyta), *Asterionella formosa* (Bacillariophyta), *Triceratium favus* (Bacillariophyta), *Volvox carteri* (Chlorophyta); *Neomeris annulata* (Chlorophyta), *Ulva lactuca* (Chlorophyta), *Fucus vesiculosus* (Phaeophyta), *Laminaria saccharina* (Phaeophyta), *Macrocystis pyrifera* (Phaeophyta). The approximate sizes are indicated on a logarithmic scale.

technology primarily utilizes small, lab-suited species, this review deals mainly with (eukaryotic) microalgae. Genetic engineering of prokaryotic algae (Cyanobacteria) has been reviewed elsewhere (Koksharova and Wolk 2002; Vioque 2007). A number of other reviews describe topics of (eukaryotic) algal biology, molecular biology and biotechnology (Pulz 2001; McHugh 2003; Olaizola 2003; Franklin and Mayfield 2004; León-Banares *et al*. 2004; Pulz and Gross 2004; Ball 2005; Grossman 2005; Montsant *et al*. 2005; Qin *et al*. 2005; Walker *et al*. 2005a, 2005b; Chan *et al*. 2006; Spolaore *et al*. 2006).

UTILIZATION OF ALGAE – A HISTORICAL VIEW

In the distant past, long before the advent of biotechnology, let alone algal biotechnology, utilization of algae as a human food source began inconspicuously. Edible blue-green microalgae, including *Nostoc*, *Spirulina*, and *Aphanizomenon* species, have been used as a nutrient-dense food for many centuries in Asia, Africa and Mexico (Jensen *et al*. 2001; Olaizola 2003). The first traceable use of microalgae by humans dates back 2000 years to the Chinese, who used *Nostoc* to survive during famine (Spolaore *et al*. 2006). The use of macroalgae as food has been traced back to the fourth century in Japan and the sixth century in China (McHugh 2003). The first report on collection of a macroalga, "nori", i.e. algae of the genus *Porphyra*, dates back to the year 530. The first known documentation of cultivation of this alga occured in 1640 (Pulz and Gross 2004). At about the same time, in the year 1658, people in Japan started to process collected *Chondrus*, *Gelidium*, and *Gracilaria* species to produce an agar-like product (Pulz and Gross 2004). In the eighteenth century, iodine and soda were extracted from brown algae, like *Laminaria*, *Macrocystis* and *Fucus*. At that time, attempts to cultivate these species on-site began. In the 1860s, Alfred Nobel invented dynamite by using diatomaceous earth (diatomite), which consists of the fossil silica cell walls of diatoms, to stabilize and absorb nitroglycerine into a portable stick (Dolley and Moyle 2003); so dynamite was, in all respects, one of the most effective algal products. With less publicity, polysaccharide hydrocolloids of alginates became suitable for industrial use at the beginning of the last century (Pulz and Gross 2004). In the 1940s, microalgae became more and more important as live feeds in aquaculture (shellfish or fish farming). After 1948, applied algology developed rapidly, starting in Germany and extending into the USA, Israel, Japan, and Italy, with the aim of using algal biomass for producing protein and fat as a nutrition source (Burlew 1953); the push for this development came from statistical data about population development and predictions of an insufficient protein supply in the future (Spolaore *et al*. 2006). At that time, the idea of using microalgae for wastewater treatment was launched and the systematic examination of algae for biologically active substances, particularly antibiotics, began (Borowitzka 1995). In the 1960s, the commercial production of *Chlorella* as a novel health food commodity was a success in Japan and Taiwan (Kawaguchi 1980) and, in the USA, interest grew in developing algae as photosynthetic gas exchangers for long term space travel (Borowitzka 1999). The energy crises in the 1970s triggered considerations about using microalgal biomasses as renewable fuels and fertilizers. An environmental technology from the USA aimed at improving the quality of wastewater through microalgae and the subsequent fermentation of the resulting biomass to methane (Pulz and Scheibenbogen 1998; Spolaore *et al*. 2006). In addition, in the 1970s, the first large-scale *Spirulina* production plant was established in Mexico (Borowitzka 1999). In the 1980s, there were already 46 large-scale algae production plants in Asia mainly producing *Chlorella*, large scale production of Cyanobacteria began in India, and large commercial production facilities in the USA and Israel started to process the halophilic green alga *Dunaliella salina* as a source of β-carotene (Spolaore *et al*. 2006). In the 1980s, the use of microalgae as a source of common and fine chemicals was the beginning of a new trend (de la Noue and de Pauw 1988). In the 1990s in the USA and India, several plants started with large-scale production of *Haematococcus pluvialis* as a source of the carotenoid astaxanthin, which is used in pharmaceuticals, nutraceuticals, agriculture, and animal nutrition (Olaizola 2000; Spolaore *et al*. 2006).

Particularly during the past two or three decades, algal biotechnology grew steadily into an important global industry with a diversified field of applications, and more and more new entrepreneurs began to realize the potential of algae.

UTILIZATION OF ALGAE – THE PRESENT SITUATION

Nowadays, about 10^7 tons of algae are harvested each year by algal biotechnology industries for different purposes. A number of commercial companies selling algae or algal products are listed in **Table 1**. Today's commercial algal biotechnology is still a non-transgenic industry that basically produces food, feed, food and feed additives, cosmetics, and pigments. Due to the marine and aquatic applications, algal biotechnology is sometimes also called blue biotechnology. The following subsections (and **Table 2**) review the actual applications of non-transgenic algae. This description of the present situation also outlines the immediate application areas of future genetically optimized transgenic algae.

Human food

In spite of the fact that biotechnological or gene-technological exploitation of the biological diversity of algae is, and will be, hampered by safety regulations for human consumption, there are already several suitable fields of application.

The microalgal market is dominated by *Chlorella* and *Spirulina* (Becker 2004; Pulz and Gross 2004), mainly because of their high protein content, nutritive value, and not least, because they are easy to grow. The biomass of these algae is marketed as tablets, capsules, and liquids.

Macroalgae are utilized as food in China, Japan, Korea, the Philippines, and several other Asian countries. The largest producer is China, which harvests about 5 million wet tonnes/year (McHugh 2003). For example, "nori", actually *Porphyra* spp., which is used e.g. for making sushi, currently provides an industry in Asia with a yearly turnover of ~US$ 1 x 10^9 (Pulz and Gross 2004). Other species used as human food are *Monostroma* spp., *Ulva* spp., *Laminaria* spp., *Undaria* spp., *Hizikia fusiformis*, *Chondrus crispus*, *Caulerpa* spp., *Alaria esculenta*, *Palmaria palmata*, *Callophyllis variegata*, *Gracilaria* spp. and *Cladosiphon okamuranus* (**Table 2**). Algae provide a large profile of natural vitamins, minerals, and essential fatty acids and thereby positively affect human health.

Animal feed

Many evaluations have shown the suitability of algal biomass as a feed supplement (Becker 2004). Mainly the microalgae *Spirulina* and, to some extent, *Chlorella* are used in this domain for many types of animals: cats, dogs, aquarium fish, ornamental birds, horses, poultry, cows and breeding bulls (Spolaore *et al*. 2006). Within the same animal spectrum, macroalgae like *Ulva* spp., *Porphyra* spp., *Palmaria palmata*, *Gracilaria* spp., and *Alaria esculenta* are used as feed (**Table 2**). All of these algae are able to enhance the nutritional content of conventional feed preparations and hence, they positively affect the physiology of these animals.

Aquaculture

Microalgae are utilized in aquaculture as live feeds for all growth stages of bivalve molluscs (e.g. oysters, scallops,

Table 1 Commercial companies selling algae or algal products.

Company	Products	Company	Products
Acroyali Holdings Qingdao Co. Ltd., China	agar	Ina Food Industry Co. Ltd., Japan	agar, carrageenan
Agar del Pacifico S.A., Chile	agar	Indonesian Seaweed Industry Association (APBIRI), Indonesia	agar
Agarmex S.A., Mexico	agar	Industria Pesquera Costa Azul, Chile	biomass (*Gelidium*)
Algas de Asturias S.A., Spain	agar	Industrias Roko S.A., Spain	agar
Algas Marinas S.A., Chile	agar	Ingredients Solutions Inc., USA	carrageenan
Algas Vallenar S.A., Chile	biomass (*Gracilaria*, brown macroalgae)	Innovalg, France	biomass (*Odontella*)
		ISP Alginates Ltd., United Kingdom	alginates
Algas, Cultivos, Exportaciones - Acex S.A., Chile	biomass (*Gracilaria*)	Jiangsu Cibainian Nutrition Food Co. Ltd., China	biomass (*Chlorella*, *Spirulina*)
Algatech, Israel	astaxanthin, microalgae-derived products	Jiangsu High Hope Int'l Group Tong Yuan Imp. & Exp. Co. Ltd., China	agar
Algisa, Compania Industrial de Alginatos S.A., Chile	alginates	Jiangxi Boyuan Spirulina Co. Ltd., China	biomass (*Chlorella*, *Spirulina*)
Alimentos Multiexport S.A., Chile	biomass (*Gracilaria*, brown macroalgae)	K. Tanaka Corp., Japan	agar
		Kimica Corporation, Japan	alginates
Alsheriff Trading, Singapore	agar	Kingland Seaweed Fertilizer Co. Ltd., China	macroalgae extract fertilizers
Bali Seaweed Company, Indonesia	agriculture products		
Billmont 169 A T/A Art Cosmetics & Art Marketing, South Africa	macroalgae based skin treatment cosmetics	Klötze, Germany	biomass (*Chlorella*)
		Kompak Indopola, Indonesia	biomass (microalgae), microalgae extracts
Bintang Mas Sportindo CV, Indonesia	biomass (*Gracilaria*, *Eucheuma*)		
Bluebio Bio-Pharmaceutical Co. Ltd., China	biomass (*Chlorella*, *Spirulina*)	Lianyungang Saknox Seaweed Industrial Co. Ltd., China	alginates
Ceamsa, Spain	carrageenan	LVMH group, France	cosmetics
China Ocean University Organism Project Development Co. Ltd., China	macroalgae extracts	Lyg Seaweed Ind., China	alginates, mannitol, iodine
		Marcel Carrageenan Corporation, Philippines	carrageenan
China Seaweed Industrial Association, China	alginates	Marine Science Co. Ltd., Japan	carrageenan
		Marokagar S.A., Morocco	agar
Chocosuc Partner S. R. O., Slovakia	agar	Martek Biosciences Corporation, USA	fatty acids
Coast Biologicals Ltd., New Zealand	agar	Matsuki Agar-Agar Industrial Co. Ltd., Japan	agar
Cobra Chile S.A., Chile	agar		
Codif Recherche & Nature, France	cosmetics	Maxdragon BioChem Ltd., China	agar
Cognis Nutrition and Health, Australia	β-carotene	Mera Pharmaceuticals, USA	astaxanthin
		MicroGaia, USA	astaxanthin
Comercial Cisandina Chile Ltd., Chile	biomass (brown macroalgae)	Midesa S.A.C., Chile	biomass (*Gelidium*, *Gracilaria*)
		Mingfu Fujian Agar Co. Ltd., China	agar
CP Kelco ApS, Denmark	carrageenan	Myeong Shin Chemical Ind. Co. Ltd., Korea	agar, carrageenan
Cyanotech, USA	biomass (*Spirulina*), microalgae extracts	Nanjing General Spirulina Developing Corporation, China	biomass (*Spirulina*)
Dainippon Ink and Chemicals, Japan	pigments		
Danisco Cultor, Denmark	carrageenan	Nantong Ding-bu-er Seaweed Food Co. Ltd., China	biomass (macroalgae)
Degussa Texturant Systems, Germany	alginates, carrageenan		
Earthrise Nutritionals, USA	biomass (*Spirulina*), microalgae extracts	Nantong Haida Aquatic Food Co. Ltd., China	biomass (macroalgae)
Exsymol S.A.M., Monacco	cosmetics	Nantong Xinshi Corporation, China	biomass (macroalgae)
Far East Bio-Tec Co. Ltd., Taiwan	biomass (*Chlorella*, *Spirulina*), microalgae extracts, health care products, cosmetics	Nature Beta Technologies, Israel	β-carotene
		Necton Sa, Portugal	biomass (microalgae)
		Nikken Sohonsha Corporation, Japan	microalgae products
Far East Microalgae Ind Co., Ltd., Taiwan	biomass (*Chlorella*, *Spirulina*)	Nutrinova, Germany	fatty acids
		Ocean Nutrition, Canada	biomass (*Chlorella*), microalgae extracts
FMC Biopolymer, USA	alginates, carrageenan		
Fortune Life Enterprise Co. Ltd., Taiwan	biomass (macroalgae)	P.T. Agarindo Bogatama, Indonesia	agar
		P.T. Asia Sumber Laut Indonesia, Indonesia	carrageenan
Fuji Chemical Industry Co. Ltd., Japan	alginates		
		P.T. Gumindo Perkasa Industri, Indonesia	carrageenan
Fuqing King Dnarmsa Spirulina Co. Ltd., China	biomass (*Spirulina*)		
		Payam Bazar, Iran	astaxanthin (microalgae)
Fuzhou Haifu Seaweed Developing Co. Ltd., China	agar, agarose	Penglai Dengzhou Seaweed Co. Ltd., China	alginates, mannitol, iodine
Gelymar S.A., Chile	carrageenan	Pentapharm, Switzerland	cosmetics
Glory Karya Anugerah, Indonesia	biomass (macroalgae)	Phycotransgenics, USA	transgenic microalgae (*Chlamydomonas*)
Guangdong Provincial Foods Enterprises Co., China	agar, carrageenan		
		Prodoctora de Agar S.A., Chile	agar
Hainan Simai Pharmacy Co. Ltd., China	biomass (Spirulina), microalgae extracts	PT. Agarindo Bogatama, Indonesia	agar
		PT. Java Seaweed, Indonesia	biomass (macroalgae)
Hangzhou Xiaoshan Jinxiang Gelatin Co. Ltd., China	agar	Putian Cheng Xiang Zone Fuli Agar Co. Ltd., China	agar
Henan Boom Gelatin Co. Ltd., China	agar, carrageenan, alginates	Qingao Gather Great Ocean Seaweed Industry Co. Ltd., China	alginates
Hispanagar S.A., Spain	agar, carrageenan		
Hx Export Company, Vietnam	agar, biomass (macroalgae)	Qingdao Bright Moon Seaweed Group Co. Ltd., China	alginates, mannitol, iodine, macroalgae fertilizer
Iberagar S.A., Portugal	agar, carrageenan		

Table 1 (Cont.)

Company	Products	Company	Products
Qingdao CoDo International Ltd., China	agar, hydrocolloids, biomass (*Porphyra*, *Laminaria*, *Ulva*, *Undaria*)	Taiwan Chlorella Manufacturing Co. Ltd., Taiwan	biomass (*Chlorella*)
Qingdao Dacon Trading Co., Ltd., China	biomass (*Laminaria*), macroalgae extracts	Taurus Products Ltd., South Africa	biomass (*Gelidium*, *Gracilaria*), alginates
Qingdao Fuhua Seaweed Co. Ltd., China	alginates	TBK Manufacturing Corp., Philippines	carrageenan
Qingdao Jiaonan Bright Moon Seaweed Industrial Co., China	alginates, mannitol, iodine, agar, carrageenan, macroalgae fertilizer	Trung Nam Son Co. Ltd., Vietnam	biomass (macroalgae)
Qingdao Nanshan Seaweed Co. Ltd., China	alginates, macroalgae extracts	Viet Delta Industrial Co. Ltd., China	agar
Qingdao Richstar Seaweed Industrial Co. Ltd, China	food additives, pharmaceutical chemicals	Water Ingredients B. V., Netherlands	agar
Rhodia Food, France	carrageenan	Wudi Xinhui Chlorella Co. Ltd., China	biomass (*Chlorella*)
Rishon Biochem Co. Ltd., China	macroalgae extracts	Wuhan Sunrise Biotech Co. Ltd., China	biomass (*Spirulina*), microalgae extracts
Seaweeds & Agar Company Pacific Ltda., Chile	agar, carrageenan, alginates, biomass (*Lessonia*, *Macrocystis*, *Gracilaria*, *Gigartina*)	Xiamen Fortune-Wide Solar Energy Technology Co. Ltd., China	biomass (*Sargassum*)
Setexam S.A., Morocco	agar	Xiamen Guangyun Trading Co. Ltd., China	agar
Sinar Kentjana, Indonesia	biomass (*Gelidium*)	Xiamen Topusing Chemical Co. Ltd., China	agar
Sinochem Shanghai Corporation, China	agar	Yantai Heatex Biochemical & Technology Co. Ltd., China	macroalgae extract fertilizers
Sobigel S.A., France	agar	Yantai Liancheng Seaweed Co. Ltd., China	biomass (macroalgae)
Soriano S.A., Argentina	agar, carrageenan	Zhengzhou Hongli Chemical Co. Ltd., China	agar
Spectra Stable Isotopes, USA	stable isotope biochemicals		
Subitec GmbH, Germany	fatty acids		

Table 2 Biotechnologically utilized algal species. Species with running or completed genome projects or with established transformation systems are indicated.

Species	Lineage	Genome project	Transformation	Utilization	Reference
Green algae					
Chlamydomonas reinhardtii	Chlorophyta; Chlorophyceae; Volvocales; Chlamydomonadaceae	✓	✓	biomass from transgenics for animal health and feed; bioremediation, environmental monitoring; production of recombinant proteins	Walker *et al.* 2005b
Dunaliella salina	Chlorophyta; Chlorophyceae; Volvocales; Dunaliellaceae	✓	✓	β-carotene and other carotenoids for health food, dietary supplements, cosmetics, and feed	Pulz and Gross 2004
Dunaliella bardowil	Chlorophyta; Chlorophyceae; Volvocales; Dunaliellaceae			β-carotene for health food, dietary supplements and cosmetics	Walker *et al.* 2005b
Haematococcus pluvialis	Chlorophyta; Chlorophyceae; Volvocales; Haematococcaceae		✓	astaxanthin for health food, pharmaceuticals, and feed additives	Pulz and Gross 2004
Chlorella vulgaris	Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae	✓	✓	biomass for health food, dietary supplements, and feed surrogates; extracts for cosmetics	Pulz and Gross 2004
Chlorella spp.	Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae			polysaccharides for dietary supplements	Walker *et al.* 2005b
Ulva spp.	Chlorophyta; Ulvophyceae; Ulvales; Ulvaceae		✓	biomass for food (“aonori”) and feed; extracts for cosmetics, wastewater treatment	Nisizawa *et al.* 1987
Monostroma spp.	Chlorophyta; Ulvophyceae; Ulvales; Monostromataceae			biomass for food (“aonori”); wastewater treatment	Nisizawa *et al.* 1987
Caulerpa spp.	Chlorophyta; Ulvophyceae; Bryopsidales; Caulerpaceae			biomass for food (sea grapes, green caviar)	McHugh 2003
Porphyra spp.	Rhodophyta; Bangiophyceae; Bangiales; Bangiaceae	✓	✓	biomass for food (nori) and feed; extracts for cosmetics	McHugh 2003; Nisizawa *et al.* 1987
Porphyridium spp.	Rhodophyta; Bangiophyceae; Porphyridiales; Porphyridiaceae		✓	polysaccharides for pharmaceuticals, cosmetics, and nutrition; phycocyanin and phycoerythrin for health food, pharmaceuticals, and cosmetics	Pulz and Gross 2004; Spolaore *et al.* 2006
Kappaphycus spp.	Rhodophyta; Florideophyceae; Gigartinales; Solieriaceae		✓	kappa carrageenan for dairy products, industrial gums, and food; thickening and stabilizing agent	McHugh 2003
Eucheuma spp.	Rhodophyta; Florideophyceae; Gigartinales; Solieriaceae			iota carrageenan for dairy products, industrial gums, and food; thickening and stabilizing agent	McHugh 2003
Betaphycus gelatinum	Rhodophyta; Florideophyceae; Gigartinales; Solieriaceae			kappa carrageenan for dairy products, industrial gums, and food; thickening and stabilizing agent	McHugh 2003

Table 2 (cont.)

Species	Lineage	Genome project	Transformation	Utilization	Reference
Chondrus crispus	Rhodophyta; Florideophyceae; Gigartinales; Gigartinaceae			biomass for food (irish moss); kappa and lambda carrageenan for dairy products, other food, and industrial gums; thickening and stabilizing agent; extracts for cosmetics	McHugh 2003
Gigartina spp.	Rhodophyta; Florideophyceae; Gigartinales; Gigartinaceae			kappa carrageenan for dairy products, other food, and industrial gums; thickening and stabilizing agent	McHugh 2003
Mazzaella spp.	Rhodophyta; Florideophyceae; Gigartinales; Gigartinaceae			carrageenan for dairy products, other food, and industrial gums; thickening and stabilizing agent	McHugh 2003
Sarcothalia spp.	Rhodophyta; Florideophyceae; Gigartinales; Gigartinaceae			kappa and lambda carrageenan for dairy products, other food, and industrial gums; thickening and stabilizing agent	McHugh 2003
Callophyllis variegata	Rhodophyta; Florideophyceae; Gigartinales; Kallymeniaceae			biomass for food ("carola")	McHugh 2003
Mastocarpus stellatus	Rhodophyta; Florideophyceae; Gigartinales; Petrocelidaceae			extracts for cosmetics	McHugh 2003
Phymatolithon spp.	Rhodophyta; Florideophyceae; Corallinales; Corallinaceae			biomass / extracts as fertilizer and soil conditioner	McHugh 2003
Gelidium spp.	Rhodophyta; Florideophyceae; Gelidiales; Gelidiaceae			hydrocolloid agar for food, feed, and use in microbiological, molecular biological or medical laboratories	McHugh 2003
Gelidiella acerosa	Rhodophyta; Florideophyceae; Gelidiales; Gelidiaceae			hydrocolloid agar for food	McHugh 2003
Palmaria palmata	Rhodophyta; Florideophyceae; Palmariales; Palmariaceae			biomass for food ("dulse") and feed	McHugh 2003
Gracilaria spp.	Rhodophyta; Florideophyceae; Gracilariales; Gracilariaceae		✓	biomass for food ("ogo", "ogonori", sea moss) and feed; hydrocolloid agar for food, feed, and use in microbiological, molecular biological or medical laboratories	McHugh 2003
Ahnfeltia spp.	Rhodophyta; Florideophyceae; Ahnfeltiales; Ahnfeltiaceae			hydrocolloid agar for food	McHugh 2003
Brown algae					
Laminaria spp.	Phaeophyta; Phaeophyceae; Laminariales; Laminariaceae		✓	alginate for food, pharmaceuticals, and cosmetics; biomass for food ("konbu") and feed; wastewater treatment	McHugh 2003; Nisizawa *et al.* 1987
Macrocystis pyrifera	Phaeophyta; Phaeophyceae; Laminariales; Laminariaceae			alginate for pharmaceuticals, cosmetics, food, and textile printing; biomass for feed and fuel; wastewater treatment	McHugh 2003
Undaria spp.	Phaeophyta; Phaeophyceae; Laminariales; Alariaceae		✓	biomass for food ("wakame")	McHugh 2003; Nisizawa *et al.* 1987
Ecklonia spp.	Phaeophyta; Phaeophyceae; Laminariales; Lessoniaceae			alginate for pharmaceuticals, cosmetics, food, and textile printing; biomass / extracts as fertilizer and soil conditioner; wastewater treatment	McHugh 2003
Lessonia spp.	Phaeophyta; Phaeophyceae; Laminariales; Lessoniaceae			alginate for pharmaceuticals, cosmetics, food, and textile printing; wastewater treatment	McHugh 2003
Alaria esculenta	Phaeophyta; Phaeophyceae; Laminariales; Alariaceae; Alaria			biomass for food (winged kelp) and feed; extracts for cosmetics	McHugh 2003
Durvillaea spp.	Phaeophyta; Phaeophyceae; Fucales; Durvillaeaceae			alginate for pharmaceuticals, cosmetics, food, and textile printing; wastewater treatment	McHugh 2003
Hizikia fusiformis	Phaeophyta; Phaeophyceae; Fucales; Sargassaceae			biomass for food ("hiziki")	Nisizawa *et al.* 1987
Sargassum spp.	Phaeophyta; Phaeophyceae; Fucales; Sargassaceae			wastewater treatment	McHugh 2003
Ascophyllum nodosum	Phaeophyta; Phaeophyceae; Fucales; Fucaceae			alginate for pharmaceuticals, cosmetics, food, and textile printing; biomass / extracts as fertilizer, soil conditioner and feed	McHugh 2003
Cladosiphon okamuranus	Phaeophyta; Phaeophyceae; Ectocarpales; Chordariaceae			biomass for food ("mozuku")	McHugh 2003
Diatoms					
Phaeodactylum tricornutum	Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Naviculales; Phaeodactylaceae	✓	✓	lipids and fatty acids for nutrition	Pulz and Gross 2004

Table 2 (cont.)

Species	Lineage	Genome project	Transformation	Utilization	Reference
Odontella aurita	Bacillariophyta; Coscinodiscophyceae; Biddulphiophycidae; Eupodiscales; Eupodiscaceae			fatty acids for pharmaceuticals, cosmetics, and baby food	Pulz and Gross 2004
Haptophytes					
Isochrysis galbana	Haptophyta; Haptophyceae; Isochrysidales; Isochrysidaceae	✓		fatty acids for animal nutrition	Pulz and Gross 2004
Eustigmatophytes					
Nannochloropsis oculata	Eustigmatophyta; Eustigmatophyceae; Eustigmatales; Monodopsidaceae			extracts for cosmetics	Spolaore *et al.* 2006
Dinoflagellates					
Crypthecodinium cohnii	Alveolata; Dinophyceae; Gonyaulacales; Crypthecodiniaceae			fatty acids for nutrition	Walker *et al.* 2005b
Cyanobacteria					
Spirulina platensis (*Arthrospira platensis*)	Cyanobacteria; Oscillatoriales	✓	✓	phycocyanin, phycoerythrin, and biomass for health food, pharmaceuticals, feed, and cosmetics	Becker 2004; Pulz and Gross 2004
Spirulina pacifica	Cyanobacteria; Oscillatoriales			biomass and extracts for nutrition, food coloring, immunological diagnostics, feed, and dietary supplements	Walker *et al.* 2005b
Lyngbya majuscule	Cyanobacteria; Oscillatoriales			immune modulators for pharmaceuticals and nutrition	Pulz and Gross 2004

clams and mussels), for the larval and early juvenile stages of abalone, crustaceans and some fish species, and for zooplankton used in aquaculture food chains (Brown 2002). Algae for aquaculture must possess several key qualities like appropriate size for ingestion, rapid growth rates up to high densities, and a good nutrient composition. In addition, growth must not be susceptible to varying culture conditions, the algae must be free of toxins and they need to be easy to digest. Such suitable species exist within the Bacillariophyta (*Nitzschia closterium*, *Chaetoceros* spp., *Thalassiosira pseudonana*, *Navicula* spp., *Phaeodactylum tricornutum*, *Amphora* spp., *Skeletonema costatum*, *Cocconeis* spp.), the Chlorophyta (*Dunaliella tertiolecta*, *Nannochloris atomus*, *Pyramimonas* spp., *Tetraselmis* spp.), the Cryptophyta (*Rhodomonas* spp., *Chroomonas salina*), the Haptophyta (*Isochrysis* spp., *Pavlova* spp.), and the Eustigmatophyta (*Nannochloropsis oculata*).

Chemicals and pharmaceuticals

Algae provide a largely untapped reservoir of novel and valuable compounds, and this also seems to be the main application area of future commercial algal transgenics. Current exploitation mainly aims to utilize fatty acids, pigments, vitamins and other bioactive compounds.

Since man, animals, and higher plants lack the necessary enzymes to synthesize long, ω3 polyunsaturated fatty acids, they have to obtain them from external sources. Not only are fish and fish oil good sources of these polyunsaturated fatty acids, but several microalgae are also good sources. Currently, algal docosahexaenoic acid (22:6 ω3, 6, 9, 12, 15, 18) produced from *Crypthecodinium coh-nii* is the only commercially available ω3 polyunsaturated fatty acid (Walker *et al*. 2005b), but others like γ-linolenic acid from *Spirulina*, arachidonic acid from *Porphyridium*, and eicosapentaenoic acid from *Nannochloropis*, *Phaeodactylum* or *Nitzschia*, have already demonstrated industrial production potential (Spolaore *et al*. 2006).

Other fatty acids or lipids are isolated from *Phaeodactylum tricornutum* as a food additive, from *Odontella aurita* for pharmaceuticals, cosmetics, and baby food, and from *Isochrysis galbana* for animal nutrition (Pulz and Gross 2004).

Macroalgae, mainly *Gelidium* spp. and *Gracilaria* spp., but also *Gelidiella* and *Ahnfeltia* spp., are used as a source of hydrocolloid agar, an unbranched polysaccharide obtained from their cell walls. The gelatinous agar (plus nutrients) is used as a standard medium in almost all microbiological, molecular biological, or medical laboratories. Moreover, agar is used in many foods (ice creams, soups, icings, jellies etc.), pharmaceuticals and feed as a gelling agent. It is also used as a vegetarian gelatin substitute, as a clarifying agent in the brewing industry and other fermentation industries, and as a laxative in addition to a couple of other purposes.

Another utilized algal polysaccharide, carrageenan, is extracted from red macroalgae including *Kappaphycus* spp., *Eucheuma* spp., *Betaphycus gelatinum*, *Chondrus crispus*, *Gigartina* spp., *Mazzaella* spp., and *Sarcothalia* spp. There are three basic types of carrageenan with somewhat different characteristics: kappa carrageenan, iota carrageenan and lambda carrageenan. Carrageenans are used as gelling agents, stabilizers, texturants, thickeners, and viscosifiers for a wide range of food products.

Alginates, the salts of alginic acid and their derivatives, are extracted from the cell walls of brown macroalgae like *Laminaria* spp., *Macrocystis pyrifera*, *Ecklonia* spp., *Lessonia* spp., *Durvillaea* spp., and *Ascophyllum nodosum*. These carboxylated polysaccharides are used for a wide variety of applications in food production as thickeners, stabilizers, emulsifier, and gelling agents. Alginates are required for production of dyes for textile printing, latex paint, and welding rods. The water absorbing properties of alginates are utilized in slimming aids and in the production of textiles and paper. Calcium alginate is used in different types of medical products, including burn dressings that promote healing and can be removed painlessly. Due to its biocompatibility and simple gelation with divalent cations, it is also used for cell immobilization and encapsulation. In addition, alginates are widely used in prosthetics and dentistry for making molds, and, in addition, they are often components of cosmetics.

Further, polysaccharides are isolated from *Chlorella* spp. for dietary supplements (Walker *et al*. 2005b) and from *Porphyridium cruentum* for pharmaceuticals, cosmetics, and nutrition (Pulz and Gross 2004).

Finally, extracts from the cyanobacterium *Lyngbya majuscule* are used as immune modulators in pharmaceuticals and nutrition management (Pulz and Gross 2004).

Pigments

Algae not only contain chlorophylls, the photosynthetic pigments, but also contain a number of other pigments which are mainly used to improve the efficiency of light energy utilization and for protection from damage by the sunlight. From a commercial point of view, the carotenoids and the phycobiliproteins seem to be the most important.

Carotenoids are a class of widespread fat-soluble pigments that form a polyene chain that is sometimes terminated by rings. In addition to their role in coloration, carotenoids act as provitamin A and as biological antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Therefore, carotenoids are utilized in pharmaceuticals, health food, dietary supplements, cosmetics, and as a feed additive. The carotenoid β-carotene and other carotenoids are produced in large scale amounts from the halophilic green microalga *Dunaliella salina*. To a lesser extent, carotenoids are isolated from *Dunaliella bardowil* (Walker *et al*. 2005b).

The hydrocarbon carotenoids are known as carotenes, while oxygenated derivatives of these hydrocarbons are known as xanthophylls. The most prominent xanthophyll is astaxanthin, which is extracted in large scale amounts from the green microalga *Haematococcus pluvialis* (Pulz and Gross 2004). Further utilized xanthophylls are lutein, canthaxanthin, and zeaxanthin.

Phycobiliproteins consist of proteins with covalently bound phycobilins, which are tetrapyrrole structures with pyrrole rings that are laid out linearly. For efficient photosynthesis, phycobiliproteins capture light energy at certain wavelengths and pass it on to the chlorophylls. The phycobiliproteins phycoerythrin and phycocyanin are isolated from *Spirulina* and *Porphyridium* and utilized for health food, pharmaceuticals, and cosmetics (Becker 2004; Pulz and Gross 2004; Spolaore *et al*. 2006). Phycobiliproteins are not only used as pigments, but have also been shown to have health-promoting properties. They are also used in research laboratories as labels for biomolecules (Spolaore *et al*. 2006).

Diatomite

Diatomite is a commercially significant derivative of diatoms. It is a sedimentary rock primarily composed of the fossilized outer casings, the silica-based cell walls, of diatoms; therefore it has a high silica content. Diatomite is used for filtration applications, as an absorbent (for oil, water and chemicals), as a cement and concrete additive, as a filler for paint and plastics, as a mild abrasive, as a horticultural potting mix and soil conditioner, as a hydroponic medium, as a lightweight building material, as a pet litter, as a mechanical insecticide, as a fertilizer, as a thermal insulator, as a refractory, and last but not least as an important component of dynamite. Diatomite production rose from 3280 tons in 1900 to 2,000,000 tons in 2005 (Kelly and Matos 2006).

Fertilizers

For many years, several macroalgae are used to fertilize land. But nowadays, macroalgal extracts and suspensions have achieved a much broader use than total macroalgae or macroalgae meal. Seaweeds like *Phymatolithon* spp., *Ecklonia* spp., and *Ascophyllum nodosum* are utilized to produce fertilizers and soil conditioners, especially for the horticultural industry (McHugh 2003).

Wastewater treatment

Algae can be used in wastewater treatment to reduce the content of nitrogen and phosphorus in sewage and certain agricultural wastes. Another application is the removal of toxic metals from industrial wastewater. Algae that are applicable to wastewater treatment must tolerate a wide variation in medium conditions (e.g. salinity). For reduction of nitrogen- and phosphorus compounds, the green macroalgae *Ulva* spp. and *Monostroma* spp. was suitable. Binding of heavy metals, such as copper, nickel, lead, zinc and cadmium, has been demonstrated for macroalgae like *Lamiaria*, *Sargassum*, *Macrocystis*, *Ecklonia*, *Ulva*, *Lessonia*, and *Durvillaea* (McHugh 2003).

Cosmetics

Components of algae are frequently used in cosmetics as thickening agents, water-binding agents, and antioxidants. Cosmetics companies claim benefits on the skin or health in general from contents like carrageenan, other algal polysaccharides, algal proteins or lipids, vitamin A, vitamin B1, iron, phosphorus, sodium, copper, magnesium, calcium, or other elements; some companies promise that algal extracts inhibit oxidative degeneration of collagen and hyaluronic acid and that they have anti-aging properties. From a scientific point of view, many of the promised effects have to be judged as not scientifically proven and unsubstantiated. Typical species that are used for cosmetics are *Chondrus crispus*, *Mastocarpus stellatus*, *Laminaria* spp., *Porphyra* spp., *Ulva lactuca*, *Ascophyllum nodosum*, *Alaria esculenta*, *Spirulina platensis*, *Nannochloropsis oculata*, *Chlorella vulgaris* and *Dunaliella salina*.

Fuel

The giant kelp *Macrocystis pyrifera* and other algae have been evaluated for biomass conversion to methane by anaerobic fermentation. Because of its high growth rate and ease of harvesting, *Macrocystis* resulted in a good methane yield in a test farm; methane could be used as a fuel, but more work is necessary to find better methods for the large scale conversion step from biomass to methane (McHugh 2003).

As mentioned above, all commercially used algae are non-transgenic, but this could change quickly. **Table 2** not only lists the biotechnologically utilized algal species, but also highlights those species with running or completed genome projects and, furthermore, it illustrates which species have already been genetically transformed. In several applications using those species, commercial companies, including some of the ones listed in **Table 1**, could quickly replace the currently used wild-type organisms by optimized transgenic organisms. These transgenics could result in new or modified products or show a reduced content of components that interfere with current production. Transgenics could also allow growth to higher densities or could permit the use of atypical, particularly cheaper, growth conditions.

THE BASIS OF ALGAL TRANSGENICS

Normally, more than just one species generates a desired product or shows another trait of interest. Therefore, careful selection of an appropriate target organism stands at the beginning of every algal transformation project. Aside from important issues like product quality and quantity, additional points have to be considered.

General aspects

One of the most obvious arguments that speak for a certain species is the ease of cultivating the organism, especially under laboratory conditions. With respect to this point, small algae that grow with a short life cycle in liquid, axenic culture in a synthetic medium under defined environmental conditions are preferred for transformation experiments. Ideally, the target species is also easily satisfied by inexpensive culture media and it grows to high densities, even under varying conditions. To allow for selection of transformed algae, a method must be established or be available that allows for regeneration of the target species

from single cells (ideally on agar plates). Most algae are photoautotrophs, so they require only light, water and basic nutrients for growth; other algae are heterotrophs and can also be grown in the dark if sugars are present in the culture medium. For transformation experiments it might also be advantageous to use a species with separate sexes and a well-known sexual life cycle that can be triggered under laboratory conditions. In this way, controlled genetic crosses can be made. It is also helpful to use an alga in which mutants can be easily generated or mutant collections exist. Not only should the repeat content of the ideal genome be as low as possible, but in most cases even haploid algae are favored. In addition, many algae have multinucleated cells, but algae with mononucleated cells should be selected for transformation experiments. Moreover, previous molecular, biochemical, physiological or ecological knowledge of the target species and a somewhat developed molecular toolbox is desirable. Finally, existence of extensive sequence information is of utmost importance for algal transgenics.

In actuality, no algal species fulfills all of these requirements, but several microalgae come quite close to this ideal of a target organism. Since sequence information is a central point and because algal sequence projects deal with organisms that are also suitable targets for transformation experiments, this aspect is reviewed in more detail.

Genome projects

Algal genome research is needed as the basis for a new level of efficiency and success in the application of biotechnology and gene technology to algae and their products. Fortunately, genomic data, not only from algae, but also from other life-forms, increases almost exponentially. There are several microalgal genome projects, of which the most advanced projects are those for the red alga *Cyanidioschyzon merolae*, the diatom *Thalassiosira pseudonana*, and the three green algae *Chlamydomonas reinhardtii*, *Volvox carteri* and *Ostreococcus tauri*; the appearance of these algae is displayed in **Fig. 1**. Sequencing and annotation of the 16.5 Mb *Cyanidioschyzon merolae* genome has been finished (Matsuzaki *et al*. 2004; Barbier *et al*. 2005) (http://merolae.biol.s.u-tokyo.ac.jp/). Similarly, sequencing and annotation of the 34 Mb *Thalassiosira pseudonana* genome has been completed (Armbrust *et al*. 2004) (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html). Also, sequencing of the ~120 Mb genome of *Chlamydomonas reinhardtii* has been completed; annotation is proceeding and should soon be finished (Grossman *et al*. 2003; Shrager *et al*. 2003; Grossman 2005) (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). The ~140 Mb-sized genome of *Volvox carteri* has been sequenced (8x coverage, unpublished, DOE Joint Genome Institute/JGI, Walnut Creek, CA) and annotation will begin in the near future. Sequencing of the 11.5 Mb-sized genome of *Ostreococcus tauri* has been finished and annotation is ongoing (Derelle *et al*. 2002; Derelle *et al*. 2006) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=12915).

Additional algal genome projects are in progress (see also http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=genomeprj), for example in *Alexandrium tamarense*, *Amphidinium operculatum*, *Aureococcus anophagefferens* (~32 Mb), *Chlorella vulgaris* (~40 Mb), *Cyanophora paradoxa*, *Dunaliella salina* (~130 Mb), *Ectocarpus siliculosus* (~214 Mb), *Emiliania huxleyi* (~220 Mb), *Euglena gracilis*, *Galdieria sulphuraria* (~12 Mb), *Guillardia theta*, *Heterocapsa triquetra*, *Isochrysis galbana*, *Karenia brevis*, *Lotharella amoeboformis*, *Micromonas pusilla* (~15 Mb), *Ochromonas danica*, *Ostreococcus lucimarinus* (~12 Mb), *Pavlova lutheri*, *Phaeodactylum tricornutum* (~30 Mb) (**Fig. 1**), *Porphyra purpurea*, and *Porphyra yezoensis*.

Finally, due to their small sized genomes, complete genome sequences from ~30 Cyanobacteria are available in different databases; among these are species like *Synechococcus elongatus* (~2.7 Mb), *Anabaena* sp. (6.4 Mb), *Nostoc punctiforme* (~7.5 Mb), *Synechocystis* sp. (~3.6 Mb), *Thermosynechococcus elongatus* (~2.6 Mb), *Gloeobacter violaceus* (~4.6 Mb), and *Prochlorococcus marinus* (1.7–2.4 Mb; size depends on ecotype).

The listing above is not complete since there are (less extensive) genomic sequences from many other algal species in GenBank and other databases. Moreover, new genome projects perpetually come along.

EST projects

Just as with genome projects, there has recently been a dramatic increase in sequenced expressed sequence tags (ESTs) in algae. Extensive EST data comes from the diatoms *Thalassiosira pseudonana* (http://avesthagen.sznbowler.com/) and *Phaeodactylum tricornutum* (http://avesthagen.sznbowler.com/) (Scala *et al*. 2002), the green algae *Chlamydomonas reinhardtii* (Shrager *et al*. 2003), *Volvox carteri* (not yet released), *Ostreococcus tauri* and *Acetabularia acetabulum* (Henry *et al*. 2004), the red algae *Porphyra yezoensis* (http://merolae.biol.s.u-tokyo.ac.jp/) (Nikaido *et al*. 2000), *Gracilaria gracilis* (Lluisma and Ragan 1997), and *Galdieria sulphuraria* (Weber *et al*. 2004), the brown alga *Laminaria digitata* (Crepineau *et al*. 2000), the haptophytes *Emiliania huxleyi* (Wahlund *et al*. 2004) and *Pavlova lutheri* (Pereira *et al*. 2004), the chlorarachniophyte *Bigelowiella natans* (Archibald *et al*. 2003), and finally the dinoflagellates *Alexandrium tamarense* (Hackett *et al*. 2004), *Lingulodinium polyedrum* (Bachvaroff *et al*. 2004), *Karlodinium micrum*, and *Amphidinium carterae* (Bachvaroff *et al*. 2004). EST data from many other algal species are available at the EST sequence databases of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/projects/dbEST/) and the Taxonomically Broad EST Database (TBestDB, http://amoebidia.bcm.umontreal.ca/pepdb/searches/welcome.php).

Other sequencing projects

Sequencing of mitochondrial and chloroplast genomes has been performed with even more algal species than the EST or genome sequencing projects, due to the much smaller size of plastid genomes. Sequences are available at the NCBI organelle database (http://www.ncbi.nlm.nih.gov/genomes/ORGANELLES/organelles.html) and the Organelle Genome Database (GOBASE, http://www.bch.umontreal.ca/gobase/gobase.html). Many organelle sequences have been generated through the Organelle Genome Megasequencing Program (OGMP, http://www.bch.umontreal.ca/ogmp/).

Many eukaryotic algae are known to be infected by viruses; some genomes of such viruses, more precisely dsDNA viruses, have been sequenced (van Etten *et al*. 2002).

Eukaryotic cells can permanently acquire chloroplasts by engulfing an alga. In most cases, little remains of the engulfed alga apart from its chloroplast, but in two groups, the cryptomonads and chlorarachniophytes, a small remnant nucleus of the engulfed alga is still present. These tiny nuclei, called nucleomorphs, have been sequenced in the cryptomonad alga *Guillardia theta* and the chlorarachniophyte *Bigellowiella natans* (Gilson and McFadden 2002).

PRODUCTION OF TRANSGENIC ALGAE

The existence of extensive sequence information for a given target alga and the fulfillment of most of the other mentioned general requirements is an excellent basis for the realization of successful and diversified transformation experiments. Before these experiments can begin, those who want to transform a new species have to think about the point of departure in his or her particular organism. In this stage, some crucial questions usually arise: Are there any close relatives that have been transformed before? Which DNA-constructs might be the most suitable for selection of my

Table 3 Transformable algal species. Nuclear transformation unless otherwise noted.

Species	Lineage	Transformation	Reference
Green algae			
Chlamydomonas reinhardtii	Chlorophyta; Chlorophyceae; Volvocales; Chlamydomonadaceae	stable	Debuchy *et al.* 1989; Kindle *et al.* 1989
Volvox carteri	Chlorophyta; Chlorophyceae; Volvocales; Volvocaceae	stable	Schiedlmeier *et al.* 1994
Dunaliella salina	Chlorophyta; Chlorophyceae; Volvocales; Dunaliellaceae	stable	Geng *et al.* 2003, 2004; Tan *et al.* 2005
Dunaliella viridis	Chlorophyta; Chlorophyceae; Volvocales; Dunaliellaceae	stable	Sun *et al.* 2006
Haematococcus pluvialis	Chlorophyta; Chlorophyceae; Volvocales; Haematococcaceae	stable	Steinbrenner and Sandmann 2006
Chlorella sorokiniana	Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae	stable	Dawson *et al.* 1997
'Chlorella' kessleri (*Parachlorella kessleri*)	Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellales incertae sedis	stable	El-Sheekh 1999
'Chlorella' ellipsoidea	Chlorophyta; Trebouxiophyceae; Trebouxiophyceae incertae sedis	transient	Jarvis and Brown 1991
Chlorella vulgaris	Chlorophyta; Trebouxiophyceae; Chlorellales; Chlorellaceae	transient	Chow and Tung 1999
Ulva lactuca	Chlorophyta; Ulvophyceae; Ulvales; Ulvaceae	transient	Huang *et al.* 1996
Red algae			
Cyanidioschyzon merolae	Rhodophyta; Bangiophyceae; Cyanidiales; Cyanidiaceae	stable	Minoda *et al.* 2004
Porphyra yezoensis	Rhodophyta; Bangiophyceae; Bangiales; Bangiaceae	stable	Cheney *et al.* 2001
Porphyra miniata	Rhodophyta; Bangiophyceae; Bangiales; Bangiaceae	transient	Kübler *et al.* 1993
Kappaphycus alvarezii	Rhodophyta; Florideophyceae; Gigartinales; Solieriaceae	transient	Kurtzman and Cheney 1991
Gracilaria changii	Rhodophyta; Florideophyceae; Gracilariales; Gracilariaceae	transient	Gan *et al.* 2003
Porphyridium sp.	Rhodophyta; Bangiophyceae; Porphyridiales; Porphyridiaceae	stable (chloroplast)	Lapidot *et al.* 2002
Brown algae			
Laminaria japonica	Phaeophyta; Phaeophyceae; Laminariales; Laminariaceae	stable	Qin *et al.* 1999
Undaria pinnatifida	Phaeophyta; Phaeophyceae; Laminariales; Alariaceae	stable	Qin *et al.* 2003
Diatoms			
Phaeodactylum tricornutum	Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Naviculales; Phaeodactylaceae	stable	Apt *et al.* 1996; Falciatore *et al.* 1999, 2000; Zaslavskaia *et al.* 2000, 2001
Navicula saprophila (*Fistulifera saprophila*)	Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Naviculales; Naviculaceae	stable	Dunahay *et al.* 1995
Cylindrotheca fusiformis	Bacillariophyta; Bacillariophyceae; Bacillariophycidae; Bacillariales; Bacillariaceae	stable	Fischer *et al.* 1999
Cyclotella cryptica	Bacillariophyta; Coscinodiscophyceae; Thalassiosirophycidae; Thalassiosirales; Thalassiosiraceae	stable	Dunahay *et al.* 1995
Thalassiosira weissflogii	Bacillariophyta; Coscinodiscophyceae; Thalassiosirophycidae; Thalassiosirales; Thalassiosiraceae	transient	Falciatore *et al.* 1999
Euglenids			
Euglena gracilis	Euglenozoa; Euglenida; Euglenales	stable (chloroplast)	Doetsch *et al.* 2001
Dinoflagellates			
Amphidinium sp.	Alveolata; Dinophyceae; Gymnodiniales; Gymnodiniaceae	stable	ten Lohuis and Miller 1998
Symbiodinium microadriaticum	Alveolata; Dinophyceae; Suessiales; Symbiodiniaceae	stable	ten Lohuis and Miller 1998
Cyanobacteria			
Spirulina platensis (*Arthrospira platensis*)	Cyanobacteria; Oscillatoriales	stable	Kawata *et al.* 2004
Anabaena sp.	Cyanobacteria; Nostocales; Nostocaceae	stable	Thiel and Poo 1989
Synechocystis sp.	Cyanobacteria; Chroococcales	stable	Dzelzkalns and Bogorad 1986

transgenic algae? Which promoters should work and result in a high level of expression? How should the DNAs be transferred into the cells? How can expression of a given gene be monitored? Answers to these fundamental questions are presented in the following subsections.

Transformed species

In the last few years, successful genetic transformation of ~25 algal species has been demonstrated (**Table 3**); most of these were achieved by nuclear transformation. Ten species of green algae have been transformed, stable transformation has been shown for seven of them, one of which was the unicellular model organism *Chlamydomonas reinhardtii* (Debuchy *et al*. 1989; Kindle *et al*. 1989), and transient transformation was demonstrated in the other three. All of these green algae are unicellular species except for *Volvox carteri*, for which stable transformation has been shown (Schiedlmeier *et al*. 1994), and *Ulva lactuca*, which was transiently transformed (Huang *et al*. 1996). The freshwater alga *Volvox carteri* represents one of the simplest multicellular organisms as it is composed of only two cell types, ~2000-4000 small biflagellate, terminally differentiated somatic cells and ~16 large asexual reproductive cells (**Fig. 1**); the multicellular marine macroalga *Ulva lactuca*, also known as sea lettuce since the thallus somewhat resembles a lettuce leaf (**Fig. 1**), has been used in many countries for years to feed domestic animals and has also been used as human food in Asia.

Six species of red algae have been transformed so far (**Table 3**), two of which are unicellular, and one of which is the ultrasmall unicell *Cyanidioschyzon merolae* (Minoda *et al*. 2004) (**Fig. 1**). All four of the multicellular species are macroalgae. Two multicellular species come from the genus *Porphyra*; stable transformation was demonstrated in one of these species, *Porphyra yezoensis* (Cheney *et al*. 2001). *Porphyra* is used to make nori, the most commonly eaten seaweed. Another one of these species, *Kappaphycus* (Kurtzman and Cheney 1991), is harvested to produce the gelling agent kappa carrageenan, which is used in industrial gums and as a smoothening agent. The fourth is *Gracilaria* (Gan *et al*. 2003), which is notable for its economic importance for producing agar, a hydrocolloid, as well as for its use as a food for humans and various species of shellfish.

Stable transformation has also been shown for two brown macroalgae from the order Laminariales, *Laminaria japonica* (Qin *et al*. 1999) and *Undaria pinnatifida* (Qin *et al*. 2003). *Laminaria* is extensively farmed in China, Korea and Japan and it is harvested from wild stands in several other countries.

Stable transformation of four diatom species has been reported (**Table 3**); another species has been transiently

transformed. Diatoms are the main producers in aquatic environments and they are responsible for the majority of the photosynthesis that occurs in fresh water and particularly in the oceans.

Finally, transformation of two dinoflagellates, *Amphidinium* and *Symbiodinium* (ten Lohuis and Miller 1998), and one euglenid, *Euglena gracilis*, has to be noted. For the latter, only chloroplast transformation has been reported so far. Also, many species of Cyanobacteria, e.g. *Spirulina*, *Anabaena*, or *Synechocystis*, can be transformed by electroporation or conjugation (Koksharova and Wolk 2002).

Transformation efficiency

The previous reports on genetic transformation of algae reveal that transformation efficiencies and total numbers of producible transformants are strongly species-dependent. For example, in *Cyanidioschyzon merolae* ~200 transformants/µg plasmid-DNA appeared when 3-4 x 10^8 cells were spread on an agar plate (Minoda *et al*. 2004). In *Porphyridium* sp. it is also possible to spread lots of cells, i.e. ~10^8, on a single plate and 2.5 x 10^{-4} transformants/µg DNA were recovered. In *Chlamydomonas reinhardtii* transformation efficiency is between 10^{-4} and 10^{-5}, and about 8 x 10^6 cells can be spread on a plate (Kindle *et al*. 1989; Kindle 1990); so there are much less cells on a plate than in *Cyanidioschyzon* or *Porphyridium* transformation experiments, nevertheless, ~1000 independent *Chlamydomonas* transformants can be recovered from a single plate. In *Volvox carteri* transformation efficiency is ~2.5 x 10^{-5} (Schiedlmeier *et al*. 1994) and thus similar to that of *Chlamydomonas*. But *Volvox carteri* is much larger than *Chlamydomonas reinhardtii* (**Fig. 1**), it is grown in liquid medium, not on plates, *Volvox* does not like high densities of organisms, and viable transformants have to be identified microscopically. So, the number of algae that can be handled at the same time is much lower than with the much smaller *Chlamydomonas* and, despite the transformation efficiency that is comparable to *Chlamydomonas*, only a few transformants result from a transformation experiment. A general but very rough rule of thumb seems to be that the larger and more complex an alga is the lower is the total number of achievable or manageable stable transformants. Incidentally, this is one important reason why most labs work with microalgae.

Selectable marker genes

The use of selectable marker genes is normally required in all experiments that aim to generate stable transgenic algae, since only a very low percentage of treated organisms are successfully transformed.

Selectable markers are often antibiotic resistance genes which are dominant markers as they confer a new trait to any transformed target strain of a certain species, no matter of the respective genotype. By far the highest number of selectable marker genes have been established for *Chlamydomonas reinhardtii*: the R100.1 plasmid/bacteriophage T4/synthetic aminoglycoside adenyltransferase gene *aadA* confers resistance to spectinomycin and streptomycin (Cerutti *et al*. 1997), the *Streptoalloteichus hindustanus ble* gene to zeomycin and phleomycin (Stevens *et al*. 1996), the mutated *Chlamydomonas reinhardtii* protoporphyrinogen oxidase gene *PPX1* to the N-phenyl heterocyclic herbicide S-23142 (Randolph-Anderson *et al*. 1998), the *Streptomyces rimosus* aminoglycoside phosphotransferase *aphVIII* (*aphH*) gene to paromomycin (Sizova *et al*. 2001), the mutated *Chlamydomonas reinhardtii* acetolactate synthase gene *ALS* to sulfonylurea herbicides (Kovar *et al*. 2002), the *Streptomyces hygroscopicus* aminoglycoside phosphortransferase *aph7″* gene to hygromycin B (Berthold *et al*. 2002), and finally, the mutated version of the *Chlamydomonas reinhardtii* ribosomal protein gene S14 (*CRY1*) to emetine and cryptopleurine (Nelson *et al*. 1994). Similarly, in the multicellular green alga *Volvox carteri*, the *Streptoalloteichus hindustanus ble* gene was shown to confer resistance to zeomycin and phleomycin (Hallmann and Rappel 1999) and the *Streptomyces rimosus* aminoglycoside phosphotransferase *aphVIII* (*aphH*) gene to paromomycin (Jakobiak *et al*. 2004; Hallmann and Wodniok 2006). In *Haematococcus pluvialis*, the modified *H. pluvialis* gene *pdsMod4.1*, coding for the carotenoid biosynthesis enzyme phytoene desaturase, confers accelerated astaxanthin biosynthesis and, as a consequence, resistance to the bleaching herbicide norflurazon (Steinbrenner and Sandmann 2006). In another unicellular green alga, *Chlorella vulgaris*, the *Streptomyces hygroscopicus* aminoglycoside phosphotransferase gene was expressed under the control of the cauliflower mosaic virus promoter (CaMV35S) for selection with hygromycin (Chow and Tung 1999); notably, the CaMV35S promoter is a typical promoter for strong expression in transgenic higher plants. A mutant form of the gene encoding acetohydroxyacid synthase [*AHAS* (*W492S*)] was established as a selectable marker for chloroplast transformation in the red alga *Porphyridium*; selection is performed with the herbicide sulfometuron methyl (Lapidot *et al*. 2002). In *Laminaria japonica* a simian virus 40 (SV40) promoter-hygromycin phosphotransferase chimeric gene confers resistance to hygromycin (Qin *et al*. 1999); SV40 is a polyomavirus that is found in both monkeys and humans, so it is quite astonishing that this promoter works in a brown alga. In the diatom *Phaeodactylum tricornutum*, the *Strepto-alloteichus hindustanus ble* gene was shown to confer resistance to zeomycin (Apt *et al*. 1996; Falciatore *et al*. 1999), the *nat* and *sat-1* genes produce resistance to the antibiotic nourseothricin (Zaslavskaia *et al*. 2000), the expressed chloramphenicol acetyltransferase gene (*CAT*) detoxifies the antibiotic chloramphenicol (Apt *et al*. 1996), and the neomycin phosphotransferase II (*npt*II) gene confers resistance to the aminoglycoside antibiotic G418 (Zaslavskaia *et al*. 2000). In another diatom, *Cylindrotheca fusiformis*, the endogenous calcium-binding glycoprotein α-frustulin *fruα3* promoter was used to drive expression of the *Streptoalloteichus hindustanus ble* gene in order to confer resistance to zeomycin (Fischer *et al*. 1999). Likewise, the *npt*II gene confers resistance to the antibiotic G418 in the diatoms *Navicula saprophila* and *Cyclotella cryptica* (Dunahay *et al*. 1995). The R100.1 plasmid/bacteriophage T4/synthetic aminoglycoside adenyltransferase gene *aadA*, which confers resistance to spectinomycin, was established as a selectable marker for chloroplast transformation in the euglenid *Euglena gracilis* (Doetsch *et al*. 2001). Transformation of the dinoflagellates *Amphidinium* sp. and *Symbiodinium microadriaticum* was shown by using the *nptII* gene driven by the *Agrobacterium nos* promoter, or the hygromycin B phosphotransferase gene (*hpt*) fused to the bidirectional *Agrobacterium* p1'2' promoter (ten Lohuis and Miller 1998).

In addition to dominant selectable markers, there are also several established recessive selectable markers for algal systems. Though recessive markers require auxotrophic mutants with mutations in the corresponding endogenous gene and the corresponding intact gene for complementation, they have the great advantage that a complete endogenous gene with its own promoter is usually used, so, in contrast to many dominant marker constructs, expression and function of the selectable marker construct in the respective organism is quite certain beforehand. A common recessive marker is the nitrate reductase gene (*nit*) which has already been used for functional complementation of nitrate reductase defective mutants of *Chlamydomonas reinhardtii* (Kindle *et al*. 1989), *Volvox carteri* (Schiedlmeier *et al*. 1994), *Dunaliella viridis* (Sun *et al*. 2006), *Chlorella sorokiniana* (Dawson *et al*. 1997), and *Ulva lactuca* (Huang *et al*. 1996). By using this gene, former *nit*$^-$ mutants gain the trait to utilize nitrate as the sole nitrogen source in transformation experiments. Nitrate reductase reduces not only nitrate, but also chlorate, the chlorine analog of nitrate, and the reduction product of chlorate, chlorite, is toxic. Because transformants are killed by chlorate in contrast to wild-type algae, chlorate can be used to re-check putative *nit*$^+$ transformants.

This characteristic is also used to obtain the required auxotrophic target organisms for *nit* transformation experiments; after random mutagenization chlorate is used as a negative selectable marker to identify *nit*$^-$ mutants.

Similar to the nitrate reductase gene, the *Chlamydomonas reinhardtii* argininosuccinate lyase gene *ASL* was shown to complement mutations in argininosuccinate lyase defective *Chlamydomonas reinhardtii* mutants by selection on arginine-free medium (Debuchy *et al*. 1989). And finally, in *Cyanidioschyzon merolae*, functional complementation of a spontaneously mutated *C. merolae* UMP synthase gene was achieved by introducing the wild-type UMP synthase gene followed by selection for uracil prototrophy (Minoda *et al*. 2004).

The remarkable repertoire of selectable marker genes that work in algal systems ought to be a basis and an impetus for all who intend to transform other algal species.

Promoters and reporter genes

Often selectable marker genes cannot be expressed under their own promoters, especially if they come from a heterologous source. Therefore, more suitable, i.e. normally strong, constitutive or inducible, and, if possible, endogenous promoters are necessary for these and other chimeric gene constructs. Furthermore, many researchers want to take advantage of transgenic organisms when they study spatial or temporal expression of their gene of interest *in situ* or *in vivo*. To allow for simple detection of this protein expression, the promoter of this particular gene is used to drive expression of a reporter gene that is easily identified and measured, in contrast to the original coding sequence that belongs to this promoter. Reporter genes often code for enzymes that convert a substrate into a colored product, or result in light emission, or the reporter gene product is a fluorescent protein itself.

So, in addition to selectable markers, suitable promoters and reporter genes for a given species are basic essentials in the molecular toolbox of a genetic engineer.

Two heterologous promoters that work in several algal species, the CaMV35S and the SV40 promoters, have been mentioned in the subsection above. In *Chlamydomonas reinhardtii*, the endogenous promoters of the *RBCS2* (ribulose bisphosphate carboxylase, small chain) gene (Stevens *et al*. 1996), a *HSP70A* (heat shock protein 70A)/*RBCS*2 fusion promoter (Schroda *et al*. 2000), a *HSP70A*/β_2*TUB* (β_2-tubulin) fusion promoter (Schroda *et al*. 2000), and especially the promoter of the *PsaD* (abundant protein of photosystem I complex) gene (Fischer and Rochaix 2001) proved to be of high value for efficient expression of chimeric constructs. Likewise, in *Volvox carteri*, the *ARS* (arylsulfatase) promoter (Hallmann and Sumper 1994), which is inducible by sulfur deprivation, the β-tubulin promoter (Hallmann and Sumper 1996), and an *HSP70*/*RBCS*3 fusion promoter (Jakobiak *et al*. 2004) were useful. In both of these algae, the endogenous *ARS* (arylsulfatase) gene (Davies *et al*. 1992; Hallmann and Sumper 1994) and a codon-optimized *GFP* (green fluorescent protein) gene (Fuhrmann *et al*. 1999; Ender *et al*. 2002) were valuable reporter genes; chromogenic substrates like 5-bromo-4-chloro-3-indolyl sulphate (X-SO_4) or *p*-nitrophenyl sulfate allow for analysis of subcellular localization and spectrophotometric quantification of arylsulfatase activity. Unlike arylsulfatase, GFP detection does not require any additives, so it can be done *in vivo*. In addition to *ARS* and *GFP* constructs, in *Chlamydomonas reinhardtii*, the codon-optimized *crluc* (*Renilla reniformis* luciferase) gene (Fuhrmann *et al*. 2004), and in *Volvox carteri*, the *HUP1* (*Chlorella kessleri* hexose/H^+ symporter) gene (Hallmann and Sumper 1996) were useful for monitoring expression of nuclear genes. A construct consisting of the CaMV35S promoter and the *Escherichia coli* β-glucuronidase (GUS) *uidA* reporter gene has been used successfully in several algal species such as *Dunaliella salina* (Tan *et al*. 2005), *Chlorella kessleri* (El-Sheekh 1999), *Chlorella vulgaris* (Chow and Tung 1999), *Porphyra yezoensis* (Cheney *et al*. 2001), and *Porphyra miniata* (Kübler *et al*. 1993); different β-glucuronidase substrates, like 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), p-nitrophenyl β-D-glucuronide or 4-methylumbelliferyl-β-D-glucuronide (MUG), allow for analysis of subcellular localization and spectrophotometrical or fluorimetrical quantification. A construct with the same CaMV35S promoter and the firefly luciferase gene was established as a reporter system for *'Chlorella' ellipsoidea* protoplasts (Jarvis and Brown 1991); the expressed luciferase is detected by *in-vitro* imaging after exposure to the substrate D-luciferin in the presence of ATP. In the seaweed *Porphyra yezoensis,* transgene expression was monitored by a CaMV35S promoter driven *GFP* reporter gene construct (Cheney *et al*. 2001). In the same algae, GFP was also expressed using promoters from two homologous genes (*RPB1* and *GAPDH*) (Cheney *et al*. 2001). A construct consisting of the SV40 promoter and the *Escherichia coli* β-galactosidase gene (*lacZ*) was shown to be a valuable reporter for *Gracilaria changii* (Gan *et al*. 2003); detection of β-galactosidase requires *in-vitro* application of the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The *lacZ* reporter gene was also expressed in sporophytes of the seaweeds *Laminaria japonica* (Jiang *et al*. 2003) and *Undaria pinnatifida* (Qin *et al*. 2003). In the diatom *Phaeodactylum tricornutum*, the firefly luciferase gene (*LUC*) was expressed under control of a promoter derived from a fucoxanthin-chlorophyll a/c-binding protein gene (*FCP*) (Falciatore *et al*. 1999), and the β-glucuronidase (GUS) *uidA* and *GFP* reporter genes were useful in transgenic *Phaeodactylum tricornutum* algae (Zaslavskaia *et al*. 2000; Falciatore and Bowler 2002). Introduction of a gene encoding glucose transporters (*glut1* or *HUP1*), turns *Phaeodactylum tricornutum* and *Volvox carteri* (Hallmann and Sumper 1996) into algae that survive on exogenous glucose in the absence of light (Zaslavskaia *et al*. 2001). In two diatoms, *Thalassiosira weissflogii* and *Cylindrotheca fusiformis*, the β-glucuronidase (GUS) *uid*A gene (Falciatore *et al*. 1999) and the *GFP* gene (Poulsen and Kröger 2005), respectively, were useful as reporter genes; in *Cylindrotheca fusiformis*, the *GFP* gene was driven by a promoter from the endogenous nitrate reductase gene (Poulsen and Kröger 2005), so expression is switched off when cells are grown in the presence of ammonium ions and is switched on when cells are transferred to medium containing nitrate.

The above subsection, which contains examples of successfully transformed algae, shows that there is a broad spectrum of proven reporter genes and promoters. Several genes and even promoters come from a heterologous source. Use of these genetic elements must be considered for transformation experiments with related (or even unrelated) previously untransformed species. Alternatively, if an endogenous genetic element is utilized, cloning and construction in a manner that is analogous to these proven constructs, using DNA from the organism of interest, might be the fastest way to successfully transform a new species.

Methods to introduce DNA into algal cells

The basis of almost all algal transformation methods is to cause, by various means, temporal permeabilization of the cell membrane, enabling DNA molecules to enter the cell. Entrance of the DNA into the nucleus and integration into the genome occurs without any external help. DNA integration mainly occurs by illegitimate recombination events, resulting in ectopic integration of the introduced DNA and, thus, culminates in stable genetic transformation. In actuality, it is not difficult to permeabilize a cell membrane in order to introduce DNA; however, the affected reproductive cell must survive this life-threatening damage and DNA invasion and resume cell division.

There are a couple of working transformation methods for algal systems that enable the recovery of viable transformants. The most popular method is micro-particle bombardment, also referred to as micro-projectile bombardment, particle gun transformation, gene gun transformation, or

simply biolistics. This method makes use of DNA-coated heavy-metal (mostly gold) micro-projectiles and allows transformation of almost any type of cell, regardless of the thickness or rigidity of the cell wall, and it also allows transformation of organelles. This course of action, which appears like an anti-algae military operation using carpet bombing, was successfully applied in *Chlamydomonas reinhardtii* (Kindle *et al*. 1989), *Volvox carteri* (Schiedlmeier *et al*. 1994), *Dunaliella salina* (Tan *et al*. 2005), *Gracilaria changii* (Gan *et al*. 2003), *Laminaria japonica* (Qin *et al*. 1999; Jiang *et al*. 2003), *Phaeodactylum tricornutum* (Apt *et al*. 1996), *Navicula saprophila* (Dunahay *et al*. 1995), *Cyclotella cryptica* (Dunahay *et al*. 1995), *Euglena gracilis* (Doetsch *et al*. 2001), *Porphyridium* sp. (Lapidot *et al*. 2002), *Cylindrotheca fusiformis* (Fischer *et al*. 1999), *Haematococcus pluvialis* (Steinbrenner and Sandmann 2006), *'Chlorella' kessleri* (El-Sheekh 1999), and *Chlorella sorokiniana* (Dawson *et al*. 1997).

Another less complex and less expensive transformation procedure involves preparation of a suspension of (micro)algae that is then agitated in the presence of micro- or macro-particles, polyethylene glycol and DNA. Several investigators have used silicon carbide (SiC) whiskers (~0.3-0.6 µm thick and ~5-15 µm long) as micro-particles; silicon carbide is a ceramic compound of silicon and carbon. These hard and rigid micro-particles allowed transformation of cells with intact cell walls including *Chlamydomonas reinhardtii* (Dunahay 1993), *Symbiodinium microadriaticum* (ten Lohuis and Miller 1998), and *Amphidinium* sp. (ten Lohuis and Miller 1998); however, cell wall reduced algae seem to be more appropriate when applying this method. In *Chlamydomonas reinhardtii*, cell wall reduced mutants are transformed through agitation in the presence of quite large glass beads (0.4-0.5 mm in diameter), polyethylene glycol and DNA (Kindle 1990); this cheap method is routinely used for transforming *Chlamydomonas*, as most investigators prefer to work with wall-reduced strains. Cell-wall free protoplasts of the green alga *'Chlorella' ellipsoidea* can be transformed without any micro- or macro-particles (Jarvis and Brown 1991); agitation of the protoplast in the presence of polyethylene glycol and DNA is sufficient. Naked cells, protoplasts, cell wall reduced mutants and other cells with thin walls can also be transformed by electroporation, in which specially designed electrodes effect voltage across the plasma membrane that exceeds its dielectric strength. This large electronic pulse temporarily disturbs the phos-pholipid bilayer of the cell membrane, allowing molecules like DNA to pass. Cells of *Chlamydomonas reinhardtii* (Brown *et al*. 1991), *Cyanidioschyzon merolae* (Minoda *et al*. 2004), *Dunaliella salina* (Geng *et al*. 2003), and *Chlorella vulgaris* (Chow and Tung 1999) have been transformed in this way. Two algal species have been genetically modified in a different way, namely by *Agrobacterium tumefaciens*-mediated transformation via tumor inducing (Ti) plasmids, which integrate semi-randomly into the genome of infected plant cells. The *Agrobacterium* infection causes tumors ("crown galls") in dicots and some monocots, and, astonishingly, some algae become infected, but they do not develop tumors. *Agrobacterium*-commended mediated transformation was demonstrated to work in the multicellular red alga *Porphyra yezoensis* (Cheney *et al*. 2001) and, most surprisingly, in the unicellular green alga *Chlamydomonas reinhardtii* (Kumar *et al*. 2004).

The subsections above show that there is a considerable spectrum of ways to introduce DNA into algal cells. With the benefit of hindsight, micro-particle bombardment might be the best method to start with if someone intends to produce transgenics with a previously untransformed alga in a background of other experimental uncertainties, like the functionality of utilized promoters and selectable markers. Though this challenging method requires an expensive particle gun and results in remarkable running expenses, it seems to work, in principle, with any type of cell regardless of the consistency and rigidity of the cell wall. The penetrating power of the micro-projectiles can be increased without difficulty, such that even the tough silica cell walls of diatoms do not form an impenetrable barrier.

EXISTING PROBLEMS

Even though the importance of algal biotechnology and genetic engineering has quickly increased, there are still difficulties, inconveniences and problems to be solved.

Problems in the field of genetic engineering

During production of transgenic algae, researchers often have to struggle with the problem that a gene construct is not expressed as desired, even though all elements required for transcription and translation have been included and the construct was integrated into the genome. This gene silencing occurs e.g. through methylation, and is caused by positional effects and epigenetic mechanisms. It is often related to the control of development and to the response of a cell to viruses, transposable elements, or other foreign DNA or unnaturally placed DNA (Cerutti *et al*. 1997; Wu-Scharf *et al*. 2000). Often, screening of a larger number of transformants for a transformant with high expression solves this problem.

Other difficulties arise when DNA constructs with a heterologous origin are used. One important point in this context is the bias in codon usage which is typical for almost every species. When common codons of the DNA donor species are rarely found in the genes of the target organism, the corresponding tRNA abundance will be low and this will be disadvantageous for translation and, as a consequence, for the expression rate. This situation is at its most extreme when a codon is not present at all in the target species. One strategy to circumvent this problem is to look for heterologous genes that have codon usage similar to genes of the target organism. In this way, for example, the *Streptoalloteichus hindustanus ble* gene was identified as a useful selectable marker for transformation of *Chlamydomonas reinhardtii* (Stevens *et al*. 1996) and *Volvox carteri* (Hallmann and Rappel 1999). Another strategy to manage this problem is to completely re-synthesize the heterologous gene by adhering to the codon usage of the target species. In this way the *Aequorea victoria GFP* gene and the *Renilla reniformis luc* gene have been converted to ideal reporter genes for expression in *Chlamydomonas reinhardtii* (Fuhrmann *et al*. 1999, 2004).

Introns pose another difficulty for transgenesis. Heterologous genes should not contain their own introns as they will likely not be spliced correctly; cDNAs should be used. However, genes without introns are often poorly expressed. This problem can be solved by introducing homologous introns into the heterologous coding region. The usefulness of such chimeric genes has been demonstrated, for example, in *Chlamydomonas reinhardtii* (Lumbreras *et al*. 1998) and *Volvox carteri* (Hallmann and Rappel 1999).

Problems also arise from heterologous flanking sequences, in particular, promoters. Even if some heterologous promoters, like CaMV35S and the SV40 promoters (see above), have been demonstrated to work in some species, inadequate recognition of the heterologous promoter region and lack of adequate regulation is the normal case; likewise, heterologous 3′ untranslated regions may cause incorrect polyadenylation and can also inauspiciously influence regulation. As a consequence, flanking sequences should come from the target species, if procurable.

Additional problems have been reported concerning insufficient DNA delivery, failure to integrate into the genome, or improper transport into the chloroplast or through the plasma membrane into the extracellular compartment. These problems and those mentioned in the subsections above are conquerable and, actually, they are not specific to algal systems since they are also known to occur with plants or other eukaryotes.

So far, *Chlamydomonas reinhardtii* is the only alga with an almost perfect repertoire of molecular tools that allows

for comprehensive genetic engineering. A couple of other species fulfill most requirements for smooth gene manipulations, but they have to catch up with respect to one or more points, like sequenced and annotated genomes, availability of molecular tools or transformation procedures (see above). The level of accessibility of a given species for gene manipulations roughly correlates with the number of groups working with a given species, so there do not seem to be general problems. Clearly more work is needed to push algal transgenics, especially in those species that are model organisms in basic research and in those that are particularly interesting for industry.

Problems in the field of biotechnology

Although algae in general and transgenic algae in particular describe a promising source for expression products and other compounds, the commercial application of wild-type algae is still limited (Borowitzka 1999) and that of transgenic algae is in its infancy.

One limiting factor for growth is light. On the one hand it is advantageous that (micro)algae grow up to high densities in photo-bioreactors or even open pond systems, on the other hand light becomes limiting in dense cultures beyond the first few centimeters and this restricts cell growth. One strategy to solve such problems is the development of special bioreactors (Morita *et al*. 2002), the use of stirred tanks, or shallow ponds. An alternative approach is the use of heterotrophic algae and addition of the required organic substrate. Another strategy is to transform photoautotrophic algae into heterotrophic algae by introducing a gene for a sugar transporter into their genome by genetic engineering. This was already accomplished in *Volvox carteri* (Hallmann and Sumper 1996) and *Phaeodactylum tricornutum* (Zaslavskaia *et al*. 2001). The diatom *Phaeodactylum tricornutum* grew heterotrophically even in the dark with glucose as the only carbon source (Zaslavskaia *et al*. 2001).

Algal cultures in bioreactors are normally axenic, but the culture volume is limited and sterilization is quite expensive. When large scale production is done in open pond systems, large starter cultures grown in closed photo-bioreactors are necessary in order to avoid overrun of the species of interest by other species in the unsterile open pond system (Walker *et al*. 2005b). Alternatively, large closed culture systems have to be developed that will, most likely, be very expensive.

Notably, open pond systems also require the development of strategies that reduce the probability and impact of gene flow between transgenic algae and their wild relatives. The likelihood that a transformed alga will escape and that the transgene will spread in the environment depends on its potential fitness impact, which is controllable to some extent by the genetic engineer.

Last but not least, harvesting of algae from an open pond is still inefficient and expensive, let alone extracting and purifying bioproducts. So, clearly more work is needed to optimize algal biotechnology for extensive commercial use.

PUBLIC ACCEPTANCE AND BIOSAFETY OF TRANSGENICS

The objective of both (algal) transgenics and traditional breeding is to genetically improve the characteristics of a certain species, so that the resulting organism has new, desirable traits. A fundamental difference between these techniques is how this goal is reached. Actually, biosafety assessments and public attention should focus on the effective properties of the modified organism and not on the process by which it was produced; but public acceptance of transgenics is much lower than that of traditional breeding, regardless of the quality and severity of the caused changes in the genome of the respective organism.

Since currently commercial companies sell algae or algal products only from wild-type organisms, there is no real public offense against algal transgenics so far. But most likely, as soon as commercial companies try to start using transgenic algae in food or feed products, opposition against transgenic algae will be much the same as it is currently against transgenic higher plants (Dale 1999). Health-related issues might concern worries about increased levels of toxic algal compounds, production of allergens, and dietary problems. Environment-related issues might concern fears about transfer of novel genes from one type of an algae to another, especially in more or less open aquatic systems, evolution of new strains in the wild, and impact on any nontarget species including humans. Besides, most groups that raise objections against transgenic plants or animals due to religious or ethical issues will also oppose against biotechnology using transgenic algae. Probably, only production of beneficial chemicals from transgenic algae in closed systems for use in drugs will encounter somewhat less public resistance.

However, matter-of-factly viewed, there is no evidence suggesting current products from transgenic organisms on the market are unsafe. More than 1,000 different transgenic foods are sold only in US supermarkets, but no case related to food allergies or other problems with transgenics has been reported. Also, the possible risk of gene flow seems to be much lower than estimated earlier; besides, the risk of gene flow through modern genetically engineered organisms is the same as with organisms modified by conventional breeding.

A basic prerequisite for public acceptance of biotechnological utilization of transgenic algae will be the efficiency and credibility of the corresponding testing and regulatory system for transgenic organisms. Fortunately, many national and international agencies already built a regulatory framework that is responsible for regulating and monitoring transgenic higher plants and animals. So, it would be no big deal to include transgenic algae in this existing system. Based on the experiences with transgenic plants and animals, transgenic algae or products from transgenics that passed the corresponding testing and regulatory system will not be harmful to health or risky for the environment.

UTILIZATION OF ALGAE – AN OPTIMISTIC FORESIGHT

Like almost all new technologies, algal transgenics and biotechnology also faces some challenges and problems as discussed above. However, these potential problems should be minimized as the technology evolves. In this context it might be encouraging for algal genetic engineers to remember the situation in the year 1995, when the first commercially grown transgenic higher plants were grown, but not many people were at that time able to imagine that there would be 70 million acres transgenic crops in the United States only four years later.

Algae have already been used in applications in many areas including nutrition, aquaculture, production of chemicals and pharmaceuticals, as discussed in the section "Utilization of Algae – the present situation". Most of these areas have the potential capacity to benefit and expand through the prospective use of optimized transgenic organisms. But algal transgenics and algal biotechnology also opens the door to exciting new areas and thus promises a much broader field of application, as outlined below.

Bioenergy technology

Through oncoming fossil fuel depletion, increasing air pollution, and global warming resulting from all causes, alternative energy resources have become most important. Algae can be utilized as such an alternative, and moreover, as a renewable energy source. One way is to use the biomass of macroalgae to produce methane as a fuel (as discussed above). Because of their high productivity and the accumulation of oils, biomass from diatoms may represent a future source for fuel (Pulz and Gross 2004). Another most promising way is to use algae to produce hydrogen. For many

years, the benefits of hydrogen as an energy carrier have been well-known. Hydrogen yields energy when it is either combusted or used with fuel cell technologies and it leaves nothing behind but water. Many photosynthetic prokaryotic and eukaryotic microorganisms evolved the ability to reduce protons to hydrogen during light absorption by the photosynthetic apparatus. Several projects are currently underway to optimize one of these species, the green model organism *Chlamydomonas reinhardtii*, by gene technology and other means, for efficient hydrogen production. The applied strategies include depletion and repletion of cultures with sulfur, screens for mutants with increased hydrogen production, genetic modifications of the light harvesting antennae complexes, forced overproduction of protons and electrons, and optimization of the hydrogenase enzyme (Melis *et al*. 2000; Kruse *et al*. 2005; Prince and Kheshgi 2005). *Chlamydomonas reinhardtii* has already been shown to produce 10 mol (20 g) H_2 per m^2 culture area per day (Melis and Happe 2001). If such high yields could also be reached in large scale production, this would be a really effective way to produce hydrogen as a renewable energy resource.

Bioremediation of water and soil

Lead, cadmium, and mercury are the most frequent water and soil polluting heavy metals. Industrial processes, including plastic manufacturing, electroplating, Ni-Cd battery production, mining and smelting industries, continuously release substantial amounts of heavy metals into the environment. A progressive way to return the environment altered by contaminants to its original condition is to use (micro)organisms, a process known as bioremediation. Algae readily take up heavy metals like cadmium from the environment and then induce a heavy metal stress response, which includes production of heavy metal binding factors and proteins. However, higher heavy metal levels obstruct other main processes (e.g. photosynthesis, growth) and finally kill the cells.

The wild-type green alga *Chlamydomonas reinhardtii* tolerates noteworthy amounts of cadmium during its rapid reproduction, but a genetically altered *Chlamydomonas*, heterologously expressing the mothbean *P5CS* gene, grows in the presence of much higher heavy metal concentrations. Expression of the *P5CS* gene, which catalyzes the first dedicated step in proline synthesis, in the genetically engineered cells results in an 80% higher free proline level and a four-fold increase in cadmium binding capacity relative to wild-type cells. Moreover, expression of this gene results in rapid growth at otherwise deadly cadmium concentrations (Siripornadulsil *et al*. 2002). One reason is that proline reduces the heavy metal stress for the cells by detoxification of free radicals produced as a result of heavy metal poisoning. Especially because this approach seems to be easily transferrable to other algae, generation of this transgenic *Chlamydomonas* is a significant step toward the use of algae for remediation of contaminated sites and waters.

Molecular farming

The idea of molecular farming (also called molecular pharming, biopharming or gene pharming) in (micro) algae is to generate biomolecules valuable to medicine or industry that are difficult or even impossible to produce in another way, or which require prohibitively high production costs in other systems.

One field of activity in this regard is the large scale production of antibodies in algal systems. Successful expression and assembly of a recombinant human monoclonal IgA antibody has already been demonstrated for *Chlamydomonas reinhardtii* (Mayfield *et al*. 2003). Achieving high expression in transgenic algae and simplification of antibody purification required optimization of the codons of the corresponding gene and fusion of the IgA heavy chain to the variable region of the light chain by genetic engineering using a flexible linker. In this way, antibody production can become not only much more convenient, but also much cheaper than expression in other systems. In addition, expression in an organism without an immune system allows expression of antibodies that would otherwise interfere with the immune system of the host animal used in conventional antibody production.

Algae have also demonstrated suitability for synthesizing vaccines. In this regard, stable expression of the heaptitis B surface antigen gene has been shown in *Dunaliella salina* (Sayre *et al*. 2001; Geng *et al*. 2003; Sun *et al*. 2003). Since *Dunaliella* is otherwise used for nutrition, there is no need for purification of the antigen, so the intact algae could be used to deliver a vaccine. A further project aims at the application of antigen producing algae in the fish industry. It is intended to use an alga-produced antigen to vaccinate fish against the hematopoietic necrosis virus (IHNV) which causes an infectious disease that kills 30% of the US trout population each year; vaccination is realized simply by feeding the fish with the algae (Banicki 2004).

Microalgae have also been shown to be useful for expressing insecticidal proteins. Because the green alga *Chlorella* is one possible food for mosquito larvae, the mosquito hormone trypsin-modulating oostatic factor (TMOF) was heterologously expressed in *Chlorella*. TMOF causes termination of trypsin biosynthesis in the mosquito gut. After feeding mosquito larvae with these recombinant *Chlorella* cells the larvae died within 72 h (Borovsky 2003). Because diseases such as malaria, dengue and west Nile fever are transmitted via mosquitoes, mosquito abatement is an expensive requirement in tropical countries. Use of such transgenic algae might be a much cheaper alternative.

The utilization of algae as an expression system is not restricted to antibodies, antigens, or insecticidal proteins. Most notably, *Chlamydomonas reinhardtii* offers a general, attractive alternative to the traditional but costly mammalian-based expression systems (Franklin and Mayfield 2004). Also, expression of proteins that harm mammalian cells on principle, or at least at higher concentrations, should be feasible in the very distantly related green algal system. Nevertheless, *Chlamydomonas* effects common posttranslational modifications like glycosylation of secreted proteins, an issue that is not or not satisfactory accomplished by common bacterial or yeast expression systems. Among other points, this characteristic makes *Chlamydomonas reinhardtii* an attractive system for expression of extracellular human therapeutic proteins.

For further biotechnological exploitation of algae, several researchers are screening extracts from a multiplicity of algal species in order to find effective organic components like secondary metabolites (Kopecky *et al*. 2000; Lubián *et al*. 2000), antifungal or antibacterial biomolecules (Piccardi *et al*. 2000), algal toxins (Piccardi *et al*. 2000), or active pharmaceutical ingredients as drug candidates (Skulberg 2000). Moreover, compounds of the primary metabolism like polysaccharides (Molton *et al*. 1980; Arad 1999; De Philippis *et al*. 2001), proteins (Molton *et al*. 1980), and fatty acids (Molton *et al*. 1980; Guil-Guerrero *et al*. 2004) are being sought and evaluated for potential pharmaceutical utilization. Identified interesting compounds can not only be used as they are, but, beyond that, they can be chemi-cally modified by adding or changing functional groups to change or enhance their bioactivity in order to produce new pharmaceuticals. The classical method would be to do this by organic chemistry, but the simplicity of generating transgenic organisms in species like *Chlamydomonas* also provides an opportunity to genetically alter the algae by using heterologous genes in such a way that they produce the modifications *in vivo*.

CONCLUDING REMARKS

There is clearly more basic research that needs to be performed before algal transgenics and algal biotechnology reach a capacity to compete with other systems. But since

many physiological, morphological, biochemical, or molecular characteristics of algae are quite different from higher plants or animals, algae can meet several requirements that other systems cannot sufficiently accomplish. This is one reason why algal systems gain more and more influence in the production of substances of economic, industrial, and pharmaceutical importance. The promising opportunity to use transgenic algae derived from a well-investigated, fast-growing species, like *Chlamydomonas*, as a bioreactor, has already resulted in several business start-ups in this field during the last few years and also some established biotechnology companies consider the use of transgenics. Genetic modifications that enhance the physiological properties of algal strains and optimization of algal production systems should further improve the potential of this auspicious technology in the future.

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