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Review article

Functional genomic and transformation resources for commercially important red macroalgae (Rhodophyta)

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ABSTRACT

Red macroalgae underpin many commercially important food, pharmaceutical and other important industries. To date, research into these species has generally focused on improving seaweed cultivation, developing new methods to extract useful compounds, or identify novel applications. Due to their economic importance, there is a requirement to develop a more complete understanding of the genome and metabolic pathways in these key seaweed species. This review describes progress in genomics, transcriptomics, protoplast isolation, and transformation approaches. It also explores the potential of genome editing using the CRISPR/Cas system to further our understanding of gene function related to different metabolic pathways and resolving unexplored aspects of macroalgal physiology traits linked to crop improvement. The application of functional genomics is essential to gain a complete understanding of both physiological and metabolomic processes, that will ultimately enhance the commercial resilience of macroalgae related industries that are subject to numerous pressures, including climate change. Although the use of genetic manipulation to alter growth characteristics or composition in seaweed will not readily apply to the macroalgae industry in the short term, it is likely to be critical for sustaining future commercial growth. The functional characterisation of macroalgal genes through the CRISPR/ Cas approach promises to open new avenues for translational research on utilising macroalgal resources for the sustainable development of these aquaculture systems.

1. Introduction

Seaweeds are the major primary producer in the coastal marine ecosystem. This environment is home to Rhodophyta, Chlorophyta and Phaeophyta, often termed red, green and brown macroalgae, respectively, of which red algae (Rhodophyta) have the most diverse habitat and cellular organisation. There are 7346 species within the phylum Rhodophyta, of which 680 are marine; compared to 6778 Chlorophyta (approximately 10 % are marine) and 2067 Phaeophyta (most of them marine) [1]. Macroalgae are a diverse group that has historically been utilised for a wide range of purposes, such as for food and animal feed, soil conditioner and fertiliser, and biorefining feedstock [2,3]. Globally, various products produced from macroalgae have an estimated total annual value of US$10.31 billion [4]. Traditionally, algae have been harvested from natural populations (1.2 million tonnes per year), but these wild stocks are limited, and over the last 50 years, there has been an increase in managed cultivation (27.3 million tonnes per year) [4]. Aquaculture allows the producer to select a cultivar based on the local environmental conditions and the intended use of the harvested material. It also generates an opportunity for macroalgae breeding through modern breeding approaches. Red macroalgae are highly abundant and diverse, they are known to possess unique biosynthetic pathways leading to the production of novel cell wall components, pigments, and bioactive compounds [5–7].

In the field of seaweed omics research, more than half of the studies focused on red seaweed are centred around the genera *Pyropia* and *Gracilaria*. Following closely were species from the *Gracilariopsis* (family Gracilariaceae) and *Porphyra* (family Bangiaceae) genera. *Pyropia* and *Porphyra* are related genera commonly used for nori in sushi rolls, while *Gracilaria* and *Gracilariopsis* species are predominantly used for agar production. The omics literature has seen relatively fewer contributions from the genera *Kappaphycus*, *Grateloupia*, *Laurencia*, *Asparagopsis*,

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Table 1
Commercially important red macroalgae and their primary products.

Species	Uses	Reference
Ahnfeltia spp.	Hydrocolloid agar for food	[11]
Callophyllis variegata	Food	[12]
Chondrus crispus	Food, κ and λ carrageenan in dairy products, thickening, stabilising agent, and cosmetics	[11]
Furcellaria lumbricalis and F. fastigiata	Furcellaran as gelling agent, colloid, viscosity control agent in food, pharmaceuticals, toothpastes, bacterial growth media	[13]
Gelidiella spp	Hydrocolloid agar in food	[14]
Gigartina spp.	Food, κ carrageenan in dairy products, thickening and stabilising agent	[15]
Gloiopeitis furcata	Funoran is used as an adhesive in the pottery and textiles industry, hair waving and dyeing or as textile and paper sizing agents.	[13]
Gracilaria spp.	Ogo, ogonori and sea moss as food or feed, and hydrocolloid agar in food, feed or laboratory reagent.	[16]
Hypnea musciformis	Hypnean as a gelling agent in food	[13]
Mastocarpus stellatus	Cosmetics	[17]
Mazzaella spp.	Food, carrageenan in dairy products, food, thickening and stabilising agent	[18]
Palmaria palmata	Food and feed	[19]
Phyllophora spp.	Phyllophoran	[13]
Phymatolithon spp.	Fertiliser and soil conditioner	[11]
Porphyra dentata	Catechol, rutin and hesperidin as anti-inflammatory agents	[20]
Py. yezoensis	Porphyran (a sulfated polysaccharide) and for food, for example, nori	[21]; [22]; [23]
Pyropia/Porphyra sp.	Phycoerythrobilin as an antioxidant agent and food	[24]
Rhodymenia palmata	Food	[13]
Sarcothalia spp.	Food, κ and λ carrageenan in dairy products, thickening and stabilising agent	[25]

Bangia, *Eucheuma*, and *Plocamium*. The publications in this area are primarily associated with the field of evolution, accounting for 46 % of the total published studies followed by fields of ecology, natural products and their biosynthesis, omics methodology, and seaweed-microbe interactions [8]. However, to fully exploit red algae as a source of food, feed, and high-value and bulk chemical feed stocks, a complete understanding of Rhodophyta genetics and the development of molecular genetic tools is required. Although genetic engineering techniques are well established in microalgae for both basic research and medical applications (such as the production of antibodies and vaccines) [5–7,9], macroalgae are significantly lagging in this respect.

Omics studies focusing on the ecology of seaweeds have significantly contributed to our understanding of the molecular mechanisms involved in stress tolerance, adaptation, and toxins. These investigations have provided valuable insights into the ecological aspects of seaweed biology, shedding light on how these organisms respond to various stressors and adapt to their environment [8]. The utilization of seaweed as a valuable source of natural products for pharmaceutical and nutraceutical purposes is expected to grow. To ensure this is done sustainably, it is important to make progress in seaweed cultivation techniques, which can be informed by genetics and other relevant biotechnologies. While such research is often motivated by industry interests, it is desirable that comprehensive databases of seaweed-derived omic data, including raw and assembled, are freely accessible to researchers. Such resources would greatly contribute to furthering our understanding of these fascinating marine organisms and enable future studies to expand our knowledge in this field.

Significant uncertainties persist regarding the construction of a precise phylogenetic tree for red seaweeds and red algae as a whole. In order to tackle this challenge, the National Science Foundation initiated the Red Algal Tree of Life (RedToL) project, which aimed to address this issue through the application of phylogenetic and genomic methods [8]. Consequently, there is a growing imperative to expand the collection of organelle and nuclear genomes specifically from red seaweeds. This endeavour will contribute to the reconstruction of the red algal tree of life, providing valuable insights into the evolutionary relationships within this group of organisms (Rhodophyta).

Here we reviewed the current status of the genetic tools and resources available for red macroalgae and analysed the gaps and bottlenecks for applied research using a Boolean search on Clarivate Web of Science covering a time-span from 1965 to 2022; the following search terms were used to identify publications on the functional genomics and transformation resources of rhodophyte macroalgae: (“Rhodophyta“ or ‘red seaweed’ or macroalgae”), (“EST or expressed sequence tag”), (“transcriptomics or RNA seq“), (protoplast isolation from macroalgae), (genetic engineering), (promoter) and (“CRISPR/Cas approach” or “genome editing”). Many research articles have been published about Rhodophytes related to protoplast isolation, EST, genomics and transcriptome and these number seem to be increasing daily. The present review summarised all the ESTs, sequence resources and transcriptomics with a future perspective of available data along with the CRISPR approach for better use of these macroalgal resources.

2. Commercially important red algae

Various species of red algae provide valuable commercial products, such as phycocolloids (gelling, thickening, emulsifying, binding, stabilising, clarifying, and protecting agents), soil additives, fertilisers, animal feed, pharmaceuticals, nutraceuticals, and cosmetics [10,11] (Table 1). *Py. yezoensis* is a commercially important red alga that can be utilised for pharmaceutical products such as porphyran (a sulfated polysaccharide) and food, for example, nori [21–23]. While Japan was the largest producer of *Porphyra/Pyropia* in 1990, its production has declined over the years from 387,000 t in 1990 to 251,000 t in 2019 [26]. However, global *Porphyra/Pyropia* aquaculture production has increased to 3 million tonnes due to expansion in China and the Republic of Korea, which account for the majority of production [26]. In terms of production value, the global *Porphyra/Pyropia* industry ranked second, with a production value of USD 2.7 billion in 2019. Despite *Kappaphycus/Eucheuma* having nearly four times the production tonnage compared to *Porphyra/Pyropia*, the latter’s production value is higher due to its significantly higher price (average USD 0.89 kg in 2019) compared to *Kappaphycus/Eucheuma* (USD 0.21/kg) [26]. Other commercially critical red macroalgae are *Kappaphycus* sp., *Eucheuma* sp., and *Chondrus* sp., which are utilised for carrageenan production. *Gracilaria* sp. and *Gelidium* sp. are used to produce agar [27].

3. EST, genome and transcriptome sequence resources in red macroalgae

Compared to microalgae, the availability of DNA sequence data in the macroalgae remains low. Red algal genome sequencing and assembly are challenging due to their large and complex genome structure. One exception is *C. crispus,* which has a relatively compact (105 Mbp) and simple nuclear genome with no indication of large-scale duplication in its evolutionary history [6]. Its genome has been sequenced, with 9606 of its genes (approximately 65 %) annotated, including those related to halogen and carbohydrate metabolism, which showed polyphyly of cellulose synthesis and mannosyl-glycerate synthase that are also found in marine bacteria [6]. Due to technical progress and the falling cost of next-generation sequencing (NGS), the number of algal species sequenced has accelerated [28,29], allowing comparative genomics to be conducted within this phylogenetic group of macroalgae. This genomic information provides important insights into the physiology, ecology, reproduction and evolution of red algae.

In 1997 the first EST from *Gracilaria gracilis* was generated, which corresponded to a carbohydrate metabolism-related gene [30]. The construction of cDNA libraries from various red algae is reported in the

Table 2
Expressed sequence tags from red macroalgae.

Species	Number of ESTs	Reference
C. crispus	2002 (protoplasts) 2052 (thallus)	[31]
Eucheuma denticulatum	311	[32]
F. lumbricalis	4971 (Atlantic Ocean) 4466 (Baltic Sea)	[33]
Gal. sulphuraria	3024 (photoautotroph) 2246 (heterotroph)	[34]
Gp. lemaneiformis	180	[35]
Grac. changii	8088	[36]
Grac. gracilis	200	[30]
Grac. tenuistipitata	3631	[37]
Grac. tenuistipitata	2112	[38]
Gri. okiensis	1104	[39]
Kappaphycus alvarezii	523	[40]
Py. haitanensis	5318	[41]
Py. yezoensis	10,154 (gametophyte)	[42]
Py. yezoensis	190	[43]
Py. yezoensis	10,625 (sporophyte)	[44]
Py. yezoensis	719	[45]

literature, for example, from *Py. haitanensis, Py. yezoensis, Griffthsia okiensis, Gracilaria changii, Grac. gracilis, C. crispus, and Galdieria sulphuraria* (Table 2). These libraries contain genes related to metabolic pathways, photosynthesis, environmental stress, regulatory mechanisms, and identification of phase-determining genes [9,30,31,34,46,47]. The whole chloroplast genome (192,266 bp) of *Py. dentata* were generated recently by Choi et al. [48], encodes 148 genes, including 92 protein-coding, 44 tRNA-coding, and 12 rRNA-coding genes, and lacks inverted repeat (IR) regions. The partial nuclear and chloroplast genome of *Grac. changii* were also sequenced which paved the way for functional studies of individual genes and resolving evolutionary relationship of red seaweeds [49]. The genome of *Grac. domingensis* provides valuable information for phycological and aquacultural research, as it is the first tropical and Western Atlantic red macroalgal genome that has been sequenced [50].

Transcriptomics can be utilised to reveal the mechanisms present in algae that allow them to adapt to a fluctuating environment [51]. Several studies have analysed the transcriptomes of different macroalgae growing in extreme environmental conditions (Table 3). For example, transcriptome data of *Ishige okamurae* (brown algae) showed that algal Rab proteins and trehalose metabolism were key to the processes of adapting to changing abiotic conditions, including temperature and ultraviolet radiation [67–69]. According to [70], 3.4 % of the modulated genes in *Porphyridium purpureum* (red microalgae) placed under salt, and chilling stress were related to solute transporters, channels and pumps; including Na^+/K^+-ATPases (*PyKPA1* and *PyKPA2*) which transport potassium into the cell with an outward flow of Na^+ using ATP [71]. Transcriptome analysis of *Py. seriata* revealed 754 transcripts which were differentially expressed under abiotic stress (heat, drought, and freezing condition) [72]. For example, heat-shock protein (HSP) 70 which is involved in stabilising proteins and maintains homeostasis was upregulated under stress in *Py. seriata* [72]. A transcriptome study in *Py. tenera* under desiccation stress showed 1160

Table 3
Genome and Transcriptome sequence resources.

Species	Data Type	Read Size	Contig Number Unigenes	Sequencing platform	Ref
Ahnfeltiopsis flabelliformis	Transcriptome	1.5 Gb	22,183	Illumina HiSeq 2000	[51]
Asparagopsis taxiformis	Transcriptome	46.5 Mb (from Guam) 83.0.6 (from California)	9,80,759 1,104,938	Illumina NovaSeq	[52]
Betaphycus philippinensis	Transcriptome	1.8 Gb	23,279	Illumina HiSeq 2000	[53]
C. crispus	Genome	1.7 Gb	925	Sanger technology	[6]
Calliarthron tuberculosum	Genome	1.6 Gb	1,19,430	Illumina Genome Analyzer Iix	[54]
Ceramium kondoi	Transcriptome	931 Mb	23,126	Illumina HiSeq 2000	[51]
Dumontia simplex	Transcriptome	1.5 Gb	18,910	Illumina HiSeq 2000	[51]
E. denticulatum	Transcriptome	1.7 Gb	24,656	Illumina HiSeq 2000	[51]
G. furcata	Transcriptome	1.3 Gb	24,860	Illumina HiSeq 2000	[51]
Gal. phlegrea	Genome	161 Mb	11,559	454 GS FLX Titanium	[55]
Gal. sulphuraria	Genome	60 Mb	117	ONT MinION	[56]
Gp. lemaneiformis	Genome	2.8 Gb	179,736	Illumina MiSeq	[57]
Gp. lemaneiformis	Genome	88.69 Mb	62,208	Illumina HiSeq 2000	[58]
Grac. blodgettii	Transcriptome	735 Mb	19,691	Illumina HiSeq 2000	[51]
Grac. changii	Nuclear genome Chloroplast genome	35.8 Mb 183,855 bp	–	Illumina Genome Analyzer	[49]
Gp. chorda	Genome	92.1 Mb	–	Illumina HiSeq 2500	[59]
Grac. chouae	Transcriptome	1.4 Gb	14,597	Illumina HiSeq 2000	[51]
Grac. domingensis	Nuclear genome	78 Mbp	–	Pacific Biosciences RS II	[50]
Grac. vermiculophylla	Transcriptome	2 Gb	13,444	Illumina HiSeq 2000	[51]
Grat. livida	Transcriptome	1.3 Gb	14,934	Illumina HiSeq 2000	[51]
Grat. turuturu	Transcriptome	1.4 Gb	15,739	Illumina HiSeq 2000	[51]
Grat.filicina	Transcriptome	1.5Gb	49,587	Illumina HiSeq 2000	[53]
Grateloupia catenata	Transcriptome	1.6 Gb	27,157	Illumina HiSeq 2000	[51]
Heterosiphonia pulchra	Transcriptome	1.5 Gb	33,225	Illumina HiSeq 2000	[51]
K. alvarezii	Transcriptome	1.9 Gb	34,095	Solexa paired-end sequencing	[60]
K. alvarezii	Transcriptome	31.89 Gb	76,871	Illumina HiSeq 2000	[61]
K. alvarezii	Transcriptome	45.16 Gb	7,58,897	Illumina Next-Seq 500	[62]
Laurencia dendroidea	Transcriptome	52 Mbp	3887	Pyrosequencing	[63]
Mazzaella japonica	Transcriptome	1.4 Gb	25,264	Illumina HiSeq 2000	[51]
Neoporphyra dentata	Mitochondrial genome	26,807 bp	–	Illumina Miseq platform	[64]
Neosiphonia japonica	Transcriptome	1.3 Gb	25,347	Illumina HiSeq 2000	[51]
Po. purpurea	Transcriptome	869.9 Mb	20,323	454 GS FLX	[53]
Po. umbilicalis	Genome	558 Gb	2126	PacBio RS	[5]
Py. dentata	Chloroplast genome	192,266 bp	–	PacBio resequencing	[48]
Py. haitanensis	Genome	53.3 Mb	–	Illumina and PacBio	[65]
Py. yezoensis	Genome	1.9 Gb	44,634	Illumina Genome Analyzer Iix	[66]
Symphyocladia latiuscula	Transcriptome	940 Mb	32,966	Illumina HiSeq 2000	[53]

Table 4
Status of protocols for the isolation of protoplasts and/or regeneration of adult red macroalgae or organised tissues from protoplasts.

Species	Success/achievement	References
Bangia atropurpurea	Isolation	[125]
C. crispus	Isolation	[126]; [127]
Gelidium robustum	Isolation and regeneration from protoplasts	[128]
Grac asiatica	Isolation and whole plant regeneration	[129]
Grac. changii	Isolation and juvenile plant regeneration	[130]
Grac. dura	Isolation	[131]
Grac. gigas	Isolation	[132]
Grac. gracilis	Isolation and regeneration from protoplasts	[133]
Grac. lemaneformis	Isolation and regeneration from protoplasts	[134]
Grac. tikvahiae	Isolation and regeneration from protoplasts	[134]
Grac. verrucosa	Isolation and regeneration from protoplasts	[135]
Grat. filicina	Isolation and leafy thalli regeneration	[136]; [137]
Grat. sparsa	Isolation and regeneration from protoplasts	[137,138]
Grat. turuturu	Isolation and microthalli regeneraton	[139]; [136]
K. alvarezii	Isolation and regeneration from protoplasts	[140]; [141]
P. palmata	Isolation and regeneration from protoplasts	[142]; [143]
Po. crispata	Isolation and leafy plant regeneration	[144]
Po. lanceolata	Isolation and plantlet regeneration	[145]
Po. linearis	Isolation and plantlet regeneration	[146]
Po. nereocystis	Isolation and calluses or differentiated blades regeneration regeneration	[145]
Po. okamurae	Isolation and thalli regeneration	[147]
Po. okhaensis	Isolation and leafy gametophytic thallus regeneration	[148]
Po. schizophylla	Isolation and plantlet regeneration	[145]
Po. tenuipedalis	Isolation and thalli regeneration	[147]
Porphyra.sp (wild)	Isolation	[149]
Py. dentata	Isolation and conchocelis-like filaments regeneration	[144]
Py. haitanensis	Isolation and plantlet regeneration	[113]
Py. leucosticta	Isolation and leafy thallus regeneration	[150]
Py. perforata	Isolation and leafy plantlets regeneration	[145,151]
Py. pseudolinearis	Isolation and thalli regeneration	[147]
Py. seriata	Isolation and thalli regeneration	[147]
Py. suborbiculata	Isolation and thalli regeneration	[147]; [152]
Py. tenera	Isolation and thalli regeneration	[153]
Py. yezoensis	Isolation, regeneration and transformation	[147]; [90]; [152]; [136]; [153]; [110]; [111]; [89]; [112]; [86]
Solieria filiformis	Isolation	[154]
Undaria pinnatifida	Protoplast regeneration from gametophytic cells	[155]

differentially expressed genes were upregulated under abiotic stress [73]. These included transcription factors, helicases, elongation factors, components of signal transduction pathways and carbohydrate metabolism, also serine/threonine-protein and MAP kinases. A candidate gene, *PtDRG2*, which was thought to be involved in salt and osmotic tolerance, was identified using transcriptome analysis of *Py. tenera* under desiccation stress. Subsequent overexpression of *PtDRG2* in *Chlamydomonas reinhardtii* strain Mut11 resulted in increased tolerance to salt and drought stress [73]. The transcriptome of *Neopyropia yezoensis* under the light/dark cycle revealed some important genes related to diurnal rhythm regulation [74]. In *Py. haitanensis,* long-term acclimation to monochromatic light was studied through transcriptome studies which showed differential expression of energy-yielding carbohydrate catabolism-related genes [75]. The transcriptome of *Gracilariopsis lemaneiformis* at the low temperature indicated that heat shock proteins play an important role in cold resistance [76]. Thien et al. [61] studied photosynthesis in *K. alvarezii* under different light (blue, green, red) and CO_2 conditions. Similar photosynthetic apparatuses were activated by different light spectra, with C3 and C4 enzymes actively transcribed under CO_2 enrichment. This study has implications for understanding carbon metabolism and optimizing red algae cultivation).

A transcriptome study of *C. crispus* under different stress conditions (high light, high temperature, and hypo/ hyperosmotic conditions) showed that 27 % of the transcripts were differentially expressed. The genes related to energy metabolism, protein synthesis and targeting to respective organs, genes encoding ribosomal proteins, antioxidant proteins, detoxifying enzymes, heat shock proteins and ribosomal proteins were differentially expressed under stress conditions [46]. In a transcriptome study of *Py. yezoensis* a range of genes related to stress tolerance was identified. For example, about 48 genes related to reactive oxygen scavenging pathways [glutathione peroxidase, catalase, superoxide dismutase (SOD)] and 208 genes related to other stress tolerance mechanisms were identified. About ten SOD genes were identified, of which five were related to Mn-SOD, four were related to Cu/Zn-SOD, and one gene was related to Fe-SOD. Other stress tolerance genes related to heat shock proteins, chaperones and ubiquitin-related genes were also identified [77]. A transcriptome study of *Grac. changii* under hyper-saline conditions identified 199 and 200 genes upregulated and down-regulated, respectively. In hypo-saline conditions, 154 and 187 genes were upregulated and downregulated, respectively, compared to the non-stressed control. In hyper-saline conditions, vanadium-dependent bromoperoxidase, vacuolar ATP synthase subunit H, transmembrane membrane protein, HSP90, and a ubiquitin-conjugating enzyme, were upregulated, and ascorbate peroxidase, catalase, water channel proteins, ABC transporter, light-harvesting proteins, adenosyl five phosphosulfate kinase, and serine acetyltransferase were down-regulated. In hypo-saline conditions, vanadium-dependent bromoperoxidase, water channel protein, ascorbate peroxidase, ABC transporter, light-harvesting protein, phycobilisome and cysteine synthase and H^+ – exporting ATPase, polyubiquitin, light-harvesting protein, phycobilisome and serine acetyltransferase were down-regulated [78].

4. Genetic transformation, protoplast isolation and promoter and terminator utilised for transformation in red algae

Although macroalgal genetic engineering was first reported approximately 30 years ago [79], significant work remains to exploit macroalgal functional genomics's advances fully. There are only a few reports of genetic transformation in macroalgae with stably transformed individuals [80]. Early attempts at genetic engineering used transient expression of reporter genes under the control of promoters previously

Table 5
Details of genetic transformation in red algae.

Species	Tissue	Expression vector	Resistance	Expression	Gene transfer method	Promoter	Terminator	Reference
C. crispus	Thallus	pCAMBIA 1301	–	Transient	*Agrobacterium* (LBA4404)	Actin/ 35S promoter	tNOS	[94]
Grac. gracilis	Thallus	pSV-β-Gal, pCMV-β-Gal and pCaMV-β-Gal	–	Transient	Particle bombardment	SV40, CMV, pCaMV	–	[101]
Grac. changii	Thallus	pSV40-lacZ	–	Transient	Particle bombardment	SV40	–	[95]
Gp. lemaneiformis	Tertiary branch tips	GBFP-PA7 PBFP-PA7	Hygromycin	Transient	Microparticle bombardment	CaMV35S promoter, the endogenous GlAct1 promoter, and the *Py. yezoensis* PyAct1	–	[102]
K. alvarezii	Callus	GFP expression vectors	–	Stable (T_0)	Electroporation	Cytomegalovirus (pCMV-GFP), cauliflower mosaic virus (pCaMV-GFP), medaka β-actin (pmBA-GFP) and Japanese fl ounder keratin (pJfKer-GFP) promoters	–	[96]
K. alvarezii	Callus	pGWB502	Hygromycin	Stable (T_0)	*A. tumefaciens* (LBA4404 and EHA101)	CaMV 35S	tNOS	[97]
K. alvarezii	Thallus	pSV-b-Galactosidase	–	Transient	Particle bombardment	SV40 promoter	–	[82]
Porphyra spp. *M. japonica* *C. ocellatus* *B. fuscopurpurea,* *G. furcata* *Grac. vermiculophylla*	Thallus	pPyAct1-PyGUS and pPyAct1-sGFP	–	Transient	Particle bombardment	PyAct1 promoter	tNOS	[108]
Py. tenera, *Py. yezoensis TU-1* *Po. okamurae* *Py. pseudolinearis* *B. fuscopurpurea*	Thallus	pCaMV35S-PyGUS,5,6 pPyAct1-PyGUS,6,7,12 and pPtHSP70-PyGUS	–	Transient	Particle bombardment	CaMV 35S, PyAct1 and PtHSP70 promoter	tNOS	[85]
Py. yezoensis	Gametophytic thallus	pEA7	Hygromycin	Stable (more than five)	Particle bombardment	Elf promoter	CrRbcS2	[100]
Py. yezoensis	Thallus	PyGUS expression vector pEA7-PyAct1:: PyGUS, AmCFP expression vector pEA7-PtHSP70::AmCFP, GLuc expression vector pEA7-PyElf1::GLuc	Hygromycin	Stable (more than five)	Particle bombardment	PyACT1, Pthsp70, Py elf1	tNOS	[98]
Py. yezoensis	Gametophyte and sporophytes	PKPA1-PyGUS	–	Transient	Particle Bombardment	pKPA1	?	[109]
Py. yezoensis	Protoplast	pYez-Rub4-GUS, pYez-Rub5- GUS and pYez-Rub6-GUS	–	Transient	Electroporation	rbcS GUS	?	[110]
Py. yezoensis	Gametophytic cells	pPyAct1-AmCFP, pPyAct1-ZsGFP, pPyAct1- sGFP, and pPyAct1-ZsYFP	–	Transient	Particle bombardment	PyAct1 promoter	?	[91]
Py. yezoensis	Gametophytic cells	pPyAct1C-PLCδ1PH-AmCFP, pPyAct1CAkt1PH-AmCFP, and pPyAct1C-BtkPH-AmCFP.	–	Transient	Particle bombardment	*P. yezoensis* actin 1 (PyAct1)	?	[88]
Py. yezoensis	Leafy gametophyte	p35S-GUS, p35SPyGUS, pGAPDH-GUS and pGAPDH-PyGUS	–		Particle bombardment	CaMV 35S, PyGAPDH	tNOS	[84]
Py. yezoensis	Protoplasts	pATubGUS (Test) pAGUSTub3 (control)	–	Transient	Electroporation	β-tubulin	tub	[111]
Py. yezoensis	Protoplasts	pYez-Rub-GUS, pYez-Rub-GFP and pYez-Rub-LUC	–	Transient	Electroporation	Ribulose-bisphosphate-	NOS	[89]

(*continued on next page*)

Table 5 (*continued*)

Species	Tissue	Expression vector	Resistance	Expression	Gene transfer method	Promoter	Terminator	Reference
						carboxylase / oxygenase (Rubisco)		
Py. yezoensis	Protoplast	pBS-GUS and pQD-GUS	–	Transient	Electroporation	CaMV 35S	NOS	[112]
Py. yezoensis	Protoplast	pBI121 and pCAMBIAI301	–	Transient	Electroporation, PEG, PEG plus electroporation and particle bombardment	CaMV 35S		[86]
Py. tenera	Leafy gametophyte	PtHSP70-PyGUS1	–	Transient	Particle bombardment	Heat shock protein 70 promoter	tNOS	[93]
Py. haitanensis	Conchospores	Plasmid pSV-b-Galactosidase	–	Transient	Glass bead agitation	SV40	NOS	[92]
Py. haitanensis	Protoplast	pCAT@3-control vector, pHR-CAT, pMAR1-HR-CAT and pMAR2-HR-CAT.	–	Transient	Electroporation	SV40	SV40	[113]

used in higher plants and microalgae [81]. In Rhodophyta, there are reports of successful transient expression of inserted genes, for example, *K. alvarezii* [79,82], *Po. miniata* [83], *Py. yezoensis* [84–91], *Py. haitanensis* [86,92], *Py. tenera* [85,87,93], *Po. okamurae, Py. onoi, Po. variegat, Py. pseudolinearis, B. fuscopurpurea* [85,87], *C. crispus* [94] and *Grac. changii* [95]. In some macroalgae, stable integration and subsequent expression of the transgenes have also been reported; for example, *K. alvarezii* [96,97], *Py. yezoensis* [98–100] and *Grac. gracilis* [101]. In *Gp.* lemaneiformis transient DNA transformation were reported recently via microparticle bombardment [102].

4.1. Methods of transformation

Various transformation methods have been successful for transient gene expression in macroalgal cells. However, there are only a few reports of stable transformation leading to the recovery of whole, viable algae. The method of transformation depends partly on the tissue to be targeted. For example, microparticle bombardment and *Agrobacterium* have been successfully used to transform thallus cells or callus cultures derived from them [79,95,97,103–105]. Electroporation or polyethylene glycol (PEG) transformations were more commonly applied for protoplasts. There is also a report of glass bead agitation of *Py. haitanensis* conchospores are leading to transient expression [106].

The biolistic transformation uses microparticles (usually tungsten or gold) coated with DNA and projected under high velocity into the desired tissue [107]. The first report of biolistics in red algae was *Grac. changii* [95] has become the most successful and commonly used method for transforming red algae (Table 5). The advantage of the biolistic method is that it is amenable to many different cell types and target organelles, for example, chloroplast, mitochondria and nucleus. The equipment needed for biolistic transformation is relatively costly but has the advantage of maintaining control over all physical and chemical parameters, such as concentration of DNA, helium pressure, chamber vacuum, travel distance etc. Each parameter can be optimised to deliver DNA according to the properties of the target algal cell [114].

Agrobacterium tumefaciens can be used to transfer relatively large DNA molecules (up to 150 kb) and depends on several virulence genes for T-DNA insertion. [115,116]. *Agrobacterium*-mediated genetic transformation has been successfully reported in *Py. yezoensis* [117,118]. The success of *Agrobacterium*-mediated transformation depends on many factors, for example, the strain of *Agrobacterium*, vectors used and the vir gene complement available [119].

Over the last 30 years, electroporation has also emerged as a viable method for the transformation of protoplasts with a very small amount of DNA at high efficiency [120,121]. The advantage of using electroporation is that it can be applied universally to cell wall free cultures of any genera and, alongside PEG transformation, has been used to transform protoplasts of several algal genera [83,86,89,111–114,122–124] (Table 4). Successful electroporation transformation parameters, such as voltage and protoplast density, must be optimised. In *Py. haitanensis* and other model organisms, higher voltages increased the permeability of cells which has led to higher transformation efficiency [156–158]. However, the viability of protoplasts decreases as voltage increases, so this must be carefully optimised and in *Py. haitanensis* the efficiency of transformation was highest when the protoplast viability was 50 % [113]. In PEG-mediated transformation, DNA gets precipitated with a high concentration of PEG and integrated into cells. Although PEG-mediated DNA transfer to cells works at relatively high efficiency, the recovery of transformed whole viable organisms depends on the regeneration capability of the protoplast [159].

Other less common methods of transformation have also been used in algae. The advantage of using glass bead agitation against electroporation and biolistic method for transformation is that there is less cell damage, and it is more repeatable, efficient, and cheaper, with no need for specialised equipment [160]. Conchospores of *Py. haitanensis* possess either thin or no cell walls and have been successfully transformed with glass bead agitation. The transformation efficiency observed was more than six transformants per 1 million conchospores [92]. The glass bead agitation method works in microalgae lacking a cell wall, or in protoplasts. The main disadvantage of glass bead agitation is that it is ineffective in cells with a thick cell wall [161].

4.2. Protoplast isolation in red algae

Protoplasts are isolated living cells from which the cell wall has been artificially removed [162]. Some protoplasts retain the ability to divide and even regenerate into undifferentiated or specialised tissues, including into new viable plants. Protoplasts that possess this totipotency can be a good source of material for transformation [163]. In general, the regeneration of macroalgae from protoplast is much easier than other explants or callus [133]. Early demonstrations of protoplast isolation from macroalgae were done by crude mechanical methods, for example, in *Bryopsis plumosa* (green macroalgae) [164], *Boergesenia forbesii* (unicellular green algae) [165] and *B. maxima* [166]. In 1979 an enzymatic method to digest the cell wall was developed for protoplast isolation in *Enteromorpha intestinalis (now Ulva intestinalis)* (green macroalgae) [167]. The isolation, yield and regeneration of protoplasts from macroalgae are affected by various factors, including treatment of tissue with proteolytic enzymes before enzymatic digestion, enzyme concentration, concentration of mannitol or sorbitol (which maintains the osmoticum of the enzyme solution), pH and temperature. Viability and totipotency of protoplasts also depend on factors relating to the explant,

such as the age of macroalgae at the time of collection, the growth state of macroalgae; or the season in which the macroalgae was collected [80,106,125,135,140,146,148,168–172]. Although various reports are available for protoplast isolation and regeneration from macroalgae (Table 4), all the conditions mentioned above may have varied from species to species or genus to genus. Although many reports describe successful protoplast isolation in macroalgae, only a few demonstrate adult, fertile macro-algae regeneration from isolated protoplast [133].

4.3. Promoters and reporter genes

Eukaryotic promoters typically possess core elements close to the transcription start site [173]. The most common of these core elements is the 8-bp AT-rich TATA box, first discovered in *Drosophila* [174]. The TATA box is not present in all genes and is more commonly found in some genera than others. In macroalgae, the core promoter elements are not well studied [175], but the regulatory machinery for gene expression appears to be quite different in green algae compared to land plants. With the help of NGS data in algae, cis-regulatory elements can be identified, which helps to locate the endogenous promoter. The use of endogenous promoters in the genetic engineering of macroalgae could enhance transcription efficiency or target expression to a particular tissue or developmental phase [176,177]. Successful expression of foreign genes in macroalgal cells needs suitable promoters, but only a few algal promoters are identified and available for heterologous gene expression. For example, the endogenous tubulin promoter was used to drive the transient expression of GusA in *Py. yezoensis*. This promoter lacks the typical cis-acting TATA and CAAT box elements [178] and has a high GC content (66.42 %); GUS expression was found to be higher than that seen with the CaMV 35S promoter.

In another study using transient expression of GUS in *Py. yezoensis,* the endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, was used [84]. This GADPH promoter also lacks a TATA box and other common motifs commonly present in higher plants. Further, according to Fukuda et al. [84], a GUS reporter gene that had been codon optimised for red algae expressed much more strongly than a non-optimised one, showing that atypical codon usage can inhibit translation in Rhodophyta. The *Py. yezoensis* codon-optimised GUS gene was also efficiently expressed under the control of a *Py. tenera* HSP70 promoter in *Py. tenera.* The PtHsp70 promoter was much more efficient than the PyGAPDH promoter for the expression of the GUS gene in *Py. tenera.*

Human and plant codon optimised genes, such as humanised Green (ZsGFP) and yellow (ZsYFP) fluorescent proteins in *Zoanthus* species, and plant-adapted sGFP (S65T) and humanised *Anemonia majano* cyan fluorescent protein (AmCFP) were successfully expressed in *Py. yezoensis* [87]. According to Uji et al. [91], the subcellular localisation of elongation factor 1 (*PyElf1*) and multiprotein bridging factor 1 (*PyMBF1*) was visualised in *Py. yezoensis* by simultaneous expression of ZsGFP and AmCFP. According to Hirata et al. [85], transient expression of *PyGUS* and *sGFP* reporters under the control of heterologous *PyAct1* promoter (*Py. yezoensis* system) were also effective in Bangiophycean algae, but promoter activity was lower compared to *Py. yezoensis*. In *Chondrus ocellatus, Gloiopeltis furcate, Grac. vermiculophylla* and *M. japonica* expression of *PyGUS* and *sGFP* under the control of *PyAct1* promoter was unsuccessful, indicating that promotors from one red algal species are not directly transferable to others [85]. The simian virus (SV40) has also been successfully used for the expression of foreign genes, such as LacZ and eGFP, in macroalgal [92,95,103,179].

5. Potential of gene editing by CRISPR/Cas

Functional genomics has often employed spontaneous or artificially induced mutants to elucidate gene function. Traditional forms of random mutagenesis have recently been superseded by methods of targeted gene editing [180]. Target sequences can be edited using a range of molecular tools such as protein-directed ZFN (zinc finger nuclease), TALEN (transcription activator-like effector nucleases), or nucleotide-directed endonucleases such as CRISPR (clustered regularly interspaced short palindromic repeats) [181–183]. Although various reports are present for successful genome editing by ZFN and TALEN, the construction of targeted arrays which result in high-efficiency editing is complicated [180].

CRISPR originated from the bacterial and archaeal immune system and contained an RNA guided CRISPR associated (Cas9) nuclease, CRISPR RNA (crRNA) (40 nucleotides) and trans-acting crRNA (tracrRNA) (guidance and activation of Cas nuclease) [184,185]. The crRNA and tracrRNA can also be fused to form single guide RNA. The Cas 9 nuclease has two catalytic subunits, RuvC and HNH. The RuvC and HNH subunits act on the non-complementary and complementary strands of DNA, respectively [186]. The CRISPR/Cas complex cuts at a specific site in the genome which is often repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR) [185]. This system requires the presence of a conserved protospacer-adjacent motif (PAM) sequence (NGG for the native Cas9) [186–189]. The NHEJ results in random insertion or deletion, whereas HDR results in targeted deletion, insertion, or correction [190–192]. Breaks are preferentially repaired via the NHEJ pathway, which is more common than the HDR pathway [193].

In addition to wild-type Cas9, the encoding DNA is often codon optimised for the relevant plant codon usage [194]. In *Arabidopsis thaliana* and *Nicotiana benthamiana,* the potato IV2 intron was inserted into Cas9 to prevent adverse effects in *Escherichia coli* cells [195]. Nuclear localisation of Cas9 protein via the addition of single or double nuclear localisation signal (NLS) further increases editing rates. The Cas9 can also be expressed with a tag, for example, a flag or GFP. Most commonly, Cas9 is expressed under the control of a CaMV promoter, but others, such as ubiquitin, have also been used in various plants [194]. The second component of CRISPR/Cas9 is the sgRNA, and it is commonly expressed under the control of a U6 or U3 promoter. The U6 and U3 promoters begin transcription at a “G” and “A”, respectively [194–197].

Gene editing technology has been successfully used in various organisms [198]. CRISPR-based gene editing was successfully applied in microalgae [199–201], but there are few reports of gene editing in macroalgae. Recently in *Gp. Lemaneiformis* (red macroalgae), CRISPR/LbCas12a (*Lachnospiraceae bacterium* ND2006) system was preliminarily established in which ribonucleoprotein (RNP) complex was transformed by bombardment in thallus tips [202]. CRISPR/Cas9 gene editing was also established in brown macroalgae *Ectocarpus* in which RNP were introduced via biolistics or microinjection, targeting the adenine phosphoribosyl transferase (APT) gene [203]. Similarly, in *Ulva prolifera* (green macroalgae) CRISPR-Cas9 RNP also targeted the APT gene using polyethylene glycol (PEG)-mediated transfection as the delivery method [204]. The implementation of CRISPR/Cas9 gene editing in macroalgae will require the optimisation of the system elements, which could be rapidly determined via transient expression in the isolated protoplast. The advantage of using protoplasts for genome editing is that millions of cells can be simultaneously targeted, but the success rate may be limited as protoplasts are fragile. Thallus tissue can be directly transformed using agroinfiltration, which is a rapid process but results in a lower expression than protoplasts [194,205].

Mutation induced by genome editing can be detected using a number of methods. For example, targeting guides to a region containing a restriction enzyme site. NHEJ often result in the loss of the restriction site. Therefore, restriction provides a rapid way of eliminating un-edited sequences and enrichment of successfully edited ones [196,206]. Another method to estimate the mutation frequency is surveyor assay in which wild type and mutated genomic DNA are denatured and then allowed to anneal together before the heteroduplex DNA is digested with T7 endonuclease [183]. The survey or assay requires a large number of mutations compared to restriction enzyme-based assays.

Various factors, in addition to codon optimisation of Cas9, influence

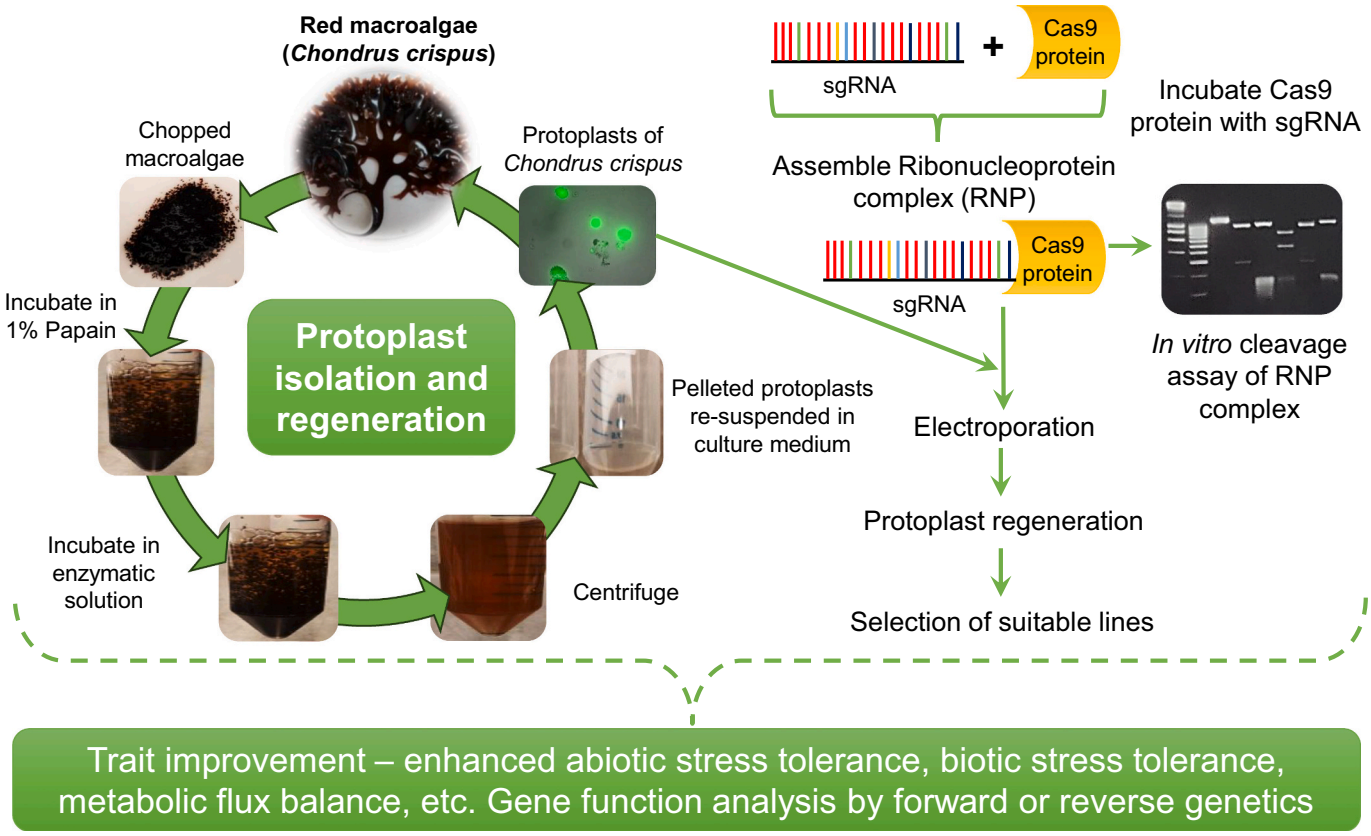

Fig. 1. Schematic representation of a potential gene editing pipeline in red macroalgae and its possible application.

the efficacy of CRISPR /Cas-based genome editing. For example, the expression efficiency of Cas9 and sgRNA, the composition of the target sequence and secondary structure off target and sgRNA. Target sequences with higher GC content lead to higher genome editing but, at the same time, increases potential off-targeting [180,207]. Further, targeted deletions may be necessary to increase the mutagenesis efficiency or remove specified regions of DNA. This can be achieved by using two sgRNA, which target a different part of a gene at the same locus. For example, in *A. thaliana,* the mutagenesis rate was increased by targeting large deletions with two sgRNA. Similarly, in *N. benthamiana* the mutagenesis rate of *NbPDS* increased by using two sgRNA 50 bp apart [195,208]. The potential for off targeting is a significant issue in CRISPR/Cas technology. The specificity of sgRNA is conferred at the 3′ end, and any mismatch at 8–10 bp out of 20 bp may result in targeting inappropriate genes.

6. Current problems and perspectives

Seaweed omics research is entering an exciting phase, integrating multiple omics approaches, and expanding to encompass a wider range of species and applications. The availability of seaweed genomes is relatively low compared to land plants and microalgae, hindering its in-depth exploration [8]. Although in recent years genomics and transcriptomics resources have also increases for red seaweeds but still, well annotated genomes and transcriptomes are still lacking. Generating high-quality reference genomes, transcriptomes, and other omics data, would serve as valuable resources for functional genomics studies. The development of such resources in red macroalgae would aid in the study of the molecular mechanisms underlying important biological processes, such as photosynthesis, nutrient uptake, and reproduction, in cultivated red macroalgae.

Functional genomics can help identify and characterize genes involved in the biosynthesis of valuable compounds, leading to a better understanding of the underlying metabolic pathways. By manipulating these pathways via the application of biotechnology, it may be possible to enhance the production of specific bioactive compounds, making red macroalgae more valuable in industries reliant on these compounds.

Future research should also focus on understanding the effects of environmental factors on seaweed communities and harnessing omics for agricultural biotechnology to improve production. Overall, the field shows significant potential for discoveries and advancements in ecological understanding and commercial applications. This knowledge can help optimize cultivation practices, including nutrient supplementation, light regimes, and growth conditions, leading to increased yields and improved cultivation efficiency. Understanding the genetic factors influencing key physiological processes under various environmental stresses, including temperature fluctuations, salinity changes, and nutrient availability can also aid in the development of sustainable cultivation methods, reducing the environmental impact of red macroalgae farming. This knowledge can be applied to develop resilient strains of red macroalgae that can thrive under suboptimal conditions, expanding their commercial cultivation range and improving overall productivity.

Red macroalgae are generally more challenging to transform compared to other organisms. Developing efficient methods for DNA transformation, and selecting transformants, remains a significant challenge in red macroalgae, and efforts to enhance DNA transformation are ongoing. In addition, genetic analysis tools to identify constitutive or tissue specific promoters, and identification of suitable terminators, pose a notable challenge in this field [102].

The development and application of gene editing technologies, such as CRISPR-Cas9, hold promise for functional genomics, and genetic improvement, in red macroalgae. These technologies can enable precise and targeted genetic modifications, enhancing our understanding of gene functions and facilitating the development of desired traits. The development of efficient CRISPR/Cas technology will help to unpick the

genetic basis of traits linked to growth rate, stress tolerance, or bioactive compound production. This could lead to development of new and improved varieties of red macroalgae with enhanced commercial value.

By improving genetic resources, optimizing transformation techniques, and leveraging emerging gene editing technologies, we can overcome these challenges and unlock the full potential of red macroalgae in various fields, ranging from agriculture to medicine and enhance environmental sustainability of its cultivation. These technologies have the potential to revolutionize red macroalgae cultivation, genetic improvement, and the production of valuable bioactive compounds, while also ensuring the sustainable and responsible management of these marine resources (Fig. 1).

7. Conclusion

A large number of red algae species are commercially important, and the increasing demands for whole and fractions of these algae are increasing. The red macroalgae are typically used as or in food, animal feed, fertilisers, nutraceutical compounds, cosmetics, and pharmaceutical compounds. The increase in these demands promotes the need to improve the strain of cultivated algae. Genetic engineering is the best option for on-shore cultivation systems to maximise algal trait improvement. The increased costs raised by farming on land may be counteracted by the benefits seen in total or selected compound yields. This, in turn, is only now possible because of the low cost of sequencing genomes and transcriptome data from valuable Rhodophyta.

CRediT authorship contribution statement

Kusum Khatri wrote the first draft and Jaykumar Patel, Jessica Adams, Huw Jones, and Dylan Phillips contributed equally throughout to editing process.

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Declaration of competing interest

Authors declare no potential conflict of interest.

Data availability

The data analysed in this review paper were obtained from previously published studies, which are all cited in the reference list.

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