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A biochemical analysis of the complex protein folding machinery in algal chloroplasts

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Miller Tran

Committee in charge:

Stephen P. Mayfield, Chair
Jack Bui
Mark Hildebrand
Michael D. Burkart
Stephen M. Hedrick
Steve P. Briggs

2012

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Chair

University of California, San Diego

2012

DEDICATION

This thesis is dedicated to my mom Cam-giang Nguyen, my dad Ba Tran, and Amy Tran. Thank you for your loving support and patience.

EPIGRAPH

“Let me tell you something you already know. The world ain't all sunshine and rainbows. It is a very mean and nasty place and it will beat you to your knees and keep you there permanently if you let it. You, me, or nobody is gonna hit as hard as life. But it ain't how hard you hit; it's about how hard you can get hit, and keep moving forward. How much you can take, and keep moving forward. That's how winning is done. Now, if you know what you're worth, then go out and get what you're worth. But you gotta be willing to take the hit, and not pointing fingers saying you ain't where you are because of him, or her, or anybody. Cowards do that and that ain't you. You're better than that!”

Rocky Balboa

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Hannon, M., J. Gimpel, **M. Tran**, B. Rasala and S. Mayfield (2010). "Biofuels from algae: challenges and potential." Biofuels 1(5): 763-784.

Tran, M. and S. P. Mayfield (2010). Expression of Full Length Monoclonal Antibodies (mAb) in Algal Chloroplast.

Tran, M., B. Zhou, P. L. Pettersson, M. J. Gonzalez and S. P. Mayfield (2009). "Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts." Biotechnol Bioeng 104(4): 663-673.

Mayfield, S. P., A. L. Manuell, S. Chen, J. Wu, **M. Tran**, D. Siefker, M. Muto and J. Marin-Navarro (2007). "Chlamydomonas reinhardtii chloroplasts as protein factories." Curr Opin Biotechnol 18(2): 126-133.

Manuell, A. L., M. V. Beligni, J. H. Elder, D. T. Siefker, **M. Tran**, A. Weber, T. L. McDonald and S. P. Mayfield (2007). "Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast." Plant Biotechnol J 5(3): 402-412.

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ABSTRACT OF THE DISSERTATION

A biochemical analysis of the complex protein folding machinery in algal chloroplasts

by

Miller Tran

Doctor of Philiosphy in Biology

University of California, San Diego, 2012

Professor Stephen P. Mayfield, Chair

Chloroplasts house the photosynthetic apparatus that allows oxygenic photosynthesis to occur. The assembly of these core complexes requires both proteins that are synthesized in the plastid as well as proteins that are translocated across the chloroplast envelope from the cytosol. In both instances, the photosynthetic subunits need to be folded or re-folded into the correct confirmation to ensure their functionality. To assist with protein folding, chloroplasts have evolved to contain a large number of molecular chaperones that prevent protein polypeptide chains from aggregating before they can achieve their native state. In addition to molecular chaperones, chloroplasts have also evolved to contain a protein disulfide isomerase that is involved in redox signaling and which can potentially be used to form disulfide bridges in complex multi-subunit proteins. Although some of the chloroplast protein

folding machinery is of a eukaryotic origin most the components still resemble those of their prokaryotic ancestors.

To increase our understanding of the complex protein folding machinery of chloroplasts we examined the ability of *Chlamydomonas reinhardtii* chloroplasts to fold and assemble complex multi-subunit proteins such as a full-length human antibody and immuotoxin proteins. The assembly of a human antibody is a complex process that requires molecular chaperones to hold proteins in a non-aggregated state while a protein disulfide isomerase catalyzes the formation of 16 disulfide bonds. The ability to assemble an antibody demonstrates biochemically that the protein disulfide isomerase in chloroplast is capable of functioning cohesively with the plastid molecular chaperones to form disulfide bridges. We were also able to produce enzymatically active immuntoxin proteins in chloroplasts and demonstrate that they were able to target and kill specific cancer cells. This ability to accumulate immunotoxin proteins demonstrates that chloroplast protein transport is unidirectional and that no proteins escape the chloroplast.

Many of the biological protein folding components have been identified in chloroplasts but little is known about how they function cohesively to allow complex proteins to fold into their native states. A thorough understanding of the biochemical nature of chloroplast protein folding and how they function together will supplement the basic knowledge of protein folding in all organisms and may also assist in the design and use of algae as a biotechnological tool to generate difficult to produce multi-subunit proteins.

Chapter 1: Introduction

The origin and evolution of chloroplast:

The endosymbiotic theory, which is widely accepted, suggests that chloroplasts originated from a prokaryotic cyanobacteria that was engulfed by a eukaryotic cell (Whatley, John et al. 1979, Kutschera and Niklas 2005). These two cells developed a symbiotic relationship whereby the cyanobacteria provided energy for the eukaryotic cell in exchange for nutrients and protection (Bedard and Jarvis 2005). Evolution caused the prokaryotic host to lose its autonomy through a process of gene transfer where a significant portion of its genome was transferred to the nuclear encoded genome of the eukaryotic cell (Baldauf and Palmer 1990). As a result, chloroplasts are believed to import greater that 90% of their protein content from the nuclear genome (Leister, 2003).

The singled celled eukaryotic green algae, *Chlamydomonas reinhardtii* has served as a model organism for understanding the biochemical and physical properties of photosynthesis (Harris 2001). This is due in part to the fact that chloroplasts of *C. reihardtii* resemble those of higher plants consisting of a photosystem II, photosystem I, plastoquinone, cytochrome b_6f, plastocyannin, and an ATP synthase (Allen, de Paula et al. 2011). As it does in land plants, the protein complexes that make up the photosynthetic apparatus of *C. reinhardtii* work in a concerted manner to allow oxygeneic photosynthesis to occur (Alric, Lavergne et al. 2010). This process

produces the reducing potential that is necessary for the Calvin (Dark) cycle to fix atmospheric CO_2 into a reduced form of carbon (Dietz and Pfannschmidt 2011). Chloroplasts are known to accumulate the proteins that make up their photosynthetic apparatus in two manners, either by importing proteins that were synthesized in the cytosol (Bedard and Jarvis 2005) or by translating their own proteins using a set of chloroplast localized 70S-like ribosomes (Manuell, Quispe et al. 2007) and elongation factors (Zerges, Girard-Bascou et al. 1997, Beligni, Yamaguchi et al. 2004). Both mechanisms of protein accumulation must be functional for an algal cell to proliferate. Here I will examine both cytosolic protein translocation into chloroplast and chloroplast translation and accumulation of proteins coded by the plastid genome. I will further dissect the role of the biochemical environment in chloroplast and how it contributes to the accumulation or proteins as well as the maintenance of protein quality.

Translocation of proteins into chloroplasts

Chloroplasts typically have two membranes, the inner envelope membrane and the outer envelope membrane (Schnell 2000) but in some instances algae such as the heterokont diatoms have additional membranes (Bedoshvili, Popkova et al. 2009). Chloroplasts also contain an additional membrane, the thylakoid membrane which harbors the photosynthetic machinery (Allen, de Paula et al. 2011). In addition to these membranes, chloroplasts also contain the inner membrane space (Heldt and Sauer 1971), which is between the inner and outer membrane, the stroma, and the lumen of

the thylakoid membrane (Goulas, Schubert et al. 2006). This complexity necessitates complex mechanisms to ensure that the correct proteins are sorted into the correct compartment (Inagaki, Fujita et al. 2000, Marques, Dudeck et al. 2003). Most nuclear encoded proteins that enter into the chloroplasts are expressed as precursor proteins that contain N-terminal transit peptides (NTP) (Blobel, Walter et al. 1979, Su, Schumann et al. 1999, Bruce 2000). These proteins are held in a confirmation that is import competent keeps them from aggregating prior to their translocation into chloroplasts. (Chen and Schnell 1999, Jarvis 2008).

Cytosolic protein components play a major role in keeping the chloroplast targeted proteins from aggregating. Most chloroplast bond precursor proteins associate with the ATP-dependent cytosolic HSP70 chaperone (Com70) (Row and Gray 2001, Shi and Theg 2010) and the translocon at the outer envelope of chloroplasts, Toc159 (Perry and Keegstra 1994) and Toc75(Ma, Kouranov et al. 1996). This pre-import protein complex appears to form for many of the chloroplast targeted proteins including Rubisco small subunit, the light harvesting complex proteins, and the ferredoxin $NADP^+$(Row and Gray 2001). Additionally, precursor proteins being targeted to the chloroplast appear to first interact with a 14-3-3 protein dimer and a Hsp90 chaperone that assist the precursor proteins in reaching TOC159 and TOC75 (Jackson-Constan, Akita et al. 2001).

Once the translocation complex is formed, precursor proteins are delivered to the chloroplast translocon. The TOC complex (Andres, Agne et al. 2010) and the translocon at the inner envelope of chloroplasts termed (TIC) (Kovacs-Bogdan, Soll et

al. 2010) mediate the transfer of proteins from the cytosol through the inner envelope space and finally into chloroplasts (Stengel, Benz et al. 2009, Strittmatter, Soll et al. 2010). Once the precursor protein is transported into the chloroplast the NTP is removed by a stromal processing peptidase (SPP) (Li and Chiu 2010). Having reached the chloroplast, the protein is either directed to another sub-compartment or it is refolded into its native state to perform its enzymatic function (Keegstra and Cline 1999).

Chloroplast Translation

The other method by which proteins accumulate in the chloroplast is by transcription and translation of their own circular genome. *C. reinhardtii* chloroplasts contain approximately 40-80 copies of their 203395-bp plastid genome that codes for 99 genes, a full repertoire of tRNAs, and a full complement of rRNAs that comprise the chloroplasts 70s ribosome (Maul, Lilly et al. 2002). As mentioned previously, gene transfer caused chloroplasts to lose a large portion of their genome (Stegemann, Hartmann et al. 2003), and thus the regulation of chloroplast translation is regulated largely in part by nuclear encoded factors (Drapier, Girard-Bascou et al. 2002, Marin-Navarro, Manuell et al. 2007). These nuclear encoded factors appear to be regulated at the transcriptional level by a family of photoreceptors (Briggs, Beck et al. 2001), while the accumulation of chloroplast encoded genes appears to be regulated at the translational level (Danon 1997, Yohn, Cohen et al. 1998, Marin-Navarro, Manuell et al. 2007). It is thus important to understand what elements of chloroplast RNA messages are regulated and what factors are responsible for that regulation.

Contribution of 5'-UTR

The process of translation in chloroplasts, like most organisms, requires ribosomes to position themselves at the start codon. In prokaryotes this ribosomal positioning is directed by mRNA sequences called Shine-Dalgarno sequences (Shine and Dalgarno 1975) and in eukaryotes, ribosomes use a scanning mechanism to identify start codons (Agarwal and Bafna 1998). Chloroplasts being of prokaryotic ancestry have several mRNAs that have Shine-Dalgarno-like sequences (Hirose and Sugiura 2004, Barnes, Franklin et al. 2005). While some messages do show the presence of Shine-Dalgarno like sequences, others chloroplast messages do not, suggesting alternative mechanisms of ribosome positioning (Hirose, Kusumegi et al. 1998).

In chloroplasts of *C. reinhardtii*, the role of 5'-UTRs and the mechanisms by which they are regulated translationally has been explored for many endogenous genes. Using chimeric reporter genes such as luciferase and GFP the 5'-UTRs of psbA and psbD have been shown to be regulated as a response to the surrounding light environment (Malnoe, Mayfield et al. 1988, Franklin, Ngo et al. 2002, Mayfield and Schultz 2004). Additionally, deletion experiments of many chloroplast 5'-UTRs has demonstrated that they are essential for the translation of chloroplast mRNAs. When the petD 5'-UTR was deleted the translation of a beta-gluuronidase which was under its control was no longer translated into a functional protein (Sakamoto, Kindle et al. 1993). This data suggest that the control of chloroplast protein accumulation is at the

translational level and that by altering key elements within the 5'-UTR, scientist can significantly impact the interaction of the chloroplast ribosome with the mutated mRNA.

RNA binding proteins

Although Shine-Dalgarno sequences do appear to exist in chloroplasts, many 5'-UTRs require the formation of an initiation complex that helps to position the ribosome at the start codon (Yohn, Cohen et al. 1996, Baecker, Sneddon et al. 2009). Additionally, some proteins that bind to RNA act as a repressor of translation. These regulatory proteins are nucelear encoded and imported into the chloroplast(Gillham, Boynton et al. 1994).

The psbA 5'-UTR has been extensively studied and a set of nuclear encoded proteins that bind to and regulate the translation of this UTR have been identified (Danon and Mayfield 1991, Barnes, Cohen et al. 2004). This complex appears to be an initiation complex consisting of RNA binding proteins 38, 47, 55, and 60 (Barnes, Cohen et al. 2004). The binding of this initiation complex is light activated and appears to respond to a change in the redox environment of the chloroplast stroma (Jackson-Constan, Akita et al. 2001). The process starts with a reduction of the protein disulfide isomerase, RB60, which leads to the reduction of RB47 which binds to the psbA 5'-UTR in its reduced state (Danon and Mayfield 1994, Alergand, Peled-Zehavi et al. 2006). The reducing potential is generated from the process of photosynthesis (Pietro 1972) and is what causes the change in the redox environment that is

responsible for the translational regulation of proteins whose accumulation is controlled by the psbA 5'-UTR (Danon and Mayfield 1994) .

Chloroplast Ribosomes and elongation factors

To understand translation in chloroplasts it is important to have an understanding of the RNA regulatory elements, the proteins that bind to them and also the ribosomes and elongation factors that are involved in protein synthesis. Ribosomes are large complexes that are comprised of both ribosomal RNAs and proteins. Bacteria and other prokaryotes have ribosomes that are 70S (determined by sedimentation coefficient) which themselves are composed of a small 30S subunit and a large 50S subunit. The 30S ribosome is made of 16S rRNA while the larger 50S ribosome consists of 5S and 23S rRNA. These ribsomomal subunits also have an extensive list of proteins they associate with which provide them with structural integrity (Klein, Moore et al. 2004). Alternatively, eukaryotic ribosomes found in the cytosol are larger than their bacterial counterparts. These ribosomes are 80S and are also made of a large and small subunit. The small subunit of the eukaryotic ribosome is 40S while the large subunit is 60S. These ribosomes also contain a large set of proteins that have been shown to associate them and provide structural integrity much like those found in prokaryotes (Armache, Jarasch et al. 2010).

Both mitochondria and chloroplasts are known to translate a set of their own proteins and as such are also known to contain their own set of unique ribosomes (O'Brien 2003, Manuell, Quispe et al. 2007). Both ribosomes appear to share

similarities with bacterial ribosomes and have a sedimentation coefficient of approximately 70S (Manuell, Quispe et al. 2007). Chloroplasts much like mitochondria are composed of proteins that share similarities to bacterial ribosomes (Schmidt, Myers et al. 1984). However, when chloroplast ribosomes from *C. reinhardtii* were isolated, there were specific proteins that were not present in either bacterial or eukaryotic ribosomes (Schmidt, Myers et al. 1984). It is not known how these extra proteins might affect ribosomal function in chloroplasts or if they alter the local protein folding environment but preliminary results suggest they may play a role in the initial interaction of the ribsome with mRNAs and the initiation of translation (Manuell, Quispe et al. 2007). Regardless, biochemical analysis has shown that chloroplast ribosomess are still sensitive to bacterial antibiotics, suggesting that these extra protein densities to not alter the functional similarities of chloroplast and bacterial ribosomes (Sugiura, Hirose et al. 1998, Beligni, Yamaguchi et al. 2004). It should be interesting to note that these ribosomes being of bacterial origin should not be affected by ribosome inactivating proteins such as ricin and gelonin which are known to hydrolyze a specific N-gycosidic bond of an adenosine on the 28s rRNA (Zamboni, Brigotti et al. 1989).

Elongation factors are another interesting component of chloroplasts protein translation and facilitate the elongation of polypeptide chains before they are folded into their native structure (Kulczycka, Dlugosz et al. 2011). One of the chloroplast elongation factors, elongation factor-Tu is plastid encoded and is known to be a bacterial homolog (Watson and Surzycki 1982). This is not surprising considering the

chloroplast ribosomes are also of bacterial origin as previously discussed. In addition to this elongation factor another plastid specific elongation factor, PSRP-7, was recently identified. PSRP-7 is a novel 65 kDa protein that associates with the 30S ribosome. This protein is unique from those found in bacteria as it has two S1 domains as well as two tandem elongation factor T domains on its carboxy end (Beligni, Yamaguchi et al. 2004). *In silico* analysis suggest that this elongation factor may be related to elongation factors found in mitochondria (Beligni, Yamaguchi et al. 2004). Still, little is known about this elongation factor and its actual origin. There are many enzymes or toxic molecules that are known to impact elongation such as diphtheria toxin from *Corynebacterium diphtheria* or exotoxin A from *Pseudomonas aeruginosa* and the expression of these proteins in chloroplasts may preset biochemical evidence as to the function of this elongation factor. What is apparent however, is that chloroplasts have a complete set of ribosomes and elongation factors that allow them to synthesize their own proteins.

Chloroplast protein quality control

Chloroplast molecular chaperones

As we have now discussed, proteins arrive in chloroplast either by translocation across the chloroplast membrane or by translation of mRNAs from chloroplast expressed genes. Regardless of the mechanism by which proteins arrive in chloroplasts, molecular chaperones and chaperonins play an important role in how

those proteins are folded. Chaperones allow hydrophic domains of nascent polypeptide chains or proteins that have almost achieved their native state sufficient time to fold into the correct confirmation and prevent protein aggregation (Leidhold and Voos 2007). This characteristic of chaperones, whereby they can prevent protein aggregation of both proteins being translocated into the chloroplast through the TIC/TOC complex and proteins being synthesized by chloroplast ribsomes, makes them essential in maintaining the integrity of the chloroplast (Schwenkert, Soll et al. 2011, Flores-Perez and Jarvis 2012). The chloroplast localized chaperones are a unique combination, consisting of those that appear to have a cyanobacterial origin (Schroda 2004) as well as chaperones such as Hsp90 that are of a eukaryotic origin (Emelyanov 2002). In addition to these chaperones, the chloroplast has also evolved to contain a set of unique co-chaperonins. Two such co-chaperonins are Cpn20 which appears to be a dimer of groES(Bertsch, Soll et al. 1992, Koumoto, Shimada et al. 1999) and Cpn10 (Koumoto, Shimada et al. 2001). The difference in the composition of the chloroplast chaperone system raises many questions including whether the chloroplast chaperones could allow chloroplast to fold complex eukaryotic proteins like those folded in the endoplasmic reticulum or if chloroplasts are more closely related to bacteria and can only fold simple single subunit proteins. Furthermore, the completion of the *C. reinhardtii* genome has allowed scientist to determine that all 5 classes of major chaperones and co-chaperones are present in chloroplasts (Schroda 2004). These families include HSp60(Peng, Fukao et al. 2011), Hsp70(Willmund, Dorn et al. 2008), Hsp90(Johnson 2012), Hsp100/Clp(Wang, Rosano et al. 2010), and

Cpn60(Peng, Fukao et al. 2011) co-chaperones GrpE, and Cpn10/20 (Koumoto, Shimada et al. 2001). All together this complex network of chaperones is essential to maintaining a functional chloroplast. First we must gain a better understanding of each type of chaperone so that we can understand how chaperones play a role in maintaining protein quality in chloroplast, and how each chaperone can potentially allow chloroplasts to fold complex recombinant proteins.

The Hsp70 family of chaperones has been identified in most known organisms and contains a highly conserved N-terminal ATPase domain as well as a conserved substrate binding domain. Generally, substrates of Hsp70 chaperones have exposed hydrophobic patches that are a characteristic of proteins that are in non-native states (Mayer and Bukau 2005). BiP, the Hsp70 chaperone was one of the first chaperones identified and is essential to the folding and assembly of full-length human antibodies (Munro and Pelham 1986). In chloroplasts, stromal HSP70B has been shown to assist in the folding of denatured proteins. In addition to its general role in helping with protein folding, HSP70B has been implicated in protection of photosystem II from irreversible photodamage (Yokthongwattana, Chrost et al. 2001). Overall the Hsp70 family of chaperones is high specialized in renaturing proteins that are in non-native states during stress situations such as heat shock and are essential for the folding and assembly of complex multi-subunit proteins (Schroda, Vallon et al. 1999).

The Hsp90 family of chaperones also represents another class of chaperones that is highly conserved. Typically, these chaperones are involved in the late folding of proteins that are in the near-native state (Wiech, Buchner et al. 1992). Also, precursor

proteins that are translocated through the TIC/TOC complex interact with an Hsp93 chaperone upon entry into the stroma (Flores-Perez and Jarvis 2012). It should also be noted that chloroplast Hsp90 chaperones appear to be more related to Hsp90 chaperones found in the endoplasmic reticulum than they do with those that are found in bacteria (Emelyanov 2002). It has been suggested that this process occurred due impart to a gene duplication which was followed by an acquisition of the chloroplast NTP (Schroda 2004). Recently, it has also been demonstrated the Hsp70 and Hsp90 form a chaperone complex termed a "foldosome" that could work in a coordinated manner to correctly fold proteins to their native state (Freeman and Morimoto 1996, Dittmar, Banach et al. 1998)

Hsp100/Clp is another chaperone complex that has homology to those that are found in bacteria and is present in both chloroplasts and mitochondria (Schroda 2004). While the mechanism of this chaperone complex has not been completely deciphered, biochemical date suggest that in chloroplasts these chaperones may interact with proteins that are being translocated across the chloroplast membrane (Constan, Froehlich et al. 2004). Potentially assisting Hsp70 chaperones with the completion of the translocation process (Rosano, Bruch et al. 2012).

Finally, the chaperonin Cpn60, which appears to be a homolog of the bacterial GroEL, was found to be localized to the stroma of chloroplasts (Viitanen, Schmidt et al. 1995). It is comprised of a tetradecameric double-ring and much like GroEL cooperates with another chaperonin Cpn10, which forms a lid for the chaperonin complex. Cpn10 is a co-chaperonin that shares homology with GroES (Viitanen,

Schmidt et al. 1995). This chaperonin complex appears to plays a role in folding and assembling mature proteins like RubisCo (Cloney, Bekkaoui et al. 1992). This stage of protein folding involving chaperonins appears to resemble those in the cytoplasm of eukaryotes, where proteins that remain partly folded after interacting with Hsp70 chaperones are sent to the groES/groEL homologues, TRiC/CCT where they either achieve a native conformation or they are sent off to be degraded (McClellan, Scott et al. 2005).

This extensive list of chaperones suggests that the protein folding machinery in chloroplasts are intricate and are more advanced than their cyanobacterial ancestors. A further biochemical analysis is essential to understanding how this network of chaperones functions cohesively to re-fold proteins being translocated from the cytosol and how they are involved in folding nascent polypeptide chains being synthesized off of chloroplast ribosomes.

Chloroplast localized proteases

Along with chaperones, proteases are the other major component that is involved in protein quality control. They are located in every compartment within the chloroplast from the thylakoid lumen(Kapri-Pardes, Naveh et al. 2007) to the stroma (Schuhmann and Adamska 2012) and are essential for ensuring the integrity of chloroplast. Of the approximately 3000 proteins that are localized to the chloroplast, it is estimated that 2-3% of the proteins are proteases (Sakamoto 2006).

Signal peptide peptidases are responsible for removing N-terminal signal peptides which would otherwise inhibit the function of many proteins that are translocated into the chloroplast (Richter and Lamppa 1998). In addition to removing the signal peptide, proteases are essential for recycling the photosynthetic proteins. Proteins such as D1 and D2, the core proteins of photosystem II are constantly photodamaged by light and need to be degraded and replaced by newly synthesized proteins to ensure the continuing function of photosynthesis. FstH a known bacterial homolog that was identified in chloroplasts has been biochemically tested for its ability to degrade the D1 protein (Lindahl, Tabak et al. 1996). Another protease that is bound to the stromal side of the thylakoid membrane, Deg2, is known to perform the initial cleavage of D1 before it is completely degraded by FstH (Haussuhl, Andersson et al. 2001). The chloroplast consist of many proteases that are of bacterial origin and one of the main complexes that is involed in general degradation of mis folded or damaged proteins is the ClpP proteolytic complex (Sokolenko, Lerbs-Mache et al. 1998). ClpP is a multisubunit enzyme that has an ATPase domain. Structurally ClpP forms a heptameric ring with a central pore where the serine protease catalytic triad is located (Wang, Hartling et al. 1997). This complex is important for chloroplasts degradation of proteins such as cytochrome b_6F, although mutants with decreased ClpP accumulation do not appear to have altered growth rates (Majeran, Wollman et al. 2000).

Although chloroplasts are thought to be evolved from cyanobacteria there are instances when proteases that are not present in cyanobacteria have been identified in

chloroplasts. One such situation was the identification of the lon protease in chloroplast of *Arabadopsis thaliana* (Ostersetzer, Kato et al. 2007). Lon is a major serine protease in *Esherichia coli* and biochemical data suggest that the co-localization of lon in mitochondria and chloroplast is due to a shared N-terminal signal peptide (Ostersetzer, Kato et al. 2007). Another instance where a novel protease was discovered in chloroplast was the identification of the aspartyl protease, CND41, in tobacco (Murakami, Kondo et al. 2000). Understanding chloroplast proteases as important complexes that recycle amino acids and which degrade misfolded or photodamaged proteins is important to our understanding of the biochemical environment in chloroplasts. Proteases are important regulators of how much a protein is allowed to accumulate. This can be seen in the protease deficient *E. coli*, BL21 where a deficiency for both ompT and lon allows these cells to accumulate larger quantities of recombinant protein (Grodberg and Dunn 1988).

Chloroplast protein folding

Protein folding of complex disulfide bond proteins in eukaryotes

To completely understand how protein folding and how the biochemical components of chloroplast work together to correctly fold proteins, it is important to understand how proteins are folded both in prokaryotes and eukaryotes. Protein folding in eukaryotes is essential to a healthy functioning cell and when mutations occur that affect the function of otherwise normal chaperones, neurodegenerative

diseases can occur (Barral, Broadley et al. 2004). As discussed previously chaperones interact with nascent polypeptide chains that are being translasted off of the eukaryotic ribosome. Peptide chains are folded by either Hsp70 chaperones or they are delivered to the TRiC chaperonin comlex to achieve their native state. However, some complex proteins require the assembly of multiple subunits. This is the case with monoclonal antibodies. Antibodies require two heavy chain protein subunits and two light chain protein subunits to be assembled together into a functional complex (Hebert and Gierasch 2009). These protein units are held together by disulfide bridges between cysteine amino acids on each of the subunits (McAuley, Jacob et al. 2008). The formation of the disulfide bridges in the endoplasmic reticulum are catalyzed by a protein disulfide isomerase PDI (Roth and Koshland 1981). It is important to note that a cell lacking a PDI will not be able to efficiently assemble multiple protein subunits through the formation of disulfide bonds. Chaperones prevent proteins from aggregating allowing PDI sufficient time to form disulfide bonds (Mayer, Kies et al. 2000). This complex concerted action of chaperones and PDI allows eukaryotic cells to fold and assemble complex proteins that prokaryotic systems cannot.

Protein folding of disulfide bond proteins in prokaryotes

The initial process of protein folding in bacteria is very similar to those that occur in a eukaryotic cell. A nascent polypeptide chain is synthesized off of a ribosome and hsp70 chaperones prevents the protein from aggregating and allow the protein sufficient time to fold into its native conformation. If the protein is unable to achieve it native state, it is delivered to a chaperonin complex that consist of GroES

and GroEL subunits (Hartl, Bracher et al. 2011). In eukaryotes complex chaperones can be found in the endoplasmic reticulum where a protein disulfide isomerase also resides to assist in assembling complex proteins. In prokaryotes where an endoplasmic reticulum does not exist, chaperones are only present in the cytosol, where no protein disulfide isomerase is present. Thus proteins that have disulfide bonds are not able to efficiently fold in the cytosol of bacteria and are often times produced as insoluble aggregates. When a disulfide bond is necessary for a protein to achieve its native confirmation, one strategy to produce such a protein in prokaryotes is to secrete the protein into the periplasmic space where the disulphide isomerase, DsbC, and the oxidase, DsbA, are able to form disulfide bonds. The major drawback of this strategy is the lack of molecular chaperones in the periplasmic space requiring scientist to co-express chaperones in an attempt to fold disulfide rich proteins (Outchkourov, Roeffen et al. 2008). This characteristic of prokaryotes makes them less than ideal when considering a protein expression platform.

Chloroplast protein disulfide isomerase

Although chloroplasts are of prokaryotic origin they have evolved a unique protein folding environment. Chloroplasts of the green algae *C. reinhardtii* contain a protein disulfide isomerase, RB60. RB60 is a key component of the redox regulation that occurs in chloroplast which initiates the translation of the chloroplast psbA gene (Danon and Mayfield 1994) . Although this protein has been shown to have homology

to a protein disulfide isomerase, it has not previously been shown to be able to work concertedly with chloroplast chaperones to fold and assemble complex proteins such as human antibodies that require multiple subunits to be held together by the formation of disulfide bonds. Having a compartment that has complex chaperones of eukaryotic and prokaryotic origins in addition to a novel protein disulfide isomerase gives *C. reinhardtii* chloroplast the potential to be a highly effective protein production platform.

Chloroplast protein production platform

Eukaryotic green algae have recently been exploited as a source of renewable biofuels for their minimal growth requirements, needing only trace elements, fertilizer, Light and CO_2 to grow. These reasons, along with those previously mentioned, make the green algae *C. reinhardtii* a potentially viable platform for the production of complex recombinant proteins. To transform chloroplasts with a recombinant gene, genetic tools had to be first developed. Chloroplasts have a recombinase that allows them to perform homologous recombination, giving scientist the ability to direct recombinant genes into silent sites in the genome where no open reading frame exists (Oppermann, Hong et al. 1989). To develop genetic tools to transform the plastid genome, a set of endogenous promoters and UTRs were analyzed for their ability to express and accumulate a recombinant Green fluorescent protein in chloroplasts (Franklin, Ngo et al. 2002). Additionally, a knock-out of the endogenous psbA gene

with a recombinant gene of interest allowed the heterologous proteins to accumulate to 10% total soluble protein (Manuell, Beligni et al. 2007). With a full repertoire of genetic tools at our disposal it was now possible to test the biochemical protein folding environment of the chloroplast and determine if this machinery is capable of functioning in a synergistic fashion to allow complex proteins to be folded and assembled into their native state.

Chapter 2: Synthesis and assemby of a full-length human monoclonal antibody in algal chloroplasts

(Adapated from: Miller Tran, Bin Zhou, Par L. Pettersson, Maria J. Gonzalez, Stephen P. Mayfield. "Synthesis and Assembly of a full-length human monoclonal antibody in algal chloroplasts". Biotechnology and Bioengineering. 2009)

Introduction

Monoclonal antibodies can be effective therapeutics against a variety of human diseases, but currently marketed antibody-based drugs are very expensive compared to other therapeutic options. Here, we show that the eukaryotic green algae Chlamydomonas *reinhardtii* is capable of synthesizing and assembling a full-length IgG1 human monoclonal antibody (mAb) in transgenic chloroplasts. This antibody, 83K7C, is derived from a human IgG1 directed against anthrax protective antigen 83 (PA83), and has been shown to block the effects of anthrax toxin in animal models. Here we show that 83K7C heavy and light chain proteins expressed in the chloroplast accumulate as soluble proteins that assemble into complexes containing two heavy and two light chain proteins. The algal-expressed 83K7C binds PA83 in vitro with similar affinity to the mammalian-expressed 83K7C antibody. In addition a human IgG1 and a mouse IgG1 were also expressed and properly assembled in algal chloroplast. These

results show that chloroplasts have the ability to fold and assemble full-length human mAbs, and suggest the potential of algae as a platform for the cost effective production of complex human therapeutic proteins.

The use of antibodies for treatment of infectious diseases dates back more than 100 years (Zeitlin, Cone et al. 2000, Casadevall, Dadachova et al. 2004). However, the use of this so called “serum therapy” decreased in the 1930’s with the discovery of sulphonamide and other small molecule drugs for the treatment of infections (Cutting and Gebhardt 1941). Antibody therapy again came to the forefront of medicine with the advent of technologies for monoclonal antibody production, and the realization that antibody therapy was effective against diseases that were not treatable by small molecule drugs alone. Today several commercially available preparations of human immunoglobulins are used to treat viral infections (Marasco and Sui 2007). In addition to treating viral infections, passive antibody administration can potentially provide immunity to bacterial pathogens, including those related to bio-terrorism such as Anthrax or Botulism (Zeitlin, Cone et al. 2000, Casadevall 2002). Despite the potential of mAbs for the treatment of both viral and bacterial infections (Marasco and Sui 2007, Wu, Pfarr et al. 2008), few antibodies are marketed for this purpose, mainly due to the high cost and limited availability of such drugs.

Bacillus anthracis (anthrax) infects and kills its mammalian host using a tripartite protein toxin. Anthrax spores can be aerosolized making this bacterium a prime candidate as an agent of biological warfare (Riedel 2005). During an anthrax infection, three protein components are secreted from the bacteria, protective antigen

83 (PA83), edema factor (EF) and lethal factor (LF) (Mourez 2004). To prevent an anthrax infection from becoming lethal, it is possible to neutralize the toxin before its entry into the cell. Antibodies against EF and LF have been shown to block their binding to the Protective antigen 63 (PA63) pore, thus eliminating the toxic effects of these factors (Little, Leppla et al. 1990). Antibodies against PA83 have also been shown to prevent binding of PA83 to the cell, and to prevent the cleavage and formation of PA63 pores (Little, Leppla et al. 1988), again blocking the toxic effects of these factors. Monoclonal antibody 83K7C binds to PA83 and has been shown to neutralize the anthrax toxin in cell based assays and to protect rats from anthrax toxicity in *vivo* (Wild, Xin et al. 2003). By targeting and successfully neutralizing the different components of the anthrax toxin it should be possible to reduce cell death and allow the host organism to clear the infection, thus decreasing the mortality rate of anthrax. Anti-anthrax antibodies have been used as passive immunotherapy to prevent anthrax toxicity and death in both rats and mice (Wild, Xin et al. 2003, Krakauer, Little et al. 2005), and this same strategy could potentially work in humans.

Monoclonal antibodies are composed of two heavy chain and two light chain proteins that are assembled within the endoplasmic reticulum of mammalian cells. During assembly 16 disulfide bonds are formed that covalently link the four subunits together. This complexity has generally limited the production of monoclonal antibodies to eukaryotic expression systems, and all marketed antibodies are currently made in mammalian cell culture. Plants have been used for the expression of human therapeutic proteins (Giddings, Allison et al. 2000) including complex mammalian

antibodies (Hiatt, Cafferkey et al. 1989, Ma, Hiatt et al. 1995, Ko and Koprowski 2005), and it is estimated that therapeutic proteins can be produced in plants for a fraction of the cost of production in traditional fermentation systems (Dove 2002). Antibodies expressed in higher plants have been targeted to the endoplasmic reticulum for processing and secretion from the cells. In these cases, it was assumed that the necessary machinery for antibody assembly and disulfide bond formation were provided by plant ER chaperones and protein disulfide isomerases (Jurgens 2004). Plant chloroplasts have also been used for recombinant protein expression and a variety of proteins have been expressed including vaccines (Walmsley and Arntzen 2003), hormones (Staub, Garcia et al. 2000), and reporter proteins (Heifetz and Tuttle 2001). Expression of some recombinant proteins in higher plant chloroplasts has reached very high levels, well above 10% of total protein (Watson, Koya et al. 2004), suggesting that expression of recombinant proteins in the chloroplast could provide a means for the cost effective production of protein therapeutics. Algae, like vascular plants, have the ability to produce proteins in an efficient manner, as algae grow on minimal media using sunlight as an energy source and CO_2 as a carbon source (Harris 2001). Algae can also be grown in complete containment making them ideal candidates for the production of therapeutic proteins (Franklin and Mayfield 2004). Previously, we have shown that chloroplasts of the eukaryotic algae *C. reinhardtii* have the ability to express recombinant proteins including functional single chain antibodies (Mayfield, Franklin et al. 2003), reporter proteins like GFP (Franklin, Ngo et al. 2002) and luciferase (Mayfield and Schultz 2004), and mammalian proteins that

stimulate mucin production in gut epithelial cells (Manuell, Beligni et al. 2007). The chloroplast of C. *rienhardtii* contains a wide range of protein chaperones and protein disulfide isomerases that could assist in the folding of complex recombinant protein therapeutics (Kim and Mayfield 1997, Schroda 2004). Although chloroplasts are capable of forming disulfide bonds, they lack the capacity to glycosylate proteins, and any antibody expressed in chloroplasts is expected to be aglycosylated. There are a variety of situations where an antibodies ability to bind an antigen is desirable but where the ability to fix complements or cause antibody dependent cell cytotoxicity (ADCC) is unwanted (Sawada-Hirai, Jiang et al. 2004). Aglycosyated antibodies should lack the ability to fix complement or recruit killer cells, and therefore the production of full-length antibodies in chloroplast would offer a unique and desirable novel type of monoclonal antibody.

Here we show for the first time that a properly assembled and functional IgG1 antibody can be produced in, and purified from chloroplasts of *C. reinhardtii.* This demonstrates that chloroplasts have the necessary machinery to synthesize and assemble complex human antibodies. Comparison of the exact same antibody expressed in algal chloroplasts and in mammalian cell culture show that the systems produce functional antibodies with almost identical antigen binding characteristics, and that the chloroplast antibody appears to lack glycosylation, as expected.

Results

Synthesis of human 83K7C heavy and light chain antibody genes in C. *reinhardtii* in chloroplast codon bias

To test whether chloroplast of C. *reinhardtii* had the capacity to express, fold, and assemble full length human monoclonal antibodies, we selected a human antibody that had previously been expressed, purified, and characterized from traditional mammalian cell culture (Wild, Xin et al. 2003). This antibody, 83K7C, was selected from a panel of antibodies that were identified from human donors who had been vaccinated against anthrax with an attenuated strain of B. *anthracis.* 83K7C expressed in CHO cells was previously shown to provide protection from anthrax toxicity, in both cell based assays and animal models (Wild, Xin et al. 2003).

We have previously shown that codon optimization of recombinant genes maximized protein expression in the chloroplast of *C. reinhardtii* (Franklin, Ngo et al. 2002, Mayfield, Franklin et al. 2003, Mayfield and Schultz 2004). We therefore synthesized the heavy chain (HC) and light chain (LC) genes of a human monoclonal antibody 83K7C by PCR gene assembly (Stemmer, Crameri et al. 1995) in codons that were optimized to reflect codons usage in abundant endogenous mRNAs of *C. reinhardtii* chloroplast. The DNA sequences of the codon optimized HC and LC genes, along with the amino acid sequences of the mature proteins, are shown in Supplemental figure 1A and B.

Construction of chimeric *C. reinhardtii* chloroplast antibody expression cassettes

Transformation of the *C. reinhardtii* chloroplast genome proceeds by homologous recombination, thus sequence homology between the vector and the chloroplast genome is required to facilitate integration of the recombinant gene. For antibody expression it is necessary to integrate both a HC and LC gene. Two independent vectors were constructed as shown in Figure 1A. The flanking genomic sequences of the *psbA* locus were included at both the 5' and 3' ends of the chimeric gene, allowing for the integration of the HC gene as a replacement of the endogenous *psbA* gene (Figure 1A). This strategy has been shown to result in robust expression of mammalian proteins in chloroplast (Manuell, Beligni et al. 2007). For LC integration we used the flanking sequences around the *psbH* gene to allow for integration of the LC gene at a silent site at the *psbH* locus (Figure 1B). Integration of the HC gene should result in replacement of the *psbA* gene making the strain non-photosynthetic (Manuell, Beligni et al. 2007). Since both the LC and the HC gene were co-transformed into *C. reinhardtii* with a plasmid p228 that contains a point mutation in the 16s rRNA sequence that confers spectinomycin resistance, homoplasmic strains were grown on media containing spectinomycin and acetate due to the deletion of the endogenous psbA gene (Gorman and Levine 1965).

Analysis of HC and LC gene integration in transgenic chloroplast

Colonies that grew on TAP spectinomycin plates were screened by Southern blot and PCR analysis for the presence of the 83K7C HC and LC genes. Chloroplasts

contain approximately 50 copies of their genome, and selection of homoplasmic lines, in which all 50 copies contain the recombinant gene, is essential to obtaining stable transgenic strains. A homoplasmic transformant (65.11) was selected that contained both the HC and LC genes integrated at the expected sites. DNA corresponding to the HC and LC genes were detected by Southern blots of genomic DNA from strain 65.11. (Figure 2A). Neither gene was detected in the wt strain. PCR analysis also confirmed the presence of both the HC and LC genes in transgenic strain 65.11 (Figure 2B)

To determine if the transformed strains were homoplasmic for both the HC and LC genes at their respective integration sites, PCR analysis was performed. To verify that the chloroplast was homoplasmic for the HC gene in strain 65.11, primers were used to generate a product that overlapped the 5'UTR of the *psbA* gene to an internal site in the coding region of the psbA gene. DNA amplification using these primers reveals the absence of the *psbA* gene in transformed chloroplast, demonstrating that the chloroplast is homoplasmic for the HC gene at the *psbA* integration site. (Figure 2C) To determine if the chloroplast of 65.11 was homoplasmic for the LC gene at the *psbH* locus, primers were used to amplify the genomic DNA around the LC integration site. The lack of an amplification product at the *psbH* locus in 65.11 demonstrates that the strain is homoplasmic for the LC gene (Figure 2C).

Accumulation of 83K7C light chain and heavy chain mRNAs in transgenic chloroplasts

Northern blot analysis of transgenic strain 65.11 was performed to determine whether transcripts of the HC and LC genes accumulated in algal chloroplast. A HC ^{32}P-labled probe hybridized with an mRNA of approximately 2.5 kb, while a LC ^{32}P-labled probe hybridized with an mRNA of approximately 1.6 kb. Both RNAs are of the predicted size for the corresponding genes (Figure 3). No signal is observed in the wt lanes for either mRNA. Hybridization with a *psbA* probe identifies a 1.4 kb band in the wt RNA as expected, while the *psbA* signal is lacking in the transgenic strain (Figure 3). No *psbA* signal should be observed in strain 65.11, as HC integration should result in deletion of the *psbA* gene and elimination of the corresponding mRNA.

Detection of HC and LC Protein Accumulation in transgenic *C. reinhardtii*

To determine whether the 83K7C HC and LC mRNAs found in transgenic line 65.11 were translated into HC and LC proteins, 65.11 was assayed for antibody protein accumulation by immunoblot analysis using an anti-gamma HC or an anti-kappa LC antibody, respectively. As shown in Figure 4A, both HC and LC proteins accumulate in chloroplast in transgenic line 65.11 as soluble proteins. No detectable signal was observed in wt *C .reinhardtii* cells (data not shown), or in the membrane (insoluble) fraction of strain 65.11 (Figure 4A).

Algal expressed HC and LC were then compared with 83K7C expressed in mammalian cell culture (CHO) (Alexion Pharmaceutical, San Diego, CA (Wild, Xin et al. 2003)). Immunoblot analysis revealed that the LC proteins of 83K7C, expressed in either CHO or chloroplast, accumulate as the expected 24-kDa species (Figure 4B). The majority of the HC protein accumulated as the expected 49-kDa monomer in both chloroplast and CHO, and a faint band was seen at the expected size (98kDa) of a HC dimer in both samples (Figure 4B.). Some of the HC proteins expressed in chloroplast appear to be cleaved by endogenous chloroplast proteases, resulting in a band of approximately 34 kDa that cross reacted with the HC antisera (Figure 4B). The chloroplast expressed HC protein is approximately 2 kDa smaller than the CHO expressed HC protein, which is expected as chloroplast do not glycosylate proteins. To confirm that the difference in size between the CHO and chloroplast expressed HC was due to glycosylation, we treated the CHO expressed 83K7C with PNGase F, an enzyme that removes the sugars from proteins, and then compared CHO and chloroplast expressed antibodies by immunoblot. Removal of sugar residues from CHO expressed HC resulted in a shift in molecular mass of the protein to that of the HC expressed in *C. reinhardtii.* (Figure 4C).

C. reinhardtii **expressed 83K7C HC and LC assembles into complex antibodies in chloroplasts**

IgG1 antibodies require 4 protein subunits, two HCs and two LCs, as well as 16 disulfide bridges in order for the antibody to assemble correctly (Figure 5). To

determine if chloroplast expressed 83K7C HC and LC proteins assembled into complex antibodies containing the correct disulfide bonds, non-reducing SDS-PAGE gels and immunoblot analysis was performed. The algal expressed antibodies assembled into complexes that migrated at approximately 150-kDa, the expected size of a correctly assembled heterotetrameric antibody containing two HC and two LC proteins (Figure 6A). The CHO expressed 83K7C also appeared as a complex of approximately 150-kDa when visualized with an anti-kappa LC antibody (Figure 6A). Similarly, visualization of 83K7C with anti-gamma HC antibodies revealed complexes of the predicted 150-kDa size for the algal expressed antibody, while the CHO expressed complex showed a larger amount of heterogeneity. For the CHO expressed antibody there was HC protein in a complex of 150 kDa, in addition to signals in smaller complexes that may have resulted from partially assembled HC proteins (Figure 6A.).

Using a variety of protein purification techniques we were able to further demonstrate that the HC and LC assembled into full length antibody complexes. Protein G binds the Fc domain which consist of constant domains 2 and 3 of IgG1s and is commonly used to purify monoclonal antibodies (Erntell, Myhre et al. 1988). Co-purification of stoichiometric amounts of 83K7C LC and HC protein from chloroplast of *C. reinhardtii* using Protein G purification suggests that chloroplast efficiently assemble full-length human monoclonal antibodies (Figure 6B). Size exclusion chromatography was then used to separate a majority of the degradation from the fully assembled antibody (Figure 7). Using size exclusion chromatography,

stoichometric amounts of the full length non-degraded HC and LC co-purified while the degradation product of the HC was separated, again suggesting that the antibody is correctly assembled.

Algal expressed 83K7C binds to protective antigen 83 at similar levels as Mammalian expressed antibodies

To determine whether the anti-anthrax 83K7C antibody expressed in chloroplast was capable of binding its target antigen PA83, ELISA assays were performed on the purified CHO and *C. reinhardtii* expressed antibodies. Purified 83K7C expressed in the chloroplast was able to bind to PA83 in a linear manner up to saturation at 10ug/ml (Figure 8). Soluble proteins from wt *C. reinhartdii* cells showed no binding (data not shown). CHO expressed and purified 83K7C also bound to PA83 that was fixed to a microtiter plate at similar levels as previously reported (Wild, Xin et al. 2003), and at levels similar to that of the algal expressed antibody. To confirm that LC proteins are present with HC proteins in the algal expressed antibody that binds to PA83, LC binding was also assayed using an anti-kappa LC antibody for detection. LC protein from the algal expressed 83K7C was found bound to the PA83 antigen, confirming that an assembled 83K7C antibody bound to the target antigen in these assays (Figure 8). These data establish that chloroplast expressed monoclonal antibodies are capable of forming functional antigen binding molecules, and that the molecule binding to the target antigen is the fully assembled antibody.

Determining the kinetic constants of 83K7C expressed in algae compared to CHO expressed antibody, by Surface Plasmon Resonance

The kinetic constants of the algal and CHO expressed 83K7C IgG mAb was determined using Surface Plasmon Resonance (SPR). PA83 was immobilized onto a CM4 Chip and followed by injections of purified 83K7C at increasing concentrations. The kinetic constants, including association and dissociation equilibrium constants (K_A and K_D), and association and dissociation rate constants (k_a and k_d), were determined for both the algal and mammalian expressed antibodies (Table 1). These data show that the only significant difference between the algal and CHO IgG1 is in their k_a (Table I). This difference in binding rate, while being well within the margin of error for measuring antibody affinity, could be attributed to the presence of non-functional degradation products in the algal sample (Figure 5B). These differences would alter the calculated molar concentration of the fully assembled antibody, resulting in an apparent slower binding rate for the algal produced antibody, when in fact the rates are the same. Once bound however, the k_d is nearly identical between the two IgG1s, suggesting that their binding affinities are the same. These results show that the algal expressed IgG1 binds its target antigen with an affinity that is almost identical to the same antibody expressed in mammalian cell culture.

Additional human antibodies expressed by the algal Chloroplast

To demonstrate that 83K7C was not a unique case of antibody expression in algal chloroplast, two others antibodies were expressed, an additional human IgG1 and

a murine IgG1. The LC and HC of these antibodies were synthesized in chloroplast codon bias and a flag peptide was placed on the carboxyl terminus of the HC and LC of each antibody. The LC and HC genes of each antibody were transformed into algal chloroplasts using the same transformation vectors and protocols as above, and homoplasmic lines were isolated that contained both of the respective LC and HC genes. Immunoblot analysis was performed on these antibodies as well as on the algal expressed 83K7C. As shown in Figure 9, both of these antibodies accumulated in algal chloroplast. Purification of these antibodies by protein G resulted in the isolation of stoichiometric amounts of HC and LC proteins for both the human and murine IGg1. These results indicate that the chloroplast of *C. reinhardtii* is capable of expressing and assembling a variety of mammalian antibodies. All three of these strains have been maintained for over a year and all continue to stably express significant levels of these antibodies. By demonstrating the ability of *C. reinhardtii* to express two human IgG1 and a murine IgG1 we have shown that the algal expression system is flexible and capable of expressing a wide range of antibodies.

Discussion

We have shown that HC and LC proteins of a human IgG1 antibody expressed in chloroplasts accumulate as soluble proteins that assemble into complexes that closely resemble assembled human IgG1 antibodies expressed in mammalian cell culture. We have also shown that an algal expressed antibody binds its target antigen, PA83, at levels similar to the same antibody expressed in mammalian cells. These

data indicate that chloroplasts have the ability to synthesize and assemble functional, complex human antibodies, and that these antibodies contain the correct disulfide bonds and have affinities similar to mammalian expressed antibodies.

Chloroplasts lack the enzymes required for glycosylation, and antibodies expressed in chloroplasts are predicted to be aglycosylated. Glycosylation of HC has been shown to impact the ability of the antibody to bind to Fc receptors and complement components (Simmons, Reilly et al. 2002), but glycosylation appears to have little impact on antigen binding in an IgG1 antibody (Walker, Lund et al. 1989). Without the ability to bind the Fc receptor, chloroplast-expressed antibodies likely lack an ability to recruit killer cells or fix complement. Although binding Fc receptors and fixing complement are key aspects of antibody function in the normal immune response, neither of these functions is required for the 83K7C antibody to work as a toxin blocking agent. In addition, there are many therapeutic circumstances when recruitment of killer cells or activation of complement are not required or specifically not wanted in a therapeutic antibody, and chloroplast expressed antibodies would be ideal in these situations. Our results confirm that glycosylation appears to have little impact on antibody assembly or antigen binding, and chloroplast expressed aglycosylated antibodies may have many uses as therapeutic agents.

Previously, there had been a number of chaperones identified in chloroplast (Schroda 2004) and here we show that these chaperones and other components of algal chloroplasts are sufficient to facilitate the folding and assembly of a full-length human monoclonal antibody. Just as important, we have also shown that these antibody

complexes remain soluble and would not require the costly steps of denaturing and refolding as required for the production of full-length antibodies in bacterial systems (Simmons, Reilly et al. 2002). This places algae in a unique position as a platform for the cost effective production of full-length aglycosylated human monoclonal antibodies. There are, however, improvements that are still needed in the algal system for it to become economically viable at a large scale. One of the main improvements that will be targeted in the coming years will be to increase protein expression levels. By expressing the antibodies as a higher percentage of total protein we will be able to decrease the cost of these traditionally expensive therapeutics. We also need to identify native proteases and protease sites within antibodies to reduce the degradation that we observe in the HC proteins, which leads to a lower overall yield of full-length antibodies When we compare the expression of the human and murine IgG1 it is noticeable that the murine IgG1 does not degrade as significantly as the human IgG1 which suggest a site specific proteolytic sequence in human HC proteins. To further address this issue, mutant strains deficient in chloroplast proteases will need to be developed, and the proteases of the chloroplast more thoroughly analyzed in order to create an optimal expression strain of *C. reinhardtii.* Improving the purification protocols so that large quantities of highly purified algal expressed antibodies, that are suitable for use in animal studies, can be obtained will be extremely important for algal therapeutics to become a reality. The data presented here clearly demonstrate the unexplored potential of eukaryotic algae as a system for the rapid and cost effective

production of complex human antibodies for use in passive immunization or any number of therapeutic and diagnostic purposes.

Experimental Procedures

Plasmid Construction

All DNA and RNA manipulations were performed as described (Sambrook 1989, Cohen, Yohn et al. 1998). The variable regions for the HC and LC genes of human antibody 83K7C (Wild, Xin et al. 2003) were synthesized de novo with *C. reinhardtii* chloroplast codon bias using PCR assembly (Stemmer, Crameri et al. 1995). The variable regions were ligated into constant domains of human IgG1 yielding 657-bp LC and 1352-bp HC genes that were also synthesized with chloroplast codon bias (Suppleental Figure 1A, B). For HC expression we placed the codon optimized HC sequence downstream of the *psbA* promoter and 5' UTR. This was followed by the 3' UTR of the *rbcL* gene (Figure 2A). We included flanking genomic sequences of the *psbA* gene to allow integration of the HC gene as a replacement of the endogenous *psbA* gene (Figure 2A). For LC expression we used the *psbA* promoter 5' and 3' UTRs (Figure 2B). To ensure LC integration we used the flanking sequences around the *psbH* gene to allow for integration of the LC gene at a silent site near the *psbH* locus (Figure 2B).

Southern and Northern blot analysis

Southern blots and ^{32}P labeling of DNA for use as probes, were carried out as previously described (Franklin, Ngo et al. 2002). Probes made for Southern blots include a 2.0-kb BamHI/XhoI fragment of the psbA locus, a 1.1-kb BamHI/ScaI fragment of the psbH locus, a 1352-bp NdeI/XbaI fragment from 83K7C HC or a 657-bp NdeI/XbaI fragment from 87K7C LC. Additional probes that were used for Northern blots include the *psbA* cDNA. Southerns and Northerns were visualized using a Packard cyclone Storage Phosphor system that used OPTIQUANT software. Vectors were co-transformed with plasmid p228 that confers spectinomycin resistance from a point mutation in the 16S rRNA gene (Franklin, Ngo et al. 2002). Spectinomycin resistant colonies were screened by Southern analysis using radio-labeled probes of the coding region of both the HC and LC genes of 83K7C. To ensure that all copies of the chloroplast genome contained the desired genes, additional rounds of Southern analysis were used to identify strains in which all copies of the chloroplast genome contained both HC and LC genes. Identification of these homoplasmic lines ensures that the strains remain stable and express the desired proteins at significant levels.

PCR analysis of transgenic algae

PCR screens were performed to ensure the integration of the HC and LC genes into the chloroplast genome. A forward primer was designed to the 5' UTR of psbA gene, 5'-gtgctaggtaactaacgtttgattttt-3' and reverse primers were designed to specific

sites of both the HC, 5’-tgtgtgagttttgtcacaagatttt-3’ and LC genes, 5’-ttcaccacggttgaaactttttgta-3’. To further confirm that the transgenic strains were homoplasmic for the HC gene, PCR was performed using a forward primer against the psbA gene, 5’- ggaaggggaggacgtaggtacataaa-3’ as well as a reverse primer designed against the psbA gene, 5’-ttagaacgtgttttgttcccaat-3’. As a control forward primers, 5’-ccgaactgaggttgggttta-3’ and reverse primers, 5’-gggggagcgaataggattag-3’ were designed against the 16srRNA genomic sequence. To test the strains homplasmicity for the LC gene in the psbH locus forward primers, 5’-ctcgcctatcggctaacaag-3’ and reverse primers, 5’-cacaagaagcaacccccttga-3’ were designed against the integration site of the psbH integration vector and PCR analysis performed. Again the same primers against the 16sRNA genomic sequence were used as a control.

Determining Strain Stability

To confirm that chloroplast transformed strains were stable, strain 65.11 was re-plated each month for the past 18 months. Strains were also maintained off of selection on TAP plates containing no antibiotics to ensure stability of transformed cultures.

Antibody protein expression and characterization

Transgenic *C. reinhardtii* cultures were inoculated at 2x10^5 cells/ml and grown in the dark for 5 days to a density of 2x10^6 cells/ml. Cultures were then split into 10L at a density of 3x10^5 and grown in light (8000lux) for 48 hours before

harvesting. Cells were lysed by sonnicationin lysis buffer (50mM Tris-HCL pH8.0, 400mM NaCl, 10% sucrose containing complete protease inhibitor (Roche)). The soluble and insoluble proteins were separated using high speed centrifugation. The soluble protein was applied to protein G sepharose linked beads and eluted using IgG elution buffer (Pierce). The antibody was further purified using size exclusion chromatography to remove the degradation in the sample (Figure 7). Human IgG1 HC and LC proteins were identified using a goat anti-human gamma HC antibody and a goat anti-human kappa LC antibody (Southern Biotech). All procedures were carried out as described (Sambrook 1989). Immunoblot analysis was also done in non-reducing conditions to visualize antibody assembly.

Antigen binding ELISA assays

ELISA antigen binding assays were done in 96-well microtiter plates (Coaster) coated with 50 ul of 1ug/ml of purified anthrax PA83. Plates were blocked with PBS blocking buffer (Pierce). Increasing amounts of *C. reinhardtii* expressed 83K7C, wt *C. reinhardtii* proteins, or purified CHO expressed 83K7C were incubated in individual wells. The samples were washed three times with PBS. Secondary HRP conjugated antibodies, goat anti-human kappa LC or goat anti-human gamma HC were then applied. A [1:1] mixture of peroxide solution (H_2O_2) and peroxidase substrate (TMB, Pierce) was premixed and 50 ul was added to each sample well, followed by 50 ul of a 2M H_2SO_4 solution to stop the reaction. The plates were visualized at 450nm and

binding was quantified using a Spectra Max 250 plate reader (Molecular Devices) with SOFTmax Pro software.

Kinetic Determination Using Plasmon Resonance

The kinetic constants of 83K7C CHO expressed antibody and 83K7C algal expressed IgG1 against PA83 were determined by surface plasmon resonance (SPR) on a BIAcore™ 3000 instrument (Biacore Ab, Uppsala, Sweden) at 25 °C as described elsewhere with modification (Zhou, Carney et al. 2008). In brief, PA83 was immobilized onto a CM4 chip targeting at 500 RU using NHS/EDC coupling chemistry, while an in-line reference with NHS/EDC activated and an ethanolamine deactivated surface was set up. Various concentrations of each IgG sample prepared at 0.04, 0.2, 1, 5, and 25 nM were injected onto the chip surface, and the interaction between IgG1 and immobilized PA83 was recorded in the sensorgram. Several blank buffer injections were engaged before and after the kinetic analysis. Measurements were conducted in triplicate under HBS-EP buffer at a flow rate of 30 µL/min, and the chip surface was regenerated by 4M $MgCl_2$ for 1 minute before proceeding to the next round of analysis. The kinetic data were evaluated by fitting the sensorgram data using a BIAevaluation software (ver. 4.1) using the Bivalent Analyte Model. The kinetic constants, including association and dissociation equilibrium constants (K_A and K_D) and association and dissociation rate constants (k_a and k_d), were determined for each IgG1.

Acknowledgements

Chapter 2, in full, is a reprint of the material as it appears in Biotechnology and Bioengineering Journal, 2009. Tran, Miller; Zhou, Bin; Pettersson, Par L; Gonzalez, Maria; Mayfield, Stephen P 2009. The dissertation author was the primary investigator of this paper.

Figures

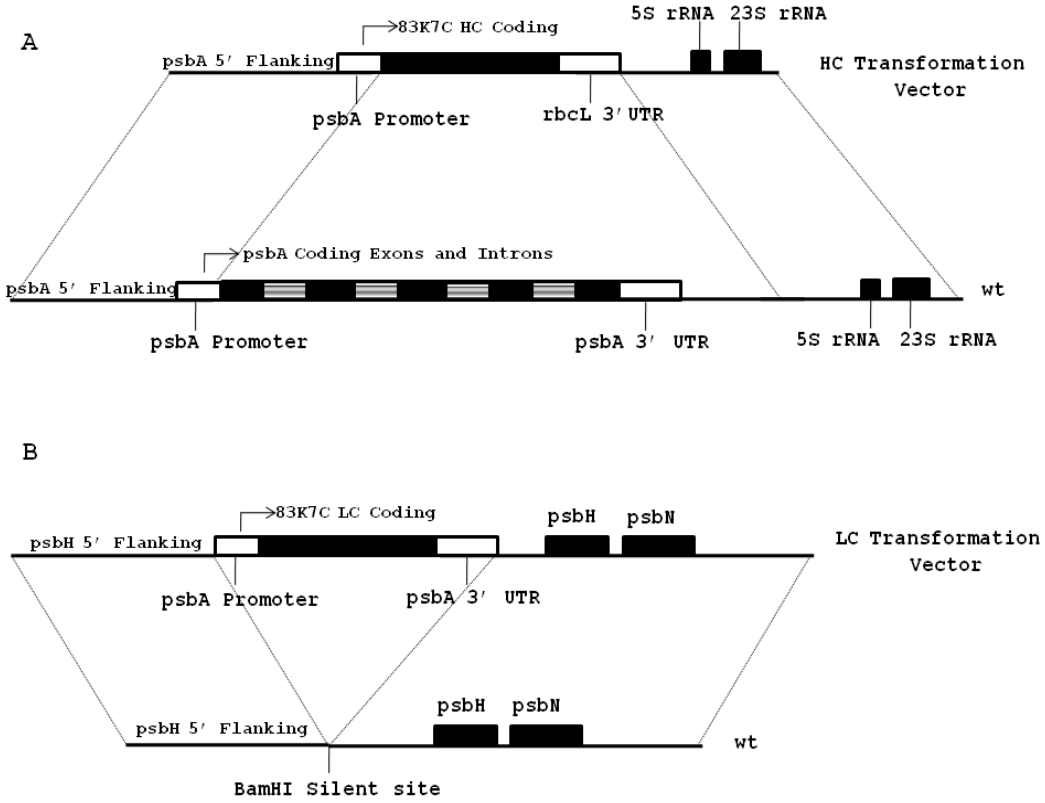


Figure 1. DNA gene constructs and sites of integration for both the HC and LC of 83K7C. (*A*) The HC gene is surrounded by DNA sequences that are homologous to the *C. reinhardtii* chloroplast genome and its expression is driven by the psbA promoter. The sites of homology on the vector allow the HC gene to integrate into the inverted repeat of the chloroplast genome and knock out the psbA gene. (B) The LC gene is placed upstream of the psbH gene and its expression is also driven by the psbA promoter. The sites of homology for the psbH gene are flanking the 5’ and 3’ UTR of the LC gene and allow for the gene to be integrated upstream of the psbH gene.

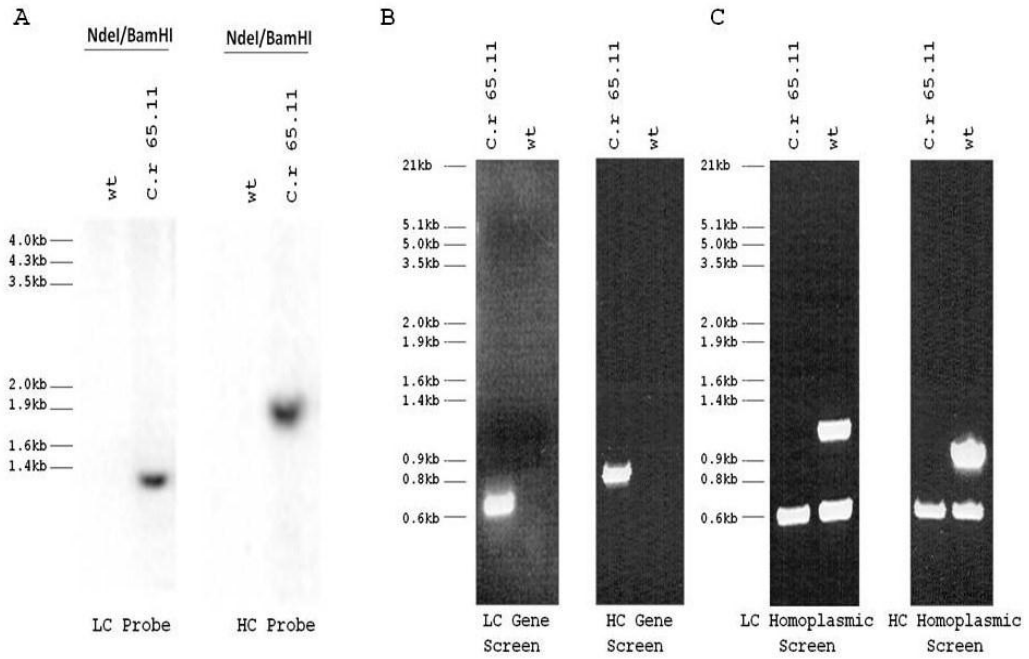


Figure 2. Southern blot and PCRanalysis of the *C. reinhardtii* genome. These blots and PCRs ensure the complete integration of the HC and LC genes of 83K7C into the chloroplast genome. (*A)* Both the HC and LC genes were recognized using ^{32}P labeled DNA probes. Both genes were shown to be homoplasmic inside the chloroplast of transgenic strain 65.11 by using a psbH ^{32}P for the LC and a ^{32}P labeled 5s rRNA/23srRNA probe for the HC. The loss of both the wt bands in strain 65.11 indicate that it is homoplasmic. (*B*)PCR was performed to ensure that strain 65.11 contained both the HC and LC genes using primers that were specific to the two genes. PCR was also performed to show that the strain 65.11 is homoplasmic using primers indicated primers. The loss of the extra band in the transgenic lanes indicates that the transformed strains are homoplasmic for both the HC and LC genes. .

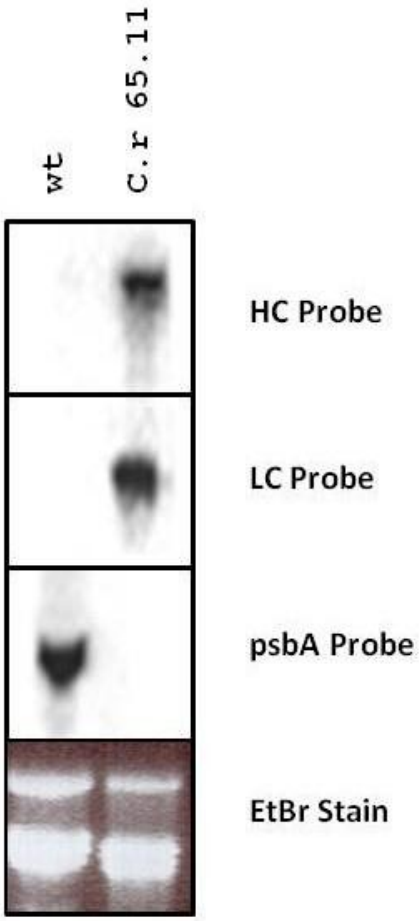


Figure 3. RNA northern blot analysis of transgenic strain 65.11. Total RNA was analyzed to show that strain 65-11 is actively transcribing both genes. An additional analysis was performed using a ^{32}P labeled psbA probe to show the complete knockout of the psbA gene.

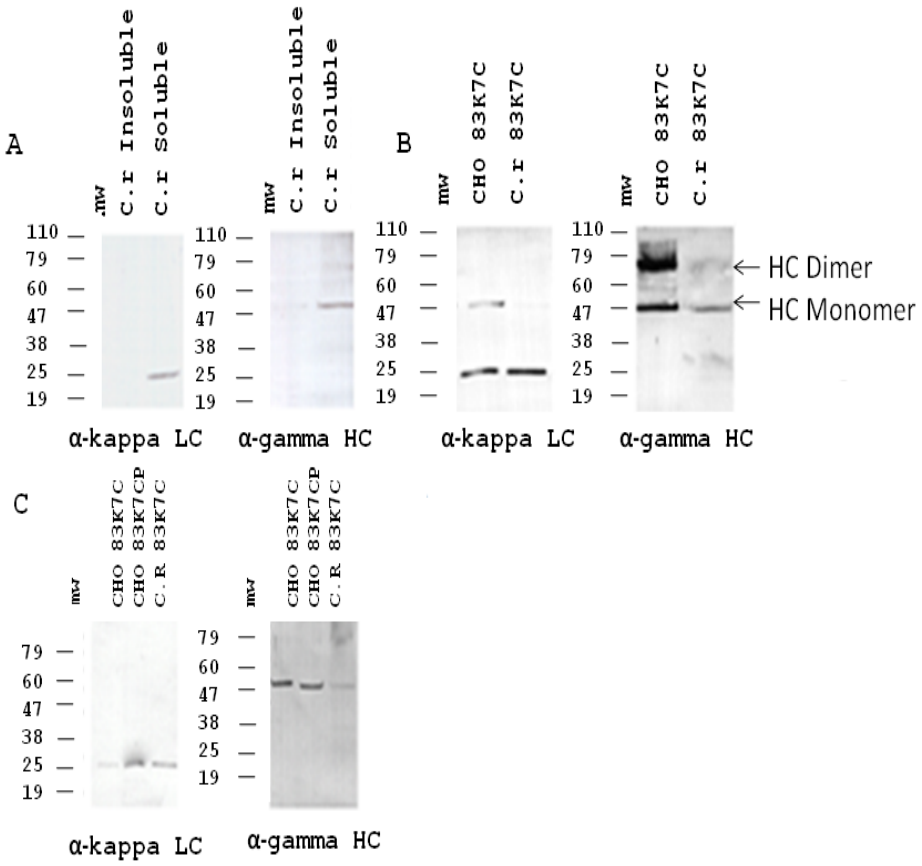


Figure 4. Transgenic algae transformed with the HC and LC genes of 83K7C are capable of expressing aglycosylated versions of the antibody. (*A*) The antibody being expressed inside of chloroplast of *C. reinhardtii* remains soluble when lysed and separated from the insoluble fraction. This is important for purification purposes, to ensure that no denaturing and refolding of the protein will be necessary. (*B)* When compared with CHO expressed 83K7C the algal expressed antibody is comparable in size, although the HC is slightly smaller due to its lack of glycosylation. The HC also suffers from partial degradation. (*C*) To show that algal expressed antibodies are not glycosylated the CHO antibody was treated with PNGase F to remove any sugars that were attached. By doing so the algal expressed antibody is shown to be the same size as the aglycosylated version of the mammalian expressed antibody.

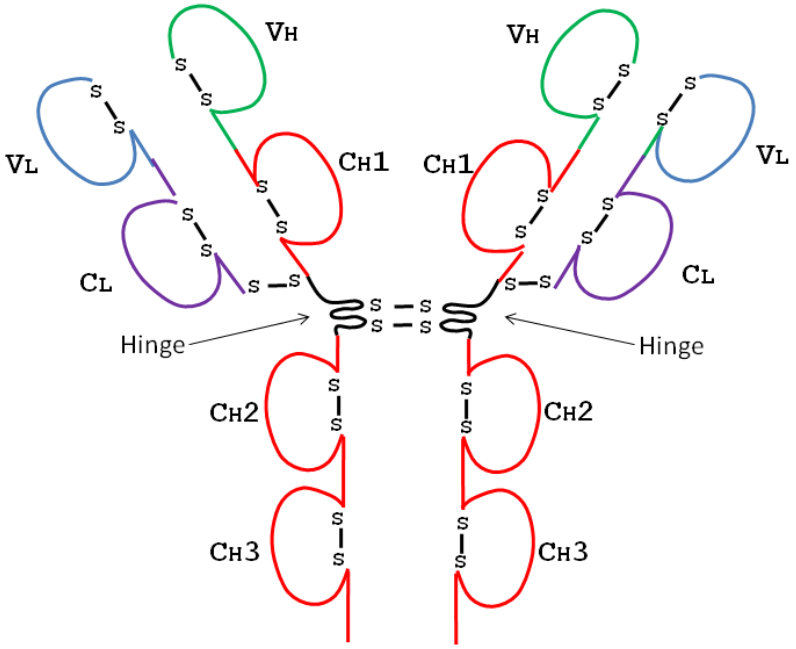


Figure 5. Necessary disulfide bond formation of an IgG1. A human IgG1 contains two HC and two LC proteins. In order for the antibody to assemble properly 16 disulfide bridges must be formed between the heavy and light chain proteins.

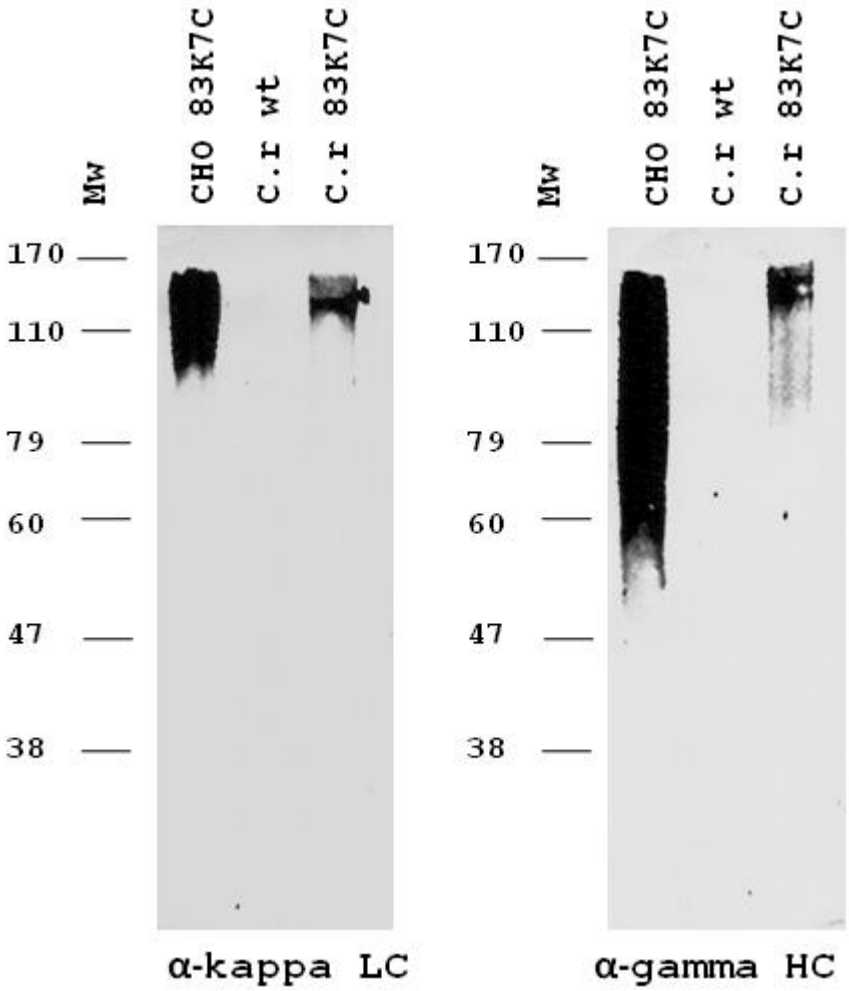


Figure 6. Transgenic strain 65.11 is capable of assembling HC and LC genes of 83K7C into full-length human monoclonal antibodies. (*A*) Non reducing westerns were run with both the CHO and algal expressed antibody to show that they assemble into tetramer complexes that are larger than the monomer subunits. These non reducing westerns confirm that algae have the necessary machinery to assemble a full-length human monoclonal antibody. (*B)* The full-length antibody was then purified from crude algal lysate using protein G, which binds to the Fc domain of the IgG1. Here it can be seen that the LC is co-purified along with the HC when using protein G, further confirming that the HC and LC are being assembled into full-length antibodies by algal chloroplast.

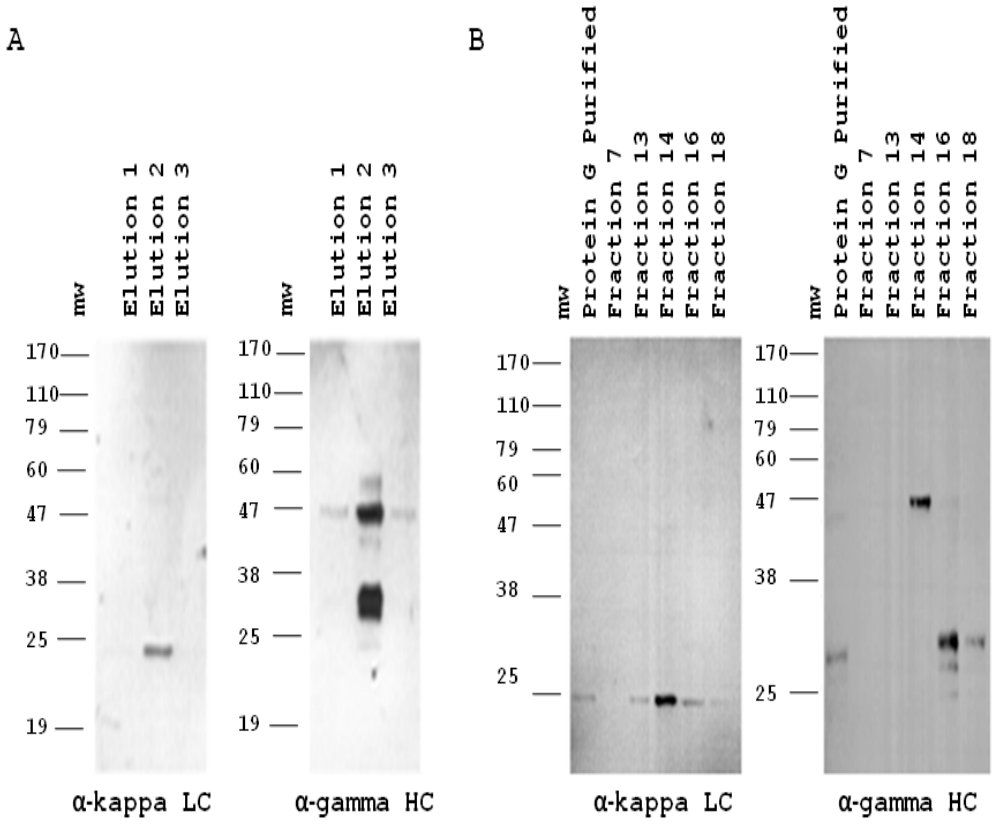


Figure 7. Separation of the degradation product from the full-length 83K7C expressed in algal chloroplast. The partial degradation of the antibody that occurs in chloroplast of *C. reinhadtii* is separated from the full-length antibody using size exclusion chromatography. This separation is important for down the road applications of the antibody as it is necessary to be able to inject pure samples into patients. The amount of LC correlates well with the amount of full-length HC that is accumulated during this chromatography step, again demonstrating that the LC assembles with the HC into a full-length human antibody

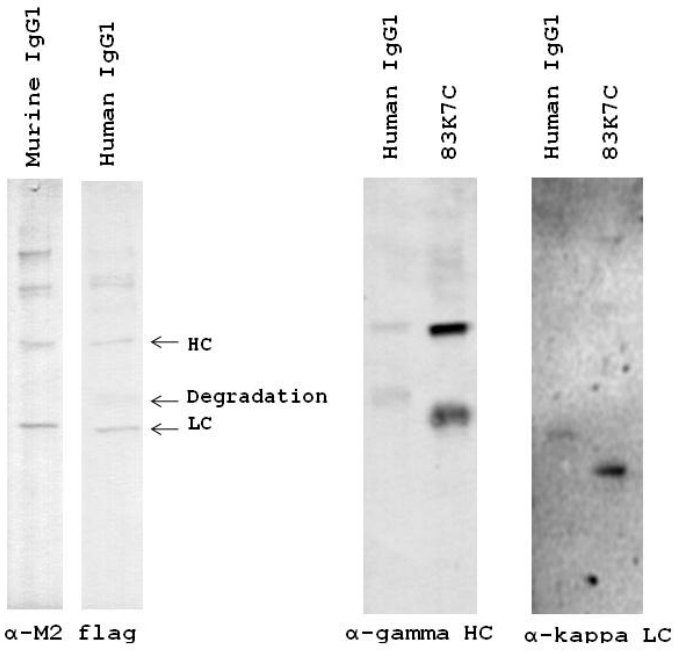


Figure 8. Algal chloroplast expression of a variety of human antibodies. This immunoblot shows in lane one an alternate IgG1, in lane 2a murine IgG1 antibody, and in lane 4 the 83K7C antibody. This blot shows that C.r chloroplast has the ability to express a wide range of antibodies and that the 83K7C expressed antibody was not an isolated incident.

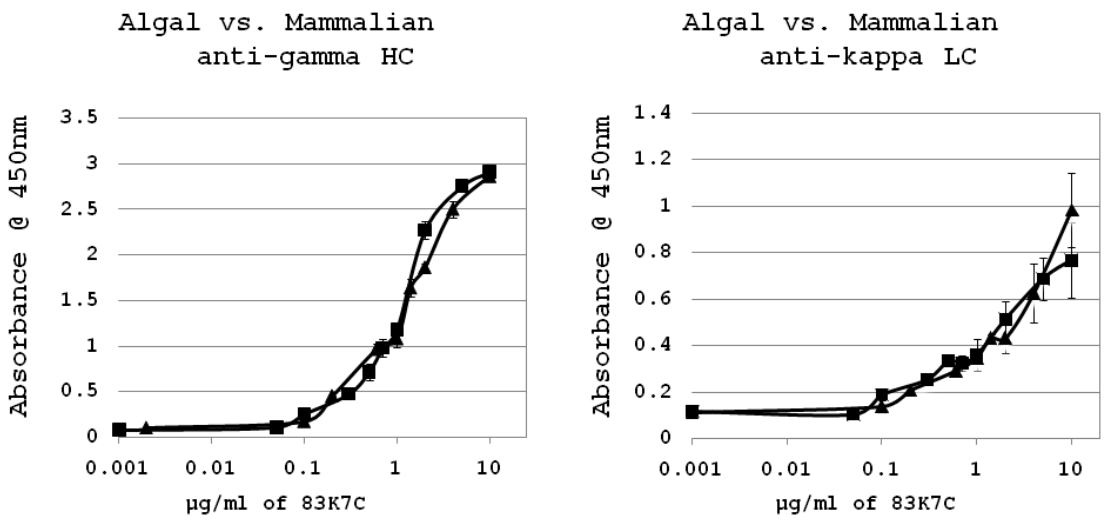


Figure 9. Binding of the target antigen and measurements of the relative affinity of algal expressed antibody against PA83. (*A*) Microtiter plates were coated with PA83 and ELISA assays were performed to show that algal expressed 83K7C is capable of binding to its target antigen. All measurements were done in triplicate from a rage of 0.001ug/ml to 10.0ug/ml for both the algal and CHO expressed antibody. The data suggest that algal expressed antibody is comparable to the CHO expressed antibody in binding capabilities.

Table 1. Surface plasmon resonance analysis. Comparison of algal expressed human antibody and CHO expressed antibody

	k_a ($M^{-1}S^{-1}$)	K_d (S^{-1})	K_D (M)	R_{max} (RU)	Chi^2
83K7C_Mam	6.64×10^5	1.22×10^{-2}	1.84×10^{-8}	127	0.238
83K7C_Algae	2.29×10^5	9.58×10^{-3}	4.18×10^{-8}	38.7	0.752

Chapter 3: Production of unique immunotoxin cancer therapeutics in algal chloroplast

(Adapated from: Miller Tran, Christina Van, Daniel J Barrera, Par L. Pettersson, Carlos D. Peinado, Jack Bui, Stephen P. Mayfield. "Production f unique immunotoxin cancer therapeutics in algal chloroplast". PNAS. 2012)

Introduction

The idea of targeted therapy, whereby drug or protein molecules are delivered to specific cells is a compelling approach to treating disease. Immunotoxins are one such targeted therapeutic, consisting of an antibody binding domains for targeting, and a toxin molecules that inhibits the proliferation of the targeted cell. One major hurdle preventing these therapies from reaching the market has been the lack of suitable production platform that allows for the cost effective production of highly effective molecules. The chloroplasts of the green algae *Chlamydomonas reinhardtii* have been shown to contain the machinery necessary to fold and assemble complex eukaryotic proteins. However, the translational apparatus of chloroplasts resembles that of a prokaryote, allowing them to accumulate eukaryotic toxins that would otherwise kill a eukaryotic host. Here we show expression and accumulation, in algal chloroplasts, of monomeric and dimeric immunotoxin proteins. These fusion proteins contain an antibody domain targeting CD22, a B-cell surface epitope, and the enzymatic domain of exotoxin A from *Pseudomonas aeruginosa* . We demonstrated that algal produced

immunotoxins accumulate as soluble and enzymatically active proteins that bind target B-cells, and efficiently kill them in vitro. We also show that treatment of mice with either the mono- or dimeric immunotoxins significantly prolongs their survival in a mice tumor model.

Microscopic eukaryotic green algae play an essential role in converting solar energy into chemical energy through the process of photosynthesis(Raghavendra 1998). Recently, these microorganisms have garnered the attention of the energy industry as a source of renewable fuel(Hannon, Gimpel et al. 2010). In addition to energy production, micro-algae have enormous potential for a variety of uses in biotechnology including as nutraceuticals and as a platform for the production of recombinant proteins including industrial enzymes and therapeutics(Rasala and Mayfield 2011, Gregory, Li et al. 2012). Using the green algae *Chlamydomonas reinhardtii* we have demonstrated that algae are capable of expressing, folding, and accumulating a range of human therapeutic proteins in the chloroplast (Tran, Zhou et al. 2009, Rasala, Muto et al. 2010, Specht, Miyake-Stoner et al. 2010). More recently we have shown that recombinant proteins can also be secreted from algae(Rasala 2012). Algae require only trace minerals, fertilizer, and sunlight to be grown at scale, giving them the potential to produce recombinant proteins, including therapeutics, very inexpensively(Franklin and Mayfield 2004). While cost can be a significant factor in the production of protein based therapies, producing unique classes of therapeutically relevant proteins is also desirable, and algae offer the potential to

produce a number of novel proteins due to the unique biochemical environment of the chloroplast(Tran, Zhou et al. 2009).

Immunotoxins, are antibodies that are either chemically(Shen, Li et al. 1988) or genetically(Mansfield, Amlot et al. 1997) coupled to a eukaryotic toxins. These chimeric proteins are used to deliver a eukaryotic toxin to a specific cancerous cell to initiate apoptosis(Bogner, Dechow et al. 2010). These molecules are currently produced as either antibodies that are expressed in CHO cells and chemically coupled to a toxin(Shen, Li et al. 1988) or as insoluble aggregates in bacteria which are then denatured and refolded to produce functional proteins(Mansfield, Amlot et al. 1997)**.** Eukaryotic expression platforms such as yeast, CHO cells, and insect cells are incapable of producing and accumulating genetically coupled immunotoxins due to inhibition of host cell proliferation by the toxin. Because of this, production of genetically coupled immunotoxins is limited to bacterial expression platforms, where the eukaryotic toxin does not inhibit the host's ability to grow. This limits the complexity of immunotoxins that are currently produced due to the nature of bacterial expression platforms, which lack the ability to efficiently fold proteins with multiple domains, or to form disulfide bonds within proteins(Yin, Li et al. 2007). These limitations reduce immunotoxin production in *E. coli* to scFv antibodies and eukaryotic toxins(Chaudhary, Queen et al. 1989) or disulfide stabilized variable domains formed *ex vivo* that are genetically linked to a eukaryotic toxin(Brinkmann, Reiter et al. 1993). For chemically linked immunotoxins, antibodies can be expressed in CHO cells and the toxin coupled in vitro, leading to functional complex proteins,

but this process results in additional chemical processing steps that leads to more expensive drug conjugates(Selyukh 2011). Each of these immunotoxin types have been demonstrated to be potent and potentially useful tools for the treatment of solid tumor(Cao, Marks et al. 2012).

C. reinhardtii is a eukaryotic algae that contains a single chloroplasts that constitutes up to 70% of the cell(Harris 2009). Chloroplasts have been shown to contain ribosomes and translation factors that resemble those of photosynthetic prokaryotes(Beligni, Yamaguchi et al. 2004, Manuell, Quispe et al. 2007). However, unlike bacteria, chloroplasts contain a wide range of chaperones(Schroda 2004), protein disulfide isomerases(Danon and Mayfield 1994), and PPIases(Breiman, Fawcett et al. 1992) that allow them to fold the complex proteins of the photosynthetic apparatus. This machinery also allows them to fold complex recombinant proteins such as full length human antibodies, that accumulate as soluble and functional molecules(Tran, Zhou et al. 2009).

To demonstrate that algae are capable of producing fully functional immunotoxins, a single chain antibody (scFv) that recognizes CD22, a B-cell surface molecule(Mansfield, Pastan et al. 1996) (Figure 1a), was genetically coupled to domains II and III of Exotoxin A (PE40) from *Pseudomonas aeruginosa* (Figure 1b). This chimeric protein is very similar to one produced in *E. coli* called αCD22PE40(Mansfield, Chiron et al. 1997). PE40 inhibits the translation of eukaryotic cells by ribosylating eukaryotic elongation factor 2 (eEF2), preventing the elongation of polypeptide chains leading to apoptosis of the targeted cell(Kondo,

FitzGerald et al. 1988). A Significant problem with immunotoxins similar to αCD22PE40 is their short serum half-life, resulting from their small size(Kreitman, Wang et al. 1999). To overcome this potential problem we also engineered a more complex chimeric immunotoxin gene that contained the hinge and CH2 and CH3 domains of a human IgG1 placed between the αCD22 scFv antibody and PE40, encoding a protein that we have termed αCD22CH23PE40 (Figure 1c). This molecule should be capable of forming a dimer through two disulfide bonds in the hinge region making it significantly larger than αCD22PE40, and doubling the number of CD22 binding domains and PE40 molecules to two.

Recently, antibody drug conjugates (ADCs) have garnered a significant amount of attention for their ability to inhibit cancer cell proliferation (Bertholjotti 2011). These ADCs are produced by first purifying an antibody made in a mammalian expression platform and followed by a chemical coupling to a toxic molecule making them expensive therapies costing upwards of $100,000(Selyukh 2011). These ADCs are engineered with special linkers that are cleaved upon acidification in the endosome following antibody binding and endocytosis(Minich 2012). PE40 provides us with a biological answer to the complexities of chemical coupling. PE40 is generated by first removing domain Ia from the full length exotoxin A. This makes PE40 more than 100-fold less toxic than the native exotoxin A due to its inability to bind to cells(Frankel 1992). Domain II of this protein is cleaved in acidified endosomes by a furin protease, liberating the cytotoxic domain III from the antibody which is targeted for degradation(Gordon, Klimpel et al. 1995). Additionally, the ability to produce

genetically coupled immunotoxins eliminates the possibilities of unstable linkers(Xie, Audette et al. 2004) and off target toxicity. Thus, immunotoxins provide a protein alternative to chemically synthesized drug molecues that are currently being linked to cancer targeting antibodies.

Here we demonstrate for the first time that a eukaryotic cell is capable of accumulating both small monomeric and larger dimeric immunotoxins. These immunotoxins are enzymatically active, bind specifically to cells displaying the CD22 molecule and are capable of causing those cells to undergo apoptosis. Furthermore, in subcutaneous Ramos cell xenograft mice models both algal produced immunotoxins are capable of significantly inhibiting tumor proliferation.

Results

Protein engineering and expression vector construction

All DNA manipulations were performed as previously described(Manuell, Beligni et al. 2007). The immunotoxin genes and subfragments were synthesized de novo using *C. reinhardtii* chloroplasts codon bias and ordered through DNA 2.0. The variable domains of a human antibody produced against the B-cell surface antigen CD22 were separated by a linker consisting of four glycines and a serine repeated four times ($4xG_4S$) to create a single chain antibody fragment (scFv) that was ligated downstream of a DNA sequence coding for a 1 x Flag peptide (DYKDDDDKS) and separated by a DNA sequence that encodes for a TEV protease cleavage site

(ENLYFQG). This gene was termed αCD22 (Figure 1a). This scFv was ligated upstream of a 2 x G_4S linker, the coding sequence for domains II and III of *Pseudomonas* exotoxin A (PE40), and the DNA sequence coding for a KDEL endoplasmic reticulum localization peptide, which has been shown to increase the activity of exotoxin A based immunotocins(Kreitman and Pastan 1995). This molecule was termed αCD22PE40 (Figure 1b). To create the larger dimeric immunotoxin, the hinge and constant domains 2 and 3 (CH23) of a human IgG1 were ligated between the scFv and PE40, separated on both its amine and carboxy end by a 2 x G_4S linker (αCD22HCH23PE40) (Figure 1c). These constructs were placed in *C. reinhardtii* chloroplast transformation cassette that contains the psbA promoter and 5' UTR upstream and the psbA 3' UTR downstream of the recombinant immunotoxin genes (Figure 2a). A kanamycin resistance gene whose expression was driven by the atpA promoter and 5'-UTR was placed downstream of the psbA 3'-UTR (Figure 2a), and was used to select algae that were transformed with the recombinant genes. Additional homology is present downstream of the 3'UTR to facilitate the integration of the immunotoxin gene into the chloroplast genome (Rasala, Muto et al. 2010).

Analysis of gene integration into the chloroplast genome

Transformation vectors were precipitated onto gold particles and transformed into wild type *C. reinhardtii* cells by particle bombardment, and selected on Tris-acetate-Phosphate (TAP) plates containing 100ug/mL of kanamycin (Figure 2b). Colonies that grew were screened for the presence of heterologous genes. Chloroplasts

contain up to 80 copies of their plastid genome and finding strains in which all 80 copies contain the heterologous gene is essential for identifying strains that stably express the desired gene(Mayfield and Schultz 2004). PCR analysis was used to identify several transgenic lines for each construct in which the αCD22, αCD22PE40, or αCD22HCH23PE40 gene was correctly integrated into the chloroplast genome at the *psbA* locus (Figure 2c) (Tran, Zhou et al. 2009).

To identify strain homoplasmic for each of these recombinant genes, additional rounds of algae cell cloning and PCR analysis was performed. Primers corresponding to the psbA 5'UTR and the coding region of the recombinant genes, or the native psbA gene were used to amplify strain homoplasmic for recombinant gene integration as previously described (Tran, Zhou et al. 2009). Control primers for the 16S rRNA region of the chloroplast genome were used for validation that the PCR reaction was successful (Tran, Zhou et al. 2009). As shown in Figure 2d homoplasmic strains were identified for all three gene constructs.

Analysis of recombinant protein accumulation in transgenic algal strains

To determine if algal chloroplasts accumulate immunotoxin proteins, western blot analysis with an anti-FLAG antibody conjugated with alkaline phosphatase was used to assay for the presence of αCD22, αCD22PE40, and αCD22HCH23PE40 in each of the respective transgenic strains (Figure 3a). αCD22, αCD22PE40, and αCD22HCH23PE40 accumulate as soluble proteins within the transgenic algae, and all migrate at the expected size when separated by reducing PAGE. When proteins

were separated on a non-reducing PAGE and assayed by Western blot analysis, αCD22HCH23PE40 accumulated as a 190 kDa species, suggesting that chloroplast assemble this protein into a dimmer (Figure 3b). The αCD22 and αCD22PE40 both migrated at the expected mass of the monomer on these non-reducing gels. These data suggest that disulfide bonds are formed between the cysteine residues found in the hinge region of a human IgG1 resulting in dimerization of αCD22HCH23PE40 within algal chloroplasts.

ADP-ribosyltransferase assays of algal expressed immunotoxins

An ADP-ribosylation assay was used to identify if algal expressed αCD22PE40 and αCD22HCH23PE40 molecules are enzymatically active. Biotinylated-NAD+ (nicotinamide-adenine dinucleotide, Sigma) the substrate for ribosyltransferases, was incubated with eEF2 and purified, algal produced immunotoxins (Figure 4a). Active PE40 molecules are capable of transferring biotinylated-ADP molecules from biotinylated-NAD+ onto EF2 (Figure 4b). Successful transfer can be visualized by separating the EF2 protein on a polyacrylamide gel, and detecting the biotin on the EF2 by Western blot analysis with an anti-biotin AP conjugated antibody (Rockland) (Figure 4c). Presence of a 92kDa ribosylated eEF2 demonstrates the presence of an enzymatically active PE40 molecule. As shown in Figure 4c, both αCD22PE40 (Lane 2) and αCD22HCH23PE40 (Lane 3) are capable of ADP-ribosylating eEF2 while the control protein αCD22 does not ADP-ribosylate eEF2 (Lane 1). These data demonstrate that algal expressed immunotoxins containing PE40 are enzymatically

active, demonstrating that algal chloroplasts are capable of expressing and accumulating active eukaryotic toxins. This is a somewhat surprising result given that even a single molecule leaking from the chloroplast into the cytosol would be sufficient to kill the eukaryotic expression host. These data demonstrate that protein translocation in chloroplast is strictly unidirectional, and that chloroplast produced proteins do not appear to transit into the cytoplasm.

Algal produced immunotoxins bind specifically to target tumor cells

Flow cytometry was used to determine if algal produced immunotoxins are capable of binding to their target cells. CA-46 B-cells, Ramos B-cells, and Jurkat T-cells were incubated with αCD22PE40 and αCD22HCH23PE40 and subsequently fixed with sodium azide. Following the initial immunotoxin binding, cells were incubated with anti-ETA antibody (produced in rabbits) and then with an anti-rabbit dylight 488 conjugated antibody (Thermo). The cells were then subject to FACs analysis on a BD influx (Becton Dickinson). CA-46 B-cells treated with either αCD22PE40 or αCD22HCH23PE40 showed a fluorescent shift demonstrating that they were bound by the immunotoxin proteins. This shift was again present in Ramos B-cells that were incubated with algal produced immunotoxin proteins (Figure 5b). Both of the algal produced immunotoxins fail to bind to human Jurkat T-cells which are deficient for the CD22 antigen (Figure 5c). These data show that algal produced immunotoxins bind specifically to their target cells and not to other cell types that lack the target antigen, thereby ensuring their specificity in cell killing.

Algal expressed immunotoxins are cytotoxic against B-cells lymphoma cell lines

CA-46, Ramos cells, and Jurkat cells were treated in triplicate with either αCD22, αCD22PE40, or αCD22HCH23PE40, in a dose dependent manner. Cells treated with PBS+0.2% Human Serum Albumin (HAS) were used as a negative control to determine the baseline for 100% survival. Cells were also treated with 10µg/mL of cycloheximide, which completely inhibits protein synthesis resulting in 100% cell death, as the positive control. A wst-8 assay was used to measure cell viability as previously described (Du, Beers et al. 2008). The αCD22 scFv lacking the PE40 toxin did not inhibit B-cells or T-cells from proliferating while αCD22PE40 and αCD22CH32PE40 inhibited CA-46 B-cell (Figure 6a) and Ramos B-cell (Figure 6b) proliferation in a dose dependent manner. The αCD22 scFv, αCD22PE40, and αCD22CH23PE40 did not inhibit Jurkat T-cell proliferation (Figure 6c). In cell based assays the monomeric αCD22PE40 killed CA-46 cells with an IC_{50} of 0.246nM and Ramos cells with an IC_{50} of 1.39nM (Figure 6d). The divalent αCD22CH23PE40 killed CA-46 cells with an IC_{50} of 0.011nM and Ramos cells with an IC_{50} of 0.042nM (Figure 6d) In comparison αCD22CH23PE40 was 22-fold more effective at killing CA-46 cells and 33-fold more effective at killing Ramos cells than αCD22PE40.

These data demonstrate that algal produced immunotoxins are capable of binding to and inhibiting cell proliferation in vitro. Furthermore, the divalent αCD22HCH23PE40 is able to cause cell death of target cells at a significantly lower concentration in vitro.

Antitumor efficacy of monomeric and dimeric algal produced immunotoxins against established B-lymphoma xenografts

Immunotoxins αCD22PE40, or αCD22HCH23PE40 and the control scFv αCD22 were evaluated for their antitumor activity against established subcutaneous xenografts in Rag x gc -/- mice. Rag x gc -/- mice were injected with $1x10^7$ Ramos cells on day 0. The tumors were grown to an average diameter of 4mm which occurred five days after transplantation. The mice were treated with three doses (QOD) by i.p injection with αCD22PE40, αCD22HCH23PE40, or αCD22. Mice were treated with 240µg/Kg of each molecule in triplicate. The mice treated with either algal produced immunotoxin showed a significant inhibition of tumor propagation (Figure 6e) when compared to mice treated with the scFv lacking the PE40 toxin. This data suggest that algal expressed and purified immunotoxins have a significant effect on tumor progression in animal models.

Discussion

We have shown that chloroplasts of the green algae, *C. reinhardtii*, are capable of accumulating fully functional immunotoxin proteins that consist of an antibody binding domain targeting the B-cell surface antigen CD22, and the PE40 toxin domain of exotoxin A. We produced two different types of immnotoxins, single chain and dimeric, and both accumulated as soluble functional proteins within algal chloroplasts. Producing a eukaryotic toxin in a eukaryotic cell was achievable because chloroplasts

have a prokaryotic-like translational apparatus, resistant to the toxin, and because protein produced in chloroplast stay in the chloroplast. A single PE40 molecule escaping the chloroplast should be able to inhibit protein translation in the algal cytosol, resulting in cell death. That the algae survive demonstrates that chloroplasts sequester chloroplast-produced proteins completely within the chloroplast. In addition to sequestering the toxin, allowing for the production of immunotoxins in a eukaryotic host, chloroplasts also have the machinery necessary to assemble complex immunotoxins that contain multiple domains such as αCD22CH23PE40 into larger assembled proteins consisting of two antibody binding domains and two PE40 molecules. No other expression platform is presently capable of producing such a complex immunotoxin.

Previous studies have produced immunotoxins by expression in *E. coli*. These proteins generally need to be purified, denatured, and then refolded, since a majority of the protein product accumulates as an insoluble aggregate in *E.coli*(Brinkmann, Reiter et al. 1993). Analysis of immunotoxin proteins produced in algae show that both αCD22PE40 and αCD22CH23PE40 accumulate in algal chloroplasts as soluble correctly folded molecules that do not require additional chemistry to be functional. Accumulation as a soluble functional molecule should significantly reduce the cost of production, as fewer steps are required to produce the functional therapeutic. Several groups have engineered *E. coli* to contain complex chaperones(Haacke, Fendrich et al. 2009), protein disulfide isomerases(Frand, Cuozzo et al. 2000) and PPIases(Outchkourov, Roeffen et al. 2008) but algae already contain these complex

protein folding machinery(Kim and Mayfield 1997, Schroda 2004, Vallon 2005). The ability of chloroplast to assemble complex mammalian proteins was previously demonstrated by using chloroplasts to fold and assemble full length human antibodies into soluble molecules that bind their target antigen(Tran, Zhou et al. 2009). Here we show that *C. reinhardtii* chloroplasts can also efficiently assemble divalent immunotoxins that contain the hinge and CH2 and CH3 domains of a human IgG1. Two inter-chain disulfide bonds are formed in the hinge region of αCD22CH23PE40 allowing the protein to form a homodimer(Bloom, Madanat et al. 1997).

Immunotoxins are multifunctional and require that each individual part of the protein be operational. FACs analysis demonstrates that the antibody portion of αCD22PE40 and αCD22CH23PE40 bind specifically to target cells that express the CD22 antigen. The antibody domain on an immunotoxin is used to direct the PE40 molecule to a specific cell type so that only the cancerous cells and not healthy cells are targeted. An ADP-ribosyltransferase assay was used to demonstrate that the PE40 component of both αCD22PE40 and αCD22HCH23PE40 was also functional.

While having enzymatically active immunotoxins that bind to their target cell are crucial for functional therapies, they must also be capable of delivering the catalytic domain of PE40 into the cytosol of the target cell to inhibit its proliferation. Both CD22PE40 and αCD22CH23PE40 were tested in a cell viability assay to determine how effective they were at inhibiting target cell proliferation. Both algal produced immunotoxins showed significant cytotoxicity towards two Burkitt's lymphoma cell lines (Ramos and CA46). The 190kDa αCD22HCH23PE40

immunotoxin is 22-fold more effective at killing CA-46 cells and 33-fold more effective at killing Ramos cells than the monomeric αCD22PE40. The increase in cytotoxicity can be attributed to two factors. First, divalent antibodies with multiple binding domains have been shown previously to have an increased binding avidity when compared to monovalent scFv antibodies. The second reason for this increased potency can be attributed to the delivery of two PE40 molecules from αCD22HCH23PE40 as opposed to one from αCD22PE40. These two factors appear responsible for the increased cytotoxicity of αCD22HCH23PE40 in cell based in vitro assays.

Importantly, both immunotoxin molecules appear to impact tumor growth, cause significant inhibition to tumor growth and significantly prolonging mice survival in a tumor challenge assay. While αCD22HCH23PE40 may be slightly better at inhibiting tumor growth, the increased effectiveness of αCD22HCH23PE40 appears to be far less in vivo than it does in vitro. Previously, it was demonstrated that increasing the valency and half-life of an immunotoxin does not always lead to an increased in vivo effectiveness for certain types of cancers(Bera, Williams-Gould et al. 2001), however in some instances it appears to have a dramatic effect(Ribbert, Thepen et al. 2010). The effectiveness of larger immunotoxins with increased valency in vivo appears to be cancer type specific.

Algae have recently garnered much attention for their potential use as a source of biofuels, but algae also appear ready to play a greater role in the production of next generation protein therapeutics. The ability to fold, assemble, and accumulate multiple

domain proteins as soluble molecules is a significant advantage. However what truly distinguishes algae from other recombinant expression platforms are the presence of chloroplasts and the ability to produce and accumulate immunotoxin proteins in these compartments. No other recombinant protein production system has been shown to be capable of accumulating these complex toxic molecules as soluble and enzymatically active proteins. This trait set algae apart from other expression platforms. The potential of immunotoxins as potent and specific anti-cancer therapeutics is enormous. The use of antibody drug conjugates, using small molecule drugs, to target and kill cancer cells and minimize the exposure of healthy cells is already a reality(Alley, Okeley et al. 2010) and many of these therapies are in late stages of clinical trials(Beck, Haeuw et al. 2010, Lash 2010) or are already FDA approved(Younes, Yasothan et al. 2012). Protein toxins have also been shown to be highly effective at inhibiting cancer cell proliferation, however their production is limited to bacterial expression platforms that require the protein to be denatured and subsequently refolded(Pastan, Hassan et al. 2007), processes that add to both the time and cost of developing these drugs. Algae provide another avenue for the production of these immunotoxins and add the ability to create more complex molecules then can presently be produced. While additional work needs to be done to determine if larger or smaller immunotoxins are more effective for specific cancers, the CD22PE40 and αCD22HCH23PE40 we have produced demonstrate that *C. reinhardtii* chloroplasts are capable of producing complex immunotoxins and provides a potentially significant new avenue to produce these next generation therapeutics.

Experimental Procedure

Construct design

All DNA and RNA manipulations were performed as previously described (Kontermann 2010). DNA encoding the variable regions of the heavy chain and light chain genes of the antibody RFB4(Shen, Li et al. 1988) were synthesized in *C. reinhardtii* chloroplast codon bias (DNA 2.0), and fused genetically with a linker sequence coding for 4 x (G_4S). This sequence was ligated downstream of a sequence coding for a 1x flag peptide and a TEV protease cleavage site yielding a 834-bp scFv gene termed αCD22. The αCD22 gene was ligated upstream of a *C. reinhardtii* chloroplast codon optimized sequence coding for a 2 x (G_4S) linker and domains 2 and 3 of *pseudomonas aeruginosa* exotoxin A, yielding a 1947-bp gene termed αCD22PE40. To generate a dimeric immunotoxin, a *C. reinhardtii* chloroplast codon optimized gene coding for the hinge and constant domains 2 and 3 of a human IgG1 (HCH23) was ligated between the coding regions of αCD22 and PE40, again separated by a 2 x (G_4S) linker on both sides of the gene, yielding a 2664-bp gene termed αCD22HCH23PE40. All three genes were ligated into the *psbA* transformation cassette (Manuell, Beligni et al. 2007).

Algal transformations and Selection of homoplasmic strains

DNA transformation plasmids were precipitated onto gold particles (Seashell Technology) and particle bombardment used to transform chloroplasts of algal strain CC-125 (Duke University) as previously described (Kontermann 2010, Rasala, Muto et al. 2010). Transformed cells were selected on Tris-acetate phosphate (TAP) plates containing 100µg/ml of kanamycin sulfate. Subsequently, transformed colonies were patched onto a TAP plate containing 150 µg/ml of kanamycin sulfate to drive the transformants to homplasmy. PCR analysis was performed to determine strains that contained the gene of interest as previously described(Tran, Zhou et al. 2009). In short, a forward primer was made against the *psbA* 5'-UTR (5'-gtgctaggtaactaacgtttgattttt-3') and reverse primer was made against the αCD22 gene (5'- tggaggtggaggtagtggtggtgg-3') that sequence is present in all the transformation constructs and the production of amplicons of 500 bp suggest that genes were integrated into the *psbA* locus. To identify strains that are homoplasmic, forward primers (5'-ggaaggggaggacgtaggtacataaa-3') and reverse primers (5'-ttagaacgtgttttgttcccaat-3') were designed against the *psbA* gene. A control primer set with forward (5'-ccgaactgaggttgggttta-3') and reverse (5'- gggggagcgaataggattag-3') primers was designed against the genomic region coding for the 16S rRNA to be used in the homoplasmic screen. This control primer set ensures that the apparent loss of the *psbA* gene is not merely a failed PCR reaction. Homoplasmic strains were identified for each recombinant gene.

Accumulation analysis, purification and characterization of algal produced immunotoxins

Accumulation of immunotoxins from transgenic strains of *C. reinhardtii* was determined by western blot analysis using anti-flag antibodies as described previously(Tran, Zhou et al. 2009). Transgenic *C. reinhardtii* cultures were inoculated at 2 $x10^5$ cells/mL and grown to a density of $2x10^6$ cells/mL in dim light (200lux). 250 mls of culture was used to inoculate 20L carboy (VWR) at a density of 2 $x10^4$ cells/mL and grown in light (10,000 lux) and mixed using bubbled air for 96 hours prior to harvesting. Cells were lysed by sonication in lysis buffer (50mM Tris-HCL pH8.0, 500mM NaCl, 0.5% tween 20 containing complete protease inhibitors (Roche)). The soluble and insoluble proteins were separated using high-speed centrifugation at 20,000 x g. The soluble protein was applied to an anti-flag M2 affinity gel (Sigma-Aldrich) and eluted with a flag elution buffer (100 mM glycine-HCL pH 3.5 and 500mM NaCl). Proteins were further purified using size exclusion chromatography to remove any degradation products. Immunotoxin and scFv protein were identified using a mouse anti-flag alkaline phosphatase conjugate antibody (Sigma). All procedures were carried out as described (Sambrook 1989). Western blot analysis was also done in reducing conditions to visualize assembly of dimeric immunotoxins. Proteins were then concentrated and buffered exchanged into phosphate buffered saline (PBS, 3.2mM Na2HPO4, 0.5mM KH2PO4, 1.3mM KCl, 135mM NaCl pH7.4) using a concentrating column (GE healthcare).

ADP-ribosyltransferase assay

Determination of the enzymatic functionality of the algal produced PE40 proteins was determined by ADP-riobsyltransferase assay. 12.5 µM Biotinylated-NAD+ (Sigma) was placed in a reaction with 50mM Tris-HCl pH 7.8, 400 ng of purified eEF2 (Company), 1mM DTT, 1mM EDTA, and 500 ng of purified αCD22, αCD22PE40, or αCD22HCH23PE40 protein in a 20ul reaction volume. Each reaction was incubated at 25°C for 30 mins. Samples were then separated by PAGE and blotted onto a nitrocellulose membrane. Once blotted, an anti-biotin-alkaline phosphatase conjugated antibody (Rockfield) was used to identify the presence of biotin molecules on the 95 kDa eEF2 protein, indicating the presence of ribosylated eEF2.

Flow cytometry cell binding assay

CA-46 B-cells, Ramos B-cells, and Jurkat T-cells were incubated in the presence of algal produced αCD22PE40 or αCD22HCH23PE40 in PBS + 0.01% Sodium Azide for 1 hour at 4°C. After primary binding, cells were incubated with an anti-exotoxin A antibody produced in rabbit (Sigma) and diluted 1:20,000 in PBS (Sigma) for 1 hour. After secondary binding, cells were incubated with an anti-Rabbit dylight 488 conjugated antibody and analyzed by flow cytometry using a BD influx (Becton Dickinson). Data was analyzed using FlowJo software.

Cytotoxic cell viability assay

100 µL of CA-46 B-cells, Ramos B-cells and Jurkat T-cells (5 x10^4 cells/ml) were seeded into each well of a 96-well tissue culture plate (corning) for 24 hours in a humidified incubator at 37°C containing 10% CO_2. After incubation, increasing concentrations of αCD22, αCD22PE40, or αCD22HCH23PE40, diluted in PBS containing 0.2% HSA, were added to each well. PBS + 0.2% HSA was used as a negative control for cell death and represented 100% cell survival. Cylcoheximide at a concentration of 10µM was used as a positive control for cell death and represented 0% cell survival. Cells were incubated with algal produced αCD22, αCD22PE40, αCD22HCH23PE40, or control reagents for 72 hours. After incubation with immunotoxins and controls, 10 ul of wst-8 reagent (Dojindo) was added to each well. Assay was allowed to develop for 4 hours and absorbance read at 450nm on a plate reader (Tecan). IC_{50} was calculated using Grafit software (Erithacus).

Antitumor efficacy of algal expressed immunotoxins against established B-lymphona xenografts

Female RAG2-/- x gc -/- mice (Taconic Farms), which lack adaptive immunity and natural killer cells were used for the establishment of human lymphoma xenografts. 3 x10^7 Ramos cells were transplanted into the subcutaneous space of the mice. When the tumors reach a mean diameter of 5 mm (typically 4 days after transplant), mice were injected with 240 ug/kg with either αCD22, αCD22PE40, or αCD22CH23PE40. Tumors were measured everyday up to 25 days to determine the

tumor size and survival of the treated mice. The results shown are representative of 3 independent experiments.

Acknowledgments

Chapter 3, in full is a reprint of material as it appears in the proceedings of the National Academy of Sciences 2012. Tran, Miller; Van, Christina; Daniel Barrera, Daniel J; Pettersson Par L; Carlos Peinado, Jack Bui and Stephen Mayfield, 2012. The dissertation author was the primary investigator and author of this paper

Figures

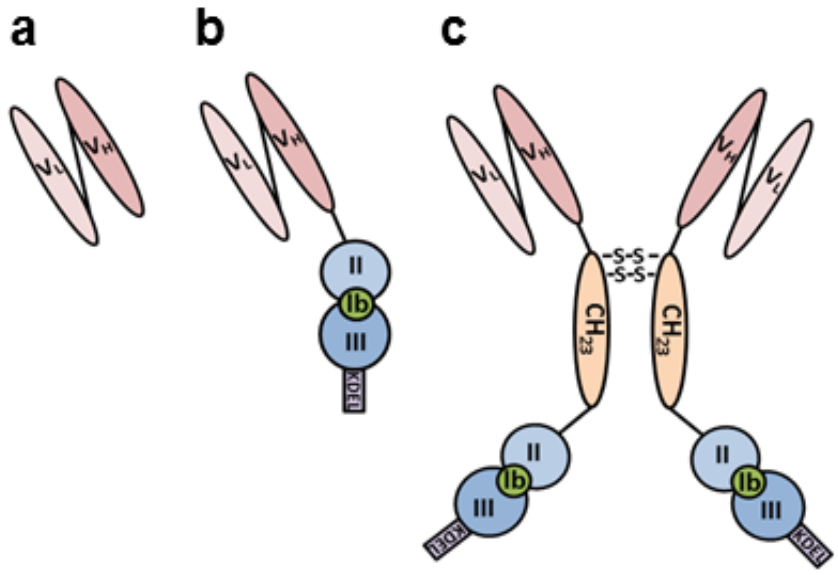


Figure 1. Depiction of algal expressed immunotoxin proteins (**a**) Single chain antibody (scFv) directed against the CD22 cell surface antigen made by linking the variable domains of the heavy and light chain antibodies with a glycine-serine linker. (**b**) The CD22-scFv is genetically linked to *Pseudomonas aeruginosa* exotoxin A domains 2 and 3. Removal and replacement of domain Ia from exotoxin A with an antibody allows cancer cells to be specifically targeted. (**c**) The CD22-scFv genetically fused to the hinge and constant domains of an IgG1 and to exotoxin A domains 2 and 3 to create a construct that forms a homodimer through disulfide bonds formed between hinge regions. This fusion allows the molecule to have two binding domains as well as two toxin molecules.

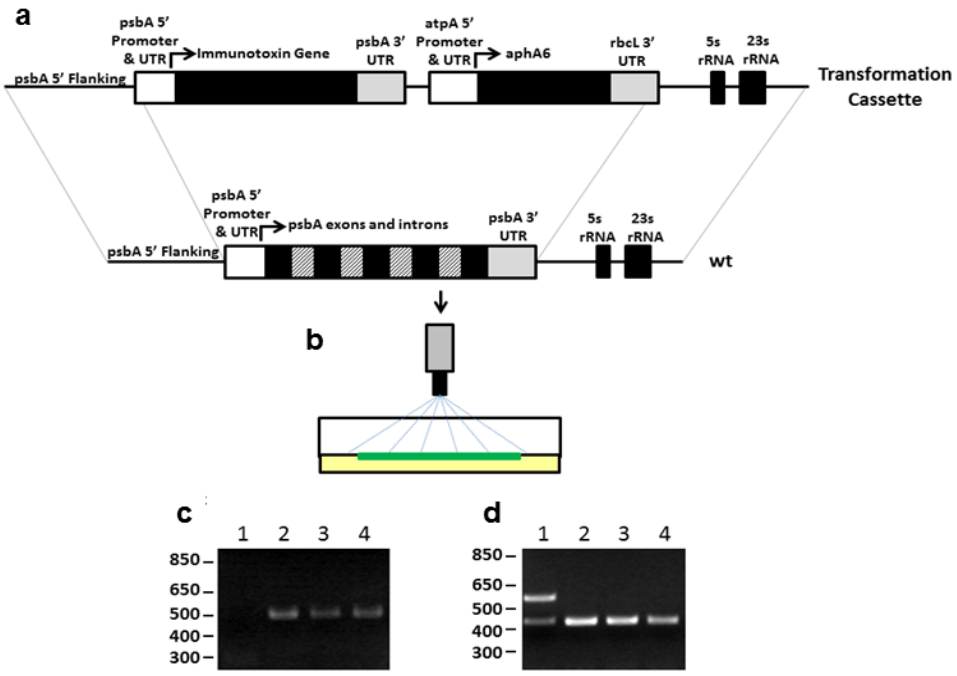


Figure 2. Integration of genes into chloroplast genome by homologous recombination. (**a**) Immunotoxin genes are ligated downstream of the *psbA* promoter and 5'-UTR and upstream of the *psbA* 3'-UTR. This construct is placed upstream of an aphA6 gene that confers kanamycin resistance to transformed cells of algae. Regions of chloroplast genome are placed at either end of the transformation vector to allow for homologous integration of the entire transformation cassette into the chloroplast genome. (**b**) Transformation plasmids are precipitated onto gold particles and delivered into algal chloroplasts by particle bombardment where they recombine into the plastid genome. (**c**) PCR analysis using primers specific to the αCD22 scFv gene and the *psbA* 5'-UTR demonstrate that coding sequences for immunotoxins have been integrated into the psbA locus. (Lane 1) contains PCR from wt algal cells. (Lane 2) contains strains transformed with αCD22. (Lane 3) contains strains transformed with αCD22-PE40. (Lane 4) contains strains transformed with αCD22-CH23-PE40. **(d)** PCR analysis is used to confirm homoplasmicity of transformed strains of algae. Primers are used to amplify a control region of the algal chloroplast genome as well the endogenous *psbA* gene. Loss of the *psbA* gene (upper band in lane 1) demonstrates homoplasmicity of the transgenic lines.

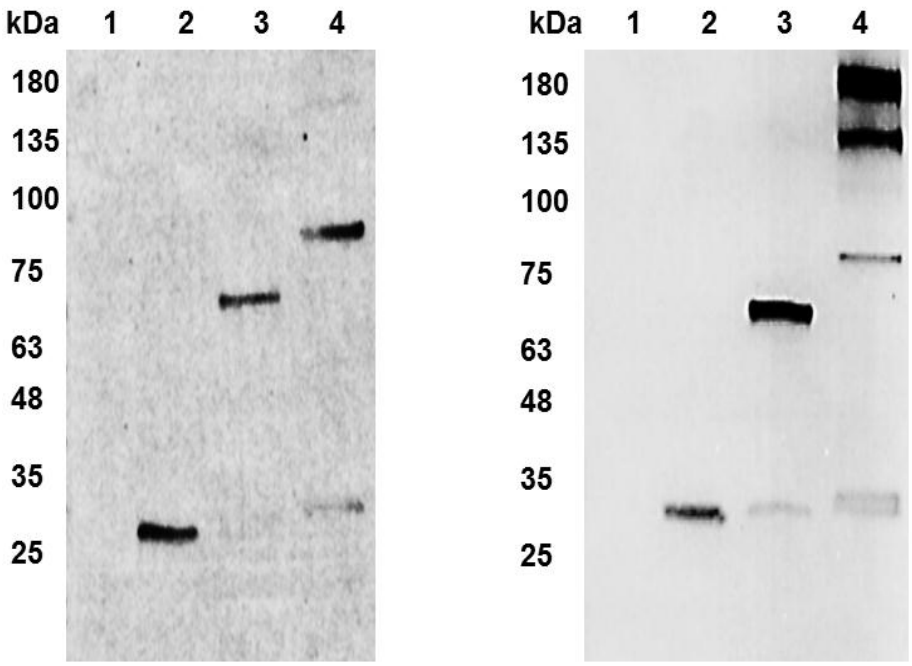


Figure 3. Western blots demonstrating the accumulation of immunotoxin proteins. (a) Samples were separated on a SDS-PAGE gel under reducing conditions, transferred to a nitrocellulose membrane and probed with an anti-flag antibody that was conjugated with alkaline phosphatase. The lanes contain the following samples: (Lane 1) wt insoluble lysate, (Lane 2) wt soluble lysate, (Lane 3) αCD22, (lane 4) αCD22PE40, (Lane 5) αCD22CH23PE40. (b) The identical samples were separated on a SDS-PAGE gel under non-reducing conditions to keep disulfide bonds intact. This demonstrates that algae produce αCD22CH23PE40 as a dimer, making it a divalent protein containing two exotoxin A molecules.

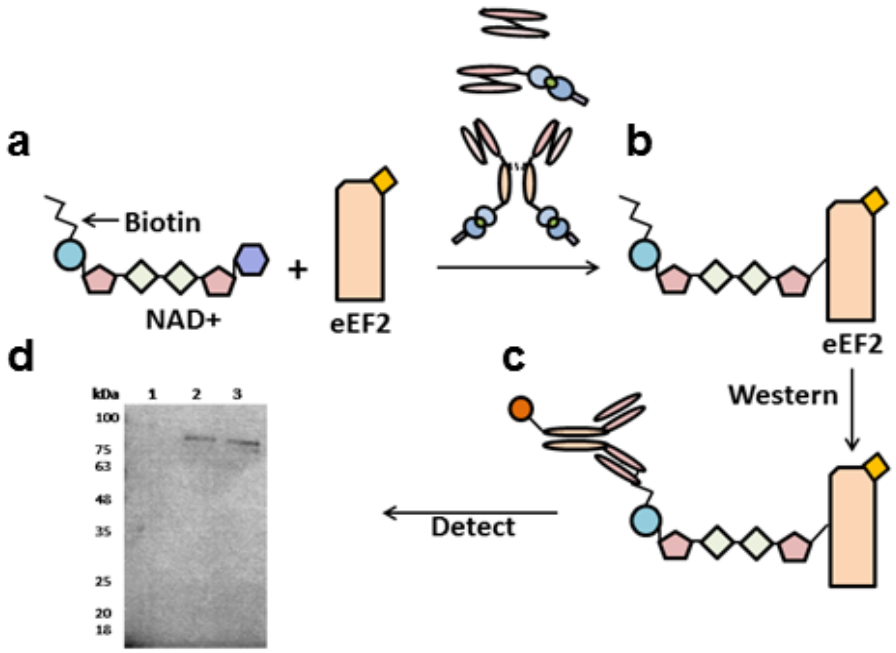


Figure 4. ADP-ribosyltransferase assay demonstrates that algal chloroplasts accumulate enzymatically active immunotoxin proteins. (**a**) Biotinylated NAD+ is mixed with eEF2, and purified αCD22, αCD22PE40, or αCD22CH23PE40. (**b**) Biotinylated ADP is transferred to eEF2 by enzymatically active exotoxin A. After reaction completion samples are separated on SDS-PAGE and blotted onto nitrocellulose membranes. **(c)** An anti-biotin antibody that is conjugated with alkaline phosphatase is used to detect eEF2 that has been ribosylated with ADP-biotin. (**d)** Western blot demonstrates that αCD22 does not ribosylate eEF2 (Lane 1) but that αCD22PE40 (Lane 2) and αCD22CH23PE40 (Lane 3) have enzymatically active PE40 and do ribosylate eEF2.

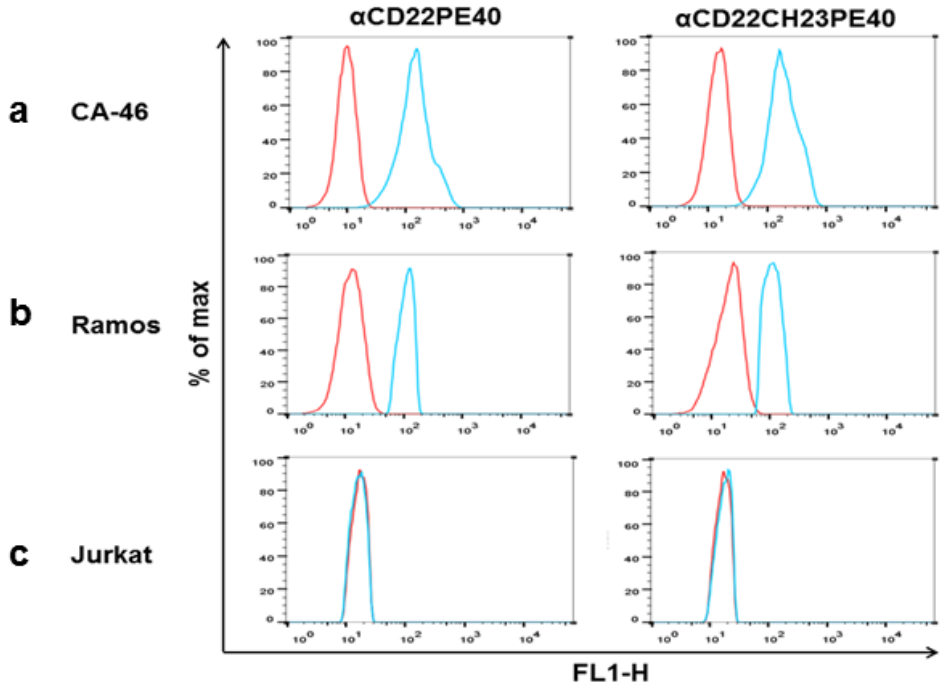
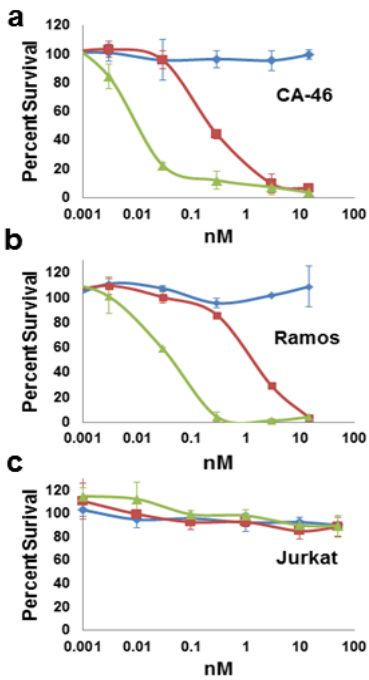


Figure 5. Flow cytometry demonstrate specific binding of algal produced immunotoxins. αCD22PE40 and αCD22CH23PE40 were incubated with CA-46 B-cells, Ramos B-cells or Jurkat T-cells. After primary incubation cells were subsequently incubated with anti-exotoxin A produced in Rabbit and finally with anti-rabbit-dylight 488. After incubation cells were analyzed by flow cytometery (a) A shift in the fluorescence spectra demonstrates that αCD22PE40 and αCD22CH23PE40 binds to CA-46 B-cells. (**b**) Fluorescence analysis also demonstrates that αCD22PE40 and αCD22CH23PE40 bind to Ramos B-cells. (**c**) A lack of fluorescence shift demonstrates that algal produced immunotoxins do not bind nonspecifically to Jurkat T-cells.



d

	Cell Type	αCD22PE40 IC_{50}	αCD22CH23PE40 IC_{50}
CA46	B-cell Lymphoma	0.246 ± 0.031nM	0.011 ± 0.003nM
Ramos	B-cell Lymphoma	1.39 ± 0.099nM	0.042 ± 0.007nM
Jurkat	T-Cell Leukemia	Does Not Kill	Does Not Kill

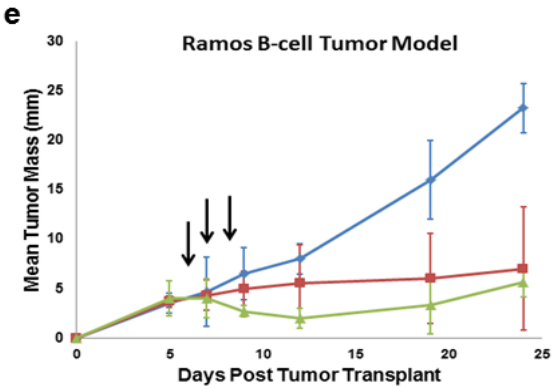


Figure 6. In vitro and in vivo analysis of the effectiveness of algal expressed immunotoxin against cancer cells. αCD22 (blue), αCD22PE40 (red), and αCD22CH23PE40 (green) were incubated with CA-46 B-Cells, Ramos B-cells, and Jurkat T-cells for 72 hours in vitro to determine their effectiveness. (**a**) αCD22PE40 and αCD22CH23PE40 were effective at killing CA-46 B-cells while αCD22 alone was incapable of killing CA-46 cells. (**b**) Additionally, αCD22PE40 and αCD22CH23PE40 were able to kill Ramos Cells while αCD22 was unable to inhibit Ramos cell proliferation. (**c**) αCD22, αCD22PE40, and αCD22CH23PE40 were unable to kill Jurkat T-cells. (**d**) IC_{50} for each immunotoxin against each cell line was calculated to determine how effective each was at inhibiting cancer cell proliferation. Both immunotoxins were capable of killing B-cells but Dimeric αCD22CH23PE40 was more effective at killing targeted cells in vitro when compared to αCD22PE40. (**e**) $1x10^7$ ramos Cells were tansplanted subcutaneously into Rag- mice until they established tumors of a mean diameter of 4mm. Mice were then treated each day for three days with 160µg/kg of either αCD22, αCD22PE40, and αCD22CH23PE40. Both αCD22PE40 and αCD22CH23PE40 were able to inhibit tumor proliferation when compared to mice treated with αCD22 alone.

Chapter 4: Algae chloroplasts as an ideal environment for the production of gelonin based immunotoxins

Introduction

Chloroplast of eukaryotic green algae have been used as a recombinant protein expression platform but the true potential of this expression platform remains a mystery. To explore a potential niche of chloroplast, we have demonstrated that chloroplast are capable of producing genetic fusion of an single chain antibody targeting CD22, a B-cell surface epitope, and the eukaryotic ribsome inactivating protein, gelonin, from *Gelonium multiflorm.* These unique molecules are termed immunotoxins and are a genetic version of the emerging antibody drug conjugate therpaies that deliver a drug molecule to a targeted cell. We have also shown that by adding an Fc domain to this protein, chloroplasts are capable of assembling them into divalent proteins that have two toxic gelonin molecules. Additionally we have demonstrated that these algal expressed proteins are capable of targeting and killing B-cell lymphomas while leaving T-cells that lack CD22 unharmed. No other protein expression platform has been shown to be capable of folding and accumulating these immunotoxins as soluble and enzymatically active proteins.

Green algae are capable of converting solar energy into energy dense molecules and producing large quantities biomass and these characteristics have made it an organism that is being investigated as a potential renewable biofuel. While energy is certainly important, the ability to accumulate biomass makes algae a promising organism for the production of protein therapeutics. To demonstrate its full potential as a protein production platform, eukaryotic green algae have been engineered to produce a wide range of recombinant proteins. There has been a focus of producing industrially relevant products such as xylanases and other industrial enzymes (Rasala 2012) to supplement the large quantity or algal biomass that will be generated from the biofuels sector but relatively little attention has been paid to algae as an organism that has the ability to produce therapeutically relevant proteins. The chloroplast of the eukaryotic green algae, *Chlamydomonas reinhardtii*, has recently been used to produce a wide range of therapeutically relevant proteins ranging from vaccines (Gregory, Li et al. 2012, Jones, Luong et al. 2012), hormones(Rasala, Muto et al. 2010), or full length human antibodies (Tran, Zhou et al. 2009). *C. reinhardtii* is also being exploited for its ability to secret proteins which will allow for an ease in protein processing which should impact the cost of protein purification. This versatility makes algae promising platform for the production of therapeutically relevant proteins. Algae have minimal growth requirements only needing trace minerals, fertilizer, and sunlight produce biomass making producing of protein therapeutics in them potentially very inexpensive. Furthermore, algal chloroplasts

provide a unique opportunity to produce very complex therapeutically relevant proteins because of the biochemical nature of chloroplasts.

Although chloroplasts are prokaryotic in origin, they are unique because of their complex protein folding machinery. Bacteria lack the ability to fold complex proteins because of the compartmentalization of the protein folding machinery. The periplasmic space of bacteria contain protein disulfide isomerases (Denoncin, Vertommen et al. 2010) that are required to form disulfide bonds and assemble proteins with multiple subunits but they lack the chaperones and chaperonins that are essential in preventing protein aggregation so that the protein disulfide isomerases have time to function. These chaperones are present in the cytosol and there have been attempts to co-express these chaperones in the periplasmic space along with a recombinant protein of interest with moderate success (Outchkourov, Roeffen et al. 2008). When proteins are expressed and secreted from mammalian cell culture they are expressed and assembled in the endoplasmic reticulum where chaperones such as BiP, an HSP70 chaperone works with PDI to allow proteins to have sufficient time to fold and assemble into functional complexes. The chloroplast unlike bacteria house both an array of chaperones (Schroda 2004) and protein disulfide isomerases in the stroma (Danon and Mayfield 1994) and it has been demonstrated that this compartmentalization allows complex human antibodies to be folded and assembled into functional molecules capable of binding to their target antigen (Tran, Zhou et al. 2009). Although it has a complex protein folding machinery, the chloroplast still has a translational apparatus, both ribosomes (Manuell, Quispe et al. 2007) and elongation

factors (Beligni, Yamaguchi et al. 2004) that resemble those of a prokaryote. These characteristics make chloroplast an ideal compartment to produce multi-subunit proteins that contain components that inhibit the proliferation of eukaryotic expression host.

One of the major advances in cancer therapies in recent years has been the advent of antibodies that are conjugated to chemotherapy agents that prevent cancer cells from divding and causing the target cell to undergo apoptosis (van de Donk and Dhimolea 2012). These drugs are currently marketed and administered to cancer patients (Pro, Advani et al. 2012). A major drawback of this technique is the high cost that is associated with these therapies due to the need to first purify the monoclonal antibody, chemically coupling it, and subsequently re-purifying the conjugated molecule (Selyukh 2011). Another approach to making antibody conjugates has been to produce single chain (scFv) antibodies that are genetically coupled to a eukaryotic toxin. The scFv antibody is able to specifically target cancer cells just like a full length antibody and deliver toxin molecules which then cause the cell to undergo apoptosis. The only systems that are currently able to produce and accumulate these molecules are prokaryotic due to the ability of these toxins to inhibit the proliferation of eukaryotic protein expression platforms. Bacterial expression platforms that currently produce these fusion proteins are hampered by their inability to produce molecules that are more complex than scFv toxin fusions. Additionally, the scFv-toxin fusions are produced as insoluble aggregate which complicates the purification process,

requiring these proteins to be denatured and re-folded ex vivo. These limitations make the bacterial expression platform less than ideal.

It was previously demonstrated that chloroplast of eukaryotic algae have the protein folding machinery (Tran, Zhou et al. 2009) that allows them to fold and assemble complex mammalian proteins as well as complex malarial proteins (Jones, Luong et al. 2012). Furthermore, chloroplasts of *C. reinhardtii* have also been shown to have translational machinery that resembles that of a prokaryote (Beligni, Yamaguchi et al. 2004, Manuell, Quispe et al. 2007) which would not be harmed by eukaryotic toxins that generally target either ribosomes (Singh, Singh et al. 1999) or elongation factors of eukaryotic cells (Mansfield, Pastan et al. 1996). These unique qualities of chloroplast make them a potentially valuable expression platform for the production of complex immunotoxin proteins.

To determine if chloroplast of *C. reinhardtii* are capable of producing functional and complex immunotoxin proteins we genetically fused a recombinant gene that codes for a scFv antibody that recognizes CD22, a B-cell surface epitope that is known to rapidly endocytose into target cells once bound by an antibody (Du, Beers et al. 2008), to gelonin, a ribosome inactivating protein (Figure 1B). This molecule was termed αCD22Gel. Gelonin is typically produced and stored in the seeds of *Gelonium multiflorm* and inhibits translation by cleaving the 28srRNA of eukaryotic ribosomes. Gelonin has also been shown to exhibit moderate DNase activity (Nicolas, Beggs et al. 1997). Fusions of gelonin to scFv antibodies that are produced in bacteria have been shown to be highly effective at inhibiting the proliferation of targeted

cancer cells (Pirie, Hackel et al. 2011). In addition to this fusion we placed the Fc domain of a human IgG1 between the scFv antibody and gelonin (Figure 1C). Since chloroplasts have PDIs that allow disulfide bridges to form, placing the Fc domain into the protein would allow the chloroplast to assemble the protein molecule into a dimer with two protein binding domains as well as two toxin molecules. This protein was termed αCD22CH23Gel. One of the major problems with the small scFv-toxin molecules is that their size greatly reduces their serum half-life. By adding an Fc domain and making a dimer of the immunotoxin the protein half-life will be dramatically increased (Saito, Kreitman et al. 1994). In addition to the increased protein half-life, delivering more toxin molecules may make gelonin immunotoxins more effective as there is a threshold of gelonin molecules that need to enter a cell before the cell undergoes apoptosis (Goldmacher, Scott et al. 1989). The scFv antibody was produced as a control molecule and termed αCD22.

To exploit the untapped potential of algal chloroplast we demonstrate that the eukaryotic green algae, *C. reinhardtii* are capable of accumulating monovalent and larger divalent gelonin based immunotoxins as soluble molecules that are enzymatically active and capable of binding to target cancer cells and causing those cells to undergo apoptosis.

Results

Expression vector construction and engineering of immunotoxin genes

All DNA manipulations were performed as previously described (Sambrook 1989, Cohen, Yohn et al. 1998). The coding sequence of a single chain antibody was codon optimized (DNA2.0) to produce a protein product that resembled a αCD22scFv (Figure 1A). To produce a monovalent immunotoxin gene a codon optimized gene sequence for an αCD22scFv was placed upstream of a codon optimized gelonin gene and separated by a genetic sequence coding for a 2x(4GlySer) linker. The resulting protein product would resemble αCD22Gel (Figure 1B). Finally to produce a divalent immunotoxin gene a codon optimized hinge, CH2 and CH3 of a human IgG1 (CH23) was placed between genes coding for αCD22 and gelonin and separated by a genetic sequence coding for a 2x(4GlySer) on either side to produce a protein product that would resemble αCD22CH23Gel (Figure 1C). These three immunotoxin genes were then placed into chloroplast transformation vectors. Plasmid vectors were engineered to contain regions of homology to the chloroplast genome to allow for integration by homologous recombination as it has been previously demonstrated (Manuell, Beligni et al. 2007). Immunotoxins genes were placed downstream of the psbA promoter and 5'-untranslated region (UTR). This construct was placed upstream of the psbA3' UTR. Additionally, a genetic construct containing the atpA promoter and 5'-UTR, the aphA6 gene, and the rbcL3'-UTR was placed downstream of the psbA 3'-UTR to

allow algal transformations to be selected for their ability to be grown on plates containing 100µg/mL of kanamycin sulfate (Figure 2A).

Integration of recombinant genes into chloroplast plastid genome

Transformed *C. reinhardtii* cells were selected on TAP plates containing 100ug/mL of kanamycin sulfate. Colonies that grew were patched onto plates that contained 150ug/mL of kanamycin sulfate. Once patches were grown, PCR was used to verify the presence of the immunotoxin genes. The presence of αCD22 was confirmed in transformants of αCD22, αCD22Gel and αCD22CH23Gel (Figure 2B). Transformed patches of *C. reinhardtii* were also screened for the presence of the gelonin gene which was present in αCD22Gel and αCD22CH23Gel (Figure 2C). Finally, transformants were screened for the presence of the CH23 which was present in only αCD22CH23Gel (Figure 2D). Gene positive colonies were subsequently screened for homoplasmicity to ensure that all copies of the chloroplast plastid genome no longer contained the M-SAA gene. All transgeneic strains were homoplasmic and no longer contained the M-SAA gene (Figure 2E).

Heterologous protein accumulation in transgenic algal strains

After the transgeneic strains of algae were shown to have their respective heterologous gene integrated into the plastid genome western blot analysis was used to

determine if *C. reinhardtii* could accumulate gelonin based immunotoxins. To detect the protein of interest western blot analysis was done with both an anti-M2 Flag antibody as well as an anti-Gelonin antibody. Non-reducing westerns were all tested to determine of the algal cells could accumulate αCD22CH23Gel into a larger dimeric complex. Detection with an anti-M2 flag antibody that was conjugated with alkaline phosphatase demonstrates that *C. reinhardtii* chloroplasts can accumulate the 29kDa αCD22, the 58kDa αCD22Gel, and the 85kDa αCD22CH23Gel (Figure 3A). Furthermore, non-reducing westerns detected with an anti-M2 flag antibody demonstrate the chloroplasts of *C. reinhardtii* are capable of assembling two subunits of αCD22CH23Gel into a dimeric 170kDa complex (Figure 3A, black arrow). Western blots detected with an anit-gelonin antibody produced in rabbit confirms the ability of chloroplasts to accumulate both αCD22Gel, and αCD22CH23Gel immunotoxins as well as their ability of to assemble two subunits of αCD22CH23Gel into a molecule that contains two antigen binding domains as well as two gelonin toxin domains (Figure 3B).

DNAse activity of algal expressed gelonin immunotoxins

Previously, it was demonstrated that ribosome inactivating proteins have additional DNAse activity which resulted in supercoiled plasmids being nicked and linearized. To determine if the algal produced ribosome inactivating protein, gelonin, has this activity, 1.0µg of puc19b supercoiled plasmid was incubated in the presence

of either 1µg of αCD22, increasing concentration of αCD22Gel, or increasing concentrations of αCD22CH23Gel. To serve as a control 1.0µg of puc19b plasmid linearized with HindIII was used. The results of the DNAse activity assay demonstrates that increasing concentrations of both αCD22Gel (Figure 4A), and αCD22CH23Gel (Figure 4B) increases the proportion of DNA that is either nicked or linearized. These results confirm that the gelonin based immunotoxins do have additional DNase activity.

Binding of algal immunotoxins to CD22 positive B-cells

To verify that the αCD22 scFv portion of the immunotoxins are capable of binding to target cells, Ramos B-cells and CA-46 B-cells were stained with both αCD22Gel and αCD22CH23Gel algal produced immunotoxins. Immunotoxins were incubated with either cell line and subsequently incubated with an rabbit α-gelonin antibody (MD Anderson) and finally incubated with an goat-anti-rabbit dylight 488 antibody. Both the monovalent αCD22Gel and the divalent αCD22CH23Gel were capable of binding to Ramos cells as shown by the fluorescent shift in the cell binding assay (Figure 5A). Additionally, both algal produced gelonin immunotoxins were able to bind to CA-46 B-cells (Figure 5B). This data demonstrates that algal expressed immunotoxins have functional antigen binding domains that allows them to bind to target cells.

Cytotoxicity of algal produced immunotoxins towards B-cell lymphoma cell lines

Algal expressed immunotoxins were tested for their ability to specifically target and kill B-cell lymphomas that are known to contain the CD22 antigen while at the same time leaving other cells unharmed. As a control, Jurkat T-cells were used to demonstrate the the algal produced immunotoxins were incapable of killing cells that lack the CD22 antigen. Cells were incubated at 37ºC with 5% CO_2 in a humidified chamber for 72 hours. Monovalent αCD22 by itself was incapable of killing, CA46 cells, Ramos Cells and Jurkat Cells (Figure 6A-C). Monovalent αCD22gel was capable of killing CA46 cells with an IC_{50} of 2.316 ± 0.471nM and Ramos calls with an IC_{50} of 21.051 ± 9595nM. The monovalent αCD22gel immunotoxin was incapable of killing jurkat T-cells (Figure 6). Divalent αCD22CH23Gel was able to kill CA46 cells with an IC_{50} of 0.093 ± 0.024nM and Ramos cells with an IC_{50} of 1.421 ± 0.298nM (Fig. 6A-C). The divalent αCD22CH23Gel did not kill jurkat T-cells (Figure 6C)

Discussion

Over the last few decades, genetic tools have been developed to allow scientist to metabolically alter *C. reinhardtii* and to use it as an alternative expression platform. More recently, this algae has been used to accumulate complex human antibodies that are capable of binding to their target antigen with an avidity that is similar to that of an antibody expressed in Chinese hamster ovary cells(Tran, Zhou et al. 2009). Here, we

demonstrate the algal chloroplast are not merely capable of doing what other expression platforms are capable of, but that they are uniquely positioned to be an expression system for the production of complex immunotoxin proteins that can target and eliminate cancer cells. Additionally, by producing gelonin in chloroplast we further demonstrate that no protein molecules leave the chloroplast, as a single gelonin molecule leaving the chloroplast and entering the cytosol would be sufficient to inhibit the translation of the algal cell and cause the cell to die.

The accumulation of gelonin based immunotoxins helps to increase our understanding of the protein folding machinery in chloroplast. By showing that algal chloroplast was capable folding multiple domain proteins such as αCD22Gel and assembling two subunits of αCD22CH23Gel into a dimeric molecule with two CD22 antibody binding domains and two gelonin molecules, we demonstrated the complexity that exist in chloroplast. The protein folding machinery consist of proten disulfide isomerases (Danon and Mayfield 1994), chaperones (Schroda 2004), chaperonins (Suzuki, Nakanishi et al. 2009) and PPIases (Breiman, Fawcett et al. 1992). While we have known that this machinery exist in chloroplast, little was known about the ability of these components to work cohesively to fold and accumulate complex heterologous proteins. Showing that these complex immunotoxins could accumulate in chloroplast as soluble proteins demonstrates that the protein folding complexes in chloroplast work in concert to ensure that proteins do not aggregate at they do in bacteria. The inability of existing expression systems to

accumulate immunotoxin proteins as soluble molecules uniquely positions algae as a system of choice for their production.

Studies with immunotoxin proteins produced in *E. coli* have demonstrated that these molecules have the potential to be used as highly effective treatments against cancer. However the method of production of these molecules leaves much to be desired. Currently, gelonin based immuotoxins are produced in *E. coli* as an insoluble aggregate which are then denatured and refolded (Pirie, Hackel et al. 2011). The biochemical protein folding environment in bacteria is insufficient to produce immunotoxins as soluble enzymatically active molecules. To demonstrate that the algal produced immunotoxins were effective at targeting cancer cells we choose two B-cell lymphoma cell lines, Ramos and CA-46 and demonstrated my flow cytometry that immunotoxins purified from chloroplast were capable of causing the population of B-cells to shift once they were bound.

More importantly, we demonstrated that in an in vitro cell viability assay algal expressed immunotoxins were able to specifically kill Ramos and CA-46 B-cells while leaving Jurkat T-cells unharmed. When directly compared, the divalent αCD22CH23Gel was able to kill CA-46 cells nearly 25-fold more effectively than the monovalent αCD22Gel and approximately 15-fold more better when targeting Ramos cells. This demonstrates that the divalent immuotoxins may play a vital role in decreasing the effective dosage of this form of therapy that might otherwise cause vascular leak syndrome (Liu, Pop et al. 2012). The increase in killing efficiency can be attributed to the increased in valency which has been shown to increase binding

avidity and to the increase in toxin molecules which increase the toxic load of each molecule that is endocytosed into the target cancer cell. Additionally, the larger size of αCD22CH23Gel can potentially increase the proteins half-life giving the molecule more time to be effective whereas smaller immuntoxins similar to αCD22Gel often times suffer from extremely short serum half-lives.

Antibody drug conjugates appear poised to play a major role in cancer treatment in the decades to come but their cost of production remains relatively high. By producing these antibody conjugates as genetic fusions we can potentially dramatically decrease the cost of production. Algal chloroplast appear uniquely positioned as a protein production platform to produce these molecules as genetic fusions. The ability of algal chloroplast to fold and assemble complex multi-subunit proteins allows them to fold immunotoxins into soluble molecules and the origin of the translational apparatus of chloroplast allows these molecules to accumulate within the cell without inhibiting the proliferation of the expression host. These unique traits make algae a truly unique expression platform.

Experimental Procedures

Plasmid Construction

Algal transformation vectors were first constructed in Escherichia coli as previously described. Three individual vectors were constructed for each heterologous gene that was to be tested. The DNA sequence of the heavy and the light chain genes of an antibody RFB4 (Wayne, Kreitman et al. 2010) were synthesized in *C. reinhardtii*

chloroplast codon bias (DNA 2.0, Gene Designer). *C. reinhardtti* chloroplast codon bias was obtained from http://www.kazusa.or.jp/codon/cgibin/showcodon.cgi?species=3055.chloroplast. These individual sequences were genetically fused with a DNA sequence coding for a 4x (G_4S) linker and ligated downstream of a DNA sequence coding for a 1xflag peptide (DYKDDDDK) and a TEV protease recognition site (GENLYFQG) that were designed in *C. reinhardtii* chloroplast codon bias and yielding a834-bp single chain antibody (scFv) termed αCD22. The αCD22 gene was ligated upstream of a 2x(G_4S) linker, a chloroplast codon optimized gene coding for gelonin and aDNA sequence coding for a KDEL protein targeting sequence. The final gene product was 1626-bp and was termed αCD22GEL. To produce divalent immunotoxins the hinge and constant domains 2 and 3 of a human IgG1 (CH23) was ligated between the αCD22 and Gelonin genes, and separated by a 2x(G_4S) linker on both sides of CH23. The final gene product was 2340-bp and termed αCD22CH23GEL. Each gene was placed downstream of the psbA-5' promoter and UTR and upstream of the psbA 3' UTR. A kanamycin resistance cassette was placed downstream of the psbA 3'-UTR as previously described.

Preparation of Algal Strains for Chloroplast Transformations

Strain w1.1, which previously had its psbA gene replaced by a gene coding for M-SAA (Manuell, Beligni et al. 2007) was grown in 250mL Tris-acetate-phosphate

(TAP) media until a density of $2x10^6$cells/mL was achieved. Cells were then spun down at 3000 rpm and re-suspended in TAP media to a cell density of $3x10^7$cells/mL. 500µL of the resuspended cells were plated onto TAP-AGAR plates containing 100µg/mL of Kanamycin.

Particle bombardment transformations of algal chloroplasts

To transform algal chloroplasts, DNA was first precipitated onto gold particles. This was done by first mixing 125µL of binding buffer (Seashell Technology, San Diego) with 25µg plasmid DNA from each transformation vector. To this mixture 2.5µg of 1000d gold (Seashell Technology, San Diego) was added and allowed to incubate on ice for 1 minute. Following incubation, 200µL of cold precipitation buffer was added to the DNA-Gold mixture. Sample preparations were vortexed to mix and followed by a centrifugation for 1 minute at 13,000rpm. The supernatant from each sample was decanted and the gold pellets were washed with 600µL of ice cold 100% ethanol. After the wash each sample was centrifuged for 1 minute at 13,000rpm. The samples were decanted and followed by an addition of 300µL of ice cold 100% ethanol. Tefzel tubing (Bio-rad laboratories) was cut in 3inch pieces and connected to a 5mL syringe by a 1/6” plastic tube. DNA-Gold sample preparations were resuspended by a brief 1-2 second pulse using a sonnicator set on 10% amplitude. Immediately following sonication, place the end of the tefzel tubing into the sample and draw up the gold mixture into the tefzel tubing being careful not to pull the sample

into the connecting tubing. Lay the syringe with the tubing on its side for 5 minutes to allow the DNA-gold mix to settle into one side of the tefzel tubing. Once the DNA-gold mixture has settled pull the syringe plunger to remove the ethanol from the sample leaving only the DNA-gold mixture in the tefzel tubing. Allowing tefzel tubing to completely dry and then cut the tefzel tubing with a tubing cuter (Bio-rad) which is designed to cut tefzel tubing into appropriate cartridge size.

The cartridges containing the DNA-gold mixtures were loaded into a cartridge holder and placed into a helios gene gun (Bio-rad). The helios gene gun was connected to a helium tank by a helios hose assembly and through the use of a regulator, the helios gene gun was primed by opening the helium tank to 375-psi. Once primed, the gun was held 4.0cm above plated algae and fired. This process was repeated 4times for each transformation construct.

PCR analysis of transgeneic algae

Colonies that grew after chloroplast transformations were patched onto a plate containing 150µg/ml of kanamycin sulfate. Once patched, PCR analysis was performed to determine if transformed strains of *C. reinhardtii* contained the transgenic genes of interest. PCR screening protocols for chloroplast transformations were performed as performed as previously described. To determine if transformants were positive for the αCD22 gene a forward primer made against the psbA 5’-UTR (5’-GTGCTAGGTAACTAACGTTTGATTTTT-3’) and a reverse primer made

against the αCD22 (5'- TGGAGGTGGAGGTAGTGGTGGTGG-3') gene were used. To determine if transformants for positive for gelonin forward (5'-GGTTTAGATACAGTTTCATTTTCAA-3') and reverse primers (5'-CAAGTTTTTTGATGCCGATGCGAAG-3') against the gelonin gene product were used in the PCR analysis. To determine if strains were homoplasmic for the gene of interest two sets of primers were used. First, a psbA 5'-UTR forward primer (5'-GTGCTAGGTAACTAACGTTTGATTTTT-3') and M-SAA reverse primer (5'-GATACCTTGAATAGTTTCTCTAGCG-3') were used to determine if the M-SAA gene product was no longer present. Also in this PCR mix was a control set of primers to ensure that the PCR reaction did not fail. This primer set included a forward primer (5'- CCGAACTGAGGTTGGGTTTA-3') and reverse primer (5'-GGGGGAGCGAATAGGATTAG-3') against the DNA coding for the 16srRNA.

Determining immunotoxin protein accumulation and biochemical characterization of algal produced gelonin based immunotoxins

Accumulation of gelonin immunotoxins from homplasmic strains of *C. reinhardtii* was determined by western blot analysis. Homoplasmic strains of *C. reinhardtii* inoculated into 250mL TAP liquid cultures at a density of $2x10^5$cells/mL and grown to a density of $2x10^6$cells/mL in dim light (200lux). The same 250mL cultures of transgenic *C. reinhardtii* were used to inoculate 20L carboys (VWR) at a density of $2x10^4$cells/mL. Carboys were grown in light (10,000 lux) and connected to

air hoses that allowed ambient air to be bubbled into the cultures. Ambient air was passed through a 0.22µM filter and used to continuously mix the algal cultures. Samples were grown for 96 hours in continuous light and harvested using a continuous flow centrifuge (Brand and maker). Once harvested, cells were lysed in a lysis buffer (50mM Tris-HCL pH8.0, 500mM NaCL, 0.5% tween 20, one roche protease inhibitor tablet) in 10mL of buffer per gram of harvested algal cells using sonication (Sonic Dismembrator, Fisher Scientific) for 30 seconds at 25% amplitude followed by a 45 second rest phase. This sonication cycle was repeated for a total of 8 minutes of sonication time. Following sonication, samples were centrifuged for 20 minutes at 21,000g to separate the soluble and insoluble lysate. Soluble lysate was mixed mixed with 1mL of anti-M2-flag agarose resin (Sigma) and tumbled at 4ºC for 1 hour. After one hour samples were centrifuged at 800g to pellet flag resin. Unbound protein was removed and flag resin washed with 50 column volumes of lysis buffer. An additional wash with 50 column volumes of TBS was done prior to resin being loaded onto a chromatography column (Bio-rad). An elution buffer (100mM glycine-HCL pH 3.5, 500mM NaCl) was applied to flag resin to separate algal expressed immunotoxin proteins from the anti-M2 Flag antibody. Samples were eluted into 1M Tris-HCL pH 8.0 to neutralize acidic elution buffer. Following elution samples were buffer exchanged into phosphate buffered saline. Immunotoxin proteins, purified from algal lysate, were separated on reducing and non-reducing PAGE gel. Once separated samples were transferred onto a nitrocellulose membrane. Nitrocellulose membranes

were probed with an alkaline phosphatase conjugated anti-M2-Flag antibody for the presence of algal produced immunotoxin proteins.

Gelonin Immunotoxin Cell Binding Assay

CA-46 B-cells, Ramos B-cells, and Jurkat T-cells were incubated in the presence of algal produced αCD22, αCD22Gel, and αCD22CH23Gel in a buffer containing PBS and 0.01% sodium azide for 1 hour at 4ºC. Cells were washed with PBS 3 times to remove residual protein that did not bind to the cell lines. Following the primary binding with immunotoxin proteins cells were incubated with an α-Gelonin antibody produced in rabbit (MD Anderson, Dr. Michael Rosenblum) that had been diluted 1:5000 in PBS for 1 hour. After secondary binding the cells were washed three times with PBS. Cells were then incubated with an anti-Rabbit dylight 488 conjugated antibody (Sigma) that was diluted 1: 5000 in PBS for 1 hour. Cells were then washed 3 time in PBS and analyzed by flow cytometry using a BD influx (Becton Dickinson). FlowJo software was used to analyze data.

Gelonin Activity Assay

To determine if algal chloroplast has produced an enzymatically active protein, a DNAse activity assay was done (Melchior and Tolleson 2010). Puc18 plasmid (1ug) was incubated in a RIP buffer (50mM Tris-HCl pH 7.5, 50mM KCl and 10mM $MgCl_2$) and 0.01, 0.05, 0.1, 0.5 and 1.0 µg of αCD22, αCD22Gel, or αCD22CH23Gel

for 1hour at 37ºC. Samples were separated on a 1% agarose gel and stained with 0.05% gel red. DNA with no immunotoxin protein was used as a control along with plasmid digested with BamHI restriction enzyme.

Gelonin Cell Viability Assay

Using a 96-well tissue culture plate, 100ul of Ramos B-cells, CA-46 B-cells and Jurkat T-cells were seeded into each well at concentration of $5x10^4$cells/mL. Cells were incubated for 24 hours at 37ºC in a humidified incubator with 10% CO_2. Following the incubation, αCD22, αCD22Gel, and αCD22CH23Gel diluted in PBS with 0.2% human serum albumin (HSA) were added to the cell lines in increasing concentrations. As a positive control for cell death, cycloheximide was added to untreated cells at a concentration of 10µM. As a negative control for 100% cell survivl, PBS+0.2%HSA was added to untreated cells. Cells were then incubated for 72 hours after the addition of immunotoxin samples and controls. Following the incubation, 10µL of wst-8 reagent (Dojindo) was added to each well (Kawa, Onda et al. 2011). The cell viability assay was allowed to develop for 4 hours and the absorbance was measure at 450nm on a plate reader (Tecan). Cell viability curves were deteremined from the absorbance measurements and the IC_{50} for each protein was calculated using Grafit software (Erithacus).

Acknowledgments

Chapter 4 is being prepared for publication. Tran, Miller; Siefker, David; Henry, Ryan E; Van, C; Newkirk, G; Kim, J; Jack Bui and Stephen Mayfield, 2012. The dissertation author was the primary investigator and author of this paper

Figures

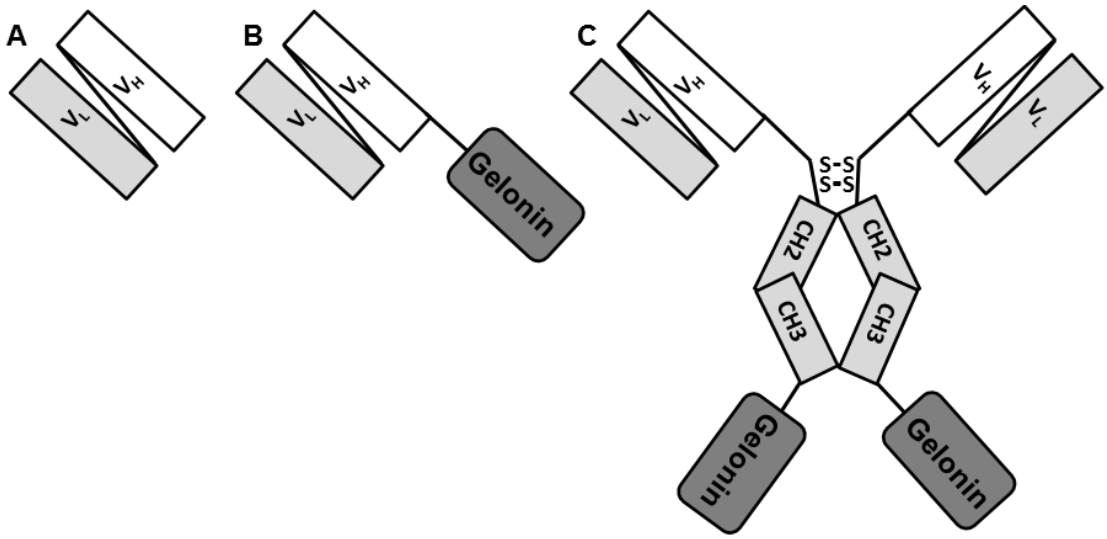


Figure 1. Depiction of algal expressed immunotoxin proteins (**a**) Single chain antibody (scFv) directed against the CD22 cell surface antigen made by linking the variable domains of the heavy and light chain antibodies with a glycine-serine linker. (**b**) The CD22-scFv is genetically linked to gelonin *Geloniun multiflorum*. (**c**) The CD22-scFv genetically fused to the hinge and constant domains of an IgG1 and to gelonin to create a construct that forms a homodimer through disulfide bonds formed between hinge regions. This fusion allows the molecule to have two binding domains as well as two toxin molecules.

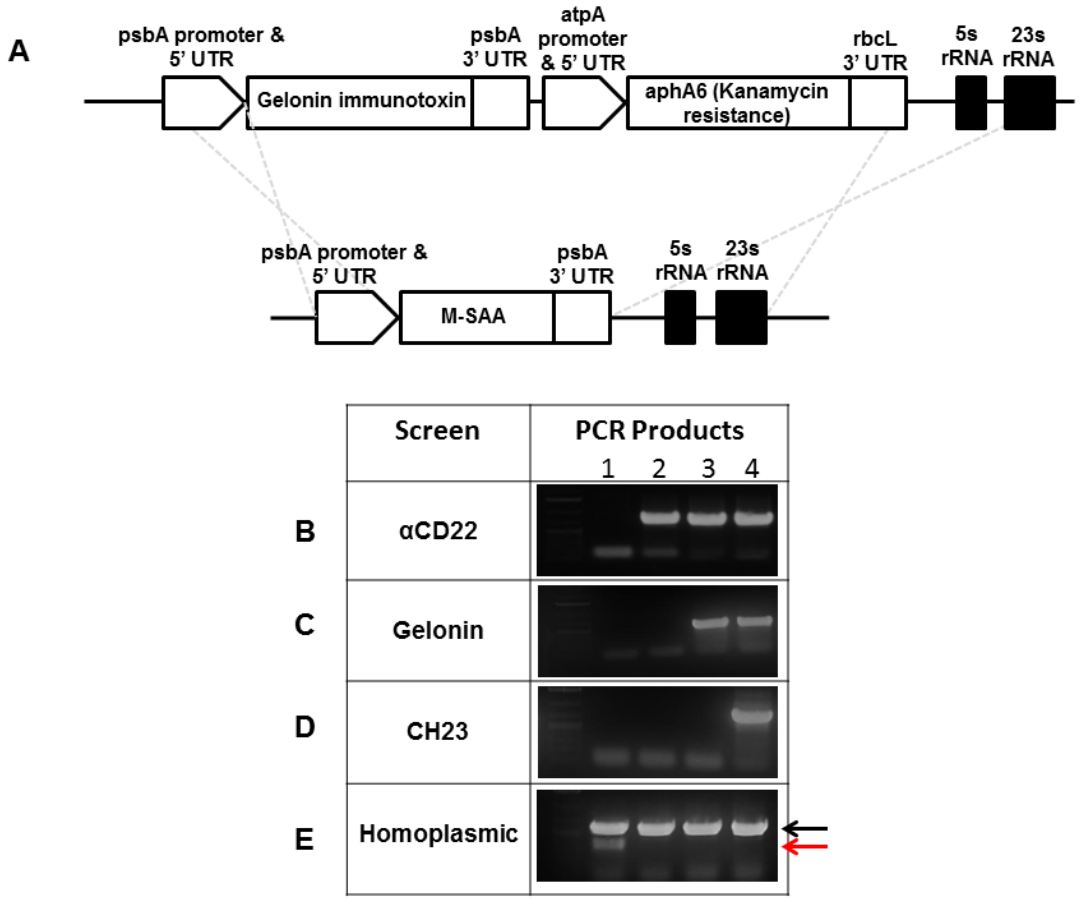


Figure 2.Integrations and Idtentification of C. reinhardtii transformants (A) Immunotoxin genes are ligated downstream of the *psbA* promoter and 5'-UTR and upstream of the *psbA* 3'-UTR. This construct is placed upstream of an aphA6 gene that confers kanamycin resistance to transformed cells of algae. Regions of chloroplast genome are placed at either end of the transformation vector to allow for homologous integration of the entire transformation cassette into the chloroplast genome PCR analysis using primers specific to the (B) αCD22 scFv gene, (C) gelonin gene, (D) CH23, and (E) primers to identify homoplasmic strains

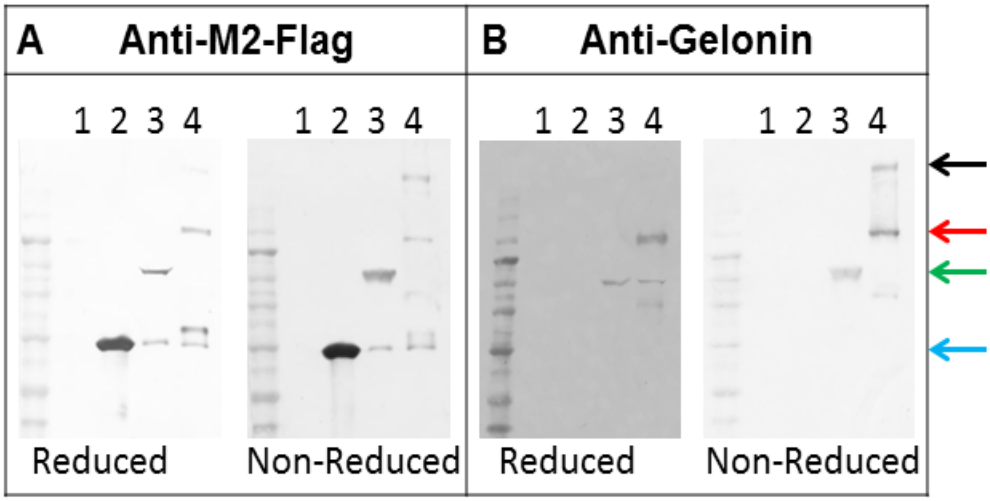


Figure 3. Western blots demonstrating the accumulation of immunotoxin proteins. (a) Samples were separated on a SDS-PAGE gel under reducing non reducing conditions, transferred to a nitrocellulose membrane and probed with an anti-flag antibody that was conjugated with alkaline phosphatase. The lanes contain the following samples: (Lane 1) wt soluble lysate, , (Lane 3) αCD22, (lane 4) αCD22PE40, (Lane 5) αCD22CH23PE40. (b) The identical samples were separated on a SDS-PAGE gel under reducing and non-reducing conditions transferred to a nitrocellulose membrane and probed with an anti-gelonin antibody.

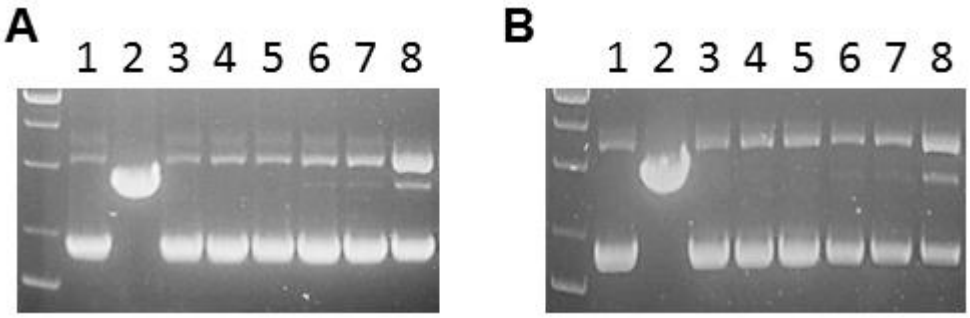


Figure 4. DNAase activity assay. (A) Lane 1: αCD22 incubated with puc18 plasmid, Lane 2: puc18 plasmid digested with BamHI, Lane 3-8: increasing concentrations of αCD22Gel. (B) Lane 1: αCD22 incubated with puc18 plasmid, Lane 2: puc18 plasmid digested with BamHI, Lane 3-8: increasing concentrations of αCD22CH23Gel.

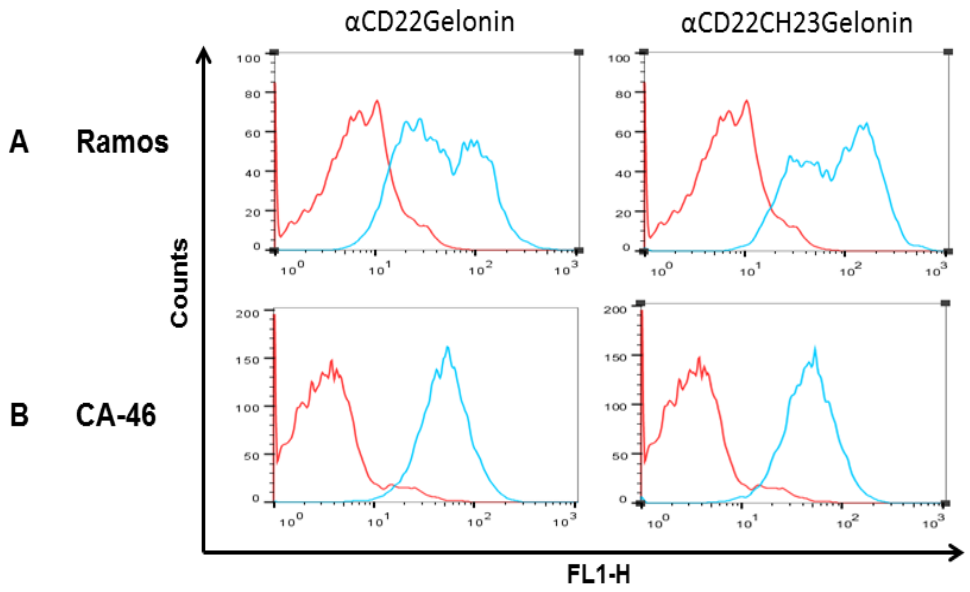
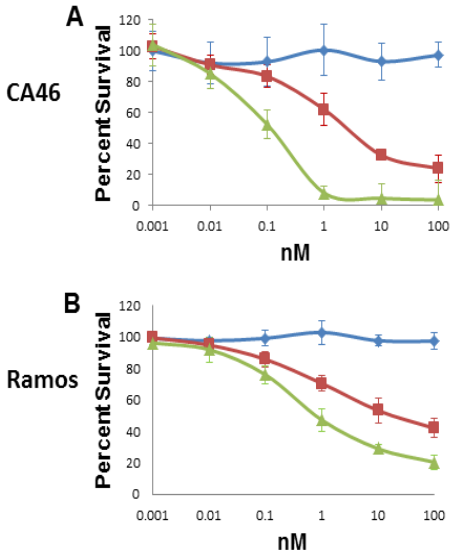
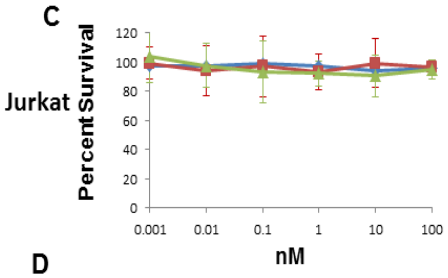


Figure 5. Flow cytometry demonstrate specific binding of algal produced immunotoxins. αCD22Gel and αCD22CH23Gel were incubated with CA-46 B-cells, Ramos B-cells or Jurkat T-cells. After primary incubation cells were subsequently incubated with anti-gelonin produced in Rabbit and finally with anti-rabbit-dylight 488. After incubation cells were analyzed by flow cytometery (a) A shift in the fluorescence spectra demonstrates that αCD22Gel and αCD22CH23Gel binds to Ramos Cells. (**b**) Fluorescence analysis also demonstrates that αCD22PE40 and αCD22CH23PE40 bind to CA-46 cells.





D

	CA46	Ramos	Jurkat
αCD22	Did Not Kill	Did Not Kill	Did Not Kill
αCD22 Gelonin	2.316 ± 0.471nM	21.051 ± 9.595nM	Did Not Kill
αCD22 CH23 Gelonin	0.093 ± 0.0236nM	1.421 ± 0.298nM	Did Not Kill

Figure 6. In vitro and in vivo analysis of the effectiveness of algal expressed immunotoxin against cancer cells. αCD22 (blue), αCD22Gel (red), and αCD22CH23Gel (green) were incubated with CA-46 B-Cells, Ramos B-cells, and Jurkat T-cells for 72 hours in vitro to determine their effectiveness. (**A**) αCD22Gel and αCD22CH23Gel were effective at killing CA-46 B-cells while αCD22 alone was incapable of killing CA-46 cells. (**B**) Additionally, αCD22PE40 and αCD22CH23PE40 were able to kill Ramos Cells while αCD22 was unable to inhibit Ramos cell proliferation. (**C**) αCD22, αCD22PE40, and αCD22CH23PE40 were unable to kill Jurkat T-cells. (**D**) IC_{50} for each immunotoxin against each cell line was calculated to determine how effective each was at inhibiting cancer cell proliferation. Both immunotoxins were capable of killing B-cells but Dimeric αCD22CH23Gel was more effective at killing targeted cells in vitro when compared to αCD22Gel.

Chapter 5: Development of *C. reinhardtii* miRNAs to target chloroplast target proteases

Introduction

Protein quality control is an essential function of all living organisms. Proteins that are no longer functional must be removed and their amino acids recycled. One of the key regulators of protein quality control are proteases. Proteases can be specific or they can be promiscuous and degrade proteins non-specifically. One of the most important advances in the production of recombinant proteins has been the development of protease deficient production host. Examples of these protease deficient production host can be seen in *E. coli* and Chinese hamster ovary (CHO) where yields have dramatically increased since these strains were identified. One of the most important developments in *E. coli* was the use of protease deficient strains of bacteria. These bacterial strains, deficient for the *lon* protease and/or the *ompT* protease (K12, BL21) (Grodberg and Dunn 1988), allowed scientists to increase recombinant protein yields. In the case of the bacterial expression of T7 RNA polymerase, intact protein could only be purified from strains deficient for both *ompT* and *lon* proteases (Grodberg and Dunn 1988). It is not currently possible to directly knock-out a gene in *C. reinhardtii* nuclear genome. However, it was recently shown

that *C. reinhardtii* has endogenous miRNAs along with the machinery to process these miRNAs into 21mers that targeted a specific gene for silencing (Molnar, Schwach et al. 2007). Additionally, artificial miRNAs have been used to silence specific genes in *C. reinhardtii* (Molnar, Bassett et al. 2009, Zhao, Wang et al. 2009). This process was used to target a broad range of proteases that are localized to the chloroplast to identify ones that limit overall protein accumulation.

Initially, a broad range of silencing constructs were designed to target 15 different proteases. This number was reduced to a more manageable 5 proteases after initial algal transformations as an initial test screen (Table 1). These proteases were selected based on homology to other proteases that have been shown to degrade proteins either similar to our recombinant protein of interest(Murakami, Kondo et al. 2000, Sakamoto 2006, Kuroda, Kitagawa et al. 2007, Baranek, Grabsztunowicz et al. 2011), their homology to other known proteases involved in non-specific degradation (Ostersetzer, Kato et al. 2007), or because of their involvement in the turnover of proteins involved in photosynthesis (Sakamoto 2006, Grabsztunowicz, Lucinski et al. 2011). This list is shown in Table 1. Two artificial miRNAs were designed to target different regions of the gene to ensure our success in silencing the targeting protease. To date we verified the silencing of three out of five proteases and demonstrated that one protease, deg2, appears to play a role in the degradation of recombinant human IgG protein expressed in chloroplasts. A qPCR analysis was done to demonstrate that USER miRNA vectors did silence deg2 and actin was used as a reference gene. After

confirming deg2 silencing, a western blot analysis was performed to determine if there was a greater accumulation of recombinant human IgG or a

Results

Proteases were targeted for miRNA silencing

Five chloroplast localized proteases were targeted for silencing (Table 1). These proteases are believed to be localized to the stroma of chloroplast where our recombinant proteins are also known to accumulate (Figure 1). Design of miRNA precursors was based on previously established protocols (Molnar, Bassett et al. 2009) Transformants were selected for colonies that grew on paromycin and bleomycin. All miRNA constructs grew demonstrating that the silencing construct was not lethal to *C. reinhardtii*.

Seamless USER cloning to produce miRNA silencing vectors

A primary vector was first created that contains a bleomycin resistance gene who expression is controlled by the regulatory elements of β-tubulin 5’ and 3’-UTR upstream of the artificial miRNA precursor. Downstream of the artificial miRNA precursor is a paromycin resistance gene whose expression is controlled by the regulatory elements of the psaD 5’ and 3’-UTR (Figure 2). Seamless USER cloning was used to construct all miRNA containing vectors (Figure 2). Upon completion of

USER cloning, two miRNA precursors were designed to target each of the 5 endogenously expressed proteases.

qPCR results demonstrate the utility of seamless USER cloning in silencing nuclear encoded genes

As a test to validate the utility of using USER cloning to produce functional miRNA constructs the MAA-7 gene which codes for the beta-tryptophan synthase was targeted for silencing. RNA isolated from strains of algae were able to grow on both paromycin and zeomycin, the selectable markers on the silencing contructs was reverse transcribed and cDNA generated. A qPCR analysis of the cDNA was done to determine the degree of silencing in each strain. As a control an empty vector containing no miRNA precursor was used to ensure that paromycin and zeomycin did not impact the accumulation of MAA-7 or protease messages. Additionally, expression of MAA-7 message was standardized against the expression of actin in each strain. Greater than 80% silencing was achieved in one strain (Figure 3).

A qPCR analysis was also performed for strains where the Deg2 protease had been targeted for silencing. When compared to an empty vector control strain there were multiple strains that displayed approximately 50-80% silencing (Figure 4). These results validate that seamless USER cloning is a robust method for producing functional artificial miRNA vectors.

Silencing of MAA-7 beta-tryptopha synthase

A biochemical analysis was used to validate that artificial miRNA vectors created by seamless USER cloning were functional. It has been previously demonstrated that strains of algae that are silenced or deficient for the MAA-7 gene are able to grow on 5-fluroindole if media is supplemented with tryptophan. Normally, if a cell is grown on 5-fluroindole, the beta-tryptophan synthase converts it to 5-flurotryptophan which becomes toxic to *C. reinhardtii* cells (Zhao, Wang et al. 2009). By silencing MAA-7 and supplementing the media with tryptophan the toxic effects can be avoided. Successful silencing of MAA-7 is seen by tranformants that are able to grow on 5-fluroindole (Figure 3).

Silencing of Deg2 protease increases recombinant protein accumulation

Deg2 is a serine type protease that is localized to the stroma and thought to potentially play a role in the degradation and recycling of the photosynthetic complex (Lucinski, Misztal et al. 2011). Strains of *C. reinhardtii* that were expressing a full length human antibody, both heavy and light chain proteins, were silenced for Deg2 (Figure 4). Once a decrease in message expression was confirmed by qPCR (Figure 4), Deg2 silenced strains were grown up and the accumulation of the recombinant human antibody was assayed by western blot analysis. When a comparison with a strain that was not silenced for Deg2 was made, it was clear the strains where Deg2 was silenced were able to accumulate more protein than the unsilenced control. These results show the potential of developing protease deficient strains of algae.

Discussion

It is estimated that of the approximately 3000 proteins that are imported into the chloroplast 2-3% of those proteins are proteases, suggesting a major role of these proteases in maintaining the protein environment of the chloroplasts. Degradation of heterologously expressed proteins has been a major concern for many expression systems (Grodberg and Dunn 1988, Tojo, Miyagi et al. 2008) and is one that has not been addressed in the algal chloroplast expression platform. A robust system of homologous recombination and an insertional library do not exist in algal chloroplast thus the only means to determine which proteases play a major role in protein accumulation was to perform artificial miRNA silencing on a strain of algae that accumulates a heterologous protein and which is also suffers from degradation. Previously, we had demonstrated that human IgGs expressed in algal chloroplast suffer from a specific cleavage (Tran, Zhou et al. 2009) and appeared to be an ideal candidate for artificial miRNA silencing to increase recombinant protein accumulation. A more robust cloning strategy than the ones that had been previously suggested was needed to produce a large amount of miRNA silencing constructs (Molnar, Bassett et al. 2009, Zhao, Wang et al. 2009).

Previous work has demonstrated that seamless USER cloning was a plausible strategy to assemble a large amount of a large amount of artificial miRNA silencing constructs (Nour-Eldin, Geu-Flores et al. 2010). Here we created a total of 12 artificial miRNAs, two each to target five proteases and the tryptophan beta synthase. USER

cloning facilitated the rapid construction of each construct and allowed the project to progress.

Once transformed with the artificial miRNA, *C. reinhardtii* transformants were screened for their ability to survive on TAP-agar plates containing both paromycin and bleomycin. This ensured the integration of the entire miRNA construct into the nuclear genome. By analyzing the expression of the target genes it was clear that the USER cloned artificial miRNA constructs are effective at silencing the targeted gene. Also, growth analysis of strains transformed with an artificial miRNA targeting MAA-7 demonstrated that the gene was indeed silenced and that they were capable of surviving on 5-fluoroindole. Silencing of one particular protease, Deg2, demonstrated that silencing chloroplast localized proteases was a viable strategy for achieving high levels of protein accumulation.

The use of miRNAs to silence proteases may be ideal due to the importance of many proteases in the development and maintenance of chloroplasts (Sakamoto 2006). A complete knock-out of these quality control proteins could result in a lethal phenotype. Simply decreasing the amount of proteases in chloroplast may be sufficient to significantly increase protein accumulation. A more complete analysis of proteases and their potential role in inhibiting protein accumulation will be necessary but we have demonstrated that by using a miRNA based silencing system, we may be able to identify which proteases affect the accumulation of our recombinant proteins the most.

Experimental Procedures

Selection of Target sequence to silence nuclear encoded proteases

Initially, a broad range of silencing constructs were designed to target 15 different proteases. This number was reduced to a more manageable 5 proteases after initial algal transformations as an initial test screen. These proteases were selected based on homology to other proteases that have been shown to degrade proteins either similar to our recombinant protein of interest(Murakami, Kondo et al. 2000, Sakamoto 2006, Kuroda, Kitagawa et al. 2007, Baranek, Grabsztunowicz et al. 2011), their homology to other known proteases involved in non-specific degradation (Ostersetzer, Kato et al. 2007), or because of their involvement in the turnover of proteins involved in photosynthesis (Sakamoto 2006, Grabsztunowicz, Lucinski et al. 2011). This list is shown in Table 1. Two artificial miRNAs were designed to target different regions of the gene to ensure our success in silencing the targeting protease.

Design of miRNA 21mer

The website http://wmd.weigelworld.org/cgi-bin/mirnatools.pl was used to design 21mers that specifically targeted our genes of interest for silencing.

Seamless USER cloning of *C. reinhardtii* miRNA silencing vectors

USER cloning is a seamless cloning technique that allows a DNA construct to be made without any genetic scars in the middle of a construct. Previous work to

produce artificial miRNA for *C. reinhardtii* focused on either the cumbersome process of overlap PCR or the placement of a restriction site in the middle of an endogenous miRNA (Molnar, Bassett et al. 2009). In addition to the internal scar in the artificial miRNA, this process proved to be costly because of the large oligos that were required to complete the miRNA. To remove this internal scar USER cloning was performed(Nour-Eldin, Geu-Flores et al. 2010). An initial backbone vector was created that contains a PacI restriction site with a Nt.BbcI nicking site. These sites were placed downstream of the HSP70rbcs2 tandem promoter and UTR (Figure 2). The vector was digested and nicked with the previously mentioned enzyme (Figure 2). Oligos containing a uracil were ordered to amplify both the artificial miRNA and the DNA that encodes for the miRNA precursor. Once amplified the PCR products are treated with the USER enzyme which removes the uracil and all DNA bases upstream of it. This leaves a 6 base pair overhang corresponding to the digested and nicked vector backbone (Figure 2). These pieces are allowed to anneal at room temperature and are then transformed into E. coli to amplify the vector (Figure 2). We were able to construct 30 miRNA, two miRNAs for 15 unique proteases. We also created an artificial miRNA to target MAA-7 which encodes for the tryptophan beta synthase to demonstrate that USER cloning of miRNA vectors was a valid method to produce these silencing vectors

Transformation and selection of miRNA silenced strains of *C. reinhardtii*

Initially, 1µg of plasmid vector from each silencing construct was digested with the restriction enzymes Ale I and Psi I for 4 hours at 37ºC to liberate the bacterial backbone from the *C. reinhardtii* artificial miRNA construct. Once liberated DNA was mixed with 250µL of *C. reinhardtii* cells at a concentration of $3x10^8$cells/mL and placed at 4ºC for 10minutes.Cells were then transferred to 4mm electroporation cuvettes. Cells were electroporated using previously estabilished protocols (Rasala 2012) and recovered in 10mL TAP medium containing 40mM sucrose. Sucrose gives the recovery medium enough osmotic pressure to prevent cells from lysing. Cells were allowed to recover in TAP+sucrose overnight. The following day cells were plated on TAP-agar plates containing 20µg/mL of paromycin. After 7 days colonies that grew were patched onto plates that contained 20µg/mL of paromycin and 10ug/mL of zeomycin. Double selection ensures that transformation vectors were not harmed by DNAses in addition to confirming that the entire transformation construct was integrated into the nuclear genome.

qPCR analysis of silenced strains of *C. reinhardtii*

To determine if artificial miRNA silencing constructs impacted expression of the targeted genes qPCR analysis was done. Silenced cultures as well as empty vector cultures, where no silencing construct was present, were grown in 50mL TAP cultures for 48 hours at 5000Lux and then harvested. Harvested cells were resuspended in 0.5mL cold Plant RNA Reagent (Life technologies). Samples were incubated for

5minutes at 25ºC and followed by centrifugation at 12,000g for 2 minutes. The supernatant was transferred to a new eppendorf tube and 0.1mL of 5M NaCl added to the samples followed by 0.3mL of chloroform. Samples were mixed and centrifuged for 10 minutes at 12,000g. The aqueous phase of the samples was transferred to a new tube and nucleotides precipitated with an equal volume of isopropanol. Samples were centrifuged for an additional 10minutes at 12,000g. Pelleted nucleotides are then washed with 1mL of ice cold 70% ethanol. Samples are centrifuged for 5 minutes at 12,000g, decanted and allowed to dry. Once dried, washed samples are re-suspended in 40µL of nuclease free water.

After RNA isolation 1µg of RNA was reverse transcribed into cDNA using the Verso cDNA synthesis kit (Thermo). Following cDNA synthesis, primer sets were designed (http://www.quantprime.de/) to measure expression of silenced genes. All primer sets were tested to ensure that primer efficiencies were in the range of 95-110%, the standard acceptable range of the MIQE guidelines (Taylor, Wakem et al. 2010).

Expression analysis was done using a CFX96 (Bio-rad) and expression of silenced genes standardized to the expression of actin. All samples were compared to the empty vector controls to determine the degree of silencing that was caused by the artificial miRNA constructs.

Silencing of MAA-7 beta-tryptophan synthase

The tryptophan beta synthase converts 5-fluroindole into 5-flurotryptophan which leads to the production of proteins that are not functional which ultimately causes the algal cell to die. Only by silencing the tryptophan beta synthase will the algal cells be able to survive on plates that contain 5-fluroindole (van Dijk and Sarkar 2011). We created artificial miRNAs targeting MAA7 and were able to demonstrate that strains in which this gene was significantly silenced were able to survive on plated containing 5uM 5-fluorindole (Figure 3). This demonstrated that USER cloning was a viable method for the production of *C. reinhardtii* artificial miRNA vectors.

Western blot analysis of silenced strains of *C. reinhardtii*

To determine if the silencing of the endogenous Deg2 protease altered the accumulation of a chloroplast expressed full length monocolonal human antibody, cells from silenced and empty vector strains of *C. reinhardtii* were inoculated into 50mL liquid TAP cultures. Samples were inoculated at a density of $3x10^5$ cells/mL and allowed to grow in 5000lux for 48 hours. Once the samples were grown cells were harvested by centrifugation.

Cells were then resuspended in a 500µL of lysis buffer (Tris-HCl pH8.0, 150mM NaCl, 0.01% tween 20) and sonnicated for 30 seconds at 4ºC. Once lysed, samples were centrifuged to separate both soluble and insoluble proteins. Insoluble protein was discarded and the concentration of soluble protein was measure by Bio-rad DC assay. 20µg of soluble protein from each Deg2 silenced sample was then

separated on a Polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with a sample containing 5% milk and probed with either an alkaline phosphatase conjugated α-M2-Flag antibody or an alkaline phosphatase α-kappa light chain antibody. Membranes were developed and visualized.

Figures

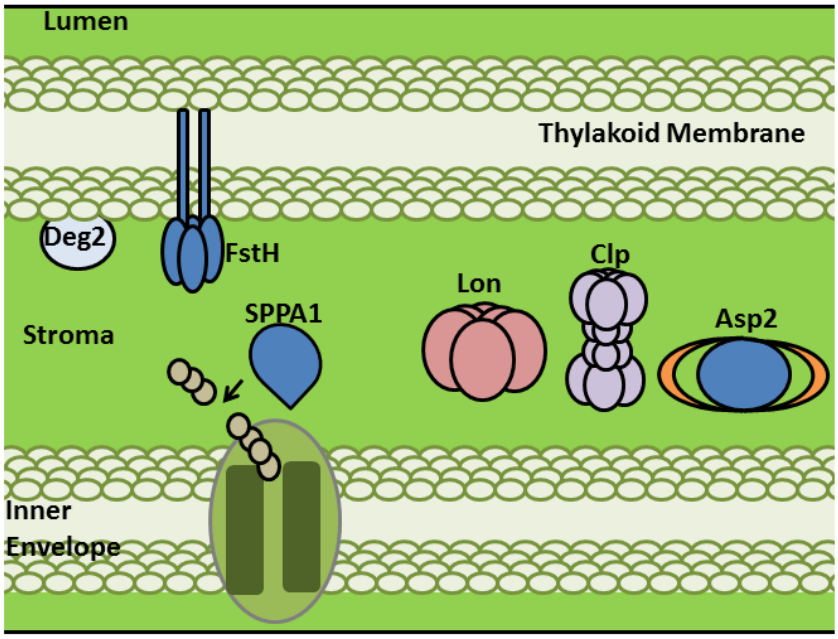


Figure 1. Stromal Chlorplast proteases. The picture depicts a list of chloroplast localized proteases the were targeted for silencing. All proteases are localized to the stroma of chloroplasts

Table 1. List of chloroplast proteases target for silencing

Target Gene	Gene Function	# of miRNAs	Primers Efficiency
Asp2	Aspartyl protease	2	Yes 102%
Deg2	Membrane serine protease (psbA and thylakoid membrane proteins)	2	Yes 92%
Ftsh1	Membrane protease (psbA and thylakoid membrane proteins)	2	No
Lon	ATP-dependent protease (house keeping)	2	Yes 100%
SPPA1	Serine protease distinct from SPP, more closely related to ClpP	2	Almost

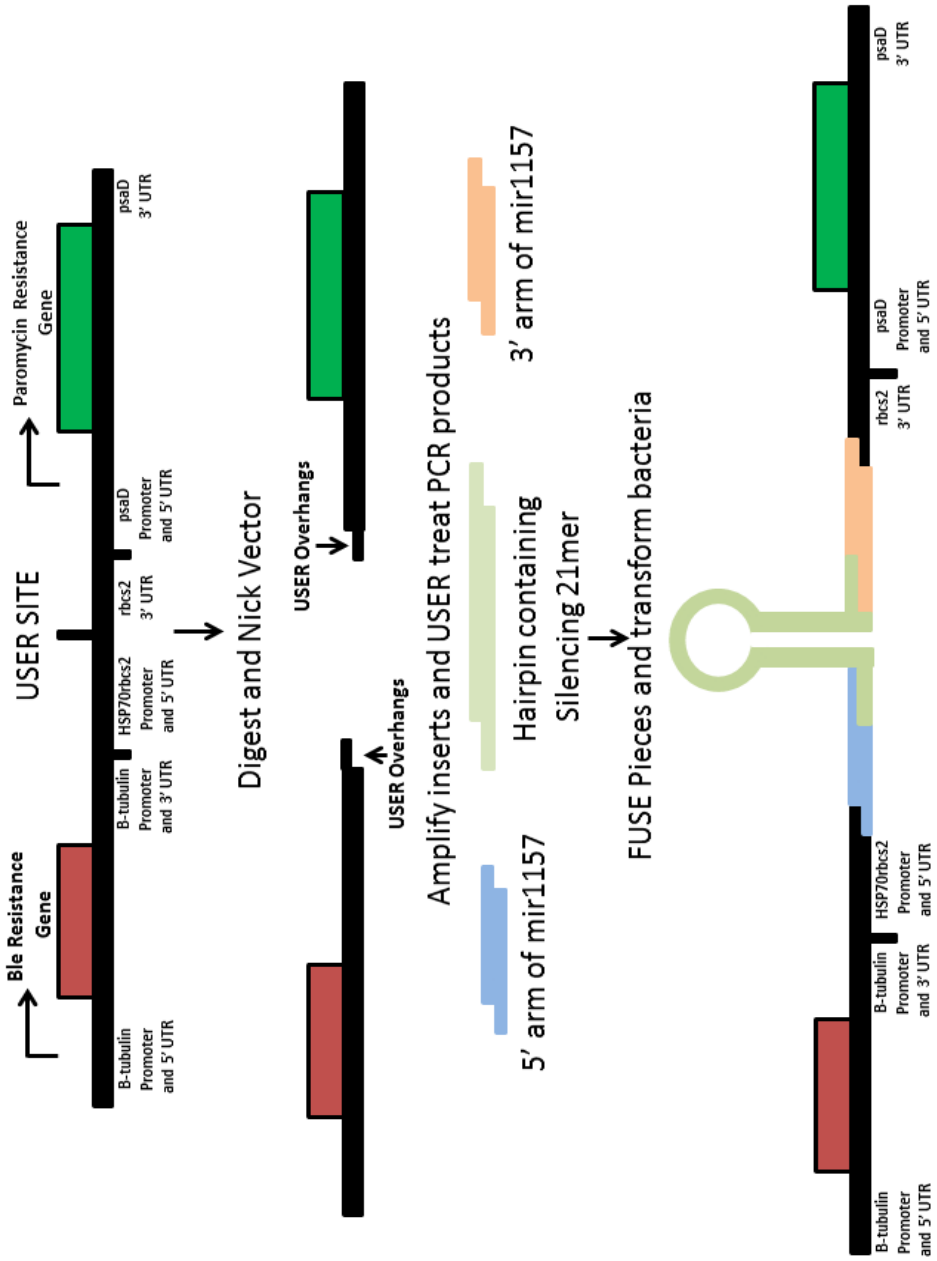


Figure 2. Depiction of seamless USER cloning to produce artificial miRNA
Constructs contain Bleomycin and Paromycin resistance genes to silence *C. reinhardtii* nuclear genes

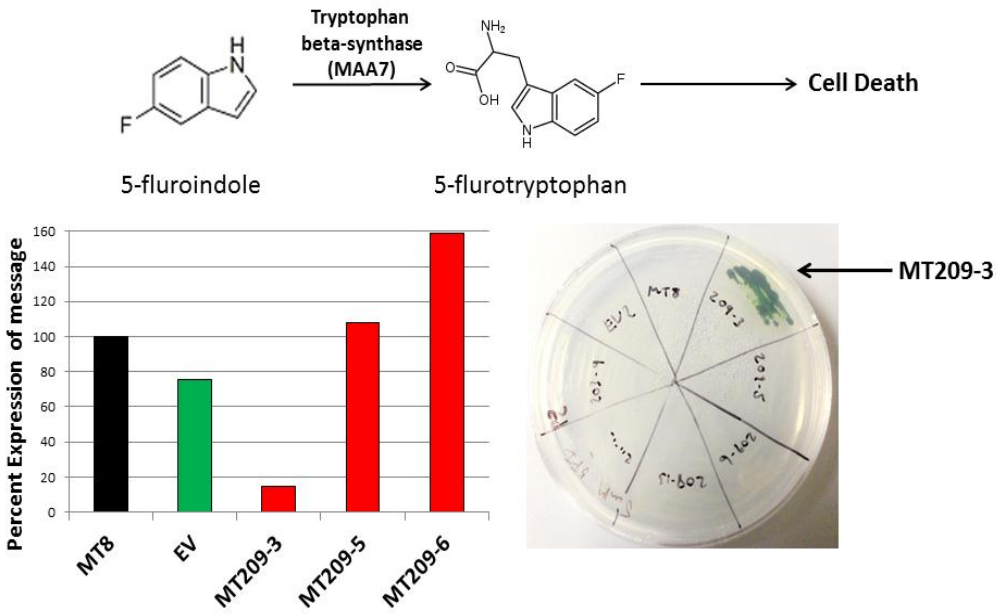


Figure 3. Conversion of 5-fluroindole into the toxic analogue 5-flurotryptophan. Artificial miRNAs were used to silence the MAA-7 gene. qPCR analysis confirms that MAA-7 genes are silenced. *C. reinhardtii* cultures silenced for the MAA-7 gene are able to grow on plates with 5-fluroindole and tryptophan

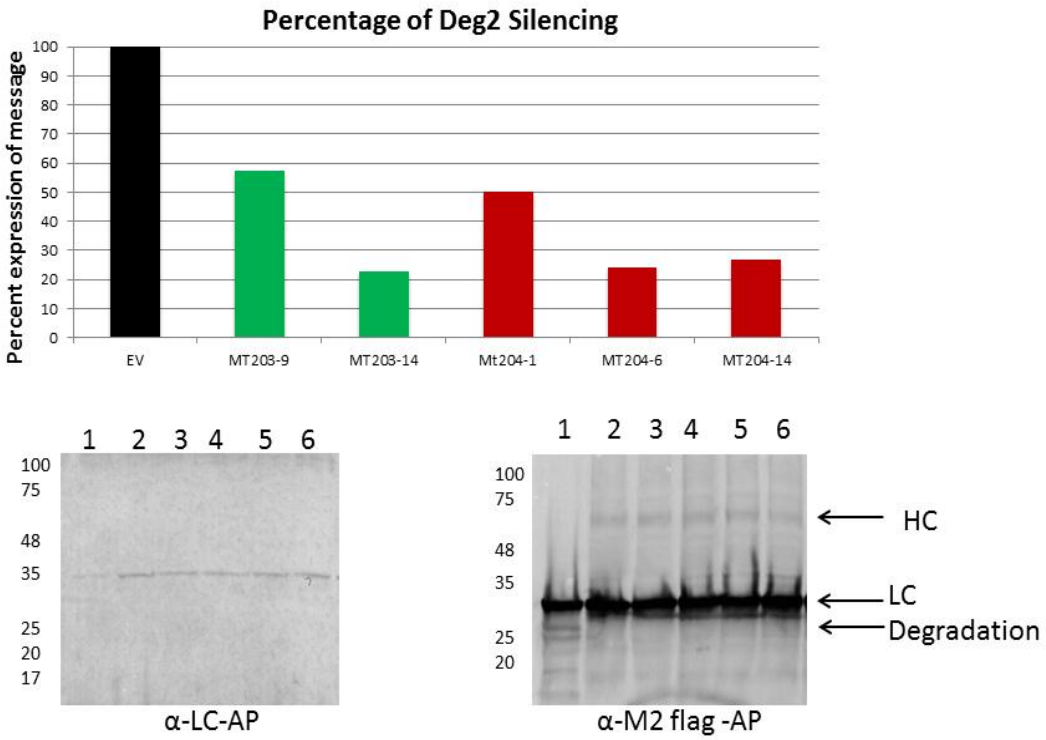


Figure 4. Silencing of the Deg2 protese increased protein accumulation. qPCR analysis confirms that artificial miRNAs decrease expression of the targeted Deg2 protease. Anit-light chain and anti-m2-flag westerns demonstrate increased recombinant protein accumulation in strains of algae where Deg2 is being silenced

Chapter 6: Recombinant protein accumulation in chloroplasts is influenced by micronutrients

Introduction

Micro and macro nutrients play a major role in the development of eukaryotic algae. Depending on their environment, different nutrients are rate limiting. In the oceans one of the major rate limiting factors of algal growth is iron (Morrissey and Bowler 2012). Scientist have demonstrated that by simply pouring large quantities of iron into the ocean large algal blooms will emerge (Smetacek, Klaas et al. 2012). This is also the case for diatoms or brown algae who often times lack silica, which is a main component of their cell wall (Pondaven, Gallinari et al. 2007). This is true not just for cell walls but for many parts of an algal cell. Sulfur is an important component of many structurual proteins (Gonzalez-Ballester, Casero et al. 2010) as well as proteins that play a vital role in the redox regulation of chloroplasts (Danon 1997). Iron is a key component in the photosynthetic apparatus and plays an important role in shuttling electrons from one photosynthetic subunit to the next (Sandmann and Malkin 1983). This is a major problem when algae are considered as a source of renewable fuel where, much like land plants, large quantities of fertilizer that contain nitrogen, potassium, phosphate and various trace elements will be required. Our understanding of how micro and macro nutrients affect the chemistry in algal cells remains largely in

its infancy but if we are able to increases our understanding of how the biology of the cells is affected by varying concentrations of this nutrients we may be able to greatly impact both the generation of biofuels and recombinant proteins.

Harnessing the potential of micro and macro nutrients is a standard practice in other recombinant protein expression platforms. By altering the contents of the growth medium for Chinese hamster ovary cells scientist were able to drastically increase the expression titers of recombinant proteins over 100-fold. (Wurm 2004, Schaub, Clemens et al. 2010). One of the key aspects of protein expression is being able to manipulate the expression of any given gene. This is a common practice in bacteria where the expression of genes are regulated by the addition of isopropyl-beta-thiogalactopyranoside to the growth medium (Li, Zhao et al. 2012). However, this is not just the case with recombinant or heterologous genes but also the case for many endogenous genes.

The importance of nutrients to the growth of the green algae, *C. reinhardtii* cannot be understated. As with all organisms this algae has developed special metal transporters to ensure that it has sufficient nutrients to metabolically functioning (Blaby-Haas and Merchant 2012) Often times when these nutrients become rate limiting,, *C. reinhardtii* will secrete proteins that assist it in scavenging for their nutrients. This is the case when *C. reinhardtii* is deficient for iron and it ecretes FEA1, and FEA2 to ensure that is can maintain its metabolic function (Allen, del Campo et al. 2007). Additional complexity is added when the expression of different cellular components are regulated by the availability of micronutrients as can be seen

with the *C. reinhardtii* hydrogenase encoding HYDA1 gene whose expression is regulated by the copper response regulator (Pape, Lambertz et al. 2012)

Continuing work has been done to demonstrate that altering the concentration of media components such as copper, iron, and other trace elements can dramatically alter the expression profile of endogenous proteins (Eriksson, Moseley et al. 2004, Allen, Kropat et al. 2007, Long, Sommer et al. 2008). While it is known that altering the media components of an algal culture can greatly affect its expression profile, little has been done to analyze how varying trace element composition could affect recombinant protein accumulation. As of today, a synthetic non-endogenous promoter and UTR have not been developed for the expression of genes in *C. reinhardtii* chloroplast and as such the expression of heterologous genes remains under the control of the identical regulatory elements or the endogenous gene. This can be seen in the light activation of all proteins that are under the control of the regulatory elements that dictate the expression of the psbA gene (Manuell, Beligni et al. 2007, Long, Sommer et al. 2008, Tran, Zhou et al. 2009, Rasala, Muto et al. 2010, Terauchi, Peers et al. 2010, Page, Allen et al. 2012). Also because we have many heterologous genes whose expression are controlled by endogenous regulatory elements we will examine how these regulatory elements are affected by varying trace element composition by analyzing changes in protein accumulation. By using recombinant proteins as a read out for the impact of trace elements we may be able to build upon our knowledge base of regulatory elements that dictate recombinant protein accumulation in chloroplasts.

As has been shown in the expression of antibodies from CHO cells, optimization of the media can dramatically increase the final yields of purified protein (Wurm 2004, Schaub, Clemens et al. 2010). Thus, it will be essential that optimal growth media be developed for *C. reinhardtii* to consistently improve yields of recombinant proteins to 5-10% of total soluble protein, the estimated concentration where economic competitiveness can be achieved (Manuell, Beligni et al. 2007). To date we have taken the initial steps to optimize this media by analyzing the effect of increasing iron concentrations in our standard Tris-Acetate-Phosphate (TAP) media. Since the promoters and UTRs that are often times used in our transformation vectors are derived from photosynthetic gene they should be regulated similarly by differenced in trace elements. Iron has been shown to increase the expression of photosynthetic genes such as psaD (Allen, del Campo et al. 2007), and it was hypothesized that recombinant protein expression would also increase by increasing iron concentrations.

By changing the expression profile of other endogenously expressed genes it might also be possible to indirectly influence the accumulation of heterologously expressed proteins. It was previously demonstrated that the reducing potential that is generated from photosynthesis is a product of reducing potential coming from photosystem I (Moseley, Allinger et al. 2002) The variability in the concentration of photosystem I can be attributed to the high iron concentration within this photosynthetic apparatus. It was demonstrated that in a low iron state *C. reinhardtii* cells will over express ferritin I which strips photosystem I of its iron content which

causes the photosynthetic apparatus to fall apart (Figure 1) (Busch, Rimbauld et al. 2008, Long, Sommer et al. 2008). In high iron concentrations ferritin I is not expressed but rather ferritin II is expressed. Ferritin II appears to play a role in donating reduced iron ions to newly synthesized photosystem I (Figure 2) (Busch, Rimbauld et al. 2008, Long, Sommer et al. 2008) . The expression of the psbA gene has been shown to be a product of a redox regulation that occurs from this increase in reducing potential (Barnes and Mayfield 2003). Thus by increasing the amount of photosystem I we may be able to directly impact the amount of reducing potential being generated and thus increase the accumulation of our recombinant protein of interest. Depicted below is at hypothetical process of how reducing potential initiates expression of recombinant proteins driven by the psbA promoter and 5'-UTR in a psbA deficient mutant (Figure 3).

Results

Effects of increased iron concentrations on heterologous proteins

In this experiment we grew strains of algae accumulating the recombinant αCD22PE40 whose expression is driven by the psbA promoter and 5' UTR in medium that contained either no iron, normal iron, or twice the amount of normal iron. Cells were allowed to grow in the dark for 4 days and subsequently shifted into the light for 48 hours. Samples were taken at initially when cells were shifted into light, 24 hours after light shift and 48 hours after light shift. Westerns were done to analyze the expression of both psaA and the recombinant αCD22PE40. Our experiments indicate

that increased iron conditions both increase the accumulation of the recombinant αCD22PE40 and psaA, a component of photosystem I (Figure 4). Additional research needs to be done to determine if there is a correlation between increased accumulation of photosystem I, reducing potential and recombinant proteins that are expressed using psbA translational elements. However, it seems clear that by simply increasing the concentration of iron in our standard TAP medium, we were able to dramatically increase the accumulation of our recombinant αCD22PE40 without negatively affecting the growth of algal cells.

Discussion

Micro and macro nutrients play an essential role in all living organisms. They assist with transporting oxygen to essential organs in mammals (Hemmingsen 1965) and allow many redox regulated reactions to occur in both prokaryotes (Rouhier, Couturier et al. 2010) and eukaryotes (Petti, McIsaac et al. 2012). Often times a rate limiting factor in algal growth in the wild is the availability of micronutrients that cells need to perform many of their basic biological functions. The importance of trace elements is also seen in many recombinant protein expression platforms. The simple optimization of media can lead to drastic increases in recombinant protein titers (Kim, Kim et al. 2012).

Here we examined the effects of the trace element iron and the effects that it had both on cell growth, accumulation of an important photosynthetic subunit and the accumulation of a recombinant protein of interest, αCD22PE40. Although our cells

were no longer photosynthetic because we replaced psbA with our recombinant gene of interest it was clear that the micronutrient iron was essential for their survival. Interestingly, iron depleted cells were able to grow in the dark and it was only upon shift into high light did the cells start to die. When we compare the growth phenotype of a psbA knock-out grown with normal Hutner's trace elements to cells grown with twice the amount of iron, we see that cells that were grown with twice the iron had a slightly faster growth rate. Although cultures grown with twice the iron did grow slightly faster it should be noted that both cultures had a similar maximal density.

The optimization of growth media for increased recombinant protein accumulation in *C. reinhardtii* has not been attempted. However, results from other expression platforms clearly demonstrate that slight alterations in media can dramatically increase the overall yield of the protein of interest. In this preliminary study we were able to increase the overall accumulation of αCD22PE40 and the photosystem I subunit psaA. Further analysis will have to be done to determine if in fact increasing photosystem I accumulation leads to a greater increase in reducing potential which leads to a greater accumulation of proteins under the control of the psbA promoter and UTR. It should also be noted that these cells are no longer photosynthetic due to the replacement of the psbA gene with our recombinant gene of interest and thus no reducing potential is being generated from photosystem II. It has been hypothesized that a cyclic electron flow occurs either around photosystem I or plastoquinone (Johnson and Alric 2012). Our cells are grown on TAP medium with contains acetate that the cells can use as a reduced carbon source. It is also possible

that starch metabolism generates the reducing potential that is then fed into photosystem one which leads to the reduction of translational elements that initiate the translation of genes under the control of the psbA 5'-UTR.

It should be noted that simply increasing trace elements may not be sufficient to increase recombinant protein titers. Different trace elements alter different metabolic pathways and some of those pathways may have a negative impact of protein accumulation. It is entirely conceivable that increasing a specific trace element could increase the concentration of proteases that degrade our recombinant protein. Thus a full comprehensive study will have to be done to determine which components of trace elements are essential and which ones have the most dramatic effect on protein accumulation. Due to the positive results that The potential to increase recombinant protein accumulation by altering trace element composition seems

Experimental Procedures

Transformation and selection of algal strain used in iron analysis

To test the effects of iron on the accumulation of a recombinant protein, an immunotoxin gene coding for a single chain antibody genetically fused to the toxin domain of *Pseudomonas aeroginosa* , termed αCD22PE40 was used as a reporter. A transformation vector was constructed to integrate this immunotoxin gene into algal chloroplasts. To build a transformation construct, αCD22PE40 was placed downstream of the psbA promoter and 5'-UTR. This ensures that the expression of αCD22PE40 will be controlled by the same regulatory elements as those that control the endogenous

psbA gene. The psbA 3'-UTR was placed downstream of the αCD22PE40gene. A flag tag was placed onto the 5'-end of the αC22PE40 gene for ease of protein detection. Following the 3'-UTR a selection cassette containing an aphA6 gene, whose expression was controlled by the atpA promoter and 5'-UTR, was used to confer resistance to kanamycin to transformed strains of algae. On either end of the construct, regions of homology were used to direct the integration of αCD22PE40 into the psbA locus. Once integrated strains of algae were screen for the presence of the immunotoxin gene and for a loss of the endogenous psbA gene (Jones, Luong et al. 2012). This would ensure the loss of the endogenous psbA gene which is known to regulate the expression and accumulation of its own message and protein (Manuell, Beligni et al. 2007). Once homoplasmic, the strain of *C. reinhardtii* was termed αCD22PE40.

Iron supplementation and depletion in algal media

Hutner's trace element recipe was used as the standard amount of micronutrients in this experiment. 18µM of iron is the standard amount of iron used in normal TAP and HSM media. To determine the effects of altering the amount of iron, two other trace elements were produced. One that contained no iron and one that contained twice the amount (36µM) of iron. One 250mL culture of αCD22PE40 was grown to a density of $2x10^6$cells/mL and split into 3 cultures containing with 0µM, 18µM, or 36µM of iron. Cells were grown for an additional 5 days to assimilate them into their new media and to remove trace amounts of iron from the first culture. Once cells were conditioned in their varying medias they were re-inoculated into fresh

media with the identical concentration of iron that they were grown in either 0µM, 18µM, or 36µM. Cultures were inoculated at a density of $1x10^5$and allowed to grow in the dark for 4 days. A sample was taken after the 4 days growth in the dark and culture density determined and protein content analyzed. Cultures were shifted into light (5000lucx) and allowed to grow for 24 hours. Another sample was taken from each culture, cell density measured and protein content measured. This was repeated one more time after 48 hours of light exposure.

Western blot analysis of αCD22PE40 and psaA accumulation

At each time point 10mL of cells were harvested and spun down at 5000g. 300µL of lysis buffer was added to each sample (Tri-HCL pH8.0, 150mM NaCl, 0.01% tween 20). Samples were sonnicated for 30 seconds at 15% amplitude at 4°C to unsure protein integrity. Soluble and insoluble proteins were then separated by centrifugation at 20,000g. Soluble protein concentration measured and 20µg of total soluble protein was loaded from each sample onto a SDS- polyacrylamide gel. Proteins were separated by PAGE-gel and transferred to a nitrocellulose membrane. Membranes were blocked with a TBS solution containing 5% milk. Once blocked membranes were probed with either an αFlag antibody or with an αpsaA antibody and accumulation of each protein determined.

Figures

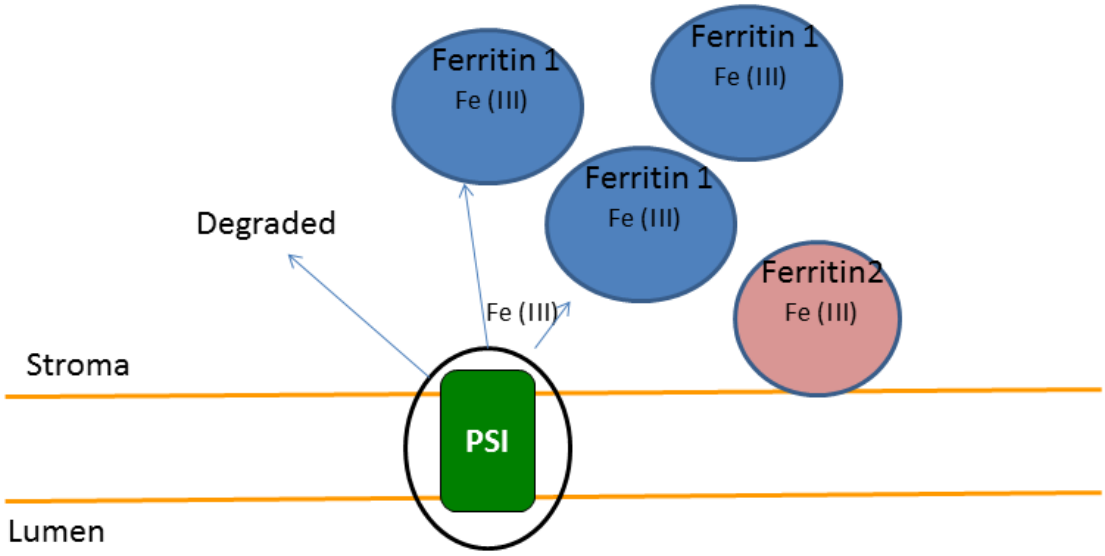


Figure 1. Depiction of low iron concentrations. In low iron concentratons, the expression of ferritin 1 is dramatically increased.Ferritin I strips photosystem I of its iron ions.

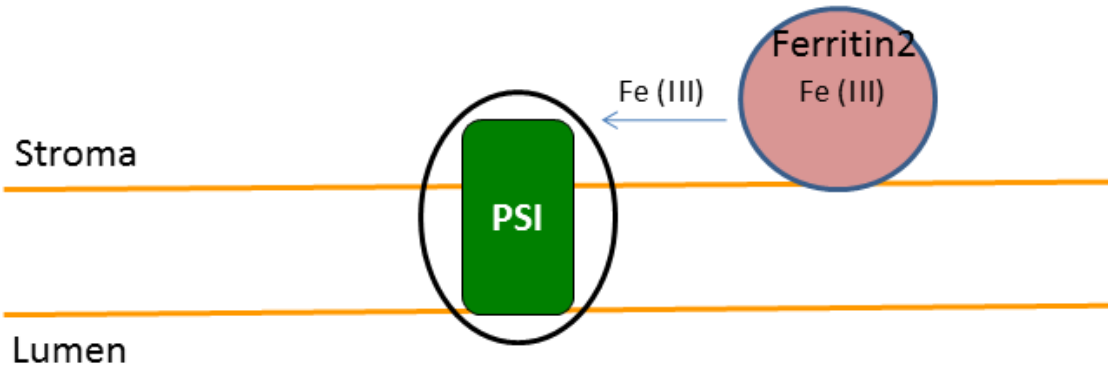


Figure 2. Depiction of high iron concentrations. In high iron concentrations ferritin 2 donates excess reduced ion to newly synthesized photosystem I

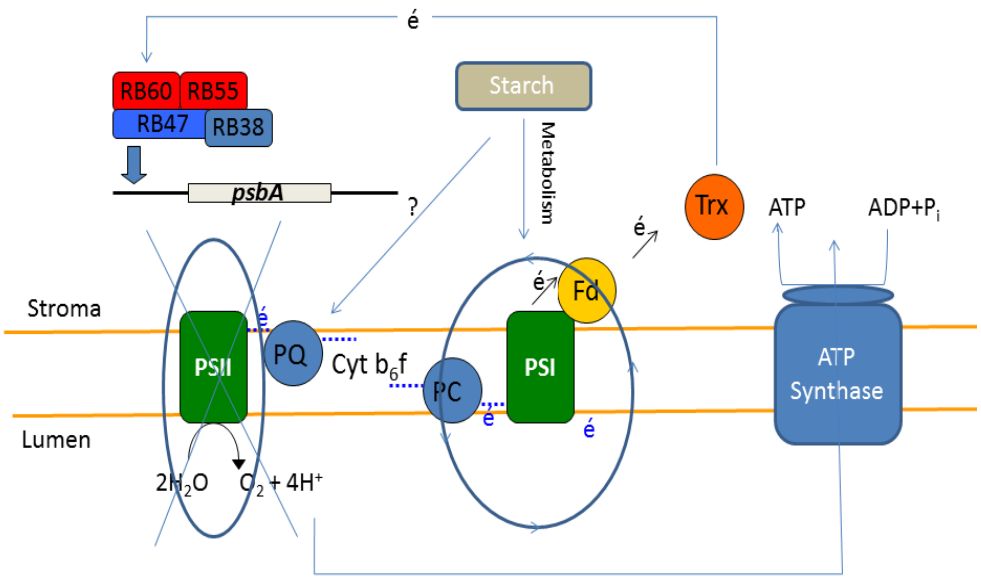
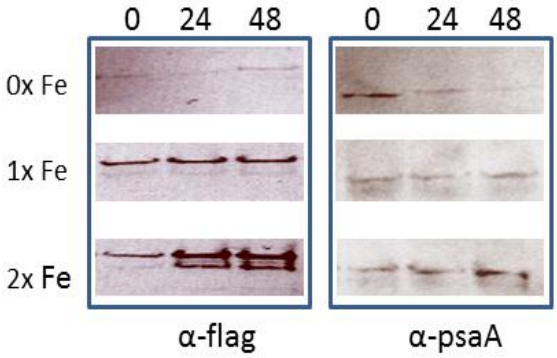


Figure 3. Schematic diagram of redox signal transduction. The redox potential generated by photosystem I reduces ferridoxin, which reduces thioredoxin, which reduces RB60, which finally reduces RB47 and causes the psbA translation initiation complex to bind to the psbA 5'-UTR. In a photosystem II knock-out reducing potential can be generated from starch metabolism



20ug of total soluble protein

	0hrs	24hrs	48hrs
0x Fe	3.7	3.7	2.8
1x Fe	7.0	26	39
2x Fe	14.5	39.6	39.5

Cell Counts (cells x 10^5/ml)

Figure 4. Effects of different iron conditions on algal cultures. Algal cultures expressing an immunotoxin protein, αCD22PE40 were treated with either no iron (0x), 18µM iron (1x), and 36µM iron. Western blot analysis was perforemed using an anti-flag or anti-psaA antibody. Rate of growth was also monitored.

Conclusion

Prior to our analysis of the biochemical protein folding environment of *C. reinhardtii* chloroplasts, there was data that suggested that a complex system of chaperones and a protein disulfide isomerase existed in the stroma (Danon and Mayfield 1991, Schroda 2004). The presence of this machinery suggested that chloroplasts could be used to fold complex mammalian proteins that contain multiple subunits held together by disulfide bonds. To determine if this machinery was capable of folding a complex mammalian protein into a native confirmation we genetically engineered strains of *C. reinhardtii* and asked if they were able to fold and assemble those proteins into functional molecules.

To determine the biochemical protein folding capabilities of algal chloroplasts, I transformed the *C. reinhardtii* plastid genome with both the heavy and light chain genes of a full length human IgG1 antibody that was made against the anthrax protective antigen 83 (PA83) . Using plasmid vectors that were previously developed (Franklin, Ngo et al. 2002, Manuell, Beligni et al. 2007), I was able to integrate both genes into different loci within the plastid genome through homologous recombination. Once these genes were stably integrated into the plastid genome I was able to show that chloroplasts could accumulate both the heavy and light chain proteins of the human IgG1. Continued biochemical studies revealed that chloroplasts were able to assemble the human IgG1 into a multimeric complex and that this complex with a fully assembled human antibody that had similar binding

characteristics as a genetically identical antibody that was expressed and purified from a Chinese hamster ovary cell expression system.

The folding of a human antibody is a complex process that requires both chaperones and a protein disulfide isomerase to ensure that the protein reaches a stable state (Mayer, Kies et al. 2000). Four separate protein subunits and 16 disulfide bonds have to be formed to ensure that the antibody can properly bind to its target antigen (Saphire, Stanfield et al. 2002). The ability of algal chloroplast to fold and assemble a full length human antibody suggest that chloroplast localized chaperones present in the stroma work synergistically with the protein disulfide isomerase, RB60, to fold complex mammalian proteins into their native confirmation. This was unexpected as RB60 had only been implicated in the redox light regulated translation of the psbA gene. Interestingly, the chloroplast localized RB60 has now been shown to co-localize to the endoplasmic reticulum, suggesting that RB60 could already play a role in folding proteins that are destined to be secreted (Levitan, Trebitsh et al. 2005). Regardless, these results demonstrate, for the first time, that chloroplasts of *C. reinhardtii* have the necessary protein folding machinery to fold and assemble a complex human antibody. This demonstrates that the biochemical protein folding contents of chloroplasts are an intriguing mix of both prokaryotic (Manuell, Quispe et al. 2007) and eukaryotic traits (Danon and Mayfield 1994, Levitan, Trebitsh et al. 2005)

To biochemically analyze the prokaryotic components of the *C. reinhardtii* chloroplast folding machinery, I attempted to fold, assemble and accumulate proteins

that contained eukaryotic toxins that would hinder only specific features of eukaryotic translation. To do this I transformed chloroplasts with genes that coded for immunotoxins. These immunotoxins are genetic fusions that contain the binding domain of a human IgG1 that is directed against CD22, a B-cell epitope present on Non-Hodgkins lymphomas (Ogura, Hatake et al. 2012), and either gelonin or exotoxin A. Gelonin is a ribosome inactivating protein originally isolated from seeds of *Gelonium multiflorum* and is known to cleave the 28S rRNA(Zamboni, Brigotti et al. 1989, Singh and Kar 1992). This toxic protein should not affect the chloroplast ribosomes since they are not comprised of any known 28s rRNA (Manuell, Quispe et al. 2007). Exotoxin A is a toxic protein from *Pseudomonas aeruginosa* and is known to ribosylate eukaryotic elongation factor 2 and prevent the elongation of polypeptide chains. Initial *in silico* studies of chloroplasts identified a plastid encoded elongation factor Tu as well as a unique elongation factor, PSRP-7. It was unclear if exotoxin A would inhibit the viability of algae cells by ribosylating PSRP-7. This was not the case and chloroplasts of *C. reinhardtii* were able to accumulate immunotoxin proteins that contained gelonin or exotoxin A. Furthermore, when these proteins were tested to determine if they were functional, both proteins remained enzymatically active. This suggests that neither the prokaryotic or eukaryotic components of protein folding in chloroplast are affected by the expression and accumulation of eukaryotic toxins. Additionally, this data suggest that not a single protein molecule escapes the chloroplasts and that protein translocation in chloroplasts is unidirectional. If a single

molecule of either gelonin or exotoxin A were able to escape the chloroplast, translation in the cytosol of *C. reinhardtii* would be completely inhibited.

To determine the biotechnological potential of *C. reinhardtii* we decided to produce these immunotoxin proteins to sufficient quantities to further study their biological properties. Immunotoxins are made to target specific cells, deliver toxin molecules to those cells, and to cause those cells to undergo apoptosis (Goldenberg and Sharkey 2012, Madhumathi and Verma 2012). It is the magic bullet theory of cancer therapy that was envisioned by Paul Ehrlich over 100 years ago (Maruta 2009). Once I produced a sufficient amount of each immunotoxin protein, I demonstrated that they were not just enzymatically active but were able to also bind specifically to their target B-cells and not non-specifically to T-cells. This demonstrates that both the carboxy and amine ends of the proteins were folded into functional molecules. It also demonstrated that chloroplasts of *C. reinhardtii* were capable of folding complex multi-domain proteins into functional molecules that remained soluble while accumulating in chloroplasts.

The production of immunotoxin proteins has previously been attempted in bacteria where the proteins were first made as insoluble aggregates and refolded into functional proteins. Once folded, it was demonstrated that immunotoxin in addition to being multi-functional, needed to be localized to the cytosol of cells to cause those cells to undergo apoptosis. Using an *in vitro* cell killing assay, we demonstrated that the algal produce immunotoxin proteins were able to kill their target B-cell lymphoma in a dose dependent manner and that they left T-cells unharmed. As a final test of the

effectiveness of algal produced immunotoxin proteins, immune deficient mice were injected subcutaneously with Ramos B-cells and tumors allowed to form. These mice were subsequently treated with algal produced immunotoxin proteins to test if these proteins were effective *in vivo*. We were able to demonstrate that mice treated with exotoxin A based immunotoxins had regressed tumors when compared to control mice. These data suggest the future viability of algae as a platform to produce therapeutically relevant immunotoxin proteins. It is important to note that no expression platform aside from algal chloroplasts has been able to accumulate a soluble enzymatically active immunotoxin protein. These proteins have either been produced as two separate molecules and later chemically linked or as insoluble aggregates inside of bacteria, suggesting a niche for the algal chloroplast protein expression platform.

By producing these complex recombinant proteins in chloroplasts we were able to gain an understanding of the intricate biochemical composition of the protein folding machinery in algal chloroplasts. In order to improve this system, we sought to understand factors that either regulate or restrict the amount of protein accumulation that can occur in chloroplasts. One of the most important components of protein quality control are proteases, which help to recycle and remove damaged or unwanted proteins in chloroplast (Janska, Kwasniak et al. 2012, Schuhmann and Adamska 2012). Since the proteins we express and accumulate in chloroplast are foreign proteins there are increasing complications with proteolysis. Other expression platforms have benefited significantly from the development of protease deficient

strains which do not degrade recombinatly expressed proteins (Kuroda, Kitagawa et al. 2007, Waegeman, De Lausnay et al. 2011, Michel and Wuthrich 2012). An effective strategy to directly target and replace nuclear encoded proteases has not been invented for *C reinhardtii* and no insertional library currently exist. Thus, a silencing technique was used to analyze which proteases played a significant role in preventing recombinant proteins from accumulating in chloroplasts. More recently, miRNAs responsible for regulating expression of nuclear genes were identified in *C. reinhardtii* (Molnar, Schwach et al. 2007). These miRNAs and their precursor messages have been developed into a useful reverse genetic tool (Molnar, Bassett et al. 2009, Zhao, Wang et al. 2009). Previously, it had only been possible to use large inverted repeats to silence *C. reinhardtii* nuclear genes (Schroda 2006), increasing the likelihood of off-site silencing. More recently, the development of artificial miRNAs has made it possible to specifically silence a gene of interest. To increase the throughput and number of miRNAs that could be targeted, we employed a USER cloning strategy (Bitinaite, Rubino et al. 2007) that allowed for the seamless construction of a large number of miRNA precursors. Using this strategy we created artificial miRNAs to target a wide range of proteases that are localized to the stroma of *C. reinhardtii* chloroplasts. The preliminary results that we have generated suggest that the use of artificial miRNA may be important in determining which proteases significantly impact protein accumulation.

Finally, the growth of algae and plants is dictated by the nutrients, both micro and macro that are available to them. Large algae blooms occur when nutrient rich

sewage is washed into the ocean and farmers add fertilizer to their soil to increase overall crop yields. There appears to be a strong correlation between growth and nutrient availability. These facts suggest that nutrients can dramatically alter metabolic pathways. The protein folding content of chloroplasts, the protein quality control apparatus, and even the redox state of a cell, can be strongly influenced by nutrient availability. Thus we sought to gain an understanding of how these micronutrients affected the accumulation of our protein of interests so that we could decipher the biochemical mechanisms that caused these variations.

Previous work has demonstrated that the translational control of the psbA gene is regulated by the redox state of the stroma (Danon and Mayfield 1991). This fluctuation is normally attributed to the onset of photosynthesis. It is known that the reducing potential that is generated by the photosystem I complex eventually causes the reduction of RB60 which activates translation of any protein under the control of the psbA 5'-UTR (Manuell, Beligni et al. 2007). It has also been demonstrated that the nutritional environment of *C. reinhardtii* cultures and the trace elements that are available could drastically affect the accumulation of different photosynthetic complexes such as photosystem I (Moseley, Allinger et al. 2002). It was hypothesized that by increasing the iron concentration in the culture medium of *C. reinhardtii* cells expressing a recombinant gene of interest, that we could drastically increase the overall accumulation of recombinant protein that was under the control of the psbA regulatory elements. Our preliminary data suggest that increasing the iron content in strains of algae deficient for psbA increases the accumulatin of photosystem I and

leads to an increase in recombinant protein accumulation. Ongoing research is being done to determine if this increase in protein accumulation is infact a result of increased reducing potential this is generated from a increasing titers of the photosystem I complex.

My research has centered on the basic understanding of the protein folding machinery in chloroplasts of *C. reinhardtii* and an analysis of how they work together to facilitate the folding of complex proteins. Our data suggest that RB60, a protein disulfide isomerase that was implicated in the redox regulated control of psbA translation, is also capable of functioning as a putative disulfide isomerase by catalyzing the formation of intra- ad inter- disulfide bonds in our plastid expressed recombinant proteins. Additionally we were able to demonstrate the chloroplasts are a unique environment that contain both prokaryotic and eukaryotic like protein folding components that allow us to generate unique classes of cancer therapies. Continued research will need to be done to elucidate the relationship between all the components of the plastid that are involved in protein folding so that we may better define how proteins are folded and accumulate in chloroplasts and what factors inhibit that process.

References

Agarwal, P. and V. Bafna (1998). "The ribosome scanning model for translation initiation: implications for gene prediction and full-length cDNA detection." Proc Int Conf Intell Syst Mol Biol **6**: 2-7.

Alergand, T., H. Peled-Zehavi, Y. Katz and A. Danon (2006). "The chloroplast protein disulfide isomerase RB60 reacts with a regulatory disulfide of the RNA-binding protein RB47." Plant Cell Physiol **47**(4): 540-548.

Allen, J. F., W. B. de Paula, S. Puthiyaveetil and J. Nield (2011). "A structural phylogenetic map for chloroplast photosynthesis." Trends Plant Sci **16**(12): 645-655.

Allen, M. D., J. A. del Campo, J. Kropat and S. S. Merchant (2007). "FEA1, FEA2, and FRE1, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with FOX1 and FTR1 in iron-deficient Chlamydomonas reinhardtii." Eukaryot Cell **6**(10): 1841-1852.

Allen, M. D., J. Kropat, S. Tottey, J. A. Del Campo and S. S. Merchant (2007). "Manganese deficiency in Chlamydomonas results in loss of photosystem II and MnSOD function, sensitivity to peroxides, and secondary phosphorus and iron deficiency." Plant Physiol **143**(1): 263-277.

Alley, S. C., N. M. Okeley and P. D. Senter (2010). "Antibody-drug conjugates: targeted drug delivery for cancer." Curr Opin Chem Biol **14**(4): 529-537.

Alric, J., J. Lavergne and F. Rappaport (2010). "Redox and ATP control of photosynthetic cyclic electron flow in Chlamydomonas reinhardtii (I) aerobic conditions." Biochim Biophys Acta **1797**(1): 44-51.

Andres, C., B. Agne and F. Kessler (2010). "The TOC complex: preprotein gateway to the chloroplast." Biochim Biophys Acta **1803**(6): 715-723.

Armache, J. P., A. Jarasch, A. M. Anger, E. Villa, T. Becker, S. Bhushan, F. Jossinet, M. Habeck, G. Dindar, S. Franckenberg, V. Marquez, T. Mielke, M. Thomm, O. Berninghausen, B. Beatrix, J. Soding, E. Westhof, D. N. Wilson and R. Beckmann (2010). "Localization of eukaryote-specific ribosomal proteins in a 5.5-A cryo-EM map of the 80S eukaryotic ribosome." Proc Natl Acad Sci U S A **107**(46): 19754-19759.

Baecker, J. J., J. C. Sneddon and M. J. Hollingsworth (2009). "Efficient translation in chloroplasts requires element(s) upstream of the putative ribosome binding site from atpI." Am J Bot **96**(3): 627-636

Baldauf, S. L. and J. D. Palmer (1990). "Evolutionary transfer of the chloroplast tufA gene to the nucleus." Nature **344**(6263): 262-265.

Baranek, M., M. Grabsztunowicz, B. Sikora and G. Jackowski (2011). "[ATP-dependent FtsH and Lon chloroplast proteases]." Postepy Biochem **57**(1): 101-108.

Barnes, D., A. Cohen, R. K. Bruick, K. Kantardjieff, S. Fowler, E. Efuet and S. P. Mayfield (2004). "Identification and characterization of a novel RNA binding protein that associates with the 5'-untranslated region of the chloroplast psbA mRNA." Biochemistry **43**(26): 8541-8550.

Barnes, D., S. Franklin, J. Schultz, R. Henry, E. Brown, A. Coragliotti and S. P. Mayfield (2005). "Contribution of 5'- and 3'-untranslated regions of plastid mRNAs to the expression of Chlamydomonas reinhardtii chloroplast genes." Mol Genet Genomics **274**(6): 625-636.

Barnes, D. and S. P. Mayfield (2003). "Redox control of posttranscriptional processes in the chloroplast." Antioxid Redox Signal **5**(1): 89-94.

Barral, J. M., S. A. Broadley, G. Schaffar and F. U. Hartl (2004). "Roles of molecular chaperones in protein misfolding diseases." Semin Cell Dev Biol **15**(1): 17-29.

Beck, A., J. F. Haeuw, T. Wurch, L. Goetsch, C. Bailly and N. Corvaia (2010). "The next generation of antibody-drug conjugates comes of age." Discov Med **10**(53): 329-339.

Bedard, J. and P. Jarvis (2005). "Recognition and envelope translocation of chloroplast preproteins." J Exp Bot **56**(419): 2287-2320.

Bedoshvili, E. D., T. P. Popkova and E. V. Likhoshvai (2009). "[Chloroplast ultrastructure in some diatoms from different classes]." Tsitologiia **51**(4): 346-357.

Beligni, M. V., K. Yamaguchi and S. P. Mayfield (2004). "Chloroplast elongation factor ts pro-protein is an evolutionarily conserved fusion with the s1 domain-containing plastid-specific ribosomal protein-7." Plant Cell **16**(12): 3357-3369.

Bera, T. K., J. Williams-Gould, R. Beers, P. Chowdhury and I. Pastan (2001). "Bivalent disulfide-stabilized fragment variable immunotoxin directed against mesotheliomas and ovarian cancer." Mol Cancer Ther **1**(2): 79-84.

Bertholjotti, I. (2011). "[Antibody-drug conjugate--a new age for personalized cancer treatment]." Chimia (Aarau) **65**(9): 746-748.

Bertsch, U., J. Soll, R. Seetharam and P. V. Viitanen (1992). "Identification, characterization, and DNA sequence of a functional "double" groES-like chaperonin from chloroplasts of higher plants." Proc Natl Acad Sci U S A **89**(18): 8696-8700.

Bitinaite, J., M. Rubino, K. H. Varma, I. Schildkraut, R. Vaisvila and R. Vaiskunaite (2007). "USER friendly DNA engineering and cloning method by uracil excision." Nucleic Acids Res **35**(6): 1992-2002.

Blaby-Haas, C. E. and S. S. Merchant (2012). "The ins and outs of algal metal transport." Biochim Biophys Acta **1823**(9): 1531-1552.

Blobel, G., P. Walter, C. N. Chang, B. M. Goldman, A. H. Erickson and V. R. Lingappa (1979). "Translocation of proteins across membranes: the signal hypothesis and beyond." Symp Soc Exp Biol **33**: 9-36.

Bloom, J. W., M. S. Madanat, D. Marriott, T. Wong and S. Y. Chan (1997). "Intrachain disulfide bond in the core hinge region of human IgG4." Protein Sci **6**(2): 407-415.

Bogner, C., T. Dechow, I. Ringshausen, M. Wagner, M. Oelsner, G. Lutzny, T. Licht, C. Peschel, I. Pastan, R. J. Kreitman and T. Decker (2010). "Immunotoxin BL22 induces apoptosis in mantle cell lymphoma (MCL) cells dependent on Bcl-2 expression." Br J Haematol **148**(1): 99-109.

Breiman, A., T. W. Fawcett, M. L. Ghirardi and A. K. Mattoo (1992). "Plant organelles contain distinct peptidylprolyl cis,trans-isomerases." J Biol Chem **267**(30): 21293-21296.

Briggs, W. R., C. F. Beck, A. R. Cashmore, J. M. Christie, J. Hughes, J. A. Jarillo, T. Kagawa, H. Kanegae, E. Liscum, A. Nagatani, K. Okada, M. Salomon, W. Rudiger, T. Sakai, M. Takano, M. Wada and J. C. Watson (2001). "The phototropin family of photoreceptors." Plant Cell **13**(5): 993-997.

Brinkmann, U., Y. Reiter, S. H. Jung, B. Lee and I. Pastan (1993). "A recombinant immunotoxin containing a disulfide-stabilized Fv fragment." Proc Natl Acad Sci U S A **90**(16): 7538-7542.

Bruce, B. D. (2000). "Chloroplast transit peptides: structure, function and evolution." Trends Cell Biol **10**(10): 440-447.

Busch, A., B. Rimbauld, B. Naumann, S. Rensch and M. Hippler (2008). "Ferritin is required for rapid remodeling of the photosynthetic apparatus and minimizes photo-oxidative stress in response to iron availability in Chlamydomonas reinhardtii." Plant J **55**(2): 201-211.

Cao, Y., J. D. Marks, Q. Huang, S. I. Rudnick, C. Xiong, W. N. Hittelman, X. Wen, J. W. Marks, L. H. Cheung, K. Boland, C. Li, G. P. Adams and M. G. Rosenblum (2012). "Single-chain antibody-based immunotoxins targeting Her2/neu: design optimization and impact of affinity on antitumor efficacy and off-target toxicity." Mol Cancer Ther **11**(1): 143-153.

Casadevall, A. (2002). "Antibodies for defense against biological attack." Nat Biotechnol **20**(2): 114.

Casadevall, A., E. Dadachova and L. A. Pirofski (2004). "Passive antibody therapy for infectious diseases." Nat Rev Microbiol **2**(9): 695-703.

Chaudhary, V. K., C. Queen, R. P. Junghans, T. A. Waldmann, D. J. FitzGerald and I. Pastan (1989). "A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin." Nature **339**(6223): 394-397.

Chen, X. and D. J. Schnell (1999). "Protein import into chloroplasts." Trends Cell Biol **9**(6): 222-227.

Cloney, L. P., D. R. Bekkaoui, M. G. Wood and S. M. Hemmingsen (1992). "Assessment of plant chaperonin-60 gene function in Escherichia coli." J Biol Chem **267**(32): 23333-23336.

Cohen, A., C. B. Yohn, B. R. K. and S. P. Mayfield (1998). Methods in Enzymology

Constan, D., J. E. Froehlich, S. Rangarajan and K. Keegstra (2004). "A stromal Hsp100 protein is required for normal chloroplast development and function in Arabidopsis." Plant Physiol **136**(3): 3605-3615.

Cutting, W. C. and L. P. Gebhardt (1941). "Inhibitory Effects of Sulfonamides on Cultures of Actinomyces Hominis." Science **94**(2450): 568-569.

Danon, A. (1997). "Translational regulation in the chloroplast." Plant Physiol **115**(4): 1293-1298.

Danon, A. and S. P. Mayfield (1991). "Light regulated translational activators: identification of chloroplast gene specific mRNA binding proteins." EMBO J **10**(13): 3993-4001.

Danon, A. and S. P. Mayfield (1994). "Light-regulated translation of chloroplast messenger RNAs through redox potential." Science **266**(5191): 1717-1719.

Denoncin, K., D. Vertommen, E. Paek and J. F. Collet (2010). "The protein-disulfide isomerase DsbC cooperates with SurA and DsbA in the assembly of the essential beta-barrel protein LptD." J Biol Chem **285**(38): 29425-29433.

Dietz, K. J. and T. Pfannschmidt (2011). "Novel regulators in photosynthetic redox control of plant metabolism and gene expression." Plant Physiol **155**(4): 1477-1485.

Dittmar, K. D., M. Banach, M. D. Galigniana and W. B. Pratt (1998). "The role of DnaJ-like proteins in glucocorticoid receptor.hsp90 heterocomplex assembly by the reconstituted hsp90.p60.hsp70 foldosome complex." J Biol Chem **273**(13): 7358-7366.

Dove, A. (2002). "Uncorking the biomanufacturing bottleneck." Nat Biotechnol **20**(8): 777-779.

Drapier, D., J. Girard-Bascou, D. B. Stern and F. A. Wollman (2002). "A dominant nuclear mutation in Chlamydomonas identifies a factor controlling chloroplast mRNA stability by acting on the coding region of the atpA transcript." Plant J **31**(6): 687-697.

Du, X., R. Beers, D. J. Fitzgerald and I. Pastan (2008). "Differential cellular internalization of anti-CD19 and -CD22 immunotoxins results in different cytotoxic activity." Cancer Res **68**(15): 6300-6305.

Emelyanov, V. V. (2002). "Phylogenetic relationships of organellar Hsp90 homologs reveal fundamental differences to organellar Hsp70 and Hsp60 evolution." Gene **299**(1-2): 125-133.

Eriksson, M., J. L. Moseley, S. Tottey, J. A. Del Campo, J. Quinn, Y. Kim and S. Merchant (2004). "Genetic dissection of nutritional copper signaling in chlamydomonas distinguishes regulatory and target genes." Genetics **168**(2): 795-807.

Erntell, M., E. B. Myhre, U. Sjobring and L. Bjorck (1988). "Streptococcal protein G has affinity for both Fab- and Fc-fragments of human IgG." Mol Immunol **25**(2): 121-126.

Flores-Perez, U. and P. Jarvis (2012). "Molecular chaperone involvement in chloroplast protein import." Biochim Biophys Acta.

Frand, A. R., J. W. Cuozzo and C. A. Kaiser (2000). "Pathways for protein disulphide bond formation." Trends Cell Biol **10**(5): 203-210.

Frankel, A. D., Ed. (1992). Genetically engineered Toxins New York, Mercel Dekker Inc.

Franklin, S., B. Ngo, E. Efuet and S. P. Mayfield (2002). "Development of a GFP reporter gene for Chlamydomonas reinhardtii chloroplast." Plant J **30**(6): 733-744.

Franklin, S. E. and S. P. Mayfield (2004). "Prospects for molecular farming in the green alga Chlamydomonas." Curr Opin Plant Biol **7**(2): 159-165.

Freeman, B. C. and R. I. Morimoto (1996). "The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding." EMBO J **15**(12): 2969-2979.

Giddings, G., G. Allison, D. Brooks and A. Carter (2000). "Transgenic plants as factories for biopharmaceuticals." Nat Biotechnol **18**(11): 1151-1155.

Gillham, N. W., J. E. Boynton and C. R. Hauser (1994). "Translational regulation of gene expression in chloroplasts and mitochondria." Annu Rev Genet **28**: 71-93.

Goldenberg, D. M. and R. M. Sharkey (2012). "Using antibodies to target cancer therapeutics." Expert Opin Biol Ther **12**(9): 1173-1190.

Goldmacher, V. S., C. F. Scott, J. M. Lambert, G. D. McIntyre, W. A. Blattler, A. R. Collnhson, J. K. Stewart, L. D. Chong, S. Cook, H. S. Slayter and et al. (1989). "Cytotoxicity of gelonin and its conjugates with antibodies is determined by the extent of their endocytosis." J Cell Physiol **141**(1): 222-234.

Gonzalez-Ballester, D., D. Casero, S. Cokus, M. Pellegrini, S. S. Merchant and A. R. Grossman (2010). "RNA-seq analysis of sulfur-deprived Chlamydomonas cells reveals aspects of acclimation critical for cell survival." Plant Cell **22**(6): 2058-2084.

Gordon, V. M., K. R. Klimpel, N. Arora, M. A. Henderson and S. H. Leppla (1995). "Proteolytic activation of bacterial toxins by eukaryotic cells is performed by furin and by additional cellular proteases." Infect Immun **63**(1): 82-87.

Gorman, D. S. and R. P. Levine (1965). "Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of Chlamydomonas reinhardi." Proc Natl Acad Sci U S A **54**(6): 1665-1669.

Goulas, E., M. Schubert, T. Kieselbach, L. A. Kleczkowski, P. Gardestrom, W. Schroder and V. Hurry (2006). "The chloroplast lumen and stromal proteomes of Arabidopsis thaliana show differential sensitivity to short- and long-term exposure to low temperature." Plant J **47**(5): 720-734.

Grabsztunowicz, M., R. Lucinski, M. Baranek, B. Sikora and G. Jackowski (2011). "[Chloroplast Deg proteases]." Postepy Biochem **57**(1): 109-114.

Gregory, J. A., F. Li, L. M. Tomosada, C. J. Cox, A. B. Topol, J. M. Vinetz and S. Mayfield (2012). "Algae-produced Pfs25 elicits antibodies that inhibit malaria transmission." PLoS One **7**(5): e37179.

Grodberg, J. and J. J. Dunn (1988). "ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification." J Bacteriol **170**(3): 1245-1253.

Haacke, A., G. Fendrich, P. Ramage and M. Geiser (2009). "Chaperone over-expression in Escherichia coli: apparent increased yields of soluble recombinant protein kinases are due mainly to soluble aggregates." Protein Expr Purif **64**(2): 185-193.

Hannon, M., J. Gimpel, M. Tran, B. Rasala and S. Mayfield (2010). "Biofuels from algae: challenges and potential." Biofuels **1**(5): 763-784.

Harris, E. H. (2001). "Chlamydomonas as a Model Organism." Annu Rev Plant Physiol Plant Mol Biol **52**: 363-406.

Harris, E. H. (2009). Chlamydomonas Sourcebook Introduction to Chlamydomonas and its laboratory Uses New York Academic Press Inc

Hartl, F. U., A. Bracher and M. Hayer-Hartl (2011). "Molecular chaperones in protein folding and proteostasis." Nature **475**(7356): 324-332.

Haussuhl, K., B. Andersson and I. Adamska (2001). "A chloroplast DegP2 protease performs the primary cleavage of the photodamaged D1 protein in plant photosystem II." EMBO J **20**(4): 713-722.

Hebert, D. N. and L. M. Gierasch (2009). "The molecular dating game: an antibody heavy chain hangs loose with a chaperone while waiting for its life partner." Mol Cell **34**(6): 635-636.

Heifetz, P. B. and A. M. Tuttle (2001). "Protein expression in plastids." Curr Opin Plant Biol **4**(2): 157-161.

Heldt, H. W. and F. Sauer (1971). "The inner membrane of the chloroplast envelope as the site of specific metabolite transport." Biochim Biophys Acta **234**(1): 83-91.

Hemmingsen, E. A. (1965). "Accelerated Transfer of Oxygen through Solutions of Heme Pigments." Acta Physiol Scand Suppl: SUPPL 246:241-253.

Hiatt, A., R. Cafferkey and K. Bowdish (1989). "Production of antibodies in transgenic plants." Nature **342**(6245): 76-78.

Hirose, T., T. Kusumegi and M. Sugiura (1998). "Translation of tobacco chloroplast rps14 mRNA depends on a Shine-Dalgarno-like sequence in the 5'-untranslated region but not on internal RNA editing in the coding region." FEBS Lett **430**(3): 257-260.

Hirose, T. and M. Sugiura (2004). "Functional Shine-Dalgarno-like sequences for translational initiation of chloroplast mRNAs." Plant Cell Physiol **45**(1): 114-117.

Inagaki, J., Y. Fujita, T. Hase and Y. Yamamoto (2000). "Protein translocation within chloroplast is similar in Euglena and higher plants." Biochem Biophys Res Commun **277**(2): 436-442.

Jackson-Constan, D., M. Akita and K. Keegstra (2001). "Molecular chaperones involved in chloroplast protein import." Biochim Biophys Acta **1541**(1-2): 102-113.

Janska, H., M. Kwasniak and J. Szczepanowska (2012). "Protein quality control in organelles - AAA/FtsH story." Biochim Biophys Acta.

Jarvis, P. (2008). "Targeting of nucleus-encoded proteins to chloroplasts in plants." New Phytol **179**(2): 257-285.

Johnson, J. L. (2012). "Evolution and function of diverse Hsp90 homologs and cochaperone proteins." Biochim Biophys Acta **1823**(3): 607-613.

Johnson, X. and J. Alric (2012). "Interaction between starch breakdown, acetate assimilation, and photosynthetic cyclic electron flow in Chlamydomonas reinhardtii." J Biol Chem **287**(31): 26445-26452.

Jones, C. S., T. Luong, M. Hannon, M. Tran, J. A. Gregory, Z. Shen, S. P. Briggs and S. P. Mayfield (2012). "Heterologous expression of the C-terminal antigenic domain of the malaria vaccine candidate Pfs48/45 in the green algae Chlamydomonas reinhardtii." Appl Microbiol Biotechnol.

Jurgens, G. (2004). "Membrane trafficking in plants." Annu Rev Cell Dev Biol **20**: 481-504.

Kapri-Pardes, E., L. Naveh and Z. Adam (2007). "The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in Arabidopsis." Plant Cell **19**(3): 1039-1047.

Kawa, S., M. Onda, M. Ho, R. J. Kreitman, T. K. Bera and I. Pastan (2011). "The improvement of an anti-CD22 immunotoxin: conversion to single-chain and disulfide stabilized form and affinity maturation by alanine scan." MAbs **3**(5): 479-486.

Keegstra, K. and K. Cline (1999). "Protein import and routing systems of chloroplasts." Plant Cell **11**(4): 557-570.

Kim, J. and S. P. Mayfield (1997). "Protein disulfide isomerase as a regulator of chloroplast translational activation." Science **278**(5345): 1954-1957.

Kim, J. Y., Y. G. Kim and G. M. Lee (2012). "CHO cells in biotechnology for production of recombinant proteins: current state and further potential." Appl Microbiol Biotechnol **93**(3): 917-930.

Klein, D. J., P. B. Moore and T. A. Steitz (2004). "The roles of ribosomal proteins in the structure assembly, and evolution of the large ribosomal subunit." J Mol Biol **340**(1): 141-177.

Ko, K. and H. Koprowski (2005). "Plant biopharming of monoclonal antibodies." Virus Res **111**(1): 93-100.

Kondo, T., D. FitzGerald, V. K. Chaudhary, S. Adhya and I. Pastan (1988). "Activity of immunotoxins constructed with modified Pseudomonas exotoxin A lacking the cell recognition domain." J Biol Chem **263**(19): 9470-9475.

Kontermann, R., Dubel, S., Ed. (2010). Expression of Full Length Monoclonal Antibodies (mAb) in Algal Chloroplast. Antibody Engineering New York, Springer Lab Manuals

Koumoto, Y., T. Shimada, M. Kondo, I. Hara-Nishimura and M. Nishimura (2001). "Chloroplasts have a novel Cpn10 in addition to Cpn20 as co-chaperonins in Arabidopsis thaliana." J Biol Chem **276**(32): 29688-29694.

Koumoto, Y., T. Shimada, M. Kondo, T. Takao, Y. Shimonishi, I. Hara-Nishimura and M. Nishimura (1999). "Chloroplast Cpn20 forms a tetrameric structure in Arabidopsis thaliana." Plant J **17**(5): 467-477.

Kovacs-Bogdan, E., J. Soll and B. Bolter (2010). "Protein import into chloroplasts: the Tic complex and its regulation." Biochim Biophys Acta **1803**(6): 740-747.

Krakauer, T., S. F. Little and B. G. Stiles (2005). "Bacillus anthracis edema toxin inhibits Staphylococcus aureus enterotoxin B effects in vitro: a potential protein therapeutic?" Infect Immun **73**(10): 7069-7073.

Kreitman, R. J. and I. Pastan (1995). "Importance of the glutamate residue of KDEL in increasing the cytotoxicity of Pseudomonas exotoxin derivatives and for increased binding to the KDEL receptor." Biochem J **307 (Pt 1)**: 29-37.

Kreitman, R. J., Q. C. Wang, D. J. FitzGerald and I. Pastan (1999). "Complete regression of human B-cell lymphoma xenografts in mice treated with recombinant anti-CD22 immunotoxin RFB4(dsFv)-PE38 at doses tolerated by cynomolgus monkeys." Int J Cancer **81**(1): 148-155.

Kulczycka, K., M. Dlugosz and J. Trylska (2011). "Molecular dynamics of ribosomal elongation factors G and Tu." Eur Biophys J **40**(3): 289-303.

Kuroda, K., Y. Kitagawa, K. Kobayashi, H. Tsumura, T. Komeda, E. Mori, K. Motoki, S. Kataoka, Y. Chiba and Y. Jigami (2007). "Antibody expression in protease-deficient strains of the methylotrophic yeast Ogataea minuta." FEMS Yeast Res **7**(8): 1307-1316.

Kutschera, U. and K. J. Niklas (2005). "Endosymbiosis, cell evolution, and speciation." Theory Biosci **124**(1): 1-24.

Lash, A. (2010). "Make the case for antibody-drug conjugates " Pharmaceuticals **28**(11): 32-38.

Leidhold, C. and W. Voos (2007). "Chaperones and proteases--guardians of protein integrity in eukaryotic organelles." Ann N Y Acad Sci **1113**: 72-86.

Levitan, A., T. Trebitsh, V. Kiss, Y. Pereg, I. Dangoor and A. Danon (2005). "Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum." Proc Natl Acad Sci U S A **102**(17): 6225-6230.

Li, C. L., Y. C. Zhao, X. Y. Song, X. X. Huang and W. D. Zhao (2012). "Molecular Cloning, Expression and Characterization of the Porcine beta Defensin 2 in E. coli." Protein Pept Lett.

Li, H. M. and C. C. Chiu (2010). "Protein transport into chloroplasts." Annu Rev Plant Biol **61**: 157-180.

Lindahl, M., S. Tabak, L. Cseke, E. Pichersky, B. Andersson and Z. Adam (1996). "Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants." J Biol Chem **271**(46): 29329-29334.

Little, S. F., S. H. Leppla and E. Cora (1988). "Production and characterization of monoclonal antibodies to the protective antigen component of Bacillus anthracis toxin." Infect Immun **56**(7): 1807-1813.

Little, S. F., S. H. Leppla and A. M. Friedlander (1990). "Production and characterization of monoclonal antibodies against the lethal factor component of Bacillus anthracis lethal toxin." Infect Immun **58**(6): 1606-1613.

Liu, X. Y., L. M. Pop, J. Schindler and E. S. Vitetta (2012). "Immunotoxins constructed with chimeric, short-lived anti-CD22 monoclonal antibodies induce less vascular leak without loss of cytotoxicity." MAbs **4**(1): 57-68.

Long, J. C., F. Sommer, M. D. Allen, S. F. Lu and S. S. Merchant (2008). "FER1 and FER2 encoding two ferritin complexes in Chlamydomonas reinhardtii chloroplasts are regulated by iron." Genetics **179**(1): 137-147.

Lucinski, R., L. Misztal, S. Samardakiewicz and G. Jackowski (2011). "The thylakoid protease Deg2 is involved in stress-related degradation of the photosystem II light-harvesting protein Lhcb6 in Arabidopsis thaliana." New Phytol **192**(1): 74-86.

Ma, J. K., A. Hiatt, M. Hein, N. D. Vine, F. Wang, P. Stabila, C. van Dolleweerd, K. Mostov and T. Lehner (1995). "Generation and assembly of secretory antibodies in plants." Science **268**(5211): 716-719.

Ma, Y., A. Kouranov, S. E. LaSala and D. J. Schnell (1996). "Two components of the chloroplast protein import apparatus, IAP86 and IAP75, interact with the transit sequence during the recognition and translocation of precursor proteins at the outer envelope." J Cell Biol **134**(2): 315-327.

Madhumathi, J. and R. S. Verma (2012). "Therapeutic targets and recent advances in protein immunotoxins." Curr Opin Microbiol **15**(3): 300-309.

Majeran, W., F. A. Wollman and O. Vallon (2000). "Evidence for a role of ClpP in the degradation of the chloroplast cytochrome b(6)f complex." Plant Cell **12**(1): 137-150.

Malnoe, P., S. P. Mayfield and J. D. Rochaix (1988). "Comparative analysis of the biogenesis of photosystem II in the wild-type and Y-1 mutant of Chlamydomonas reinhardtii." J Cell Biol **106**(3): 609-616.

Mansfield, E., P. Amlot, I. Pastan and D. J. FitzGerald (1997). "Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors." Blood **90**(5): 2020-2026.

Mansfield, E., M. F. Chiron, P. Amlot, I. Pastan and D. J. FitzGerald (1997). "Recombinant RFB4 single-chain immunotoxin that is cytotoxic towards CD22-positive cells." Biochem Soc Trans **25**(2): 709-714.

Mansfield, E., I. Pastan and D. J. FitzGerald (1996). "Characterization of RFB4-Pseudomonas exotoxin A immunotoxins targeted to CD22 on B-cell malignancies." Bioconjug Chem **7**(5): 557-563.

Manuell, A. L., M. V. Beligni, J. H. Elder, D. T. Siefker, M. Tran, A. Weber, T. L. McDonald and S. P. Mayfield (2007). "Robust expression of a bioactive mammalian protein in Chlamydomonas chloroplast." Plant Biotechnol J **5**(3): 402-412.

Manuell, A. L., J. Quispe and S. P. Mayfield (2007). "Structure of the chloroplast ribosome: novel domains for translation regulation." PLoS Biol **5**(8): e209.

Marasco, W. A. and J. Sui (2007). "The growth and potential of human antiviral monoclonal antibody therapeutics." Nat Biotechnol **25**(12): 1421-1434.

Marin-Navarro, J., A. L. Manuell, J. Wu and P. M. S (2007). "Chloroplast translation regulation." Photosynth Res **94**(2-3): 359-374.

Marques, J. P., I. Dudeck and R. B. Klosgen (2003). "Targeting of EGFP chimeras within chloroplasts." Mol Genet Genomics **269**(3): 381-387.

Maruta, H. (2009). "From chemotherapy to signal therapy (1909-2009): A century pioneered by Paul Ehrlich." Drug Discov Ther **3**(2): 37-40.

Maul, J. E., J. W. Lilly, L. Cui, C. W. dePamphilis, W. Miller, E. H. Harris and D. B. Stern (2002). "The Chlamydomonas reinhardtii plastid chromosome: islands of genes in a sea of repeats." Plant Cell **14**(11): 2659-2679.

Mayer, M., U. Kies, R. Kammermeier and J. Buchner (2000). "BiP and PDI cooperate in the oxidative folding of antibodies in vitro." J Biol Chem **275**(38): 29421-29425.

Mayer, M. P. and B. Bukau (2005). "Hsp70 chaperones: cellular functions and molecular mechanism." Cell Mol Life Sci **62**(6): 670-684.

Mayfield, S. P., S. E. Franklin and R. A. Lerner (2003). "Expression and assembly of a fully active antibody in algae." Proc Natl Acad Sci U S A **100**(2): 438-442.

Mayfield, S. P. and J. Schultz (2004). "Development of a luciferase reporter gene, luxCt, for Chlamydomonas reinhardtii chloroplast." Plant J **37**(3): 449-458.

McAuley, A., J. Jacob, C. G. Kolvenbach, K. Westland, H. J. Lee, S. R. Brych, D. Rehder, G. R. Kleemann, D. N. Brems and M. Matsumura (2008). "Contributions of a disulfide bond to the structure, stability, and dimerization of human IgG1 antibody CH3 domain." Protein Sci **17**(1): 95-106.

McClellan, A. J., M. D. Scott and J. Frydman (2005). "Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways." Cell **121**(5): 739-748.

Melchior, W. B., Jr. and W. H. Tolleson (2010). "A functional quantitative polymerase chain reaction assay for ricin, Shiga toxin, and related ribosome-inactivating proteins." Anal Biochem **396**(2): 204-211.

Michel, E. and K. Wuthrich (2012). "High-yield Escherichia coli-based cell-free expression of human proteins." J Biomol NMR **53**(1): 43-51.

Minich, S. S. (2012). "Brentuximab vedotin: a new age in the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma." Ann Pharmacother **46**(3): 377-383.

Molnar, A., A. Bassett, E. Thuenemann, F. Schwach, S. Karkare, S. Ossowski, D. Weigel and D. Baulcombe (2009). "Highly specific gene silencing by artificial microRNAs in the unicellular alga Chlamydomonas reinhardtii." Plant J **58**(1): 165-174.

Molnar, A., F. Schwach, D. J. Studholme, E. C. Thuenemann and D. C. Baulcombe (2007). "miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii." Nature **447**(7148): 1126-1129.

Morrissey, J. and C. Bowler (2012). "Iron utilization in marine cyanobacteria and eukaryotic algae." Front Microbiol **3**: 43.

Moseley, J. L., T. Allinger, S. Herzog, P. Hoerth, E. Wehinger, S. Merchant and M. Hippler (2002). "Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus." EMBO J **21**(24): 6709-6720.

Mourez, M. (2004). "Anthrax toxins." Rev Physiol Biochem Pharmacol **152**: 135-164.

Munro, S. and H. R. Pelham (1986). "An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein." Cell **46**(2): 291-300.

Murakami, S., Y. Kondo, T. Nakano and F. Sato (2000). "Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells." FEBS Lett **468**(1): 15-18.

Nicolas, E., J. M. Beggs, B. M. Haltiwanger and T. F. Taraschi (1997). "Direct evidence for the deoxyribonuclease activity of the plant ribosome inactivating protein gelonin." FEBS Lett **406**(1-2): 162-164.

Nour-Eldin, H. H., F. Geu-Flores and B. A. Halkier (2010). "USER cloning and USER fusion: the ideal cloning techniques for small and big laboratories." Methods Mol Biol **643**: 185-200.

O'Brien, T. W. (2003). "Properties of human mitochondrial ribosomes." IUBMB Life **55**(9): 505-513.

Ogura, M., K. Hatake, K. Ando, K. Tobinai, K. Tokushige, C. Ono, T. Ishibashi and E. Vandendries (2012). "Phase I study of anti-CD22 immunoconjugate inotuzumab ozogamicin plus rituximab in relapsed/refractory B-cell non-Hodgkin lymphoma." Cancer Sci **103**(5): 933-938.

Oppermann, T., T. H. Hong and S. J. Surzycki (1989). "Chloroplast and nuclear genomes of Chlamydomonas reinhardtii share homology with Escherichia coli genes for DNA replication, repair and transcription." Curr Genet **15**(1): 39-46.

Ostersetzer, O., Y. Kato, Z. Adam and W. Sakamoto (2007). "Multiple intracellular locations of Lon protease in Arabidopsis: evidence for the localization of AtLon4 to chloroplasts." Plant Cell Physiol **48**(6): 881-885.

Outchkourov, N. S., W. Roeffen, A. Kaan, J. Jansen, A. Luty, D. Schuiffel, G. J. van Gemert, M. van de Vegte-Bolmer, R. W. Sauerwein and H. G. Stunnenberg (2008). "Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice." Proc Natl Acad Sci U S A **105**(11): 4301-4305.

Page, M. D., M. D. Allen, J. Kropat, E. I. Urzica, S. J. Karpowicz, S. I. Hsieh, J. A. Loo and S. S. Merchant (2012). "Fe Sparing and Fe Recycling Contribute to Increased Superoxide Dismutase Capacity in Iron-Starved Chlamydomonas reinhardtii." Plant Cell.

Pape, M., C. Lambertz, T. Happe and A. Hemschemeier (2012). "Differential expression of the Chlamydomonas [FeFe]-hydrogenase-encoding HYDA1 gene is regulated by the copper response regulator1." Plant Physiol **159**(4): 1700-1712.

Pastan, I., R. Hassan, D. J. FitzGerald and R. J. Kreitman (2007). "Immunotoxin treatment of cancer." Annu Rev Med **58**: 221-237.

Peng, L., Y. Fukao, F. Myouga, R. Motohashi, K. Shinozaki and T. Shikanai (2011). "A chaperonin subunit with unique structures is essential for folding of a specific substrate." PLoS Biol **9**(4): e1001040.

Perry, S. E. and K. Keegstra (1994). "Envelope membrane proteins that interact with chloroplastic precursor proteins." Plant Cell **6**(1): 93-105.

Petti, A. A., R. S. McIsaac, O. Ho-Shing, H. J. Bussemaker and D. Botstein (2012). "Combinatorial control of diverse metabolic and physiological functions by transcriptional regulators of the yeast sulfur assimilation pathway." Mol Biol Cell **23**(15): 3008-3024.

Pietro, A. S. (1972). "Generation of reducing potential in photosynthesis." Biochem J **128**(1): 28P-29P.

Pirie, C. M., B. J. Hackel, M. G. Rosenblum and K. D. Wittrup (2011). "Convergent potency of internalized gelonin immunotoxins across varied cell lines, antigens, and targeting moieties." J Biol Chem **286**(6): 4165-4172.

Pondaven, P., M. Gallinari, S. Chollet, E. Bucciarelli, G. Sarthou, S. Schultes and F. Jean (2007). "Grazing-induced changes in cell wall silicification in a marine diatom." Protist **158**(1): 21-28.

Pro, B., R. Advani, P. Brice, N. L. Bartlett, J. D. Rosenblatt, T. Illidge, J. Matous, R. Ramchandren, M. Fanale, J. M. Connors, Y. Yang, E. L. Sievers, D. A. Kennedy and A. Shustov (2012). "Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: results of a phase II study." J Clin Oncol **30**(18): 2190-2196.

Raghavendra, A. S. (1998). Photosynthesis: A Comprehensive Teatise. Cambridge, Cambridge University Press

Rasala, B. A., Lee, P.A., Shen, Z., Briggs, S.P., Mendez, M., Mayfield, S.P. (2012). "Robust expression and secretion of xylanase1 in Chlamydomonas reinhardtii by

fusion to a selection gene and processing with the FMDV 2A peptide " PLoS One **In Press**.

Rasala, B. A. and S. P. Mayfield (2011). "The microalga Chlamydomonas reinhardtii as a platform for the production of human protein therapeutics." Bioeng Bugs **2**(1): 50-54.

Rasala, B. A., M. Muto, P. A. Lee, M. Jager, R. M. Cardoso, C. A. Behnke, P. Kirk, C. A. Hokanson, R. Crea, M. Mendez and S. P. Mayfield (2010). "Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of Chlamydomonas reinhardtii." Plant Biotechnol J **8**(6): 719-733.

Ribbert, T., T. Thepen, M. K. Tur, R. Fischer, M. Huhn and S. Barth (2010). "Recombinant, ETA'-based CD64 immunotoxins: improved efficacy by increased valency, both in vitro and in vivo in a chronic cutaneous inflammation model in human CD64 transgenic mice." Br J Dermatol **163**(2): 279-286.

Richter, S. and G. K. Lamppa (1998). "A chloroplast processing enzyme functions as the general stromal processing peptidase." Proc Natl Acad Sci U S A **95**(13): 7463-7468.

Riedel, S. (2005). "Anthrax: a continuing concern in the era of bioterrorism." Proc (Bayl Univ Med Cent) **18**(3): 234-243.

Rosano, G. L., E. M. Bruch, C. V. Colombo and E. A. Ceccarelli (2012). "Toward a unified model of the action of CLP/HSP100 chaperones in chloroplasts." Plant Signal Behav **7**(6): 672-674.

Roth, R. A. and M. E. Koshland (1981). "Role of disulfide interchange enzyme in immunoglobulin synthesis." Biochemistry **20**(23): 6594-6599.

Rouhier, N., J. Couturier, M. K. Johnson and J. P. Jacquot (2010). "Glutaredoxins: roles in iron homeostasis." Trends Biochem Sci **35**(1): 43-52.

Row, P. E. and J. C. Gray (2001). "Chloroplast precursor proteins compete to form early import intermediates in isolated pea chloroplasts." J Exp Bot **52**(354): 47-56.

Saito, T., R. J. Kreitman, S. Hanada, T. Makino, A. Utsunomiya, T. Sumizawa, T. Arima, C. N. Chang, D. Hudson, I. Pastan and et al. (1994). "Cytotoxicity of recombinant Fab and Fv immunotoxins on adult T-cell leukemia lymph node and blood cells in the presence of soluble interleukin-2 receptor." Cancer Res **54**(4): 1059-1064.

Sakamoto, W. (2006). "Protein degradation machineries in plastids." Annu Rev Plant Biol **57**: 599-621.

Sakamoto, W., K. L. Kindle and D. B. Stern (1993). "In vivo analysis of Chlamydomonas chloroplast petD gene expression using stable transformation of beta-glucuronidase translational fusions." Proc Natl Acad Sci U S A **90**(2): 497-501.

Sambrook, J., Fritsch, E. F., Maniatas, T (1989). Molecular Cloning: A Laboratory Manual Plainview, New York Cold Spring Harbor Lab. Press.

Sambrook, J., Fritsch, E.F., Maniatas, T. (1989). Molecular cloning: A Laboratory manual. New York Cold Spring Harbor Lab. Press.

Sandmann, G. and R. Malkin (1983). "Iron-sulfur centers and activities of the photosynthetic electron transport chain in iron-deficient cultures of the blue-green alga aphanocapsa." Plant Physiol **73**(3): 724-728.

Saphire, E. O., R. L. Stanfield, M. D. Crispin, P. W. Parren, P. M. Rudd, R. A. Dwek, D. R. Burton and I. A. Wilson (2002). "Contrasting IgG structures reveal extreme asymmetry and flexibility." J Mol Biol **319**(1): 9-18.

Sawada-Hirai, R., I. Jiang, F. Wang, S. M. Sun, R. Nedellec, P. Ruther, A. Alvarez, D. Millis, P. R. Morrow and A. S. Kang (2004). "Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed." J Immune Based Ther Vaccines **2**(1): 5.

Schaub, J., C. Clemens, P. Schorn, T. Hildebrandt, W. Rust, D. Mennerich, H. Kaufmann and T. W. Schulz (2010). "CHO gene expression profiling in biopharmaceutical process analysis and design." Biotechnol Bioeng **105**(2): 431-438.

Schmidt, R. J., A. M. Myers, N. W. Gillham and J. E. Boynton (1984). "Chloroplast ribosomal proteins of Chlamydomonas synthesized in the cytoplasm are made as precursors." J Cell Biol **98**(6): 2011-2018.

Schmidt, R. J., A. M. Myers, N. W. Gillham and J. E. Boynton (1984). "Immunological similarities between specific chloroplast ribosomal proteins from Chlamydomonas reinhardtii and ribosomal proteins from Escherichia coli." Mol Biol Evol **1**(4): 317-334.

Schnell, D. J. (2000). "Functions and origins of the chloroplast protein-import machinery." Essays Biochem **36**: 47-59.

Schroda, M. (2004). "The Chlamydomonas genome reveals its secrets: chaperone genes and the potential roles of their gene products in the chloroplast." Photosynth Res **82**(3): 221-240.

Schroda, M. (2006). "RNA silencing in Chlamydomonas: mechanisms and tools." Curr Genet **49**(2): 69-84.

Schroda, M., O. Vallon, F. A. Wollman and C. F. Beck (1999). "A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after photoinhibition." Plant Cell **11**(6): 1165-1178.

Schuhmann, H. and I. Adamska (2012). "Deg proteases and their role in protein quality control and processing in different subcellular compartments of the plant cell." Physiol Plant **145**(1): 224-234.

Schwenkert, S., J. Soll and B. Bolter (2011). "Protein import into chloroplasts--how chaperones feature into the game." Biochim Biophys Acta **1808**(3): 901-911.

Selyukh, A. (2011). "Seattle Genetics cancer drug may top $100,000." from http://www.reuters.com/article/2011/08/22/us-seattlegenetics-idUSTRE77L5EB20110822

Shen, G. L., J. L. Li, M. A. Ghetie, V. Ghetie, R. D. May, M. Till, A. N. Brown, M. Relf, P. Knowles, J. W. Uhr and et al. (1988). "Evaluation of four CD22 antibodies as ricin A chain-containing immunotoxins for the in vivo therapy of human B-cell leukemias and lymphomas." Int J Cancer **42**(5): 792-797.

Shi, L. X. and S. M. Theg (2010). "A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss Physcomitrella patens." Plant Cell **22**(1): 205-220.

Shine, J. and L. Dalgarno (1975). "Terminal-sequence analysis of bacterial ribosomal RNA. Correlation between the 3'-terminal-polypyrimidine sequence of 16-S RNA and translational specificity of the ribosome." Eur J Biochem **57**(1): 221-230.

Simmons, L. C., D. Reilly, L. Klimowski, T. S. Raju, G. Meng, P. Sims, K. Hong, R. L. Shields, L. A. Damico, P. Rancatore and D. G. Yansura (2002). "Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies." J Immunol Methods **263**(1-2): 133-147.

Singh, V. and S. K. Kar (1992). "Properties of a ribosome-inactivating protein, gelonin, purified using three different methods." Indian J Biochem Biophys **29**(1): 31-41.

Singh, V., R. C. Singh, R. K. Dubey and A. Alam (1999). "Purification and characterisation of gelonin from seeds of Gelonium multiflorum." Indian J Biochem Biophys **36**(4): 258-265.

Smetacek, V., C. Klaas, V. H. Strass, P. Assmy, M. Montresor, B. Cisewski, N. Savoye, A. Webb, F. d'Ovidio, J. M. Arrieta, U. Bathmann, R. Bellerby, G. M. Berg, P. Croot, S. Gonzalez, J. Henjes, G. J. Herndl, L. J. Hoffmann, H. Leach, M. Losch, M. M. Mills, C. Neill, I. Peeken, R. Rottgers, O. Sachs, E. Sauter, M. M. Schmidt, J.

Schwarz, A. Terbruggen and D. Wolf-Gladrow (2012). "Deep carbon export from a Southern Ocean iron-fertilized diatom bloom." Nature **487**(7407): 313-319.

Sokolenko, A., S. Lerbs-Mache, L. Altschmied and R. G. Herrmann (1998). "Clp protease complexes and their diversity in chloroplasts." Planta **207**(2): 286-295.

Specht, E., S. Miyake-Stoner and S. Mayfield (2010). "Micro-algae come of age as a platform for recombinant protein production." Biotechnol Lett **32**(10): 1373-1383.

Staub, J. M., B. Garcia, J. Graves, P. T. Hajdukiewicz, P. Hunter, N. Nehra, V. Paradkar, M. Schlittler, J. A. Carroll, L. Spatola, D. Ward, G. Ye and D. A. Russell (2000). "High-yield production of a human therapeutic protein in tobacco chloroplasts." Nat Biotechnol **18**(3): 333-338.

Stegemann, S., S. Hartmann, S. Ruf and R. Bock (2003). "High-frequency gene transfer from the chloroplast genome to the nucleus." Proc Natl Acad Sci U S A **100**(15): 8828-8833.

Stemmer, W. P., A. Crameri, K. D. Ha, T. M. Brennan and H. L. Heyneker (1995). "Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides." Gene **164**(1): 49-53.

Stengel, A., J. P. Benz, B. B. Buchanan, J. Soll and B. Bolter (2009). "Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in Pisum sativum." Mol Plant **2**(6): 1181-1197.

Strittmatter, P., J. Soll and B. Bolter (2010). "The chloroplast protein import machinery: a review." Methods Mol Biol **619**: 307-321.

Su, Q., P. Schumann, C. Schild and A. Boschetti (1999). "A processing intermediate of a stromal chloroplast import protein in Chlamydomonas." Biochem J **344 Pt 2**: 391-395.

Sugiura, M., T. Hirose and M. Sugita (1998). "Evolution and mechanism of translation in chloroplasts." Annu Rev Genet **32**: 437-459.

Suzuki, K., H. Nakanishi, J. Bower, D. W. Yoder, K. W. Osteryoung and S. Y. Miyagishima (2009). "Plastid chaperonin proteins Cpn60 alpha and Cpn60 beta are required for plastid division in Arabidopsis thaliana." BMC Plant Biol **9**: 38.

Taylor, S., M. Wakem, G. Dijkman, M. Alsarraj and M. Nguyen (2010). "A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines." Methods **50**(4): S1-5.

Terauchi, A. M., G. Peers, M. C. Kobayashi, K. K. Niyogi and S. S. Merchant (2010). "Trophic status of Chlamydomonas reinhardtii influences the impact of iron deficiency on photosynthesis." Photosynth Res **105**(1): 39-49.

Tojo, N., I. Miyagi, M. Miura and H. Ohi (2008). "Recombinant human fibrinogen expressed in the yeast Pichia pastoris was assembled and biologically active." Protein Expr Purif **59**(2): 289-296.

Tran, M., B. Zhou, P. L. Pettersson, M. J. Gonzalez and S. P. Mayfield (2009). "Synthesis and assembly of a full-length human monoclonal antibody in algal chloroplasts." Biotechnol Bioeng **104**(4): 663-673.

Vallon, O. (2005). "Chlamydomonas immunophilins and parvulins: survey and critical assessment of gene models." Eukaryot Cell **4**(2): 230-241.

van de Donk, N. W. and E. Dhimolea (2012). "Brentuximab vedotin." MAbs **4**(4): 458-465.

van Dijk, K. and N. Sarkar (2011). "Selectable and inheritable gene silencing through RNA interference in the unicellular alga Chlamydomonas reinhardtii." Methods Mol Biol **765**: 457-476.

Viitanen, P. V., M. Schmidt, J. Buchner, T. Suzuki, E. Vierling, R. Dickson, G. H. Lorimer, A. Gatenby and J. Soll (1995). "Functional characterization of the higher plant chloroplast chaperonins." J Biol Chem **270**(30): 18158-18164.

Waegeman, H., S. De Lausnay, J. Beauprez, J. Maertens, M. De Mey and W. Soetaert (2011). "Increasing recombinant protein production in Escherichia coli K12 through metabolic engineering." N Biotechnol.

Walker, M. R., J. Lund, K. M. Thompson and R. Jefferis (1989). "Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors." Biochem J **259**(2): 347-353.

Walmsley, A. M. and C. J. Arntzen (2003). "Plant cell factories and mucosal vaccines." Curr Opin Biotechnol **14**(2): 145-150.

Wang, B., J. M. Rosano, R. Cheheltani, M. P. Achary and M. F. Kiani (2010). "Towards a targeted multi-drug delivery approach to improve therapeutic efficacy in breast cancer." Expert Opin Drug Deliv **7**(10): 1159-1173.

Wang, J., J. A. Hartling and J. M. Flanagan (1997). "The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis." Cell **91**(4): 447-456.

Watson, J., V. Koya, S. H. Leppla and H. Daniell (2004). "Expression of Bacillus anthracis protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop." Vaccine **22**(31-32): 4374-4384.

Watson, J. C. and S. J. Surzycki (1982). "Extensive sequence homology in the DNA coding for elongation factor Tu from Escherichia coli and the Chlamydomonas reinhardtii chloroplast." Proc Natl Acad Sci U S A **79**(7): 2264-2267.

Wayne, A. S., R. J. Kreitman, H. W. Findley, G. Lew, C. Delbrook, S. M. Steinberg, M. Stetler-Stevenson, D. J. Fitzgerald and I. Pastan (2010). "Anti-CD22 immunotoxin RFB4(dsFv)-PE38 (BL22) for CD22-positive hematologic malignancies of childhood: preclinical studies and phase I clinical trial." Clin Cancer Res **16**(6): 1894-1903.

Whatley, J. M., P. John and F. R. Whatley (1979). "From extracellular to intracellular: the establishment of mitochondria and chloroplasts." Proc R Soc Lond B Biol Sci **204**(1155): 165-187.

Wiech, H., J. Buchner, R. Zimmermann and U. Jakob (1992). "Hsp90 chaperones protein folding in vitro." Nature **358**(6382): 169-170.

Wild, M. A., H. Xin, T. Maruyama, M. J. Nolan, P. M. Calveley, J. D. Malone, M. R. Wallace and K. S. Bowdish (2003). "Human antibodies from immunized donors are protective against anthrax toxin in vivo." Nat Biotechnol **21**(11): 1305-1306.

Willmund, F., K. V. Dorn, M. Schulz-Raffelt and M. Schroda (2008). "The chloroplast DnaJ homolog CDJ1 of Chlamydomonas reinhardtii is part of a multichaperone complex containing HSP70B, CGE1, and HSP90C." Plant Physiol **148**(4): 2070-2082.

Wu, H., D. S. Pfarr, G. A. Losonsky and P. A. Kiener (2008). "Immunoprophylaxis of RSV infection: advancing from RSV-IGIV to palivizumab and motavizumab." Curr Top Microbiol Immunol **317**: 103-123.

Wurm, F. M. (2004). "Production of recombinant protein therapeutics in cultivated mammalian cells." Nat Biotechnol **22**(11): 1393-1398.

Xie, H., C. Audette, M. Hoffee, J. M. Lambert and W. A. Blattler (2004). "Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (huC242-DM1), and its two components in mice." J Pharmacol Exp Ther **308**(3): 1073-1082.

Yin, J., G. Li, X. Ren and G. Herrler (2007). "Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes." J Biotechnol **127**(3): 335-347.

Yohn, C. B., A. Cohen, A. Danon and S. P. Mayfield (1996). "Altered mRNA binding activity and decreased translational initiation in a nuclear mutant lacking translation of the chloroplast psbA mRNA." Mol Cell Biol **16**(7): 3560-3566.

Yohn, C. B., A. Cohen, C. Rosch, M. R. Kuchka and S. P. Mayfield (1998). "Translation of the chloroplast psbA mRNA requires the nuclear-encoded poly(A)-binding protein, RB47." J Cell Biol **142**(2): 435-442.

Yokthongwattana, K., B. Chrost, S. Behrman, C. Casper-Lindley and A. Melis (2001). "Photosystem II damage and repair cycle in the green alga Dunaliella salina: involvement of a chloroplast-localized HSP70." Plant Cell Physiol **42**(12): 1389-1397.

Younes, A., U. Yasothan and P. Kirkpatrick (2012). "Brentuximab vedotin." Nat Rev Drug Discov **11**(1): 19-20.

Zamboni, M., M. Brigotti, F. Rambelli, L. Montanaro and S. Sperti (1989). "High-pressure-liquid-chromatographic and fluorimetric methods for the determination of adenine released from ribosomes by ricin and gelonin." Biochem J **259**(3): 639-643.

Zeitlin, L., R. A. Cone, T. R. Moench and K. J. Whaley (2000). "Preventing infectious disease with passive immunization." Microbes Infect **2**(6): 701-708.

Zerges, W., J. Girard-Bascou and J. D. Rochaix (1997). "Translation of the chloroplast psbC mRNA is controlled by interactions between its 5' leader and the nuclear loci TBC1 and TBC3 in Chlamydomonas reinhardtii." Mol Cell Biol **17**(6): 3440-3448.

Zhao, T., W. Wang, X. Bai and Y. Qi (2009). "Gene silencing by artificial microRNAs in Chlamydomonas." Plant J **58**(1): 157-164.

Zhou, B., C. Carney and K. D. Janda (2008). "Selection and characterization of human antibodies neutralizing Bacillus anthracis toxin." Bioorg Med Chem **16**(4): 1903-1913.