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Review article

Green giant—a tiny chloroplast genome with mighty power to produce high-value proteins: history and phylogeny

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Summary

Free-living cyanobacteria were entrapped by eukaryotic cells ~2 billion years ago, ultimately giving rise to chloroplasts. After a century of debate, the presence of chloroplast DNA was demonstrated in the 1960s. The first chloroplast genomes were sequenced in the 1980s, followed by ~100 vegetable, fruit, cereal, beverage, oil and starch/sugar crop chloroplast genomes in the past three decades. Foreign genes were expressed in isolated chloroplasts or intact plant cells in the late 1980s and stably integrated into chloroplast genomes, with typically maternal inheritance shown in the 1990s. Since then, chloroplast genomes conferred the highest reported levels of tolerance or resistance to biotic or abiotic stress. Although launching products with agronomic traits in important crops using this concept has been elusive, commercial products developed include enzymes used in everyday life from processing fruit juice, to enhancing water absorption of cotton fibre or removal of stains as laundry detergents and in dye removal in the textile industry. Plastid genome sequences have revealed the framework of green plant phylogeny as well as the intricate history of plastid genome transfer events to other eukaryotes. Discordant historical signals among plastid genes suggest possible variable constraints across the plastome and further understanding and mitigation of these constraints may yield new opportunities for bioengineering. In this review, we trace the evolutionary history of chloroplasts, status of autonomy and recent advances in products developed for everyday use or those advanced to the clinic, including treatment of COVID-19 patients and SARS-CoV-2 vaccine.

Introduction

Chloroplasts have sustained life on this planet for one billion years by providing carbohydrates, amino acids, lipids for human nutrition and O_2 to breathe through the process of photosynthesis. Chloroplasts synthesize the most abundant protein on Earth (RuBisCO) and utilize CO_2, thereby playing a key role in reducing atmospheric CO_2, and moderating global warming. Although non-Mendelian inheritance was recognized in early 1900s (Baur, 1909; Correns, 1909), direct evidence for chloroplast DNA was not established until 1960s (Kirk, 1963; Ris and Plaut, 1962). In the past three decades, chloroplast genomes have been genetically modified to introduce agronomic traits mostly in model systems or to produce therapeutic products or enzymes used in everyday life. The typical maternal inheritance (with rare paternal or biparental inheritance) of chloroplast genomes offers gene containment when foreign genes are integrated into chloroplast genomes. The large copy number of chloroplast genomes (up to 10,000 in each plant cell) offers high levels of expression of introduced foreign genes. Site-specific integration of foreign genes reduces the number of transgenic lines to be evaluated. In addition, the lack of gene silencing or position effects in chloroplast genomes offers uniform expression of foreign genes. Thus, modern-day chloroplasts could play additional major roles in human health and well-being well beyond providing food, feed, fuel and oxygen.

Integration of foreign genes (>300) into chloroplast genomes resulted in their highest levels of expression and conferred the highest level of tolerance or resistance reported to date. Insulin was expressed in chloroplasts up to 70% of leaf protein or higher than RuBisCO (Ruhlman *et al*., 2010), and a high-value polymer, pHBA, was expressed up to 26.5% of leaf dry weight (Viitanen *et al*., 2004). The Cry2A operon produced folded Bt crystals within chloroplasts (DeCosa *et al*., 2001). Chloroplast expression

of Tic40 triggered the nucleus to make all inner membrane proteins, resulting in 8-20 layers of the inner envelope membrane (Singh *et al*., 2008). Although these are early examples of using very small human proteins or genes of bacterial origin, most of the challenging problems with engineered plastids remained unaddressed until recently. Failure to adequately express human or viral genes in chloroplasts has been addressed recently by developing new algorithms that relied on a large number of sequenced chloroplast genomes (Kwon *et al*., 2016; Kwon *et al*., 2018). When the largest human blood protein (FVIII, 185 kDa monomer, with pentamer assembly) was expressed in the chloroplast, the level reached industry expectations for commercial scale production and clinical evaluation (Kwon *et al*., 2018). Codon optimised expression of angiotensin-converting enzyme 2 not only helped to treat pulmonary hypertension, but has also now been tested in the clinic to treat COVID-19 patients (Daniell, 2020), as discussed in depth below. Codon-optimized expression of a novel human insulin-like growth factor in lettuce chloroplasts promoted musculoskeletal cell proliferation, differentiation and diabetic bone fracture healing (Park *et al*., 2020). The expression level of enzymes in chloroplasts and the efficacy exceeded current products made in fermentation systems using almost all evaluation criteria, without any need for purification (Daniell *et al*., 2019c; Kumari *et al*., 2019). Most importantly, a selectable antibiotic resistance gene has been removed from transplastomic chloroplast genomes in edible crops (Daniell *et al*., 2019b; Kumari *et al*., 2019; Park *et al*., 2020). Production of protein drugs in cGMP facilities, long-term stability of drugs in freeze-dried plant cells (Daniell, 2020; Daniell *et al*., 2019b; Daniell *et al*., 2019c; Park *et al*., 2020; Su *et al*., 2015), evaluation of drug substance by toxicology and pharmacokinetic studies (Daniell *et al*., 2019c) and recent FDA approval of PDs orally delivered peanut cells to treat allergy (Tilles and Petroni, 2018; Vickery *et al*., 2018) all represent major recent advances in this field. Based on these recent advances, the next decade should be particularly promising for the common use of proteins made in chloroplasts to enhance human health and well-being.

This review offers a brief overview of the history, changes in chloroplast autonomy, the use of plastid DNA sequence data to reconstruct plant evolutionary history, as well as recent advances and future use of chloroplast genomes for biotechnology applications. This summary is not intended to provide a complete list of all chloroplast genomes sequenced or foreign genes expressed but our goal is to focus on a few selected products advanced beyond laboratories for daily use or in clinical development. In addition, philosophical questions on the loss of chloroplast genes during evolution and status of chloroplast autonomy are discussed.

Female dominance and transgene containment

In 1909, Baur and Correns described the revolutionary concept of non-Mendelian inheritance factors located outside the nucleus in the protoplasm (Baur, 1909; Correns, 1909; Table 1). In crosses between variegated, yellow and green *Mirabilis jalapa* plants, branches always gave rise to the same colour seedlings, without any Mendelian segregation (Correns, 1909). Studies in the following decades showed different mechanisms for exclusion of male chloroplast DNA during sexual fusion. The generative cells divide unequally during pollen formation and do not receive any chloroplasts. In angiosperms, each generative cell forms two sperm cells, one of which fuses with a female gamete to form a zygote and the other of which unites with polar nuclei to produce endosperm, that is the process referred to as 'double fertilization'. In this process, male chloroplasts or DNA is specifically excluded or degraded, assuring maternal inheritance of the chloroplast genome in both the vast majority of angiosperms and other land plants as well, with a few noteworthy exceptions (Daniell, 2002; Daniell, 2007; Hagemann and Schroeder, 1989).

Role of maternal inheritance of chloroplast genomes gained greater attention when foreign genes were introduced into chloroplast genomes. Transgene escape via pollen and the possibility of weedy relatives capturing this valuable trait have been a major concern in nuclear transgenic crops. Therefore, integration of the herbicide resistance gene via the chloroplast genome and maternal inheritance of transgenes was considered a major accomplishment, and this invention was featured on the cover of *Nature Biotechnology* (Daniell *et al*., 1998). Subsequently, maternal inheritance of numerous foreign genes integrated into the chloroplast genome has been documented (Daniell *et al*., 2016a; Daniell *et al*., 2016b; Jin and Daniell, 2015; Table 1).

In addition to transgene containment via maternal inheritance, chloroplast engineering produces products in leaves, facilitating their harvest before appearance of any reproductive structures, thus offering complete containment. Therefore, field tests of products were conducted a decade ago for biopharmaceuticals expressed in chloroplasts (Arlen *et al*., 2007) (Table 1). More recently, USDA-APHIS certifies that 'transplastomic lines do not fit the definition of a regulated article under USDA-APHIS regulations 7 CFR part 340, because there are no plant pest components' (Kwon and Daniell, 2015). These advantages should facilitate field production of products engineered via the chloroplast genome.

Entrapment and semi-autonomous state of chloroplasts

The concept of cyanobacteria as evolutionary precursors of chloroplasts was proposed a century ago (Mereschkowsky, 1905), and a cyanobacterial origin of all plastids is now well established (Delwiche *et al*., 1995). About 1.5–2 billion years ago, free-living cyanobacteria were entrapped by early eukaryotic cells. This entrapment process has been experimentally demonstrated in laboratories through the uptake of algal chloroplasts by plant protoplasts or of green chloroplasts by albino protoplasts, and regeneration of green or variegated plants (Bonnett, 1976; Bonnett and Eriksson, 1974). Subsequent to this ancient entrapment event, there was a massive transfer of cyanobacterial genes to the nuclear genome over the long course of evolutionary history of green plants. In *Arabidopsis,* 18% of the nuclear genes originated from the ancestral plastid genome (Martin *et al*., 2002). Therefore, most of the plastid proteins are nuclear-encoded and genetically dependent on the host cell, and chloroplasts are no longer obligate endosymbionts but organelles (Cavalier-Smith, 1985).

There has been an enormous reduction in gene content from the ancestral cyanobacteria to the plastid genomes found in photosynthetic eukaryotes. The genome of the cyanobacterium *Synechocystis* PCC 6803 is 3,573 kbp and contains ~ 3,200 genes, and the plastid genome in the red alga *Porphya purpurea* is only 191 kbp, with ~ 250 genes (Reith and Munholland, 1995). Plastids of red algae and glaucocystophytes encode genes for several biosynthetic pathways, nitrogen assimilation and metabolic regulation, in addition to genes involved in protein synthesis and photosynthesis (Delwiche, 1999). Indeed, the plastid genome of the glaucophyte *Cyanophora paradoxa* contains genes for

Table 1 Milestones in plant chloroplast genome and genetic engineering/biotechnology (first reports and subsequent validation by further research). First reports on chloroplast transformation in different crop species, without useful products (only aadA gene), although important milestones, are not listed here due to inadequate follow-up studies and space limitations

Year (s)	Milestone Developments	Author/group/References
1909	Non-Mendelian inheritance	Baur (1909), Correns (1909)
1929	'Plastome' as hereditary factor	Renner (1929)
1962	Chlamydomonas Cp DNA – visual evidence	Ris and Plaut (1962)
1963	Broadbean cp DNA—visual evidence	Kirk (1963), Leff *et al* (1963)
1974-1976	Uptake of isolated chloroplasts by protoplasts and plant regeneration	Bonnett and Eriksson (1974), Bonnett (1976)
1983-1986	Synthesis of thylakoids, macro-grana functional PSI, PSII in isolated chloroplasts	Daniell *et al*. (1983, 1984, 1986)
1986	Complete sequence of the first plant chloroplast genome (tobacco)	Shinozaki *et al*. (1986)
1987	First foreign genes (chloramphenicol acetyltransferase, B-lactamase) expressed in isolated chloroplasts –	Daniell and McFadden (1987)
1990	First chloroplast vectors using the psbA regulatory sequences subsequently used in large majority of transgene expression studies in chloroplasts	Daniell *et al*. (1990, 1991)
1990	First foreign gene expression in plant chloroplasts (chloramphenicol acetyl transferase, B-glucuronidase) using the gun powder or helium gene gun – the most reproducible gene delivery system for chloroplast transformation	Daniell *et al*. (1990, 1991), Ye *et al*. (1990)
1991	The aadA gene as chloroplast selectable marker- subsequently used in most transgenes integrated into chloroplast genomes.	Goldschmidt-Clermont (1991)
1993	The aadA gene integration into the tobacco chloroplast genome in the large single-copy region	Svab and Maliga (1993)
1995	Cry1Ac gene expression in chloroplasts to confer resistance to insects, followed by subsequent reports in other genes	McBride *et al*. (1995), Kota *et al* (1999), Chakrabarti *et al* (2006)
1998	First transgene integration into the Inverted Repeat regions of the chloroplast genome, subsequently confirmed by other labs to significantly enhance transgene expression by doubling the copy number, accelerate integration due to copy correction and used in large majority of transgene expression studies	Daniell *et al*. (1998), Krichevsky *et al* (2010)
1998	Maternal inheritance of herbicide resistance gene, reported in most subsequent studies for this or several other traits	Daniell *et al*. (1998), Lutz *et al* (2001), Ye *et al* (2001)
2000	Marker-free transplastomic tobacco from the large single-copy region, followed by excision in soybean transplastome	Iamtham and Day (2000), Dufourmantel *et al*. (2007)
2000	First biopolymer, biopharmaceutical subsequently, numerous biopharmaceuticals have been expressed in chloroplasts	Guda *et al*. (1999); Staub *et al*. (2000)
2001	First foreign operon (Cry2A) and Bt crystals in chloroplasts, several operons were expressed subsequently	DeCosa *et al*. (2001), Malhotra *et al*. (2016), Fuentes *et al*. (2016)
2001	First vaccine antigen (CTB) in chloroplasts followed by several vaccine antigens challenged with toxins or pathogens in animal models against anthrax, cholera, dengue, HIV, malaria, plague, polio, tetanus, etc.	Daniell *et al*. (2001), Koya *et al* (2005), Arlen *et al* (2008), Davoodi-Semiromi *et al* (2010), Gonzalez-Rabade *et al* (2011), Chan *et al* (2016), van Eerde et al (2019), Daniell *et al* (2019b), Tregoning et al (2005)
2001	First antimicrobial peptide followed by several other studies	DeGray *et al*. (2001), Oey *et al*. (2009), Gupta *et al*. (2015), Lee *et al*. (2011)
2003	First cell wall degrading enzyme in chloroplasts followed by enzyme cocktails for plant biomass hydrolysis, detergent or textile applications	Leelavathi *et al*. (2003), Verma *et al*. (2010), Agrawal *et al* (2011), Petersen and Bock (2011), Daniell *et al* (2019), Kumari *et al* (2019)
2003	First phytoremediation using chloroplasts followed by several subsequent studies	Ruiz *et al*. (2003, 2011), Hussein *et al* (2007)
2004	First engineering via somatic embryogenesis and non-green carrot plastids, conferred salt–tolerance, followed by demonstration of soybean with agronomic traits	Kumar *et al*. (2004), Dufourmantel *et al*. (2004), Dufourmantel *et al*. (2007)
2004	First metabolic engineering in chloroplasts followed by several other products	Viitanen *et al*. (2004), Harada *et al*. (2014), Apel and Bock (2009), Pasoreck *et al*. (2016)
2004	First RuBisCO nuclear SSU expression in chloroplasts followed by carboxysomes	Dhingra *et al*. (2004), Sharwood *et al* (2008), Long *et al*. (2018)
2005-2008	Complete chloroplast genome sequences of common crops—Potato, tomato, soybean, carrot, coffee, grape, orange, cotton, cassava, cocoa, lettuce, etc.	Saski *et al* (2005), Daniell *et al*. (2006, 2008), Jansen *et al*. (2006, 2008), Bausher *et al* (2006), Samson *et al* (2007), Lee *et al* (2006), Ruhlman *et al* (2006)

Table 1 Continued

Year (s)	Milestone Developments	Author/group/References
2006	First biopharmaceutical in edible crop – lettuce followed by several protein drugs	Ruhlman *et al*. (2007, 2010), Boyhan and Daniell (2011), Su *et al* (2015), Kwon *et al* (2016, 2018), Park *et al* (2020), Daniell et al (2020)
2007	First field production of cp biopharmaceuticals followed by enzyme field production	Arlen *et al*. (2007), Schmidt *et al*. (2019)
2008	First membrane protein expressed in chloroplasts increased inner envelope up to 19 layers, followed by 8 layers with TMT expression	Singh *et al*. (2008), Jin and Daniell (2014)
2010	First oral tolerance induction to treat allergies to protein drugs, followed by FVIII, Pompe	Ruhlman *et al* (2007), Verma *et al*. (2010), Sherman *et al* (2014), Kwon *et al*. (2018), Su *et al*. (2015)
2015	First DS RNA expression in chloroplasts confers protection against insects, followed by subsequent studies	Zhang *et al*. (2015), Jin *et al*. (2015), He *et al*. (2020)
2015	cGMP production and evaluation of protein drugs made in chloroplasts	Su *et al*. (2015), Park *et al* (2020), Daniell et al (2020)
2016	Evaluation of chloroplast polio vaccine by CDC, FDA, Gates Foundation team	Chan *et al*. (2016), Xiao and Daniell (2017), Daniell *et al*. (2019a)
2016	Codon optimization using > 130 chloroplast genomes and expression of the largest human protein in lettuce chloroplasts	Kwon *et al*. (2016, 2018), Chan *et al* (2016), Park *et al* (2019), Daniell et al (2020)
2017	Lettuce chloroplast FIX protects haemophilia dogs from anaphylaxis -Novo Nordisk industry evaluation	Herzog *et al*. (2017)
2019	First marker-free enzymes/biopharmaceutical in lettuce chloroplasts, integrated into the inverted repeat region, followed by other reports	Daniell *et al*. (2019c), Kumari *et al* (2019), Park *et al* (2020), Daniell et al (2020)
2019	Commercial launch of chloroplast enzyme products for textile/detergent—by PhylloZyme	Daniell *et al*. (2019c), Kumari *et al*. (2019)
2019	First Investigational New Drug studies of biopharmaceutical made in chloroplasts and advanced to human clinical trials –COVID-19	Daniell (2020), Daniell *et al* (2020)
2019	First chloroplast transformation using carbon nanotubes	Kwak *et al*. (2019)
2020	First Cas9/gRNA chloroplast genome editing, followed by subsequent studies;	Yoo *et al*. (2020), Zhan *et al*. (2019)

biosynthesis of the peptidoglycan wall, and this species retains a cyanobacterial cell wall (Löffelhardt and Bohnert, 1994). Loss of the peptidoglycan wall during the evolution of land plants is a significant irreversible step towards reducing plastid survival outside the plant cell. Reduction of plastid genome size continues (up to 113 kbp) with loss of both the inverted repeat region and *ndh* genes in land plants (Sanderson *et al*., 2015). Most land plant plastid genomes contain 110-130 genes, with ~ 80 genes coding for proteins involved in photosynthesis and other processes. In parasitic plants, photosynthetic genes are lost, but the chloroplast genomes retain genes essential for protein synthesis and origin of DNA replication (Banerjee and Stefanović, 2019).

Despite the heavy loss of genes from the ancestral cyanobacterial genome, isolated chloroplasts still retain the ability to perform protein synthesis because they contain > 50% of the total ribosomal complement of photosynthetic cells, DNA, DNA polymerase, RNA polymerase and tRNAs (Ellis, 1977). Use of S^{35} methionine and specific protein synthesis inhibitors for chloroplast (chloramphenicol) and cytosolic (cycloheximide) ribosomes made it possible to distinguish proteins synthesized in each compartment (Ellis, 1977). Isolated intact chloroplasts were shown to synthesize proteins, capable of photosystem I activity with cyclic phosphorylation (Daniell *et al*., 1983) and form macrograna (Rebeiz *et al*., 1984) with photosystem II activities (Daniell *et al*., 1984; Daniell and Sarojini, 1984) during greening in vitro. It is remarkable that protein synthetic capacity is retained in parasitic plants when all photosynthetic genes were lost.

Plastome—a century of progress

Renner coined the term 'plastome' to describe plastid hereditary factors (Renner, 1929, 1934) (Table 1). Although the presence of DNA within plastids was debated for decades, Ris and Plaut (1962) were the first to convincingly demonstrate the presence of DNA in *Chlamydomonas* chloroplasts using electron micrographs with 25 A microfibril size, sensitivity to nuclease digestion and Feulgen reaction and yellow-green fluorescence of acridine orange staining. Subsequently, DNA was shown in broad bean (Kirk, 1963) and other chloroplasts and was referred to as 'satellite DNA' (Leff *et al*., 1963) (Table 1).

Two decades after discovery of organellar DNA, the complete chloroplast genome of the first chloroplast genome (tobacco) was published by Shinozaki et al in 1986 (Table 1). Only a few crop chloroplast genome sequences were published in the following decade, and this paucity of data was a major limitation in engineering crop chloroplast genomes. The misconception was that chloroplast genome sequences are highly conserved among crop chloroplast genomes. However, ~50% of the chloroplast genome contains coding sequences which are highly conserved, but the intergenic sequences that are essential for transgene integration or that contain regulatory sequences are not conserved. Among species of Solanaceae, only four of > 150 intergenic sequences are conserved, including the *trnI*/*trnA* spacer region used in our chloroplast vectors (Daniell *et al*., 2006). Not even a single spacer region is conserved among sequenced grass

chloroplast genomes (Saski *et al*., 2007). Transgene cassettes are introduced into the chloroplast genome intergenic spacer regions, using native genes to facilitate homologous recombination. However, when tobacco chloroplast genome flanking sequences were inserted into the lettuce chloroplast genome, unique nucleotides were completely eliminated or modified to achieve 100% homologous recombination, dramatically reducing transformation efficiency (Ruhlman *et al*., 2010). Likewise, when tobacco *psbA* regulatory sequences (promoter, 5′, 3′ UTR) were used in lettuce chloroplasts, there was 80-97% reduction in translation when compared to the endogenous regulatory sequences (Ruhlman *et al*., 2010). As a result, species-specific chloroplast vectors with endogenous genes and regulatory sequences are required for efficient foreign gene expression.

Based on the work noted above, in the last decade, significant efforts were made to sequence crop chloroplast genomes that are used in everyday life including soybean (Saski *et al*., 2005), other legumes (Jansen *et al*., 2008), potato (Daniell *et al*., 2006), tomato (Daniell *et al*., 2006), grape (Jansen *et al*., 2006), coffee (Samson *et al*., 2007), cotton (Lee *et al*., 2006), orange (Bausher *et al*., 2006), cassava (Daniell *et al*., 2008), carrot (Ruhlman *et al*., 2006) and cereals (Saski *et al*., 2007). For a current list of edible crop chloroplast genomes, readers are referred to FAO: http://www.fao.org/fileadmin/templates/ess/documents/world_census_of_agriculture/appendix3_r7.pdf. - and Table 2. As discussed below, chloroplast genome sequences facilitate codon optimization and offer the best regulatory sequences to enhance translation and transgene integration. Understanding the origins of economically important cultivated species facilitates breeding and prevents cross-contamination of plants used in herbal medicine. Moreover, an understanding of the diversity of chloroplast genomes, in terms of both structure and sequence, is important for developing efficient systems for genetic engineering. However, among ~ 3,000 cultivated crops, completely sequenced chloroplast genomes are available for fewer than 70 genera. Among these, <80 complete chloroplast genomes are available in the NCBI database. However, the One Thousand Plants Transcriptome Project (1KP; onekp.com), as well as other recent efforts, have contributed over 1,000 complete or nearly complete plastid genomes to global databases, most of these from plants that are not of economic importance (Gitzendanner *et al*., 2018; Leebens-Mack, 2019; Li *et al*., 2019); hence, our understanding of plastid genomes across the Tree of Life has improved dramatically in the past decade.

Fundamental tool in phylogenetics and evolution

For a number of reasons (abundance, single-copy genes, lack of recombination and appropriate rate of nucleotide evolution), the plastid genome has long been the primary workhorse for studies of plant phylogeny and evolution. The size and structure of the plastid genome have been remarkably conserved across land plant evolution (although intergenic spacer regions and regulatory sequences are not well conserved), in stark contrast to the enormous variation in size and structure of the plant mitochondrial genome, and this conservation has facilitated the use of both sequence data and plastome rearrangements in phylogenetic analyses. As noted above, transfer of genes from the plastome to the nuclear genome has reduced the size of the plastid genome over the course of green plant evolution, with chlorophytes having larger plastid genomes and more genes than streptophytes, particularly land plants. There is also evidence of some plastid gene movement to the mitochondrial genome.

Phylogenetic analyses using plastid genes have been conducted across a range of divergences from the species level and to very deep levels. Particularly at deeper levels (e.g. at divergences traditionally recognized at the family level and deeper), plastid data have been of enormous value. Initial studies employed only *rbcL* (encoding the large subunit of RuBisCO); in a landmark study showing the utility of plastid gene sequences, a collaboration of 43 investigators provided the first DNA phylogenetic framework for seed plants based on an analysis of 499 species (Chase *et al*., 1993). Most recently, next-generation sequencing has enabled the sequencing of the complete plastid genome and the assembly of large phylogenetic trees across all green plants (Gitzendanner *et al*., 2018; Ruhfel *et al*., 2014); other studies of plastid loci have focused on major subclades of green plants (Li *et al*., 2019). Plastid phylogenetics ushered in the most the fundamental changes in our understanding of plant relationships in the past 150 years, revealing the major clades of green plants, the sister group to land plants, relationships across land plants, with a major reshaping of our understanding of moss, liverwort, fern, gymnosperms and angiosperm phylogeny. Not only have these studies resulted in a clearer understanding of evolutionary relationships, they have also prompted major new classifications for the angiosperms (APG IV 2016) and ferns (Pteridophyte.Phylogeny.Group, 2016); these are groundbreaking classifications that represent dramatic changes from anything previously published based on morphology.

In some cases, the plastid genome has exhibited enough variation to be of utility in studies at the population level and also in phylogeographic analyses (Brunsfeld et al., 2001; Soltis *et al*., 1997), although not on the scale observed with mtDNA in animals. Although hybridization has long been known to be a major force in plant evolution, molecular studies using plastid genes have revealed many unsuspected past hybridization events showing that hybridization is even more prevalent in plants than thought, with hundreds of documented cases of introgression of plastid genomes. Most of our current framework of green plant phylogenetic relationships is based on plastid genome sequence data, and current classifications are largely based on plastid gene phylogenetics. Only in the past few years as nuclear gene sequencing has become more routine have comparable nuclear gene topologies been generated. Importantly, there are discordances between plastid and nuclear trees, not only at shallow levels where introgression has long been detected, but also at deep levels (Stull *et al*., 2020; Sun, 2015), indicating putative ancient reticulation.

Studies of plastid genes and genomes have also revealed the complex history of the plastid green plant clade, with secondary and tertiary endosymbiotic events (representing the capture of photosynthetic green or red algae) occurred in other lineages, including brown algae, red algae and *Euglena* (Keeling, 2004; Keeling, 2010; Palmer *et al*., 2004). Together, this increasingly large set of plastid genes and genomes from across green plant phylogeny and other clades of photosynthetic eukaryotes provides the sequence information and resources, not only for tracing plant evolution, but also for chloroplast genetic engineering.

The technical innovations (Moore *et al*., 2006; Stull *et al*., 2013; Uribe-Convers *et al*., 2014) that enabled use of the entire plastome, or at least most of the ~ 80 protein-coding genes, as well as the four tRNA genes, typical of an angiosperm plastome, in phylogenetic analyses (Gitzendanner *et al*., 2018; Jansen *et al*., 2007; Li *et al*., 2019; Moore *et al*., 2007, 2010; Ruhfel *et al*.,

Table 2 List by of edible traits of crop/vegetable/fruit/oil/herb species that have complete annotated chloroplast genome sequences

Common name	Species	Genome size	Accession No.	References
Vegetables				
Onion	*Allium cepa*	153538	NC_024813	von Kohn *et al*. (2013)
Sweet Pepper	*Capsicum annuum*	156781	NC_018552	Jo *et al*. (2011)
Chickpea	*Cicer arietinum*	125319	NC_011163	Jansen *et al*. (2008)
Broccoli	*Brassica oleracea*	153366	KR_233156	Seol *et al*. (2017)
Cucumber	*Cucumis sativus*	155293	NC_007144	Plader *et al*. (2007)
Carrot	*Daucus carota*	155911	NC_008325	Ruhlman *et al*. (2006)
Celery	*Apium graveolens*	152050	MK036045	Zhu *et al*. (2019)
Lettuce	*Lactuca sativa*	152765	NC_007578	Jansen and Palmer (1987)
Pea	*Pisum sativum*	122169	NC_014057	Magee *et al*. (2010)
Kidney bean	*Phaseolus vulgaris*	150285	NC_009259	Guo *et al*. (2007)
Radish	*Raphanus sativus*	153368	NC_024469	Jeong *et al*. (2014)
Tomato	*Solanum lycopersicum*	155461	NC_007898	Daniell *et al*. (2006)
Spinach	*Spinacia oleracea*	150725	NC_002202	Schmitz-Linneweber *et al*. (2001)
Mung bean	*Vigna radiata*	151271	NC_013843	Tangphatsornruang *et al*. (2010)
Eggplant	*Solanum melongena*	154289	KU_682719	Sciencetechnology *et al*. (2016)
Cabbages/Turnips	*Brassica rapa*	153482	DQ231548	Li *et al*. (2017)
Globe Artichoke	*Cynara scolymus*	152529	KM035764	Curci *et al*. (2015)
Asparagus	*Asparagus officinalis*.	156699	LN896355	Sheng *et al*. (2017)
Chicory	*Cichorium intybus*	152975	MK569377	Yang *et al*. (2019b)
Pumpkin	*Cucurbita pepo*	157343	MH031787	Zhang *et al*. (2018)
Garlic	*Allium sativum*	153189	MK335928	Huo *et al*. (2019)
Welsh onion	*Allium fistulosum*	153162	MK335927	Huo *et al*. (2019)
Chive	*Allium tuberosum*	154056	MK335929	Huo *et al*. (2019)
Spice Crops				
Chili pepper	*Capsicum chinense*	156807	NC_030543.1	Park *et al*. (2016)
Aniseed/Badian	*Illicium verum*	142747	NC_034689	Park *et al*. (2019)
Fennel	*Foeniculum vulgare*	153628	NC_029469	None
Nutmeg	*Torreya grandis*	136949	KY369757	Mu *et al*. (2018)
Cinnamon	*Cinnamomum aromaticum*	152754	MN173819	Xie *et al*. (2019)
Ginger	*Zingiber officinale*	162621	MH161428	Cui *et al*. (2019)
Black pepper	*Piper nigrum*	161523	NC_034692	None
Japanese pepper	*Zanthoxylum piperitum,*	158154	NC_027939	Lee *et al*. (2015)
Fruits/melons				
Pineapple	*Ananas comosus*	159636	NC_026220	Nashima (2015)
Papaya	*Carica papaya*	160100	NC_010323	Ming *et al*. (2008)
Wild strawberry	*Fragaria vesca*	155691	NC_015206	Shulaev *et al*. (2011)
Banana	*Musa textilis*	161347	NC_022926	Martin *et al*. (2013)
Vanilla	*Vanilla planifolia*	148011	NC_026778	Lin *et al*. (2015)
Kiwifruit	*Actinidia chinensis*	156346	NC_026690	Yao *et al*. (2015)
Chestnut	*Castanea mollissima*	160799	NC_014674	Jansen *et al*. (2011)
Coco plum	*Chrysobalanus icaco*	162775	NC_024061	Malé *et al*. (2014)
Sweet Orange	*Citrus sinensis*	160129	NC_008334	Bausher *et al*. (2006)
Karaka nut	*Corynocarpus laevigata*	159202	NC_014807	Atherton *et al*. (2010)
Date palm	*Phoenix dactylifera*	158462	NC_013991	Yang *et al*. (2010)
Peach	*Prunus persica*	157790	NC_014697	Jansen *et al*. (2011)
Cranberry	*Vaccinium macrocarpon*	176045	NC_019616	Fajardo *et al*. (2013)
Wine grape	*Vitis vinifera*	160928	NC_007957	Jansen *et al*. (2006)
Chinese pear	*Pyrus pyrifolia*	159922	NC_015996	Terakami *et al*. (2012)
Cultivated Apple	*Malus domestica Borkh*	160062	MH595623	Yan *et al*. (2019)
Wild Apricot	*Prunus sibirica*	158248	MN708049	Dong *et al*. (2020)
Dwarf cherry	*Cerasus humilis*	158084	MF405921	Mu *et al*. (2018)
Mango	*Mangifera indica*	157837	NC_035239	Zhao *et al*. (2019)
Chinese Cherry	*Prunus pseudocerasus*	157834	KX_255667	Cao *et al*. (2018)
Sweet Cherry	*Cerasus avium*	157987	MH_756631	Chen *et al*. (2018)
Litchi	*Litchi chinensis*	162524	KY_635881	Rabah *et al*. (2017)
Longan	*Dimocarpus longan*	160833	MG_214255	Wang *et al*. (2017)

Table 2 Continued

Common name	Species	Genome size	Accession No.	References
Avocado	*Persea americana Mill*	152723	KX437771	Song *et al*. (2016)
Date	*Ziziphus jujuba*	161466	NC_030299.1	Ma et al. (2017)
Fig	*Ficus carica L*.	160602	NC_035237	Rabah *et al*. (2017)
Key lime	*Citrus aurantiifolia*	159893	KJ865401.1	Su *et al*. (2014)
Watermelon	*Citrullus lanatus*	156699 ~ 156907	KY_430683–KY_430693	Shi *et al*. (2017)
Sweet Melon	*Cucumis melo*	156017	JF412791	Rodríguez-Moreno *et al*. (2011)
Cereals				
Pearl millet	*Cenchrus americanus*	140718	NC_024171	Mariac *et al*. (2014)
White fonio	*Digitaria exilis*	140908	NC_024176	Mariac *et al*. (2014)
Barnyard grass	*Echinochloa oryzicola*	139891	NC_024643	Ye *et al*. (2014)
Buckwheat	*Fagopyrum esculentum*	159599	NC_010776	Logacheva *et al*. (2008)
Barley	*Hordeum vulgare*	136462	NC_008590	Saski *et al*. (2007)
Rice	*Oryza sativa*	134525	X15901	Hiratsuka *et al*. (1989)
Bread wheat	*Triticum aestivum*	134545	NC_002762	Ogihara *et al*. (2002)
Rye	*Secale cereale*	114843	NC_021761	Middleton *et al*. (2014)
Maize	*Zea mays*	140384	NC_001666	Maier *et al*. (1995)
Sorghum	*Sorghum bicolor*	140754	NC_008602	Saski *et al*. (2007)
Oat	*Avena sativa*	135890	NC_027468	Saarela *et al*. (2015)
Adlay	*Coix lacryma-jobi,*	140745	FJ261955	Kang *et al*. (2018)
Foxtail millet	*Setaria italica*	135516	NC_022850	Wang and Gao (2015)
Oil Crops				
Canola	*Brassica napus*	152860	NC_016734	Hu *et al*. (2010)
Soybean	*Glycine max*	152218	NC_007942	Saski *et al*. (2005)
Sunflower	*Helianthus annuus*	151104	NC_007977	Timme *et al*. (2007)
Castor bean	*Ricinus communis*	163161	NC_016736	Rivarola *et al*. (2011)
Sesame	*Sesamum indicum*	153324	NC_016433	Yi and Kim (2012)
Tea oil plant	*Camellia oleifera*	156971	NC_023084	Shi *et al*. (2013)
Coconut	*Cocos nucifera*	154731	NC_022417	Huang *et al*. (2013)
Oil palm	*Elaeis guineensis*	156973	NC_017602	Uthaipaisanwong *et al*. (2012)
Olive	*Olea europaea*	155862	NC_015604	Besnard *et al*. (2011)
Peanuts	*Arachis hypogaea*	156395	KX257487	Yin *et al*. (2017)
Flax	*Linum usitatissimum*	156721	KY849971	de Santana Lopes *et al*. (2018)
Mustard	*Brassica juncea*	153483	NC_028272.1	Prabhudas *et al*. (2015)
Niger seed	*Guizotia abyssinica*	151762	NC_010601.1	Dempewolf *et al*. (2010)
Safflower	*Carthamus tinctorius*	153675	NC_030783.1	Lu *et al*. (2015)
Beverage Crops				
Coffee	*Coffea arabica*	155189	NC_008535	Samson *et al*. (2007)
Cacao tree	*Theobroma cacao*	160604	HQ_336404	Jansen *et al*. (2011)
Tea tree	*Camellia sinensis*	157025	MH_042531	Meng *et al*. (2018)
Starch/Sugar Crops				
Potato	*Solanum tuberosum*	155312	DQ231562	Chung *et al*. (2006)
Cassava	*Manihot esculenta*	161453	EU117376	Daniell *et al*. (2008)
Yam	*Dioscorea polystachya*	153243to 153292	MG267375 to MG267378	Cao *et al*. (2018)
Sweet potato	*Ipomoea batatas*	161303	NC_026703	Yan *et al*. (2015)
Sugar beet	*Beta vulgaris*	149637	EF534108.1	Li *et al*. (2014)
Sugar cane	*Saccharum officinarum*	141187	MN204507	Xu et al. (2019)
Sweet sorghum	*Sorghum bicolor*	141266	NC543562	Yang *et al*. (2019a)
Medicinal plants				
Guan-bai-fu/Fu-zi	*Aconitum coreanum/ Aconitum carmichaelii*	155880/ 157040	KU318669/KY407560	Park *et al*. (2017)
Ye-Xing-Ba	*Scrophularia dentata*	152553	KT428154	Ni *et al*. (2016)
Bei Mu	*Fritillaria thunbergii*	152155	KY646165	Moon *et al*. (2018)
Di Huang	*Rehmannia chingii,*	154055	KX426347	Zeng *et al*. (2016)
San Qi	*Panax notoginseng*	156324	KT001509	Zhang *et al*. (2016)
Ginseng	*Panax ginseng*	156356	KM067388	Kim *et al*. (2015)
Rough hedge parsley	*Torilis scabra*	157855	MN105615	Yao *et al*. (2019)
Chervil	*Anthriscus cerefolium*	154719	NC_015113	Downie and Jansen (2015)

Table 2 Continued

Common name	Species	Genome size	Accession No.	References
Ma Huang	*Ephedra equisetina*	109558	MH161420	Chen *et al*. (2019)
	Ephedra intermedia	109667	MH161421	
	Ephedra sinica	109550	MH161422	
Ginger	*Zingiber officinale*	162621	MH161428.	Cui *et al*. (2019)
Bush clover	*Lespedeza cuneata*	149010	MN268503	Somaratne *et al*. (2019)
Goji berry	*Lycium chinense*	155756	MK040922	Yang et al. (2019c)
Dwarf cardamom	*Amomum compactum*	163553	MG000589	Wu *et al*. (2018)

'None' in the last column means the chloroplast genome sequences of species are available in NCBI with accession number but there is no publication.

2014; Stull *et al*., 2015) continue to be instructive about the evolution of the genome itself. Despite long-standing debate about if, when and how to combine data from different sources (molecules and morphology) or different genomes (nuclear and organellar), combining all plastid genes in a single analysis, perhaps with different evolutionary parameter values, has generally received consensus, as these genes are linked and represent a single chromosome (perhaps viewed as a single character, Doyle, 1992), and most studies have indeed combined plastid genes into a single analysis. Recent evidence, however, has revealed extensive topological discordance among trees built from individual plastid genes, both across angiosperms as a whole (Walker *et al*., 2019) and within legumes (Zhang *et al*., 2020). Although differences among plastid gene trees may arise due to biological causes—for example heteroplasmic recombination and gene transfer among plastid, nuclear and mitochondrial genomes—strong conflict is unexpected, particularly given that heteroplasmy is considered rare (Sancho *et al*., 2018; Sullivan *et al*., 2017), limiting the possibility of recombination. The factors responsible for observed discordant plastid gene trees are not well understood on an empirical level, and more research is needed into heteroplasmy and intergenomic transfer, although results to date point to both stochastic and systematic error, the latter arising due to misspecifications of the evolutionary model used in the phylogenetic analysis (Walker *et al*., 2019). Regardless of the cause of gene tree conflict, plastid genes, if combined into a single analysis, may conflate multiple phylogenetic signals, muddying the overall inference of both topology and branch lengths, with consequences for downstream analyses of divergence times, diversification, character evolution and more; thus, greater exploration of plastid gene trees is needed in future studies.

Conception and advancement of chloroplast genetic engineering

At the dawn of the GMO revolution in the 1980s, introducing herbicide resistance genes into the nuclear genome, using *Agrobacterium*-mediated transformation (Shah *et al*., 1986) with the possibility of escape of transgenes via pollen, was publicly debated. Another major concern was the development of resistance in insects to bacterial insecticidal (Bt) proteins produced in plants at low expression levels via the nuclear genome. Therefore, the concept of chloroplast genetic engineering was first demonstrated in isolated chloroplasts (Daniell *et al*., 1991; Daniell *et al*., 1984; Daniell and McFadden, 1987; Daniell *et al*., 1983; Rebeiz *et al*., 1984), with the goal of reintroduction of chloroplasts into protoplasts to regenerate transplastomic lines (Bonnett, 1976; Bonnett and Eriksson, 1974) (Table 1). However, invention of the gene gun by John Sanford at Cornell University eliminated the need for the latter step and facilitated introduction of foreign DNA directly into chloroplasts of plant cells (Daniell, 1993; Daniell *et al*., 1984; Daniell *et al*., 1990; Ye *et al*., 1990) (Table 1). After 30 years of research on foreign gene expression in chloroplasts, >75% of foreign genes (Daniell *et al*., 2016a; Daniell *et al*., 2016b) use the *psbA* regulatory sequences used in this first report (Daniell *et al*., 1990). Although several methods for DNA delivery into chloroplasts have been reported, gene gun delivery is still the only reproducible method (Table 1). Goldschmidt-Clermont (1991) introduced the first selectable marker gene for chloroplasts—the aadA gene (Table 1), which was subsequently used for transforming the tobacco chloroplast genome (Svab and Maliga, 1993) and most other crops (Jin and Daniell, 2015). Although foreign genes were introduced into the transcriptionally silent spacer region (Svab and Maliga, 1993), transcriptionally active spacer region in the inverted repeat region of the chloroplast genome was proposed by Daniell *et al* (1998) and is now the most widely used spacer region for transgene integration (Daniell *et al*., 2016a, b; Daniell *et al*., 1998; Jin and Daniell, 2015).

Codon optimizer algorithm for expression of large human genes in chloroplasts

Chloroplast genomes have been engineered to express foreign genes from bacterial, fungal, protozoan or human genomes for various biotechnology applications. It is quite astounding that a bacterial operon from *Bacillus thuringiensis* was expressed utilizing bacterial regulatory sequences and achieved the highest level of Bt protein reported (DeCosa *et al*., 2001). Small human genes (insulin) are expressed at very high levels (up to 70% of leaf total protein (Boyhan and Daniell, 2011; Ruhlman *et al*., 2010)). However, when large human genes are introduced into the chloroplast genome, there are poorly translated. Therefore, a new codon optimization program was developed based on codon usage of the most highly expressed *psbA* genes from 133 sequenced chloroplast genomes (Kwon *et al*., 2016). The first iteration used a new codon optimizer algorithm that followed the codon usage hierarchy observed among sequenced *psbA* genes. Synonymous codons for each amino acid were ranked according to their frequency of use. Codon optimization of the human blood clotting factor FVIII-HC (2262 bp) modified 406 codons out of 754 amino acids. Native FVIII-HC used the CTC leucine codon 11 times but the codon-optimized HC eliminated all CTC codons. Likewise, another rare codon, TCA (serine), used 16 times in the native FVIII-HC coding sequence, was completely eliminated after codon optimization. Collectively, the codon-optimization algorithm eliminated 105 rare codons from the FVIII-HC native human

gene, resulting in enhanced expression. Similar codon optimization of the CTB-FVIII-LC gene resulted in successful expression of the largest human blood protein (FVIII, 185 kDa monomer, with pentamer assembly) in chloroplasts (Kwon *et al*., 2018), and this has now advanced to commercial production and clinical trials. Codon-optimized Ace2 is now tested in the clinic to treat COVID-19 patients (Daniell, 2020).

From conception to commercialization of leaf enzymes

The global enzyme market was valued at US $11 billion in 2018; it is used in the food/beverage (37%), cleaning (26%), bioenergy (15%) and animal feed (13%) industries. Rising demand in detergents, textile and wastewater treatment applications are propelling significant growth in the enzyme industry. Enzymes are now used as biofertilizers and soil enhancers to accelerate growth through enhancing beneficial microbes. Carbohydrase led the product segment, with 48% market share in 2018. Globally, the Asia Pacific region leads the industrial enzyme demand ($4.6 billion), followed by Europe ($2.68 billion) and the United States has the largest (38%) market share of industrial enzymes.

Waxes and pectins decrease water absorption of native cotton fibres; contrary to common understanding, natural cotton fibre does not absorb water or dyes (Lin and Hsieh, 2001). To enhance absorption of harsh chemicals, alkaline pH and other severe conditions have been used, but these are environmentally problematic in that they release toxic effluents. Therefore, pectinases are used for textile bioscouring to enhance water absorbency of cotton fibres, without causing cellulose destruction. Likewise, lipases are used in the detergent industry to remove oils or butter stains (Jaeger and Reetz, 1998). However, performance of current commercial lipases is limited by minimal activities in alkaline pH or at higher temperature (detergents are alkaline and washing machines often use hot water).

Mannans are found in chocolate, ice cream and tomato ketchup, which are unfortunately the most common cloth stains (Chauhan *et al*., 2012). Mannanase hydrolyses insoluble mannan into water-soluble smaller oligosaccharides (Dhawan and Kaur, 2007), thereby facilitating stain removal. However, current mannanase products are not active through a wide range of temperatures or pH (Sarmiento *et al*., 2015). Mannanase is also used in the paper and pulp industries, bioethanol production, oil and gas well stimulation, food and feed, nutraceutical and pharmaceutical industries (Srivastava and Kapoor, 2017; Zyl *et al*., 2010). Endoglucanases and exoglucanases are useful in enhancing colour brightness and fabric softening (Agrawal, 2017). These enzymes are used in denim biowashing, biostoning and biopolishing to remove dye from the fibril surface with minimal damage to fabrics (Anish *et al*., 2007; Araújo *et al*., 2009; Miettinen-Oinonen and Suominen, 2002). Cocktails of cellulases, hemicellulases and pectinases are used for fruit juice clarification, concentration and reducing viscosity (Brito and Vaillant, 2012; Sharma et al., 2016).

Currently, industrial enzymes are produced in microbial systems that are expensive and that rely on decades-old processes. Building fermentation facilities and their maintenance free of contamination is the first challenge. Purification of enzymes from host cells and their formulation to increase concentration, stability and cold storage/transportation are all very expensive. Therefore, several enzyme products made in leaves and expressed in chloroplasts (Agrawal *et al*., 2011; Jin *et al*., 2011; Leelavathi *et al*, 2003; Verma *et al*., 2010) were launched recently by PhylloZyme in order to replace prohibitively expensive microbial enzyme processes (Daniell *et al*., 2019a; Daniell *et al*., 2019c; Kumari *et al*., 2019). This is the first report of commercially launched protein products made in leaves (Table 1; Figure 1). Leaf pectinases, endoglucanases, lipases or mannanases were validated with 23 commercial microbial enzyme products for textile (dye binding, removal, depilling), detergent (stain removal) or juice industry (clarification) applications. Most leaf enzymes function in broad pH/temperature ranges as crude leaf extracts without the need for purification, and leaf enzymes could be stored as lyophilized plant cells at ambient temperature for several months/years, without loss of enzyme activity.

CO_2 emission by fermentation and capture by chloroplasts

Commercially produced enzymes are widely used in daily life from coffee/juice in the morning, textiles worn during the day (enhancing water absorption, dye binding), cleaning of dishes/clothes (detergents), evening beverages (wine, beer) or digesting food (fat, carbohydrates). Currently, these enzymes are produced in yeast, fungi or bacteria in fermentation systems. Life cycle analysis of β-glucosidase, used for digesting milk products, revealed that, for each kg of β-glucosidase enzyme, 52 kg of CO_2 are released from fermentation, along with 18,140 kg wastewater and > 15 kg of solid debris and carbon monoxide, methane and other toxic gasses, in addition to environmental stress caused by toxic chemicals used in cleaning the fermenters used in enzyme production (Feijoo *et al*., 2017). Considering the millions of tons of microbial biomass produced every day, dealing with global CO_2 emission and toxic effluents is a major environmental challenge.

Here, we use cellulase production with the tobacco enzyme production platform to evaluate the carbon capturing potential of leaf enzyme technology. Assuming a standard tobacco production for 12 weeks, reaching a final leaf area index (LAI) of 2.5, and average photosynthetic properties as reported in (Schmidt *et al*., 2019), respiration of 30% of the total photosynthetic CO_2 uptake (Amthor, 1989; Amthor, 2000), then the total CO_2 uptake by such a tobacco canopy to produce 1 kg cellulase will be about 570 kg. If at the end of the growth cycle, if LAI is 6 instead, a net photosynthetic CO_2 uptake of 484 kg is predicted during the production of 1 kg cellulase. Replacement of enzymes made by fermentation by leaf enzymes could capture up to 570 kg CO_2 in addition to preventing 110 kg CO_2 release for a net capture of 680 kg CO_2 for each kg of enzyme/protein produced, altogether leading to decreased anthropogenic greenhouse gas emissions.

Affordable protein drugs and vaccines—from laboratory to the clinic

Although protein drugs like insulin save millions of lives, they are not affordable for a large majority of the global population. Indeed, insulin pumps cost $6,000-$12,000 but one third of the global population earn <$2 per day. Therefore, new approaches are needed to produce and deliver protein drugs (PDs) more cost effectively. Edible plant cells offer the opportunity to express and orally deliver PDs. Upon oral delivery, PDs are protected in the stomach from digestion by enzymes because human or animal enzymes do not break beta linkage of plant cell wall polymers. However, when intact plant cells reach the gut, enzymes released by commensal microbes in the gut digest plant cell wall, releasing PDs into the gut lumen (Kumar *et al*., 2020). When tags are fused to PDs, they cross gut epithelium and reach the circulatory or immune system (Figure 2). Therefore, oral delivery of PDs

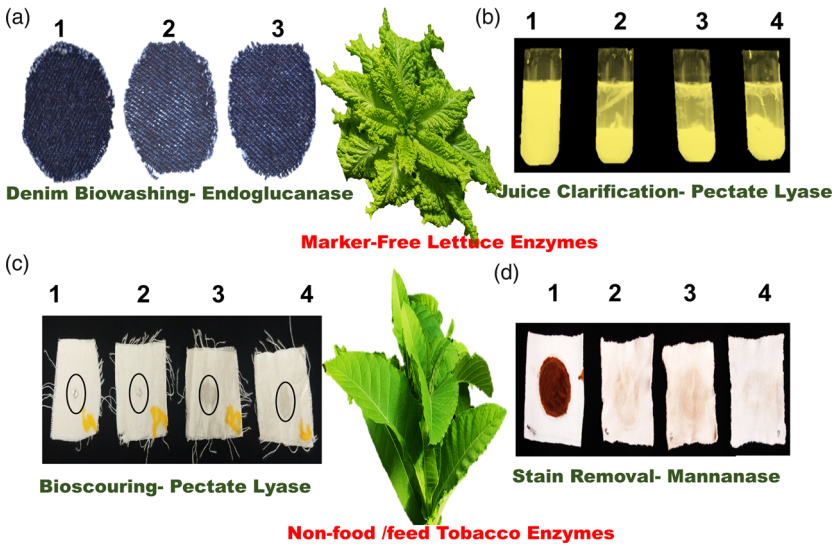

Figure 1 Comparison of chloroplast and microbial enzymes on dye or stain removal, water absorption or juice clarification: (a) Endoglucanase expressed in chloroplasts (a3) removed dye from denim fabric uniformly unlike uneven removal using microbial enzyme (a2) when compared to untreated control (a1). (b): Chloroplast pectinase (b2, b3) clarified orange juice pulp similar to microbial pectinase (b4) when compared to untreated control (b1). (c) Chloroplast pectinase (c4) increased water absorption of cotton fabric similar to microbial enzyme (c3), when compared to controls with water (c1) or untransformed plant extract (c2). (d) Chloroplast mannanase (d4) removed cholate stain more efficiently than microbial enzyme (d3) when compared to untreated control (d1) or with detergent alone (d2). Antibiotic resistance gene was removed from lettuce transplastomic lines but not from tobacco. For further details, see Daniell *et al*, 2019 or Kumari *et al*, 2019

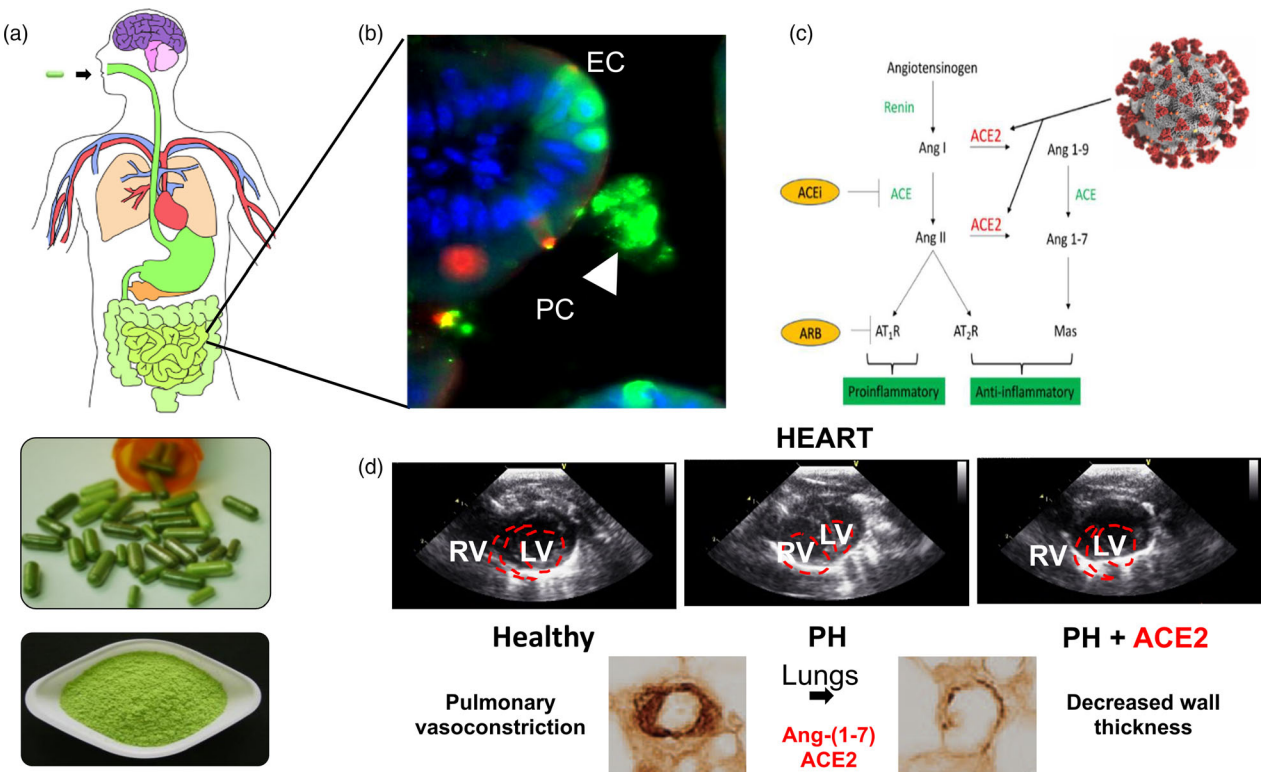

Figure 2 Oral delivery of protein drugs made in lettuce chloroplasts: (a) Production of Ace2/Ang1-7 in lettuce chloroplasts, freeze drying and oral delivery of capsules. (b) Protection of intact plant cells expressing GFP from stomach acids and enzymes and delivery to the gut. Gut microbes release enzymes to digest the plant cell wall and release GFP to gut lumen, which is taken up by gut epithelial cells. (c) SARS-CoV-2 binds to the human Ace2 receptor via the spike protein (target of several vaccines), lowers Ace 2 levels and causes lung/heart injury through inflammation by disregulation of the RAS pathway. (d) Oral delivery of angiotensin-converting enzyme 2 and angiotensin-(1-7) bioencapsulated in plant cells attenuates pulmonary hypertension and reduces right ventricular systolic pressure, right ventricular hypertrophy. Fibrosis and pulmonary vessel wall thickness, which are the symptoms observed in COVID-19 patients. For further details, see Daniell (2020).

expressed in lettuce chloroplasts are developed to treat infectious or inherited diseases in several animal models and are now advanced to the clinic.

Recent advances include expression of PDs in marker-free chloroplast genomes by removal of the antibiotic resistance gene (Park *et al*., 2020; Daniell et al 2020), production of lettuce expressing PDs in cGMP facilities (Daniell *et al*., 2019a, 2020; Park *et al*., 2020), evaluation of drug substance by toxicology and pharmacokinetic studies (Daniell et al, 2020) and recent FDA approval of PDs orally delivered in peanut cells to treat allergy (Tilles and Petroni, 2018; Vickery *et al*., 2018). Two major examples are discussed below in the context of developing SARS-CoV-2 vaccine or treating acute/lethal lung/heart failure of COVID-19 patients (Figure 2).

There is an urgent need to develop new vaccine strategies for SARS-CoV-2. There are > 50 vaccine clinical trials currently in progress, most of them using the same antigen (spike protein) but produced using DNA, RNA or recombinant proteins (Amanat and Krammer, 2020). Because all of these are injectable vaccines (except Vaxart), they will primarily produce systemic immunity with suboptimal induction of mucosal surface immunity required to protect at viral entry points (Sui and Berzofsky, 2020). Induction of mucosal immunity will likely require oral vaccination or antigen delivery via other mucosal routes. In addition, the induction of suboptimal and/or short-lived responses (as seen already in repeat COVID-19 infection) including in the elderly would require additional boosters. In this context, recent development of a polio oral booster vaccine using lettuce chloroplasts, in collaboration with FDA and CDC laboratories, funded by the Gates foundation is highly significant. Oral delivery of codon-optimized polio viral protein 1 common to all polio viral serotypes generated both IgA and IgG1 specific antibodies and conferred protection against all three polio virus serotypes (Chan *et al*., 2016; Xiao and Daniell, 2017; Daniell *et al*, 2019b). While a single-dose injectable polio vaccine (IPV) did not produce significant levels of IgA and IgG1 titres decreased quickly, oral boosters with VP1 expressed in chloroplasts maintained both IgA and IgG1 up to 400 days. In case of Sabin 1 and Sabin 2, a single dose of IPV resulted in poor seropositivity (<20% or < 40%), but showed ~ 70% seropositivity against Sabin 3. Plant cell boosting showed higher seropositivity against Sabin 1, Sabin 2 and Sabin 3 (80-100% protection). When OPV2 was withdrawn by the World Health Organization because it could revert to virulence by point mutations or recombination with other enteroviruses, most developing countries could not afford a second dose of IPV. In a very similar situation, an affordable oral booster strategy is now developed for SARS-CoV-2 oral booster vaccination, especially to develop prolonged mucosal immunity.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for coronavirus disease 2019 (COVID-19), infects host cells via the angiotensin-converting enzyme 2 (ACE2) receptor, resulting in vasoconstriction, hypercoagulability, myocardial/lung injury, fibrosis, inflammation associated with ACE2 down-regulation and/or cytokine storm (Du *et al*., 2020; Zoufaly *et al*., 2020). In healthy people, ACE2 is expressed in type II alveolar lung epithelial cells that produce surfactants and this protects alveoli from collapsing. Most importantly, ACE2 produces anti-inflammatory, cytoprotective angiotensin 1-7 (Ang 1-7) peptide via cleavage of the vasoconstrictor angiotensin II (AngII). Of potential therapeutic relevance, oral delivery of both ACE2 and Ang1-7 bioencapsulated in plant cells significantly decreased right ventricular systolic pressure and improved pulmonary blood flow and right ventricle function in diseased hypertensive animals (Daniell, 2020; Shenoy *et al*., 2014; Figure 2). Conceivably, therapeutic delivery of ACE2/Ang1-7 could also be employed to restore a more favourable balance of Ang II and Ang 1-7 in patients with COVID-19 disease. In contrast to exogenously delivered truncated (transmembrane deleted) soluble ACE2 (Zoufaly *et al*., 2020), full-length ACE2 accumulates in the lungs at 10-fold higher concentrations than in the plasma, with no evidence of toxicity (Daniell et al, 2020). Based on these observations, evaluation of therapeutic efficacy and safety of supplementing ACE2 and Ang (1-7) with this existing product in non-critically ill COVID-19 patients in the hospital and at home is in progress. Investigational New Drug application has been filed and has gone through two rounds of FDA review, by employing an integrated Phases 1 and 2 clinical trial design. This clinical trial is planned to treat a growing number of COVID-19 patients to protect them from lung/extrapulmonary injury and heart failure.

Future perspectives

Sequenced chloroplast genomes are available for fewer than 70 genera of the ~ 3,000 species of cultivated crops and of fewer than 2,000 of the ~ 350,000 species of flowering plants; thus, further efforts are needed to increase the number of sequenced plastomes. Approximately half of every chloroplast genome contains intergenic spacer regions and regulatory sequences that are not conserved, but essential for chloroplast genetic engineering. The lack of intergenic spacer sequences is an important unmet need. In addition, several attempts to transform cereal chloroplast genomes have been unsuccessful, and advances in this area are needed to apply this concept to confer desired agronomic traits. Almost all successful chloroplast genetic engineering reported here used the gene gun and the *aadA* gene. Therefore, future investigations could focus on development of new selectable markers and DNA delivery methods to achieve plastid transformation in cereal crops.

Plastid DNA sequences will continue to be an important tool in elucidating relationships and evolutionary processes across not only green plants, but also other photosynthetic lineages of life. Of particular interest in the next decade will be more studies that compare patterns of nuclear and plastid phylogenetic relationships. It is anticipated that many more discordant patterns of relationships will be uncovered across the photosynthetic tree of life, providing novel insights into a deep and recurring history of reticulate evolution. Moreover, understanding the nature of the discordance in the phylogenetic signal of different plastome-encoded genes will yield further insight into the evolutionary constraints on the plastome and thus the utility of different regions for bioengineering.

Chloroplasts play important roles in sustaining life on this planet by providing carbohydrates, amino acids and lipids as sources of animal (including human) nutrition and O_2, which supports animal life and moderates global warming by trapping CO_2. In the past two decades, chloroplasts have been called upon to produce proteins (enzymes) used in everyday life and to reduce the cost of drugs to offer affordable health care. Major advances have been made in this area in the past few years. When clinical trials currently in progress are successful, an affordable protein drug production and delivery will be launched soon using chloroplasts as bioreactors. Recent advances augur well for chloroplasts to play a central role in both medicine and in sustaining life on Earth, replacing decades-old prohibitively expensive fermentation and drug delivery technologies.

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Conflicts of interest

Henry Daniell discloses conflict of interest due to several patents on chloroplast genetic engineering and technology founder of PhylloZyme that launched chloroplast enzyme products and biopharmaceuticals research supported in the past by Bayer, Novo Nordisk, Johnson & Johnson and currently by Shire/Takeda.

Author contributions

HD conceived this topic and wrote this review. SJ contributed to sections on crop chloroplast genomes and contents of tables. X-GZ contributed the section on CO_2 emission by fermentation and capture by chloroplasts. MAG, DES and PSS contributed sections on chloroplast genome and phylogenetics.

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