# Bulked segregant analysis as a new tool for identification and cloning of genes in *Chlamydomonas reinhardtii*. Identification of *TBC1*

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A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biology at

Concordia University

Montreal, Quebec, Canada

August 2006

*Your file Votre référence*
*ISBN: 978-0-494-20726-0*
*Our file Notre référence*
*ISBN: 978-0-494-20726-0*

## ABSTRACT

Bulked segregant analysis as a new tool for identification and cloning of genes in *Chlamydomonas reinhardtii*. Identification of *TBC1*

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Although *Chlamydomonas reinhardtii* is a widely used model organism for studies of a variety of cell biological processes, the identification and cloning of genes known by mutations is still arduous. Current methods are inefficient for many of the existing mutations as their mutant alleles can either spontaneously revert or produce no selectable phenotype. With the sequencing and the annotation of the *C. reinhardtii* nuclear genome and mapping of available molecular markers, positional cloning is now possible. This thesis explored the application of bulked segregant analysis (BSA) with two types of molecular markers, amplified fragment length polymorphisms and single nucleotide polymorphisms, in order to identify and characterize the nuclear *TBC1* gene and its predicted product. TBC1 was previously shown to functionally interact with specific regions and structures in the 5' untranslated region of chloroplast *psbC* mRNA to promote its translation and the synthesis of its product, the CP43 subunit polypeptide of PSII. Using BSA, *TBC1* was mapped to an 8 map-unit region of Linkage Group VI. Complementation analysis narrowed down its position to a 41 kb region. Analyses of predicted genes in the region identified an exonuclease II orthologue as the best candidate for being *TBC1*.

## ACKNOWLEDGEMENTS

I would like to deeply thank my supervisor, Dr. William Zerges for his guidance and help throughout the course of this project. I also want to thank to my committee members Dr. Herrington for the encouragement and timely advice and Dr. Dyanandan for giving me the opportunity to use his lab equipment for SNP genotyping and helping me with ideas on the SNP analysis. Thank you to Dr. David Stern for providing us with ideas on electroporation of *C. reinhardtii.* I would also like to thank to Hamid Ashrafi for his work in constructing the bulks and to lab-mate James Uniacke.

I thank my family and especially my wife for her patience and moral support.

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# CHAPTER 1. INTRODUCTION

## 1.1 Chloroplasts

Chloroplasts are the organelle in plants and eukaryotic algae that carry out a variety of essential functions; photosynthesis, the assimilation of N, S and P, and the production of amino acids and fatty acids. The light dependent reactions of photosynthesis are carried out in the membranes of an elaborate network of membranous vesicles within chloroplasts, called thylakoids. These flattened sacks are organized in stacks called grana and as individual vesicles that extend from grana into the chloroplast stroma as "stroma thylakoids". Within thylakoid membranes two multisubunit complexes, photosystem I (PSI) and photosystem II (PSII), use light energy to generate the reducing power in the form of plastoquinol and reduced ferredoxin, respectively. An electrochemical proton gradient is generated across thylakoid membranes by the oxidation of water by PSII and the sequential reduction and oxidation of plastoquinol by PSII and by a third integral thylakoid membrane complex, the cytochrome b6/f complex. This electrochemical proton gradient is used by a fourth complex, the chloroplast ATP synthase, to generate ATP from ADP and inorganic phosphate. ATP and electrons from reduced ferredoxin are used by the light-independent reactions of photosynthesis, the "Calvin Cycle", to convert $CO_2$ to 3-phosphoglycerate for the subsequent synthesis of carbohydrates, which fuel cellular metabolism and also constitute the primary energy source of the food chain. PSII is also the primary source of $O_2$ in the biosphere for aerobic respiration by diverse species.

Chloroplasts have evolved from a photosynthetic bacterium, probably a cyanobacteria, through an endosymbiosis with a eukaryotic host (Gray 1989; Morden et al. 1992). Unlike most organelles, but like mitochondria, chloroplasts exist as semi-autonomous organelles. This means they cannot be formed *de novo*, but rather they divide by binary fission and transmit and express an organellar genome. This distinct genetic system produces at least 100 of the proteins that function in chloroplasts. The genetic system of chloroplasts consists of a circular genome, of c.a. 100-300 kb, its replication machinery, and gene expression system composed of RNA-polymerases, 70S ribosomes, tRNAs, and all of the other components. Owing to the eubacterial ancestry of chloroplast, most of the components of chloroplast genetic systems are closer to those of bacteria than to those of the nuclear-cytosolic systems in plant cells. Chloroplast RNA-polymerases and ribosomes are similar in composition and other properties to those of eubacteria. The chloroplast genome has been reduced in size during chloroplast evolution by the loss of genes or by their transfer to the nuclear genome. Chloroplast proteins encoded by these nuclear genes are synthesized by the 80S ribosomes in the cytoplasm and then imported into the chloroplast (Gutensohn et al. 2006). Indeed, most of the proteins that function in chloroplasts are encoded by the nuclear genome (Gillham et al. 1994) (Hauser et al. 1998). Chloroplast genomes, however, still encode all of the RNA components of the organellar gene expression system, e.g., tRNAs and rRNAs.

The first goal of this thesis was to identify and characterize the nuclear *TBC1* gene and its predicted product in the eukaryotic green alga *Chlamydomonas reinhardtii*. *TBC1* genetically interacts with specific regions and structures in the 5' untranslated region (UTR) of chloroplast *psbC* mRNA to promote its translation and the synthesis of

its product, the CP43 subunit polypeptide of PSII. The second goal of this thesis addressed the difficulties in locating genes on the physical map of the nuclear genome that have only been identified genetically, i.e. by the phenotypes produced by mutant alleles. Thus, this introduction reviews translation and translational control in chloroplasts, with an emphasis on previous genetic characterizations of *TBC1* function. Also reviewed are existing approaches used for mapping and identification of genes known only by genetics and the use of the unicellular eukaryotic alga *Chlamydomonas reinhardtii* as a model organism for the study of chloroplast biogenesis.

## 1.2 Translation in Chloroplasts

### 1.2.1 Chloroplast Translation Machinery

Chloroplast mRNAs are translated by 70S ribosomes that resemble bacterial ribosomes in their composition of ribosomal proteins, the sequences and structures of their rRNAs, and their sensitivity to antibiotics (Bourque et al. 1971). Most of our understanding of translation in chloroplasts is based on molecular biological approaches (e.g. site-directed mutagenesis), characterization of chloroplast ribosomes and orthologues of general translation factors in eubacterial systems, and by analogy to eubacterial translation systems. In most other organisms in which translation has been studied, the biochemical steps have been dissected using *in vitro* reconstituted translation systems. However, *in vitro* translation systems for chloroplasts have been difficult to develop and use. Consequently, there are only a few reports of their use for studies of translation in tobacco chloroplasts (Hirose and Sugiura 1996; Hirose and Sugiura 1997; Hirose et al. 1998). An *in vitro* translation system has not been developed for the *C.*

*reinhardtii* chloroplast. Yet the presence of chloroplast orthologues of all of the translation initiation, elongation and termination factors known in eubacterial systems supports the view that the biochemical steps involved in these processes are generally shared. For chloroplast mRNAs the initiation codon is usually AUG, although a few mRNAs use GUG, which is also a rare initiation codon in bacteria. *psbC* translation, for example, is initiated at a GUG initiation codon (Rochaix et al. 1989). Replacing the AUG initiation codon of *petD* and *petA* mRNA with AUU or AUA only decreased translation rates *in vivo* (Chen et al. 1995; Chen et al. 1997), unlike *psbD* where similar changes abolished its translation (Nickelsen et al. 1999).

### 1.2.2 Translation in chloroplasts has features similar to both eukaryotes and prokaryotes.

In eubacteria, translation initiation begins with direct binding of the small subunit of the ribosome to the translation initiation region by base-pairing of a Shine-Dalgarno (SD) sequence (GGAGG) located 10 – 15 nt upstream of the translation initiation codon and a complementary sequence at the 3' end of the 16S rRNA called the anti-Shine-Dalgarno sequence. The ribosomal protein S1 of the 30S small ribosomal subunit also can be involved in its recognition and binding to the mRNA via its interaction with a pyrimidine-rich sequence upstream of the SD region (reviewed by Zerges, 2000).

In eukaryotes, translation initiation is a more complex process that involves additional steps and factors (Pestova et al. 2001). A 43S preinitiation complex is formed by an interaction of initiation factors bound to the 5' $m^7G$ cap on the mRNA and the small ribosome subunit. The small subunit translocates in a 3' direction along the 5'

untranslated region (UTR) of the mRNA in a process called "scanning" because it stops at the first AUG initiation codon in the proper context for assembly of the ribosome. There, the small subunit facilitates the base-pairing of the initiation codon to the initiator tRNA, and these are joined by the large ribosome subunit to form a translation-competent ribosome.

The initiation of translation on a few cellular mRNAs and the mRNA of the picorna viruses is $m^7G$ cap-independent and occurs by a process called "internal ribosomal entry". In these cases the 40S ribosomal subunit is recruited to the 5' UTR of the mRNA by an RNA structure called an internal ribosome entry site (IRES) (reviewed Pestova et al. 2001).

In chloroplasts, features similar to both systems described above could be found (reviewed by Zerges, 2000). SD sequences could be found in many chloroplast mRNAs; however their sequence and position relative to the initiation codon varies. In many chloroplast genes no SD sequences are found within 200 nt of the initiation codon and if there are, they seem to not always be required for translation (Fargo et al. 1998). It was shown that the 16S rRNA sequence in chloroplasts, including their anti-SD sequence, is highly conserved with the 16S rRNA in eubacteria (Dron et al. 1982; Steege et al. 1982). This has raised the question of whether these SD sequences actually function in translation initiation. The ability to transform the chloroplast with chimeric genes comprised of the promoter and 5' UTR regions of chloroplast genes with altered SD-like sequences fused to a reporter gene has allowed detailed studies on the requirement of those sequences in translation initiation. Results of this approach have revealed that translation initiation of the chloroplast *petD* gene was independent of its putative SD

sequence element and that *petD* 5' UTR is sufficient for translation (Sakamoto et al. 1993; Sakamoto et al. 1994). Similarly, replacement mutagenesis of SD-like sequences of two ribosomal protein mRNAs (*rps4, rps7*) and two mRNAs that encode ATP synthase subunits (*atpB, atpE*) showed no dependency of translation initiation on this region of their 5' UTR (Fargo et al. 1998). For some mRNAs, however, it was found that mutagenesis of the SD-like sequence reduced translation (Nickelsen et al. 1999; Zerges et al. 2003), while in other cases translation was abolished (e.g., for the *C. reinhardtii psbA* mRNA and the *rps14* mRNA in tobacco chloroplasts (Mayfield et al. 1994; Hirose and Sugiura 1996). Translation of *psbC* is partially dependent on a potential SD sequence (GGAGG) that could either act as such or as a part of larger cis- acting region (Zerges et al. 2003). Similar to other chloroplast genes like *psbD,* the SD-like sequence in *psbC* 5'UTR was necessary for translation but this requirement was not absolute as a deletion of this region only partially reduced translation levels (Zerges et al. 2003).

The use of site-directed mutagenesis has identified translational *cis*-acting elements in the 5' UTRs of several chloroplast mRNAs. Some of those elements appear to function as unstructured RNA while others have secondary and tertiary structure, which are important for their function (reviewed by Zerges, 2000). Structures within these 5' UTRs seem to be analogous to IRESs in that they are required for translation and they are distant from the translation initiation region on the primary nucleotide sequence. A potential stem-loop secondary structure, for example, in *psbA* may be critical for its translational activation in response to light (Mayfield et al. 1994). A structural element in the *C. reinhardtii psbC* 5' UTR has these properties and is described below.

Although these later steps in the assembly of a translation-competent 70S ribosome at the translation initiation region (TIR) of the mRNAs of chloroplast and eubacterial mRNAs are similar, the pathway by which the small ribosome subunit reaches this region and the mechanisms of translational control appear to differ between these systems. In eubacteria and the cytosolic translation systems of eukaryotes, the control of translation initiation is exerted by the binding of proteins to specific sequences or the formation of RNA structures by the sequences in the 5' UTR that block the binding of the small subunit of the ribosome to the TIR and, thereby, repress translation initiation. Translation of chloroplast mRNAs, on the other hand, typically requires specific RNA sequences or structures in their 5' UTR and trans-acting factors for translation. An extensive deletion analysis of the 5' UTRs of the chloroplast mRNAs of *petD* and *psbC* in *C. reinhardtii*, for example, did not find any sequences that repress translation or completely eliminate the requirement for the trans-acting factors encoded by nuclear genes. Thus, translational activation appears to be a more common mode of regulation than repression in chloroplasts. However, translational repression through the cessation of activation by these factors does appear in the chloroplast of *C. reinhardtii* and this is reviewed below.

Translational activation of chloroplast mRNAs is exemplified by a control exerted by a class of factors that are encoded by nuclear genes and required for translation of specific chloroplast mRNAs. For example, *TBC1* is one such nuclear gene, the function of, which is required for translation of the chloroplast *psbC* mRNA in *C. reinhardtii*. The translation of specific chloroplast mRNAs encoding core subunits of the photosynthesis complexes requires at least one nuclear genome-encoded factor (reviewed

below). These factors have been identified by genetic approaches, and several have been characterized at the molecular level. Yet little is known about the mechanisms by which they exert translational control.

The role of *TBC1* in *psbC* translation is perhaps the best characterized at the genetic level. The only mutant allele of *TBC1*, *tbc1-F34*, was identified in a mutant called *F34*. *F34* was induced by methyl methane sulfonate and identified in a screen for photosystem II mutants, which is described later in this introduction. Another mutant isolated in this screen, *F64*, also was deficient in *psbC* translation, and its characterization and the cloning of the mutant gene led to the discovery and characterization of *TBC2*. The first classical complementation test in *C. reinhardtii* was performed using *tbc1-F34* and the original *TBC2* mutant allele, *tbc2-F64*. It revealed that the PSII deficiencies produced by both alleles are recessive: a diploid with one copy of each allele was phenotypically wild-type (Bennoun et al. 1980). *TBC1* and *TBC2* are also unlinked and, thus, they are distinct loci. The history of the discovery of *TBC1* is described in more detail below. *F34* and *F64* were originally shown to be specifically defective in *psbC* translation by a study that revealed a specific absence of the synthesis of CP43 in short radioisotope-pulse-labeling experiments, even though the *psbC* mRNA accumulated in these strains to wild-type level (Rochaix et al. 1989). Additional evidence that *TBC1* is required for *psbC* translation, and that the block in *psbC* translation in the *tbc1* mutant is the only basis for its PSII deficiency, was provided by the identification of a point mutation in the *psbC* 5' UTR that partially suppresses this phenotype of the *tbc1-F34* mutant allele (Rochaix et al. 1989). Thus, this provides evidence that *TBC1* does not control the translation of other target mRNAs. That both *TBC1* and *TBC2* function via

interactions with the *psbC* 5' UTR, and thus are required before the elongation phase of translation, was revealed by the inability of *tbc1* and *tbc2* mutants to express a chimeric reporter gene from the *psbC* 5' UTR in transgenic chloroplasts (Zerges and Rochaix, 1994). Recent results of fluorescence *in situ* hybridization revealed that the *psbC* mRNA is not properly localized in the in *tbc1* mutants, although it is localized normally in *tbc2* mutants (James Uniacke, personal communication). Thus, our current hypothesis proposes that *TBC1* functions to localize the translationally silent *psbC* mRNA to regions in the chloroplast where its translation is activated by *TBC2*. A recent dissection of the *cis*-acting elements in the 550 nt *psbC* 5' UTR revealed that a central region of 120 nt is absolutely required for translation (Munir Rahim, M.Sc. thesis, Concordia University). *TBC1* functionally interacts with this region, and sequences immediately 3' to it, which are partially required for translation because alteration or deletion of these sequences results in translation that is independent of *TBC1* (Zerges et al. 2003). A recent study revealed that this region forms a large RNA tertiary structure, composed of a pseudoknot structure and a 3-way junction (Munir Rahim, M.Sc. Thesis, Concordia University). A genetic interaction between *TBC1* and a third nuclear locus, *TBC3*, was identified by the *TBC3* mutation that partially suppresses all of the mutant phenotypes of *tbc1-F34* (Zerges et al. 1997). *TBC3* interacts with RNA sequences between the 5' end of the mRNA and the central required region of the *psbC* 5' UTR (Zerges et al. 2003).

Thus, identification of the *TBC1* gene would allow a prediction of the structure and possible functions of its product, using computer-based searches for conserved motifs, domains, and known orthologues or homologues. Having the *TBC1* gene and a complete cDNA would allow the use of the diverse collection of molecular biological,

immunological and biochemical approaches available to study the role of the *TBC1* product in *psbC* translation and its interactions with the mRNA and the product of *TBC3*. These approaches have begun to reveal some cell biological and structural properties of the protein encoded by *TBC2*, which was cloned and sequenced a few years ago (Auchincloss et al. 2002). Tbc2 has a molecular mass of 114.8 kDa (Auchincloss et al. 2002) and it localizes to the chloroplast stroma (Auchincloss et al. 2002). Its amino acid sequence has a weak similarity to that of *Cpr1* in maize, which is involved in the translational control of the mRNAs of *petA* and *petD*. Tbc2 may have other partners as immunoprecipitation showed that it co-fractionates with a 400-kD protein complex (Auchincloss et al. 2002). However, no insights are available on the *TBC1* product or the mechanism(s) by which it exerts translational control of *psbC*.

Other members of this class of chloroplast mRNA-specific translational factors have been characterized. Translation of the *psbA* mRNA (which encodes the D1 of PSII) of *C. reinhardtii* is dependant on at least one nuclear gene product, called F35 (Yohn et al. 1998). The nuclear *Nac1* and *Ac115* genes are involved in translation of the *psbD* mRNA, which codes for the D2 subunit of PSII (Kuchka et al. 1988; Kuchka et al. 1989). Similar nuclear gene products appear to function in the translation of *petD*, encoding subunit IV of the cytochrome b6/f complex (Barkan et al. 1994). *TCA1* encodes a protein that is absolutely required for the translation of *petA* (Wostrikoff et al. 2001). In maize, the *crp1* gene is required for translation of the chloroplast *petA* and *petD* mRNAs encoding subunits of the cytochrome b6/f complex (Barkan et al. 1994; Fisk et al. 1999). These factors were shown to interact with their target mRNAs via their 5'UTRs in order to exert translation control.

Like Tbc2, Crp1 and Tab2 are localized to the chloroplast stroma and function in large complexes (Fisk et al. 1999; Auchincloss et al. 2002). AC115 is suggested to be associated with a membrane due to a hydrophobic stretch of amino acids at its COOH terminus (Rattanachaikunsopon et al. 1999). Indirect function through other factors of Crp1, Tbc2, Nac2 and Tab2 was suggested by observations that they are not associated with ribosomes, although such interactions could be too transient to result in stable assoication (Fisk et al. 1999; Boudreau et al. 2000; Auchincloss et al. 2002; Dauvillee et al. 2003). Tab2 and Crp1 directly interact with their target mRNAs (Dauvillee et al. 2003; Schmitz-Linneweber et al. 2005).

One unresolved problem concerns the basis of the specificity of these factors for their mRNAs targets. This specificity may relate to a fundamental aspect in the biogenesis of the oxidoreductase complexes of electron transport chains because genetic approaches in the yeast *Saccharomyces cerevisiae* have revealed an analogous set of nuclear genes that are required for the translation of mRNAs transcribed from the mitochondrial genome and encoding subunits of cytochrome oxidase (Fox 1996; Chacinska and Boguta 2000). The current model for these factors in chloroplasts proposes that these factors mediate a negative feed-back repression by the subunit they encode on their translation (Choquet and Wollman 2002). Autofeedback repression is a common mechanism of translational control in bacteria, and typically occurs in the expression of proteins that bind RNA since this activity allows them to bind to the 5' UTR of the mRNA encoding them to repress its translation (Draper et al. 1998). Recently, the unassembled subunits of PSI, PSII and the cytochrome b6/f complex have been shown to repress the translation of the mRNAs encoding them (Choquet et al. 2001;

Pineau et al. 2004; Wostrikoff et al. 2004). As these subunits are not known to be able to bind RNA, and are too hydrophobic to exist outside of the lipid bilayer of the thylakoid membrane, ternary translation factors have been proposed to exist to mediate their ability to repress their own synthesis (Wostrikoff et al. 2001). Thus, the translational regulators of specific mRNAs, including the *TBC1* gene product, may function as these intermediate factors in pathways that sense the accumulation of a specific subunit of a photosynthesis complex in an unassembled state, and consequently inactivate their required role in the translation of the mRNA encoding that particular subunt. As these factors are *required* for translation of their target mRNAs, this repression may involve the cessation of their positive role in translation or their ability to both activate or repress translation via a molecular switch. Another possible role of some for these mRNA specific factors could be to regulate translation of their target mRNA in response to requirements for the subunit they encode for repair of the photosystem (e.g. in response to damage by photochemical reactions within the complex). In particular, translation of the *psbA* mRNA is specifically activated by exposure of cells to high intensity light to replace damaged D1 in PSII (Aro et al. 1993). Specific translational regulation of other chloroplast mRNAs that require specific translation factor(s), however, has not been described.

### 1.2.3 Common structures of the translation regulators; TPR, PPR motifs

Many of the characterized chloroplast mRNA-specific factors share conserved protein motifs. Several proteins involved in the post-transcriptional regulation of chloroplast gene expression, at the levels of mRNA stability and translation, contain

tetratricopeptide repeats (TPR). The TPR motif is a degenerate 34 amino acid repeat often arranged in tandem (Small and Peeters 2000). Despite its degenerate nature, there is a largely conserved pattern of amino acid similarity or homology in terms of size, hydrophobicity, and spacing. Eight amino acid residues seen to have a higher frequency of conservation generating a consensus at positions 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/L), and 32 (P/K/E). A TPR domain is punctuated by proline-induced turns and consists of two α-helical domains (Hirano et al. 1990; Small and Peeters 2000). Proteins containing TPR motifs are conserved in diverse organisms and seem to function in different intracellular compartments, e.g. the nucleus, the cytoplasm, mitochondria, and chloroplasts (Lamb et al. 1995). The TPR repeats are proposed to mediate specific intermolecular or intramolecular protein–protein or protein–RNA interactions (Lamb et al. 1995). TPR proteins have been described to have a variety of cellular functions (Lamb et al. 1995), such as scaffolding proteins. Many function in multisubunit complexes such as the mitochondrial import receptor complex (Lithgow et al. 1995; Moczko et al. 1997), the peroxysomal import receptor complex (Van der Leij et al. 1993; Brocard et al. 1994), a transcription repression complex (Smith et al. 1995; Tzamarias and Struhl 1995), and the anaphase-promoting complex.

Nac2 has nine TPR-like domains, eight of which are arranged in tandem and one was shown to be essential for the proper folding of the protein in a functional form (Boudreau et al. 2000). The cloning and characterization of *Mbb1*, which is required for the translation of the chloroplast *psbB* mRNA, revealed that its protein product has 10 tandem TPR-like motifs that comprise half of the protein sequence (Vaistij et al. 2000). In maize the Crp1 protein, which involved in RNA processing and translational control of

the dicistronic *petA-petD* mRNA, contains several repeats, called PPR repeats, which are similar to the TPR motif (Fisk et al. 1999; Small and Peeters 2000; Schmitz-Linneweber et al. 2005). Ycf3 is a chloroplast protein required for the stable accumulation of PSI, and it also contains TPR motifs (Boudreau et al. 1997). Thus Nac2, Mbb1 and Crp1 all functionally interact with the 5′ UTR of their target mRNAs, have tandem repeated TPR or PPR motifs and are part of high molecular weight complexes. This raises the possibility that these complexes specifically recognize their target mRNA 5' UTR through their protein structural domains formed by TPR sequences. Similarly, Tbc2 contains five copies of a quasi-conserved 39 amino acid residue PPPEW motif in its C-terminal region (Auchincloss et al. 2002).

### 1.2.4 Translation regulation in chloroplasts in response to light

*psbA* translation is activated during photo-oxidative stress induced by high intensity light exposure, in order for new D1 subunit of photosystem II to be synthesized (Bruick and Mayfield 1999) to replace D1 subunits that are damaged by photochemical reactions within PSII (Ohad et al. 1990). Similar results for D1 were obtained for pea (Kettunen et al. 1997) and *Spirodela* (Fromm et al. 1985). Another form of light regulation occurs following a transition from darkness to moderate intensity light, when translation of many chloroplast mRNAs is stimulated, as revealed by radioisotope pulse-labeling experiments in *C. reinhardtii*. These include the translation of *psbA*, *psbD* and *rbcL* mRNAs, coding for D1 and D2 subunits of PSII, and the large subunit (LSU) of ribuolose bisphosphate carboxylase-oxygenase (rubisco) (Herrin et al. 1986; Malnoe et al. 1988).

Experimental results have shown that translation control in these cases is mediated by the effects of light on photosynthesis, rather than by specialized light receptors, which are known to mediate the light regulation of nuclear gene expression in different organisms (Fankhauser and Staiger 2002). This was revealed in several cases when blocking specific steps of the photosynthetic electron transport chain or the formation of the electrochemical proton gradient across thylakoid membranes inhibited the light-dependent translation of *psbA* in *C. reinhardtii* (Trebitsh and Danon 2001) and the finding that translation is activated by light in isolated chloroplasts (Taniguchi et al. 1993; Kettunen et al. 1997; Trebitsh and Danon 2001). Thus the light perception and the transduction of this signal to the translation regulatory machinery is mediated by photosynthesis (Bruick and Mayfield 1998; Bruick and Mayfield 1999; Zerges et al. 2002). It has been proposed that the light alters biochemical "sensors" within chloroplasts, which are monitored by regulatory factors. These "sensors" include the ADP/ATP ratio in the stroma, the electrochemical proton gradient across the thylakoid membrane, and the redox states of electron carriers in the electron transport chain (Allen 1993; Allen 1993; Allen and Raven 1996; Zerges 2000); (Allen 1993; Pfannschmidt 2003; Pfannschmidt et al. 2003). Proteins that specifically bind to the *psbA* 5' UTR that have been proposed to be involved in its translation regulation in response to light have been identified in *C. reinhardtii* (Ossenbuhl et al. 2002) as well as in vascular plants (Klaff et al. 1997; Alexander et al. 1998; Nickelsen 2003).

### 1.2.5 Translation regulation plays a role in the assembly of the photosynthetic complexes

The biological function of translational control by these mRNA-specific translational regulators could be related to the fact that the synthesis of certain chloroplast proteins is tightly bound to their insertion in the thylakoid membranes, which is dependent on the availability of their assembly partner. A model called "control by epistasy of synthesis" (CES) proposed the autofeedback regulation described above for the assembly of the photosynthesis complexes in *C. reinhardtii*. It proposes that this translational regulation ensures that subunits are synthesized and inserted into a complex in a specific order and in the stoichiometric amounts, required for their assembly (Wollman et al. 1999; Choquet and Vallon 2000; Choquet et al. 2001; Choquet and Wollman 2002; Zerges 2002). In this sense translation control could guarantee that a subunit is synthesized only if its assembly partner is already available.

For example nascent D1 is synthesized and inserted only in the presence of D2 which functions as its receptor (Ohad et al. 1990; van Wijk et al. 1997; Zhang et al. 1999). Crosslinking and immunoprecipitation experiments have confirmed that there are close contacts between elongating D1 and D2 (Zhang et al. 1999; Zhang et al. 2000).

A deletion mutant lacking cytochrome *b-559* was shown to abolish translation of other PSII core subunits D1, D2, CP43 and CP47 (Morais et al. 1998). In a similar manner D1 and CP47 are not synthesized in a mutant lacking D2 (de Vitry et al. 1989; Erickson et al. 1989). Furthermore when D1 was not produced CP47 protein is undetectable (Bennoun and Spierer-Herz 1986; de Vitry et al. 1989; Erickson et al. 1989). Translation of CP43 (P6 in *Chlamydomonas*) seems to be dependent on preexisting

cytochrome *b-559* α-subunit, but not on the other subunits (de Vitry et al. 1989; Morais et al. 1998).

Control by epistasy of synthesis also occurs in photosynthetic complexes other than PSII. ATP synthase β subunit deficiency is associated with decreased levels of α-subunit synthesis (Drapier et al. 1992). In PSI, PsaB deficiency abolishes translation of its assembly partner, PsaA (Girard-Bascou et al. 1987; Stampacchia et al. 1997). PsaA deficiency, on the other hand, inhibits the synthesis of the PsaC subunit (Takahashi et al. 1996). A *rbcS* deletion mutant of *C. reinhardtii* is negatively affected in the translation of *rbcL* mRNA (Khrebtukova and Spreitzer 1996).

For the biogenesis of the cytochrome *b6/f* complex in *C. reinhardtii* the synthesis of cytochrome *f* depends on the presence of cytochrome $b_6$ and subunit IV (Kuras and Wollman 1994). *petA* translation is repressed by the absence of cytochrome $b_6$ or subunit IV, because the *petA* product cytochrome *f* cannot be assembled with these subunits. As a consequence, the free C-terminus of unassembled cytochrome *f* is exposed to the stroma where it initiates autofeedback repression of *petA* translation via the 5' UTR of this mRNA (Choquet et al. 1998). The *petA*-specific translational regulator encoded by the nuclear *TCA1* gene has been proposed to mediate this regulation (Wostrikoff et al. 2001).

The scarcity of orthologues of these mRNA-specific translational regulatory factors in plants however raises the question of whether this regulation is conserved or specific to green algae. There is not much evidence for this type of translational regulation in the chloroplasts of vascular plants, although one report in tobacco did provide evidence for CES (Monde et al. 2000). Two mRNA-specific translation factors in Maize, crp1 and Atp1 (Barkan et al. 1994; McCormac and Barkan 1999), as well as

the recent cloning of *HCF109* that is shown to encode a factor involved in translation regulation in *Arabidopsis thaliana* (Meurer et al. 2002) suggest that analogous regulatory processes occur in vascular plants.

## 1.3 *Chlamydomonas reinhardtii* as a model organism

In this study *C. reinhardtii* was used because it is a well-established model organism for studies of chloroplast biogenesis and photosynthesis, as well as a variety of other processes; light perception, flagellar structure and function, genetics of basal bodies, cell-cell recognition, cell cycle control and the assimilation of S, N and P. *C. reinhardtii* is a unicellular chlorophyte or "green" alga. Unlike many eukaryotic algae, *C. reinhardtii* is in within the phylum with vascular plants. It has been established as an amenable model system due to its relatively simple and short sexual cycle, short division time during vegetative growth, the ability to use microbial genetic techniques for isolation of mutants affected in a variety of processes, and an increasing array of available molecular tools. Its nuclear and chloroplast genome have been sequenced and annotated (as described below). Electron microscopy has shown that *C. reinhardtii* has sixteen or more chromosomes (Storms and Hastings 1977), which is consistent with the 17 linkage groups defined by genetic analysis (Kathir et al. 2003). *C. reinhardtii* has two anterior flagella which are organized by basal bodies within the cell. It has a single cup-shaped chloroplast which occupies two thirds of the cell volume (Schotz et al. 1972). The chloroplast is surrounded by an envelope, composed of inner and outer membranes. It also contains the aforementioned thylakoid membranes and typically one pyrenoid, a compartment that specializes in $CO_2$ fixation and starch synthesis.

*C. reinhardtii* is easily cultured on both solid and liquid medium without any requirements for exogenous vitamins or other co-factors (Harris 1989; Harris 2001). It has a sexual cycle that can be precisely controlled. A very important feature of *C. reinhardtii* for genetic analyses of photosynthesis and chloroplast biogenesis is that it does not require photosynthesis when supplied with an exogenous reduced carbon source (e.g. acetate). The implications of this are described in the next section.

### 1.3.1 *C. reinhardtii* lifecycle and genetics

*C. reinhardtii* cells are typically haploid and they reproduce vegetatively by mitosis. Upon deprivation of nitrogen, however, cells differentiate into gametes and the sexual cycle is initiated. Following the fusion of gametes, the zygote forms a zygospore, which is resistant to severe conditions. Gametes of opposite mating type (see below) pair in the flagellar tips, shed the cell walls by secretion of an enzyme called autolysin, and they fuse to form a zygote (Wilson et al. 1997). This zygote eventually sheds the flagella and forms a hard, impermeable zygospore wall. After a period of zygospore maturation, zygotes germinate, meiosis occurs and four haploid meiotic progeny are formed. Thus, tetrad analysis and complementation tests are possible with *C. reinhardtii*. For the latter, complementation is tested in the transient diploid zygotes (i.e., soon after gamete fusion) or in stable vegetative diploids, which arise from a low percentage of zygotes that do not undergo meiosis.

*C. reinhardtii* cells belong to one of two genetically-determined mating types defined by the mating-type (*MT*) locus. The *MT* locus is relevant to the genetic mapping of *TBC1* in this thesis and, thus, a brief description of it is provided here. *C. reinhardtii*

mating-types are indicated as mating-type plus (*mt+*) and minus (*mt-*). The *MT* locus which is complex locus of approximately 1 Mb and located at the distal end of the left arm of linkage group VI. The *mt+* and *mt-* loci each contain a distinct set of genes involved in cell recognition and fusion, zygospore maturation, and mating-type controlled inheritance of organelle genes (Ferris and Goodenough 1997; Ferris et al. 2002). Recombination is suppressed at the MT loci to maintain distinct mating types, as is the case for sex chromosomes.

*C. reinhardtii* genetics has provided a powerful set of tools to identify gene products that function in a variety of processes. The use of *C. reinhardtii* genetics to identify *TBC1* is described below. A variety of wild-type and mutant strains are available from the research community and a centralized strain center. Mutants with abnormalities in basal body function, flagella biogenesis and functioning, and phototaxis have also been identified. Conditional mutants blocked at specific points in the cell division cycle at restrictive temperature have been described (Howell and Naliboff 1973; Harper et al. 2004). Many mutants have been generated and a large collection of them is available. Unlike vascular plants which require photosynthesis beyond the seedling stages, *C. reinhardtii* can be cultured heterotrophically (i.e. without photosynthesis). This has allowed the isolation of many mutants that are affected in photosynthesis and chloroplast biogenesis. From these, a large number of genes and gene products involved in chloroplast biogenesis and photosynthesis have been identified and characterized (as described above). Vascular plants model organisms are less amenable to genetic analyses of photosynthesis and chloroplast biogenesis because they require photosynthesis for viability, except in the cases when phenotypes are analyzed in seedlings or when mutants

are maintained aseptically on media containing sucrose, a condition that is highly prone to contamination by bacteria and fungi.

Many early discoveries relating to photosynthesis and the organization of the photosystems in thylakoid membranes were made possible by the isolation and characterization of photosynthesis-deficient mutants in *C. reinhardtii*. In 1967 Levine and Bennoun developed screen for the isolation of mutants impaired in photosynthesis (Bennoun and Levine 1967) based on detection of high steady fluorescence levels due to blocked electron transport in these mutants. This screen was thereafter widely used for inducing and studying photosynthesis-deficient mutants. All the photosynthetic mutants were characterized as acetate-requiring ($ac^-$), lacking the ability to grow autotrophically, or by high-chlorophyll-fluorescence (hcf). Among the first mutants isolated with this technique was *F34* (Chua and Levine 1969). The mutant gene in *F34* was later named *TBC1* (Zerges et al. 1997). Crosses between *F34* and wild-type strains showed Mendelian inheritance of a single mutant locus, rather than uniparental inheritance characterisic of the transmission of the chloroplast genome. This revealed that *TBC1* is a nuclear locus.

*F34* was used in many genetic studies of photosynthesis. For instance, it was used in a study relating the light-induced absorbance change of photosynthetic mutants to the affected photosystem (Chua and Levine 1969). These authors suggested that since F34 exhibited only the light-induced absorbance peak of photosystem I, the *F34* mutation affected a component in electron transport chain close to photosystem II, which had only been identified by biophysical techniques and not at all characterized at the biochemical level. The readily available photosynthetic mutants in *C. reinhardtii* were also used in determining the membrane polypeptide compositions of thylakoid membranes (Chua and

Bennoun 1975). In these studies, done using *F34* and a suppressor mutant (which later was shown to be the point mutation in the *psbC* 5' UTR) revealed that a 47 kD chloroplast protein was not present in the F34 mutant compared to the wild type. This protein seemed to be associated with the normal functioning of the PSII reaction center, and later this protein was shown to be the CP43 subunit of the PSII reaction center (Rochaix et al. 1989). F34 was used in many studies investigating PSII and its structure (Joliot et al. 1973; Chua and Bennoun 1975; Olive et al. 1979; Wollman et al. 1980). The identification and sequencing of the *psbC* gene revealed that it encodes the polypeptide that is not synthesized in *F34*. This protein was originally called P6, but is now known as CP43, the name used in vascular plants (Rochaix et al. 1989). The absence of CP43 synthesis in F34 also was found to result in a destabilization of the other PSII core subunits, which is now known to be an occurrence for many multisubunit complexes. Several subsequent experiments (reviewed above) revealed that *TBC1* is required for the translation of the *psbC* mRNA.

Currently, approaches for generating mutations in *C. reinhardtii* are achieved by UV or chemical mutagenesis, or by insertional mutagenesis via transformation with exogenous DNA. A few spontaneous mutations have also been found to be induced by transposable elements (Gorinsek et al. 2005).

### 1.3.2 The *C. reinhardtii* nuclear and chloroplast genomes

The ongoing genome project is now almost complete and the approximately $1 \times 10^8$ bp of the genome has been sequenced. Computer programs are accessible online for homology searches and gene predictions (Grossman et al. 2003). The sequence is nearly

completely annotated with respect to predicted and known genes, their orthologues, the sequences of small cDNAs (called expressed sequence tags or ESTs), the genomic DNA inserts in recombinant bacterial artificial chromosomes (BACs), and known molecular markers.

The 203,828 bp circular genome in the *C. reinhardtii* chloroplast has been completely sequenced (GenBank accession number BK000554). The chloroplast genome of *C. reinhardtii* resembles those of vascular plants, e.g., in its richness in A and T residues and the presence of two large inverted repeat sequences (Boudreau et al. 1994). It encodes approximately 100 genes which encode subunits of the photosynthesis apparatus and are required for chloroplast gene expression, and a variety of other functions (Maul et al. 2002). Unlike nuclear genes, which are transmitted by Mendalian patterns of inheritance, chloroplast genes are inherited uniparentally from the *mt+* parent (Sager and Ramanis 1968).

## 1.4 Molecular Biological Tools

This thesis used a variety of molecular biological approaches, molecular markers, and recombinant plasmids that are available for research with *C. reinhardtii.* In particular, this thesis made an important contribution to a widely used transformation procedure and explored the use of bulked segregant analysis (BSA) for the positional-cloning of nuclear genes in this organism. Brief descriptions of these tools and approaches are provided in this section. Traditional molecular approaches have been established for *C. reinhardtii.* In *C. reinhardtii* both the nuclear and chloroplast genomes can be stably transformed with exogenous DNA. The nuclear genome can be

transformed by particle bombardment (Kindle et al. 1989; Mayfield and Kindle 1990), agitation of cells with DNA and glass beads (Kindle 1990; Nelson and Lefebvre 1995), and electroporation (Brown et al. 1991; Keller 1995; Shimogawara et al. 1998). Integration of exogenous DNA into the nuclear genome almost always occurs randomly by nonhomologous recombination, with homologous recombination occurring at a frequency of less that 1% (Sodeinde and Kindle 1993; Gumpel et al. 1994). Typically, transformations are carried out with strains carrying the *cw15* mutation, which produces a cell wall defect to improve transformation efficiency, or wild-type cell that have been stripped of their cell walls with "autolysin" prior to transformation (Kindle 1990). When transformants are selected for the expression of one of two genes on independent plasmids during "co-transformation" of the nuclear genome, approximately 10 % also integrate and express the non-selected gene (Kindle 1990).

The integration of transformed DNA into the chloroplast genome, on the other hand, occurs by homologous recombination. This is useful for conducting site-directed mutagenesis, disruptions of chloroplast genes or targeting of chimeric reporter genes to specific locations. Transformations of the chloroplast with *psbC* 5'UTR having different lesions fused to a reporter gene were used to determine the target sites of *TBC1*, *TBC2* and *TBC3* in *psbC* 5' UTR as described above.

Many selectable marker genes are available for the selection of transformants of both the nuclear and chloroplast genomes (Grossman et al. 2003). *ARG7* (Debuchy et al. 1989; Auchincloss et al. 1999), *NIT1* (Fernandez et al. 1989) and *ble* (conferring zeocin resistance (Stevens et al. 1996)) are regularly used as selectable markers for the transformation of the gene of interest, either on the same plasmid or by co-

transformation. The bacterial gene *aadA* (which confers resistance to spectinomycin and streptomycin) is a commonly used marker for chloroplast transformation and occasionally of the nuclear genome (Goldschmidt-Clermont 1991).

## 1.5. Molecular and genetic markers

A variety of molecular and genetic markers are also available and their application is related to this project, as appropriate molecular markers had to be chosen as part of the mapping of *TBC1*. These markers have known positions on the physical map of the nuclear genome and on the genetic map. Thus, they can be used to identify a specific genomic region with a locus from its position on the genetic map.

### 1.5.1 Restriction fragment length polymorphisms (RFLPs)

RFLPs are restriction fragments that differ in length, or their presence, across polymorphic strains. They are identified by comparing the differences in restriction fragment patterns in a particular genomic region by genomic Southern blot hybridizations using a specific probe. They were one of the first markers to be used for positional cloning in *C. reinhardtii*. Indeed the first molecular map comprising molecular markers anchored to the genetic map of the *C. reinhardtii* nuclear genome was based on the RFLPs (Ranum et al. 1988). The isolation of the exceptionally highly polymorphic *S1D2* strain helped immensely to enrich the mapped RFLP collection (Gross et al. 1988; Lefebvre and Silflow 1999). RFLPs were successfully used for mapping of genes such as *NIT1* (Fernandez et al. 1989). However their use is too laborious as it relies on isolation

of genomic DNA and Southern blot hybridizations. This has directed research to move towards PCR-based methods.

### 1.5.2 Amplified fragment length polymorphisms (AFLPs)

AFLPs were developed as a genomic fingerprinting method based on the detection of PCR-amplified genomic DNA restriction fragments (Vos et al. 1995). They have been used as molecular markers for mapping and the positional-cloning of genes in many plant species including Arabidopsis, tomato and barley (Cnops et al. 1996; Simons et al. 1997; Simons et al. 1998).

AFLPs are generated by digesting total genomic DNA with two restriction enzymes followed by the ligation to their ends of double stranded adapters with known sequence (Vos et al. 1995). Specific subpopulations of these fragments are then amplified by PCR using primers that hybridize to the sequence of the adapters and the adjacent bases remaining from the restriction sites used initially. The addition to the 3' ends of these primers of 1-3 "selective nucleotides" results in amplification of a subset of fragments from the pool that have the complementary nucleotide(s) adjacent to the restriction sites within the fragments.

AFLPs have many advantages over RFLPs. They are much easier to generate and can be used in fingerprinting of any genome without any prior knowledge of the genomic sequence, since PCR primers are based upon the sequences of the adaptors. AFLP simultaneously generates molecular markers from throughout the genome, rather than specific regions searched individually for RFLPs. The vast number of restriction fragments from which the AFLPs are generated allows the amplification of an essentially

unlimited number of markers from a single initial digestion. The use of primers with different selector bases allows the PCR amplification of 100-200 AFLPs and their visualization on a single lane of a polyacrylamide gel. The restriction enzymes can also be varied to increase the number of AFLPs obtained in a particular study. Each AFLP is considered as a locus with two allelic states (present or absent) which are easily scored. In this aspect the AFLP analysis detects only presence or absence of restriction fragments rather than differences in lengths.

Detection of the AFLPs can be achieved via radioisotope labeling (Vos et al. 1995), digoxigenin labeled primers (Vrieling et al. 1997) or even with ethidium bromide staining (Suazo and Hall 1999). Visualization using silver staining (Cho et al. 1996) was shown to closely match the sensitivity of the radio-labeling with $^{32}P$ but providing the important benefit of visualizing the AFLP directly on the gel. This feature would be very useful if AFLP fragments are to be isolated and used for further analytical procedures.

### 1.5.3 Single nucleotide polymorphisms (SNP)

One of the most important tools that can significantly increase the efficiency of positional cloning are SNPs. These markers are specific positions in a genome that differ in a single base across different strains. SNPs can be detected by using different techniques such as template directed dye-terminator incorporation with subsequent fluorescence detection (Chen and Kwok 1999; Hsu et al. 2001), SNapShot™ (Turner et al. 2002), pyrosequencing (Alderborn et al. 2000), oligonucleotide specific ligation (Tobe et al. 1996), dynamic allele specific hybridization (Prince et al. 2001), the Taq-man

system (Lee et al. 1999), mass spectrometry (Ross et al. 1998; Stoerker et al. 2000), oligonucleotide arrays (Lindblad-Toh et al. 2000).

One of the major advantages of SNPs is that they are relatively easy to find since only a single nucleotide substitution is required. In *C. reinhardtii* comparisons of the sequence of the same genomic region in S1D2 and a lab strain, on average of 2.7 substitutions are found in every 100 bp of sequence (Kathir et al. 2003). A definite advantage also is the ease and speed of some of the approaches that are used to detect SNPs.

Currently there is a collection of more than 186 SNP markers that have been identified throughout *C. reinhardtii* nuclear genome (Vysotskaia et al. 2001) and they are available at the web site of the *Chlamydomonas* genetics center at Duke Univesity (http://www.chlamy.org/chlamydb.html). One disadvantage, however, of using SNPs is that their detection is often an expensive process which also requires some special equipment and this in an obstacle in their wide use.

## 1.6 Cloning of *C. reinhardtii* genes by complementation and insertional tagging

### 1.6.1 Identification of recombinant genomic cosmids that complement a mutant phenotype

It is difficult to identify genes on the physical map of a genome if they have only been identified by mutant alleles. Complementation by recombinant genomic cosmids has been used to clone genes that produce a wild-type phenotype that is either selectable (prototrophy for an amino acid or another exogenous compound) or easily identified in screens of large numbers of colonies. Recombinant libraries with random fragments of

*C. reinhardtii* genomic DNA are available in a variety of vectors; bacteriophage λ, cosmids, yeast artificial chromosomes (YACs), and BACs. In this approach, a mutant strain for a gene of interest is transformed with a complex genomic cosmid library and rare transformants that have integrated a cosmid with a wild-type copy of the gene are identified by their wild type phenotype. In a selection scheme, conditions are used that allow the growth of only the phenotypically reverted transformants, i.e. those that have acquired a cosmid with the wild-type gene of interest. When direct selection is not possible, up to $10^4$ transformants are selected with a transformation marker (a library with the ARG7 gene in every cosmid exists for this purpose) and then screened for phenotypic reversion. For example, a single colony having lost the high chlorophyll fluorescence phenotype of PSII mutants can be rapidly identified with a video camera and the appropriate filters and software (Bennoun and Beal 1997). The established techniques for transformation and the abundance and ease of getting mutants (see 1.4.) along with the availability of cDNA, YAC clones (Vashishtha et al. 1996), BAC clones and ordered genomic libraries made cloning by complementation a preferred and straightforward process.

One problem with this approach has been that it is often difficult to retrieve the complementing cosmid from the genomic DNA of the transformant. While this has been successful in many cases, in others it was never achieved. Unfortunately, autonomously replicating vectors for *C. reinhardtii*, which can be easily isolated because they do not integrate, have not been developed. Nevertheless, these problems have been resolved by the development of indexed cosmid libraries, a set of 100 microtiter plates in which each of the 96 wells contains an *E. coli* strain with a distinct genomic cosmid. Researchers

prepare the 96 cosmids from a culture of all of the *E. coli* strains on a each plate and then test these pools individually for the ability to complement a mutant phenotype by transformation (as described above). When a pool that contains a complementing cosmid is identified, the *E. coli* strains from each row and from each column are cultured and then smaller subpopulations of cosmids are prepared and tested for the ability to complement the mutant phenotype. The well at the intersection of the row and column that test positive should contain the *E. coli* strain with the cosmid that complements the mutant allele. This is determined by yet another transformation of the mutant with that cosmid. While this approach is laborious compared to cloning by complementation with *S. cerevisiae*, it has proven to be the most efficient means for cloning by complementation in *C. reinhardtii*. The rescuing cosmid is then digested and restriction fragments are tested for complementation to identify the minimal region containing the gene of interest. DNA sequence from anywhere in the cosmid can be used in a Blast search of the genome to identify the region from which the cosmid was derived. An ordered cosmid library of 11,280 individual clones arranged in 120 microtiter plates was made (Zhang et al. 1994) and is successfully used for identification of genes (Purton and Rochaix 1994; Funke et al. 1997; Rattanachaikunsopon et al. 1999; Dauvillee et al. 2003). Purton and Rochaix (1994) prepared a cosmid library in a vector containing a wild-type copy of Arg7 gene. Arginine-prototrophic transformants of an *arg7* mutant can be selected and then screened for complementation of the original mutation. A cosmid library with cosmid vectors containing a copy of Ble (Stevens et al. 1996) (conferring zeocyn resistance) was also created (Chang et al. 2003). *TBC2* was recently identified by this approach (Auchincloss et al. 2002). The known alleles of *tbc2* are stable,

spontaneous revertants of its mutant phenotypes do not arise. Thus complementation was a reliable approach since mutations are stable and easily selectable. Pools of genomic cosmid clones from an indexed library were used to complement the PSII deficiency of *tbc2*, and after finding a pool that rescued the mutation, a single cosmid was isolate that was able to complement it, digesting and subcloning fragments of it identified Tbc2 (Auchincloss et al. 2002).

### 1.6.2 Cloning genes that are tagged by insertion mutagenesis from the insertion sequences

Insertion mutagenesis is also used to tag genes as a cloning approach. When an inserted sequence disrupts a nonessential gene, the insertion can be used as a probe for hybridization to clone the gene. PCR-based approaches also exist. This approach is favored by the ease of nuclear transformation and the nonhomologous, random integration of transforming DNA. A large number of genes required for a particular process can be identified and cloned in a large-scale project. The insertion frequencies are high enough so that the approach can be used to obtain mutants with specific phenotypes and to characterize the genes that cause them. This technique has been used to clone tens of genes so far.

Other constructs have selectable markers conferring antibiotic resistance (Nelson and Lefebvre 1995; Kovar et al. 2002). In some cases transposon tagging of genes has been used as a cloning method in *C. reinhardtii*, but transposition cannot be controlled to generate a mutagenized population of cells (Schnell and Lefebvre 1993).

Cloning by complementation cannot be used universally though. First it requires a mutant allele of the gene of interest that has wild-type phenotype that can be selected or rapidly identified in a screen of up to $10^4$ colonies. It is also inapplicable for the identification and cloning of genes for which such mutant alleles do not exist. For example, the phenotypes of most motility mutants can only be identified by microscopy or in individual liquid cultures, assays that are too laborious to carry out on thousands of transformants. In the latter case of genes identified by dominant alleles, or suppressor mutations, construction of new genomic library from the strain carrying the suppressor mutation is required.

The insertion tagging also has limitations. Because insertions almost always cause a complete loss of gene function, tagging by insertion cannot be used to identify essential genes. Insertions generate limited types of phenotypes since predominantly null mutations are generated and difficulties occur in analysis of the large deletions flanking the integration site that usually occur. It is inapplicable when suppressors of photosynthetic mutations are recovered from photosynthetic mutants. For example in the case of PS+ suppressors, a recessive suppressor would yield a PS− phenotype upon complementation, and even dominant suppressors require construction of a genomic library from the suppressed strain if they are to be cloned by complementation. Furthermore identifying a gene by complementation requires that it has a stable mutant phenotype as spontaneous phenotypic reversion could pose quite a challenge in this case (reviewed by Rymarquis et al. 2005). Although in many cases both approaches have worked for identifying of photosynthetic mutants (Gumpel et al. 1995; Boudreau et al.

2000; Auchincloss et al. 2002), they still are associated with the drawbacks described above.

Cloning of *TBC1* was attempted by complementation. However, the only mutant allele, *tbc1-F34*, reverts to wild-type at a frequency of approximately $10^{-5}$ (data not shown). Thus, direct selection of ac+ transformants of a genomic cosmid library is not possible because every petri plate had c.a. 100 wild-type spontaneous reverant colonies. When 3000 transformants were selected for the ARG7 transformation marker in the vector of a cosmid library, only 10 colonies were obtained per plate and none were wild-type for *TBC1* (W. Zerges, unpublished data).

The recent sequencing and annotation of the nuclear genome has allowed the use of mapped molecular markers more efficiently. A search for candidate genes in a region could be easily performed using the BLAST tools available at (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Chlre3&advanced=1). This is an important point in this project as the approach that was used to identify *TBC1* relied on the molecular markers (described above) and on the ability to search for potential candidate genes through the genomic sequences mapped to a certain distance from any given marker. Finding genomic BAC clones covering specific regions or predicted genes and performing complementation tests with these clones is much easier. This, along with the difficulties that would have been experienced with any of the other cloning methods due to *tbc1* reversion frequency, made us look for an alternative map based approach for mapping of genes in *C. reinhardtii* in order to identify *TBC1*.

## 1.7 Map Based Cloning

In each of the approaches described above, where other methods of identification would not be appropriate, isolation of the gene of interest could be accomplished by map-based cloning. Map-based cloning, or positional cloning, is based on finding linkage of the gene of interest to molecular markers with known physical locations in the genome.

In positional cloning genetic mapping of the mutant allele with respect to known molecular markers narrows down a genomic region containing the gene of interest. Depending on the size of the defined interval other methods may be required to accurately identify the gene. If the positional cloning points out a small region the annotated genome sequence would be sufficient to point out a candidate gene. In the case of ending up with a wider region hosting the mutated gene molecular complementation with overlapping genomic DNA fragments can be employed to further narrow down the position and determine the sequence of the gene. Positional cloning has become common for identifying genes in *Arabidopsis thaliana* that had been identified by genetic approaches (reviewed by Lukowitz et al. 2000), due to the completed genome sequence and the availability of thousands of molecular markers. It has been effectively used to clone genes in, rice, maize, and barley. In humans positional cloning is the main method for identification of mutations that cause disease (reviewed by Guo and Lange 2000). In *C. reinhardtii* a molecular map was generated (reviewed by Kathir et al. 2003) using the polymorphic strain S1D2, which compared to other lab strains has an abundance of polymorphic molecular and genetic markers. (Gross et al. 1988; Vysotskaia et al. 2001; Grossman et al. 2003). Kathir et al. (2003) published 264 molecular markers placed on the 17 linkage groups of *C. reinhardtii* genome. Currently the collection of

markers of all laboratories contributing to the *Chlamydomonas* genome project consists of 385 (Rymarquis et al. 2005). The total length of this map is 1,025 centimorgans.

The markers were anchored to the genetic map by using as reference points genes corresponding to mapped phenotypic markers or from data obtained by tetrad analysis. This combined with the recently indexed BAC clones available at Clemson University Genetics Institute (CUGI) is a base for making positional cloning in *C. reinhardtii* a preferred method for identification of genes.

## 1.8 Bulked-segregant analysis (BSA)

BSA is a rapid method for the identification of molecular markers that are linked to a mutant allele of a gene of interest. The method is based on comparison of pooled DNA of individuals from a segregating population of a single cross (Michelmore et al. 1991). The pools, or "bulks", are comprised of individuals identical for the trait/gene of interest but arbitrary for all other genes. In comparison the two bulks are genetically different in the region of interest but due to independent segregation and recombination they are both equally heterozygous in all the other regions. Due to linkage and cosegregation however, only markers that are genetically linked to the gene of interest are polymorphic between the DNA pools. The second step of this approach is the screening of the bulks for differences in their different molecular markers. Since this method was never reported on *C. reinhardtii* the reliability of different molecular markers and the precision of the approach had to be tested. Here the use of AFLP and subsequently SNP markers in combination with BSA is reported along with the precission of mapping for

each of them. These markers were chosen due to their abundance and relatively easy scoring, as described in detail in 1.5.3.

This approach has the potential to significantly simplify map-based cloning as the testing of individual progeny is no longer required.

Positional cloning in *C. reinhardtii* to date has consistently involved the screening of molecular markers in many individual progeny from a cross (reviewed by Rymarquis et al. 2005). This requires hundreds of PCR reactions in order to test for the linkage of a single molecular marker in each single progeny of each cross (Bowers et al. 2003). This is expensive in terms of the time and labor required for mapping. Although BSA was used in other organisms such as Arabidopsis, Brassica sp., Helianthus sp. (Lukowitz et al. 2000; Chen et al. 2006; Yi et al. 2006) and although it was recently proposed (Rymarquis et al. 2005), it still has not been used for map-based cloning in *C. reinhardtii*. Using bulked progeny rather than individuals would shorten the time and decrease the cost required to screen a large number of molecular markers and to map a particular gene. A major benefit is that the bulks can be generated for any genomic region and from any segregating population and that it is independent of the phenotype of the mutation of interest, as long as the mutant is viable In this aspect combining BSA with the available mapped molecular markers would allow faster identification of the many existing mutations with non-selectable phenotypes. Therefore a map-based cloning project was initiated, in which BSA was used to test for linkage of *TBC1* to AFLP and SNP markers as a new method for the positional cloning of *C. reinhardtii* genes.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1. *C. reinhardtii* strains and culture conditions

*C. reinhardtii* strains (Table 1.) were cultured on tris-acetate-phosphate (TAP) medium (Harris 1989) supplemented with 55 μg/ml arginine. Wild-type strains were cultured under bright light (c.a. 150 $\mu E/cm^2$) while PSII mutants were grown under dim light (c.a. 20 $\mu E/cm^2$) to avoid advantageous growth of spontaneous *tbc1* revertants. PSII deficiency was tested by the inability to grow on high salt minimal medium (HSM(Harris 1989)), which lacks a reduced carbon source. The arginine auxotrophy phenotype produced by *arg7-8* was tested by inability of growth on TAP medium without arginine.

| Strain name | Genotype |
|---|---|
| Harris#6 | Wild type (WT) |
| Jarvick#6 | Wild type (WT) |
| Jarvick 124 | Wild type (WT) |
| Quebec WT | Wild type (WT) |
| *C. smithii* | Wild type (WT) |
| S1D2 | Wild type (WT) |
| $4C^{+}$ | Wild type (WT) |
| cc1618 | *cw15* |
| F34.4- | *tbc1-F34, mt-* |
| F34.4+ | *tbc1-F34, mt+* |
| F34.3+ | *tbc1-F34, mt+* |
| F34.2A+ | *tbc1-F34, mt+* |
| *F34.2A+ | WT (tbc1-F34 spontaneous revertant) |
| F34.4- | *tbc1-F34, mt-* |
| WZ120 | *tbc1-F34:arg7:cw15* |
| AS74 (generated in this study) | *tbc1-F34:arg7:cw15* |
| AS76 (generated in this study) | *tbc1-F34:arg7:cw15* |
| AS82 (generated in this study) | *tbc1-F34:arg7:cw15* |
| AS86 (generated in this study) | *tbc1-F34:arg7:cw15* |

**Table 1.** ***C. reinhardtii*** **strains**

## 2.2 Bulked segregant analysis

### 2.2.1 *C. reinhardtii* genetic crosses, generation of wild-type and mutant bulks

Strains were transferred to fresh TAP media (solidified with agar) every three days several times, prior to gametogenesis. Vegetative cells were induced to form gametes by starving them for nitrogen. Cultures (10 ml) of TAP medium without N were inoculated with the two strains to be mated and incubated on a shaker (140 rpm) overnight. The two cultures were then combined and left in dim light for two hours to mate.

The mating mixture was then centrifuged at 3 - 4 K rpm for 5 minutes. Cell pellets were resuspended in approximately 200 µl of the remaining supernatant and these were plated on a TAP solidified with 4% agar in a petri plate. The plate was left in moderate light for 8 hours in order for the cells (zygotes and non-mated cells) in mating mixture to adhere to the agar and then it was wrapped in parafilm and aluminum foil and incubated for 5 days at 24°C.

Vegetative cells were then scraped off the 4% TAP plate using a sterile razorblade. Any vegetative cells left on the plate were then killed by placing the agar surface above chloroform vapors for 45 seconds. Zygotes remained adhered to the agar and they were then transferred to a fresh petri plate with TAP (solidified with 1.5% agar) supplemented with arginine and were allowed to germinate overnight at 24°C under light. The asci were then dissected and the four ascospores from each ascus were separated. Plates were incubated under appropriate conditions so that each spore forms a single isogenic colony. BSA was conducted by crossing *C. reinhardtii* strains listed in Table 2.

| Cross | Names of the generated bulks |
|---|---|
| Harris#6 x F34.4- | Harris WT (wild-type) and Harris MUT (mutant) |
| Jarvick#6 x F34.4- | Jarvick WT (wild-type) and Jarvick MUT (mutant) |
| Jarvick 124 x F34.4+ | Jarvick124 WT (wild-type) and Jarvick124 MUT (mutant) |
| Quebec WT x F34.4+ | Quebec WT (wild-type) and Quebec MUT (mutant) |
| S1D2 x F34.4+ | S1D2 WT (wild-type) and S1D2 MUT (mutant) |
| *C. smithii* x F34.4+ | *C. smithii* WT (wild-type) and *C. smithii* MUT (mutant) |

**Table 2. Crosses performed to generate bulks**

### 2.2.2 Screening for PSII mutants

Precision of the BSA required bulks of purely wild-type or *tbc1* mutant progeny. Therefore progeny were scored for two phenotypes produced by *tbc1-F34*, high chlorophyll fluorescence and the inability to grow on HSM (the *ac-* phenotype), in multiple rounds of screening and any ambiguous progeny were discarded. Bulks of approximately 150 progeny were generated by scrapping similar amounts of cells growing in patches on solidified TAP medium and then resuspending them in liquid medium. These bulks were cultured for 6-8 hours and then aliquoted and stored at -80°C.

## 2.3 Isolation of genomic DNA

For pooling genomic DNA and Southern blot hybridization experiments, genomic DNA was isolated using a method involving cetyl trimethyl ammonium bromide (CTAB). Samples of 1.5 ml from a very dense culture were centrifuged at 5,000 rpm for

5 min and the cell pellet was resuspended in 500 µl of CTAB buffer (2% CTAB, 100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA). An aliquot (58 µl) of 20% SDS was added, samples were mixed and incubated at 65°C for 1 hour. Then, several phenol:chloroform:IAA (25:24:1) extractions were performed until there were no longer visible debris in the interphase after centrifugation. Insufficient extraction sometimes resulted in degradation of the genomic DNA during subsequent restriction digests. At least two chloroform-isoamyalcohol (24:1) extractions were done followed by an isopropanol precipitation of the nucleic acids. Pelleted DNA (and RNA) was resuspended in TE (10 mM Tris HCl pH 7.0, 1 mM EDTA). When required, RNA was degraded by adding RNaseA to the buffer, followed by size exclusive PEG precipitation (Sambrook and Russell 2001). Adding beta-mercantoethanol to the CTAB buffer (as originally described) was not found to positively influence the quality of the DNA prep. In addition a previously described treatment with proteinase K was also found not detectably beneficent for the prep and was omitted.

## 2.4 Amplified fragment length polymorphisms

### 2.4.1 Generation of AFLPs, restriction digests, ligation of adapters, PCR

Restriction digests of the pooled genomic DNA extracted from the bulks were performed with pairs of enzymes: *Pst*I and *Mse*I; *Bam*HI and *Mse*I. Consecutive single digests were performed in order to provide optimal conditions for each enzyme. To ensure that both the genomic DNA was digested to completion by both enzymes, two samples were subjected to the digestion conditions, digestion was verified by agarose gel electrophoresis and staining with ethidium bromide. When digestion under both

conditions was confirmed, each sample was treated with the conditions of the other and complete digestion was assumed.

Adaptors shown in Table 3 were ligated to the generated restriction fragments (according to (Sambrook and Russell 2001).).

| Adaptor | Structure |
|---|---|
| MseI | 5'-GACGATGAGTCCTGAG-3'<br>\| \| \| \| \| \| \| \| \| \| \| \| \| \| \| \| \|<br>3'-TACTCAGGACTCAT-5 |
| BamHI | 5'-CTCGTAGACTGCGTACC-3'<br>\| \| \| \| \| \| \| \| \| \| \| \| \| \| \| \| \|<br>3'- CATCTGACGCATGGCTAG -5 |

**Table 3. AFLP adaptor structure**

Two rounds of PCR were carried out in order to generate AFLPs (Vos et al., 1995). All primers combinations used in PCR reactions generating AFLPs for this project are shown in Table 4. Primers were supplied by Operon$^{TM}$ and their sequences are given in Table 5.

Preamplification, or the PCR amplification of a very complex set of fragments with the ligated adaptors, generated a pool of templates from which different subsets of AFLPs could be amplified with primers having additional selective bases (as described in Results). This was carried out using primers with one selective base (Table 4., PCR-1). It was conducted in a final volume of 20 µl containing 5 µl template restriction digested bulk DNA, 30 ng of each primer, 2 µl 10X Fermentas PCR buffer without $MgCl_2$, 0.2

mM of the four dNTPs (0.4 µl from 10 mM dNTP mix), 1.5 mM $MgCl_2$ (1.2 µl of the 25 mM $MgCl_2$ and 0.4 U Taq Polymerase (0.08 µl from 5 U/µl Fermentas Taq Polymerase). PCR was conducted using a GeneAmp 2400 system in 20 cycles: denaturation at 94°C for 30 sec, annealing at 56°C for 60 sec, and extension at 72°C for 60 sec.

The preamplification PCR product was diluted ten fold in $ddH_2O$ and 5 µl was used as a template for the subsequent secondary PCR amplification, conducted using primers as in the preliminary PCR but with an additional selective base (Table 4., PCR-2). The second amplification was carried out in a final volume of 20 µl containing 5 µl of the diluted preliminary PCR product, 30 µg of each primer, 2 µl 10X Fermentas PCR buffer without $MgCl_2$, 0.2 mM of the four dNTPs (0.4 µl from 10 mM dNTP mix), 1.5 mM $MgCl_2$ (1.2 µl of the 25 mM $MgCl_2$ and 1 U Taq Polymerase (0.2 µl from 5 U/µl Fermentas Taq Polymerase). This secondary PCR was conducted with an initial denaturation step at 94°C for 3 minutes. Then 37 cycles were performed with the following conditions: denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec during the first two cycles, extension 72°C for 60 sec. The annealing temperature was decreased by 1.4°C every two cycles, until it reached 56.6°C. After performing two cycles with annealing temperature of 56.6°C, it was decreased to 56.0°C and additional 23 cycles were performed. A final extension step at 72.0°C for 5 minutes was performed after the last cycle.

Samples of 8-10 µl from each of the two PCR reactions (one from each bulk) were electrophoresed on a 0.8% agarose gel next to 1Kb or 100bp+ Fermentas molecular DNA marker and stained with ethidium bromide, in order to confirm the amplification.

| Bulk | AFLP PCR-1 | AFLP PCR-2 |
|---|---|---|
| **S1D2 x F34.4+ MUT/WT** | MseI-G / Bam-T | MseI-GA / Bam-T* |
| | MseI-G / Bam-A | MseI-GA / Bam-A |
| | MseI-G / Bam-G | MseI-GA / Bam-G |
| | MseI-G / Bam-C | MseI-GA / Bam-C |
| | MseI-T / Bam-T | MseI-T / Bam-T |
| | MseI-G / Bam-T | MseI-GAC / Bam-T |
| **Harris x F34.4+ MUT/WT** | MsIe-G / Bam-T | MseI-GA / Bam-T |
| | MseI-G / Bam-A | MseI-GA / Bam-A |
| | MseI-G / Bam-G | MseI-GA / Bam-G* |
| | MseI-G / Bam-C | MseI-GA / Bam-C* |
| | MseI-G / Bam-C | MseI-GAC / Bam-C |

**Table 4. PCR primers used in all amplification reactions generating AFLP**

Bulks and primers combinations used to generate AFLPs in this project. In all these cases bulks were digested with enzymes *MseI* and *BamHI*. Subpopulations of the obtained restriction fragments were amplified in two rounds. For each bulk, primers used in the first round of PCR are listed in the graph AFLP PCR-1. The product of this PCR was used as a template for a second round with the corresponding primers listed in the graph AFLP PCR-2. Primer combinations that yielded the unique AFLP bands that were sequenced and used for mapping are marked with asterisks.

| Primer name | Primer sequence 5'- 3' |
|---|---|
| MseI-G | GATGAGTCCTGAGTAAG |
| MseI-GA | GATGAGTCCTGAGTAAGA |
| MseI-GAC | GATGAGTCCTGAGTAAGAC |
| MseI-T | GATGAGTCCTGAGTAAT |
| PstI-T | GACTGCGTACATGCAGT |
| BamHI-T | GACTGCGTACCGATCCT |
| BamHI-A | GACTGCGTACCGATCCA |
| BamHI-C | GACTGCGTACCGATCCC |
| BamHI-G | GACTGCGTACCGATCCG |

**Table 5. Primers used to generate AFLPs from bulked DNA.**

Selective nucleotides are added to the 3` end of the sequence binding to the adapters ligated to the total genomic digest and the restriction site. The names of the primers consist of the names of the restriction enzymes used to cut the genomic DNA and the selective nucleotide(s) added at their 3` ends.

### 2.4.2 Detection of AFLP, PAGE, Silver-staining

The secondary AFLP PCR products were resolved on denaturing polyacrylamide gels (Sambrook and Russell 2001). Gradient polyacrylamide gels were used to resolve the initial problem of having an uneven distribution of the fragments with more AFLP

clustered at the top of the gel, which made detection more difficult and introduced a risk of contamination by other AFLPs during excision.

Samples (3 µl - 5 µl) of the secondary PCR products were denatured at 80°C for 10 minutes, then immediately transferred on ice and run on a 6% buffer-gradient denaturing polyacrylamide gel (Sambrook and Russell 2001) next to a 100 bp DNA molecular marker. AFLPs from wild-type and mutant bulks of each cross were resolved in adjacent lanes in order to easily compare and find unique bands.

After electrophoresis, the gel was fixed in 10% glacial acetic acid (200 ml glacial acetic acid in 1.8 l $dH_2O$) for ~30 minutes, until the loading dye on the gel was no longer visible (the gel could be left in the fixing solution overnight) and silver-stained using a kit (Promega) following the manufacturer's protocol.

The silver staining was performed as follows. After fixing, the gel was washed in $dH_20$ 3 times for 2 minutes each. After each washing step the gel plate was allowed to drain for 20 seconds. Immediately after that the gel plate was transferred to the staining solution (2 g silver nitrate to 2 l $dH_20$ + 3 ml 37% formaldehyde) and stained for 30 minutes. After that the gel was rinsed in $dH_20$ for 10 seconds and transferred to the developing solution (60 gm sodium carbonate (anhydrous) to 2l $dH_20$ + 3 ml formaldehyde + 400 µl sodium thiosulphate (10 mg/ml)), which had been precooled to 4°C. The developing time proved to be crucial. The gel was developed with gentle agitation of the solution until bands were clearly visible (usually 5- 7 min). Prolonged development resulted in a high, dark background. In order to terminate the developing process the gel was transferred to cooled (5 - 10°C) fix solution (10% glacial acetic acid)

for 5 min. After this the gel was washed 2 times for 5 min each in $dH_20$, air dried and analyzed.

Many different primer combinations were tested; however only certain primer/selective nucleotides combinations yielded easily identifiable bulk specific AFLPs (Table 4.). Other primers either amplified no detectable bulk specific AFLPs or the AFLPs were too close to each other to allow excision of a single AFLP.

### 2.4.3 Extraction and amplification of DNA from polyacrylamide gels

DNA from unique AFLPs to one bulk (Figure 1.) were extracted from the gel and amplified in order to generate enough DNA for sequencing. Each band was carefully excised with a sterile pre-wet syringe needle from the polyacrylamide gel, put in a 1.5 ml Eppendorf tube with 50 µl of TE and vortexed at maximum speed for 5 min. Then the tubes were centrifuged for 1 min at 13,000 rpm at 4°C. The supernatant was transferred to a new Eppendorf test tube and was used as a template for PCR amplification, after determining the optimal amount that yielded most PCR product (usually 0.005 – 0.5 µl). The PCR conditions were the same as the second AFLP PCR described above (see 2.4.1). The primers were the same as those used in the second AFLP PCR for the pool the bands were excised from.

Initial attempts to extract the DNA fragments from the gel using different protocols with elution buffers proved to be inefficient as some component of these buffers or damage to the DNA seemed to inhibit the Taq polymerase during consequent PCRs.

PCR was scaled up to amplify enough product to be purified from an agarose gel and sequenced (c.a. 0.2-1 μg). After verification of the PCR product on a 2% agarose gel, each sample was precipitated with ethanol, and pellets were resuspended in 10 – 15 μl of TE and then the entire sample was electrophoresed on a 2% agarose gel. The gel was stained using ethidium bromide and the bands corresponding to each AFLP fragment were cut from it. DNA was extracted using the Quiaex II gel extraction kit (Quiagen), following the manufacturer's protocol. Initial attempts to extract the DNA from the agarose using the phenol-freeze-fracture method (Bewsey, 1991) yielded a sufficient amount of DNA but resulted in poor subsequent enzymatic reactions (Sequencing PCR) probably due to remaining traces of phenol.

The gel purified fragments were verified by resolving them in a lane adjacent to the original pool specific bands in a subsequent denaturing PAGE. Samples with 75 ng of each amplified and gel purified unique AFLP were run on a denaturing polyacrylamide gel next to the corresponding wild-type and mutant bulks in order to confirm that co-migrate with the originally observed unique AFLP fragments. Four unique AFLPs were sequenced (Table 9.)

The concentrations of the samples were determined by ethidium bromide spot assay and 40 ng of each was used for sequencing from double-stranded templates by a commercial sequencing service.

## 2.5 AFLP sequence analysis

The sequences of the four AFLP were then compared to the *C. reinhardtii* genome using JGI *C. reihardtii* BLAST v2.0 (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=chlre2) to define identify scaffolds in the genome that contained them (data shown in Table 9.). After identification of these scaffolds, a search was done in the molecular maps prepared as part of the Chlamydomonas Genome Project by Drs. Carolyn Silflow and Paul Lefebvre at the University of Minnesota which feature the known and mapped molecular markers for each linkage group and the scaffolds on which they were found (http://www.chlamy.org/bac.html). This search showed that the scaffolds with these AFLPs (Table 9.) are all located in Linkage group VI. Furthermore using these data at least one molecular marker was identified that was in each scaffold, which by using the available molecular maps (http://www.chlamy.org/nuclear_maps.html) provided to the *C. reinhardtii* online database by the same contributors, gave an approximate idea of the relative positions of those scaffolds on the arms of the linkage group (data showed in Figure 2.).

## 2.6 Mating type PCR

The mating type loci were detected by PCR with primers and conditions previously described by Zamora (Zamora et al. 2004). Adding an initial denaturing step of 4 minutes at 94°C was shown to significantly increase the effectiveness when amplifying from total genomic DNA. In addition, for an unknown reason, when amplifying from bulks the PCR required higher amount of template, approximately 800 ng.

## 2.7 Identification and detection of SNP markers

### 2.7.1 Identification of SNPs on linkage group VI

Different markers mapped to linkage group VI were identified by searching through the available molecular map prepared by Dr. Lefebvre at the University of Minnesota (http://www.chlamy.org/bac.html; http://www.chlamy.org/nuclear_maps.html) shown in Figure 2. A search performed for the identified markers in linkage group VI in the Chlamydomonas Genetics Center Database (http://www.chlamy.org/cgi-bin/ace/searches/browser/default) showed the sequence and the polymorphic base-pair of each marker.

SNP markers were chosen and SNP primers sequences were obtained from the same source. The SNP markers used in this project and their sequences are listed in Table 6. Primers were synthesized by Operon™.

| SNP marker | PCR primers | PCR product size | SNP flank primer | SNP for strains S1D2 and F34.4+ |
|---|---|---|---|---|
| RPL41 | forward AGGAACGTGTAACGAGTCCG<br>Reverse GCAAGCTTGTCTGAAATACCG | 263 | Forward – TGGGACTGCCGGCTGCTGCTTTGGTCTGCA<br>Reverse - GTTCAGGGGTAACCGATTCGAGCGTTCTGG | F34.4+ C, S1D2 T |
| CNA9 | forward ATCTGCTGGGGACTGGGAAT<br>reverse GAATGAGCGTTGTGAAGGGC | 241 | forward – CAATCTGCCTTAACCCTGTCCCA<br>reverse CCGAGGTACGTAGTGCAAGAAGAGTGTGCG | F34.4+ T, S1D2 C |
| PF14 | forward ACAAGGCCTACACCAACCAC<br>reverse TGCTGGGTCACTGTAAGACG | 325 | forward GGCGGGSCCTTACGCGCCCTCCGCCTCGGC<br>reverse ACAAGGCCTACACCAACCACCCCGCGTT | F34.4+ G, S1D2 A |
| CNC30 | forward TGTTTACAGGTGGAGTCGCA<br>reverse CGGCAATAAGCTCTCGACAC | 151 | forward TGTTTACAGGTGAGTCGCAGCCCG<br>reverse TGCAGCCACACGAGACGTTTGAGCCCACCT | F34.4+ G, S1D2 A |
| ICL | forward TCGCCCGCCCTGTCCCTCCT<br>reverse CAGCAGAGTGACACAGACKC | 160 | forward GGTTTCCCGCATCAACAACGCCTTCCAGGT<br>reverse TGACATGTGCAAGCATGTCATCCAGCGCCT | F34.4+ A, S1D2 G |
| S813 | forward CCACTCCCTGTAAATGCCCA<br>reverse GCGTTACAGATCGCCAGTCC | 194 | forward CGCCRCCYGTTGAGAGGCGGGCGGCGT<br>reverse GCGACGCATCCTCAACACACCGCGACGCAG | F34.4+ G, S1D2 T |
| GP337 | forward AAGACCCGTCCCCGCCAGCA<br>reverse AGGACGACTCTGTGGGCAAG | 130 | forward TCCCCGCCAGCAGCTCACCATGGACGT<br>reverse GTACGACGCCCTGATGTACGGCGGCCTGCC | F34.4+ G, S1D2 A |
| CNA79 | forward ATCCCGAGGTAACCCCAA<br>reverse GATGCACTGTGGTTCCAATG | 159 | forward AGCGAAGCTATCCCATAGGTAACCCCAAAC<br>reverse GACAACCCTGGGTGCACCCCTCAGGCAACA | F34.4+ C, S1D2 T |
| GP350 | forward GGGAAGTTGGTTCAGCACAT<br>reverse CCAATGTAAGTGTTGCACGG | 380 | forward AGAAGTACAAGACCTCCCACTCGCCGCTCA<br>reverse GCCGCCAGCTGGCCCACATACGGCCGCACG | F34.4+ A, S1D2 G |

**Table 6. SNP primers and expected products.** PCR primers for each SNP used for amplifying a fragment with a corresponding size containing the SNP. Only one of either SNP flank primers was used to amplify the SNP. The expected products for both parents are shown. The first 6 markers are mapped to linkage group VI. The other three GP337, CNA79, GP350 were randomly chosen from different linkage groups (VIII, I and X resp.).

### 2.7.2 Detection of SNP markers. SNP PCR-1, purification of PCR products

Detection of the two bases for each SNP in the bulks was done in two consecutive PCR reactions. The first amplified a fragment containing the SNP. The second reaction consisted of several cycles of chain termination. In it, a primer adjacent to the position of the SNP is extended by the dideoxynucleotide corresponding to the base of that position using the fragment from the first PCR reaction as template. Thus, the second SNP PCR is essentially a chain-terminating sequencing reaction at one position. The only nucleotides added to the PCR mix are all four fluorescencntly labeled ddNTPs and only one primer hybridizing to the sequence adjacent to the SNP (SNP flank primer). Thus extension of the primer is with the addition of only the one dideoxy nucleotide that corresponds to the SNP.

The first SNP PCR was carried out using PCR primers listed in Table 6. It was conducted in a final volume of 25 µl containing 100 ng template bulk DNA (S1D2 WT or S1D2 MUT), 0.8 uM of each SNP primer (2 µl of 10 uM primer solution), 2.5 µl 10X Fermentas PCR buffer without $MgCl_2$, 0.2 mM of the four dNTPs (0.5 µl from 10 mM dNTP mix), 1.5 mM $MgCl_2$ and 2.5 U Taq Polymerase (0.5 µl from 5 U/µl Fermentas). The Taq polymerase was added to each PCR test tube after the initial denaturation step (see below).PCR was conducted using a GeneAmp 2400 System with initial denaturation at 95°C fro 8 - 10 min (hot start PCR) followed by 35 cycles each having a denaturation step at 94°C for 60 sec, annealing at 56°C for 60 sec, extension at 72°C for 1 min 30 sec.Products were electrophoresed on 1.5- 2% agarose gels in order to verify their lengths (provided by Chlamydomonas Genetics Center Database (Table 6.).

Initial optimization of the PCR conditions showed that using a "hot-start" PCR (in which Taq polymerase was added to the already denatured at 95°C DNA template) with a longer initial denaturation step greatly improved amplification from the bulks as well as from *C. reinhardtii* genomic DNA. Although a thermal cycle with a shorter initial denaturing step was more convenient because it was not necessary to add the Taq polymerase to every reaction afterwards, this worked inconsistently.

Amplification of the fragment with the ICL SNP required a high annealing temperature of 60°C due to the higher Tm of the primers used. The published size of the SNP PCR-1 product in the Chlamydomonas online database of 160bp was incorrect and the correct size is approximately 199 bp.

A sample of 20 µl of the product of the first SNP PCR was purified by adding 1 µl (20 U) Exonuclease I and 0.5 µl SAP. The mix was then incubated at 37°C for 1 hour 30 min and then at 80°C for another 20 min. The PCR thermal cycler was used for the incubation.Initial attempts to purify the fragments using gel purification and agarose gel extraction using Quiagen Quiaex II Gel Extraction kit resulted in aberrant SNP signals.

### 2.7.3 Detection of SNP markers. ABI Snapshot PCR (SNP PCR-2), purification of the PRC product

The purified products were then used as template for a second ABI Snapshot PCR (SNP PCR-2). In this PCR only one of two possible primers (SNP flank primers) was used, as the primer flanks the SNP and the PCR reaction extends it with only one nucleotide complementary to the SNP. Primer sequences are shown in Table 6 (SNP flank primer). PCR was carried out in a final volume of 10 µl with 0.2 pmol (1 µl of 2

pmol/μl stock) of either flank primer, 1 μl of 5x Sequencing buffer, 0.5 μl SNAPSHOT mix and 2 μl of the purified SNP PCR-1 product were used as template. Sequencing buffer and SNAPSHOT mix (containing labeled ddNTPs and Taq) were provided by Applied Biosystems. The thermal cycle used was 40 cycles with denaturing step at 95°C for 10 sec., annealing at 50°C for 5 sec. and extension at 60°C for 30 sec. The SNP PCR-2 product was purified adding 1 μl of SAP to the PCR test tubes and incubating first at 37°C for 45min – 1 hour and then at 80°C for 20 min.

### 2.7.4 Detection of SNP markers. Using ABI310 and Genotyper® Software v 3.7 to detect SNP signals, calculating genetic distance

A sample of 1 μl of the purified SNP PCR-2 product was added to 15 μl of HiDi and 0.05 – 0.25 μl of LIZ size standard (provided by Applied Boisystems). Samples were denatured at 95°C for approximately 4 min., and then immediately transferred to ice and loaded to an ABI PRISM® 310 DNA Sequencer. Genotyping results were then analyzed with the Genotyper® Software v 3.7, which was used for visualizing the detected SNP signals as peaks of different colors, each corresponding to a certain labeled ddNTP; red, T; black, C; green, A; blue, G. Verification of the specificity of the signal was possible as the graphic representation of the results included the size of the fragments for which the signal was detected. In all cases the signals were detected from fragments that corresponded to the size of the SNP flank primer used + 1 to 5 nucleotides. This small difference was due to the bigger size of the labeled ddNTPs labeled due to their attached fluorescent dye. The software provided numerical values for each peak height which is linearly correlated with the number of labeled fragments detected by the sequencer. For

each bulk the ratio of the two peaks generated by SNPs transmitted by the wild-type and mutant parent was used to approximately calculate genetic linkage (see 3.2.3).Conducting the SNP analysis using parental DNA followed the same protocol.

## 2.8 Complementation analysis. Identification of BAC clones.

### 2.8.1 Isolation of BAC DNA.

BAC clones were obtained from CUGI (Table 7.) as single *E. coli* colonies transformed with BAC containing a chloramphenicol resistance gene. BAC DNA was isolated using a slightly modified plasmid midi-prep protocol (Sambrook and Russell 2001). *E. coli* cells from an individual chloramphenicol resistant colony were inoculated in 100 ml of 2XYT medium supplemented with 12.5 μg/ml chloramphenicol and incubated at 37°C overnight on a rotary shaker (200 rpm). Cultures were then centrifuged at 8 000 rpm for 10 minutes. The supernatant was removed, cell pellets were resuspended in 5 ml TES (10 mM TRIS, 1 mM EDTA, 100 mM NaCl, pH 7,5) and incubated at room temperature for 5 min. Lysing solution (5 ml of 200 mM NaOH, 1% SDS) was added, the mixture was gently shaken by inversion and then it was incubated for 5 min on ice. Finally 5 ml of 3M K, 5M Acetate was added and after gentle mixing by inversion the mixture was incubated for another 5 min on ice. 3M K, 5M Acetate prepared as described by (Sambrook and Russell 2001) (Alkaline lysis solution III). Samples were then centrifuged at 10.000 rpm in a Sorval rotor for 15 min and the supernatant was transferred to a fresh 50 ml tube. This was followed by the addition of 5 μl of 10 mg/ml Ribonuclease A and incubation for 40 minutes at room temperature or overnight at 4°C. DNA was recovered by a standard isopropanol precipitation, and then by washing of the

pellet in 75% ethanol, then resuspension in 500 µl TE (10 mM TRIS, 1 mM EDTA). At least three phenol:chloroform:isoamyalcohol extractions were performed (until no debris is seen in the interphase), followed by a chloroform:isoamyalcohol extraction. BAC DNA was finally recovered by a standard ethanol precipitation and stored at -20°C .

| | BAC clones |
|---|---|
| 1 | 2K15, 33C16, 32K12, 1O23, 23M5, 40J24, 13N1, 13J7, 4N7, 29G24, 16C8, 23D2, 9E16, 7J2, 12A3, 37G13, 24O1, 6L2, 5D14, 4E6 |
| 2 | 36M13, 2J11, 28C9, 32C19, 34N12, 24J11, 6E15, 36D8, 33G9, 4E18, 6M15, 22A12, 33G23, 26G1, 39A9, 23N10, 5K8, 28I7, 14N2 |
| 3 | 1F6, 13L20, 29F12, 32C19, 32A18, 32B18 |

**Table 7. BAC clones used in complementation tests.** BAC clones used for complementation tests. 1. BACs containing genomic inserts covering the 869kb sequence of Scaffold 29 (JGI Chlamydomonas BLAST v2.0), 2. Additional BACs covering the extended 1620 Kb scaffold sequence in the new assembly (JGI Chlamydomonas BLAST v3.0), 3. BAC clones containing inserts covering portions of the genomic region covered by 28c9.

### 2.8.2 Cotransformation, selection of phenotypic *tbc1-F34* revertants

*C. reinhardtii* strain AS.76 (Table 1) was co-transformed by electroporation (see 2.8) with each BAC clone and plasmid pT7-ARG7 (Auchincloss et al. 1999) and selection for restoration of the wild-type PSII phenotype produced by *tbc1-F34* and for the arginine auxotrophy produced by *arg7-8* on a selective medium described in section

2.1. All transformations performed during the complementation analysis are listed in Appendix 1. Out of two selection options: consecutive selection for complementation of *arg7-8* followed by screening for rescue of the ac- phenotype produced by *tbc1-F34*, and double selection for complementation of both mutations, the latter was found to be more reliable (see below).

During initial transformations with the first twenty BACs (Table 7. 1; Figure 9.) no transformants were obtained. In each round of transformation, a positive control transformation, which selected only for complementation of *arg7-8*, generated the expected number of transformants. In order to rule out the possibility that the simultaneous selection for both phenotypes was placing the cells in conditions too severe to allow the selection for concurrent complementation of both *tbc1-F34* and *arg7-8*, AS.76 cells transformed with both a genomic BAC and the ARG7 transformation marker gene were initially selected only for ARG7 complementation (on a medium lacking arginine but containing acetate to rescue the *ac-* phenotype of *tbc1-F34*). The *arg+* transformants subsequently screened for an *ac+* phenotype by testing them for the ability to grow on a minimal medium lacking both arginine and acetate (HSM medium). This however was found to have the risk of reversion of *tbc1-F34* as individual transformed cells in the patches are sufficient in number to form visible colonies on selection conditions. As a result one BAC, 23m5 initially emerged as a potential carrier of *TBC1*. After analyzing the predicted genes in the BAC, doing a restriction analysis of its sequence, digesting it and isolating fragments containing single genes and testing for complementation with them it was found that none complemented. Furthermore the false positive state of the phenotypic revertants transformed with *ARG7* and 23m5 was

implicated by the fact that after transferring the colonies from TAP-R to HSM-R the patch of cells did not grow uniformly but only a number of single cells survived to create colonies covering the patch. Further streaking of those colonies on HSM-R showed the same result as many of them were not *ac*+. Other BACs (Appendix 1.) seemed to also produce variable number of single colonies after being subjected to both selection conditions. They were not taken into account as only uniform growth of the entire patched area would be an indication of true complementation rather than spontaneous reversion of single cells in the patch.

This risk of reversion suggested that transformed cells should be directly subjected to a simultaneous selection for complementation of both phenotypes. After obtaining the second set of BACs (Table 7., 2) covering a larger area of linkage group VI (Figure 10.) all transformations including the ones with the previously chosen 20 were plated directly on HSM minimal medium without arginine.

It was also found that during the simultaneous double selection which was the more stringent of the two types, certain types of contamination were able to phenotypically rescue the *tbc1-F43* mutants. This could have also lead to false interpretations of the results, so only colonies surviving selection that grew in definitely clean zones of the medium plates, i.e. away from any contamination, were considered to be rescued by complementation. Furthermore all colonies produced by transformed cells selected under the conditions above were also patched or struck on fresh HSM plates in order to confirm that they did not bring any contamination and that they were still able to grow on fresh selective medium free of any contamination whatsoever.

## 2.9 Electroporation of *C. reinhardtii*

A 100 ml culture of TAP (with arginine and 1% sorbitol) was inoculated with strain AS.76 and incubated in 24°C on a shaker for 2 - 3 days to a cell density of 2-5 x $10^6$ cells/ml. After that this "starter culture" was used to inoculate 300 ml of TAP (with arginine and 1% sorbitol) and this was cultured for another 24 hours or until the cell density reached 1 - 2 x $10^6$ cells/ml.

Cells were then collected by centrifugation at 4K rpm in for 5 minutes at 4°C. The pellet was resuspended in 1/100 volume of TAP (with arginine and 60 mM sucrose). Aliquots (250 µl) of the cell suspension and 0.1 – 1 µg of DNA were then added to electroporation cuvettes (with a 4 mm gap). The cuvettes were then incubated on ice for 15 minutes prior to transformation. Electroporation settings were: 0.8 kV, 25 uF, with the shunt resistor off. The time elapsed for the electrical pulse to be delivered under those conditions was 13 – 16 ms. The cells were then immediately plated on selective medium. Two to three weeks were usually necessary for the first colonies to appear.

### 2.9.1 Electroporation optimizations, crosses yielding electroporation efficient strains

Different methods of transformation of *C. reinhardtii* were tested. A standard method involving the use of glass beads (Kindle 1990) showed poor results; approximately ten transformants per transformation reaction were obtained and attempts to improve the efficiency by embedding in top agar were in vain. Therefore, cotransformation was done by electroporation (Shimogawara et al. 1998) with some modifications). Initial attempts to test the efficiency of the protocol by rescuing the *arg7*

phenotype of *WZ120* (*tbc1-F34:cw15:arg7*) using plasmids containing a functional *ARG7* gene yielded no significant transformation frequencies. Plasmid pT7-ARG7 (Auchincloss et al. 1999) was used. Multiple attempts to improve this transformation efficiency were unsuccessful, including, use of another selectable marker *BLE* in pSP124-ble (Lumbreras et al. 1998), conferring resistance to Zeocyn. Variations of all the electroporation conditions were also unsuccessful, and these included an overnight outgrowth of the electroporated cells prior to selection, embedding of these cells in bentonite, top agar or starch in order to avoid cell death. High transformation frequencies were obtained with strains that have a slight cell wall defect as described in Results.

Therefore, it was necessary to generate a new triple mutant stain for screening of the genomic BACs for *TBC1* by complementation, one with *tbc1-F34*, *arg7-8*, and with a weak to moderate *CW15* cell wall defect. Several crosses between WZ120 (*tbc1-F34:arg7:cw15*) and strains carrying either wild-type or the *tbc1-F34* alleles of *TBC1*, (Table 8.) were carried out and their progeny was tested for the *ac-* phenotype of *tbc1-F34.* These *ac-* strains were tested for arginine auxotrophy produced by *arg7-8.* Finally these *ac-*, *arg-* strains were tested for the cell wall defect produced by *CW15* by measuring the time elapsed for cells taken from a liquid culture to lyse in 2% SDS. After testing the severity of the *cw15* phenotype in these strains, four progeny strains with intermediate levels of CW15 were identified (AS.74, AS.76, AS.82 and AS.86 (Table 8.) and tested for high electroporation frequency. All of them transformed well and AS.76 was used in all subsequent electroporations as it regularly yielded 200 – 500 *arg+* transformants per electroporation.

| Crosses | Electroporation efficient progeny | Genotype |
|---|---|---|
| *WZ120 x F34.3+* | AS74, AS76 | *tbc1-F34:arg7:cw15* |
| *WZ120 x F34.2A+* | AS86 | *tbc1-F34:arg7:cw15* |
| *cc1618 x WZ120* | AS82 | *tbc1-F34:arg7:cw15* |
| *cc1618 x F43.4-????* | - | - |

**Table 8. Crosses done to generate electroporation efficient strains with weak to moderate *CW15* cell wall defect.** Progeny was selected for tbc1-F34:arg7:cw15. Four strains weak to moderate *CW15* cell wall defects were isolated and used in all further electroporations.

## 2.10 Southern analysis

Southern hybridization was carried out as described previously (Sambrook and Russell 2001), with minor modifications. Total genomic DNA from strains $4C^+$, WZ120 and the spontaneous *tbc1-F34* revertant to Wild Type *F34.2A+ (Table 1.) was isolated using the method described in section 2.3, and 40 µg were digested overnight with restriction enzyme *Pvu*II. Digest was precipitated by a standard ethanol precipitation and DNA was diluted in 30 µl TE buffer. Although the genomic DNA was treated with Ribonuclease A during extraction, ethidium bromide spot assay (Sambrook and Russell 2001) was preferred to measuring $OD_{260}$ with a spectrophotometer in order to determine concentration as $OD_{260}$ was influenced by any RNA contamination of the sample. 20 µg of the digest was separated by standard agarose gel electrophoresis using a 0.8% agarose

gel and transferred overnight to a Hybond N+ nylon membrane by capillary blotting using alkaline transfer according the instructions provided by the manufacturer (Amersham Biosciences).

DNA probe was generated by digesting BAC 32A18 with *Cla*I and *Xba*I (Figure 12.). The 10.7 kb *Cla*I-*Xba*I restriction fragment was gel purified using Quiagen QuiaexII gel extraction kit. It was radiolabeled by adding 50ng of DNA to 100 ng random hexamer primer and denaturing in boiling waterbath for 2 min. and immediately transferred on ice. 1X Klenow buffer (supplied by MBI Fermentas), 0.5 mM dNTP (dAGT), 1 µl DTT (100 mM), 80uCi of [$\alpha^{32}$P] dCTP (3000 Ci/mmole), 1 µl Klenow fragment and water were added up to 25 µl and the mixture was incubated at room temperature for 1 hour. Removal of unincorporated dNTPs was done by passing the reaction through a Sephadex G50 column (Sambrook and Russell 2001).

Due to the presence of repetitive sequences in *C. reinhardtii* nuclear genome an additional step of pre-annealing the probe before hybridization was introduced to reduce background in the blot hybridization. A genomic DNA driver was prepared by adding 65 µl of total genomic *C. reinhardtii* DNA (1 µg /µl) to 2.3 ml of Formamide Hybridisation buffer (described by (Sambrook and Russell 2001)) and was then sonicated for 45 sec. Combination of 50 ng of the random primed probe and the genomic driver and hybridization solution brought the final volume to 2.5 ml and the sample was denatured for 5 minutes in boiling water bath. The probe was then allowed to pre-anneal at 42 $^0$C for 4 hours. After that the probe was added to the pre-hybridized at 42$^0$C for at least 4 hours membrane and left to hybridize at 42 $^0$C for at least 10 hours. Hybridization was carried out in glass roller bottles as described by (Sambrook and Russell 2001). Washing

steps were carried out as described previously (Sambrook and Russell 2001). The hybridization signal was detected by autoradiography.

## 2.11 Bacterial transformations, plasmid preps

Transformations of $CaCl_2$ competent *E. coli* DH5α and subsequent DNA isolations of all plasmids used in this project were done as described by Sambrook and Russell (2001). All plasmids were verified by test digests before use.

# CHAPTER 3. RESULTS

## 3.1 Bulked-segregant Analysis (BSA): generation of wild-type and mutant bulks

### 3.1.1 BSA and AFLP markers were combined as a new approach to identify genes in *C. reinhardtii.* The approach identified linkage group VI as the location of *TBC1.*

BSA (see 1.8.) was initiated by generating pools or "bulks" of individual progeny that were randomized for polymorphisms throughout the genome except for those linked to *TBC1.* Since *C. reinhardtii* is typically haploid, and only has a transient diploid phase during its sexual cycle, there was no need to identify homozygous progeny. Bulks were generated by conducting a cross between two highly polymorphic strains which were also either wild-type or mutant at *TBC1.* The recent wild isolate strains S1D2 (wild type) or Harris #6 (WT) were used. S1D2 is known to be highly polymorphic with respect to all laboratory strains, which were derived from a pair of opposite mating types of the same strain many decades ago (Harris 1989). The *tbc1* mutant strain used was F34.4+, which carries *tbc1-F34* (Table 1.). As a result 300-400 haploid progeny from each cross were screened for the acetate-requiring (ac-) and high-chlorophyll-fluorescence (hcf), phenotypes produced by *tbc1-F34* due to its PSII deficiency. Approximately 130 mutant and 130 wild-type progeny were identified in multiple rounds of careful screening (see Materials and Methods) and pooled to form the mutant and the wild-type bulks. Total genomic DNA was prepared from the bulks. For convenience the wild-type and mutant bulks generated with the progeny of the cross S1D2 x F34.4+ will be called the S1D2

WT bulk and the S1D2 MUT bulk, respectively. Following the same nomenclature, the bulks of the cross Harris WT x F34.4+ will be called the Harris WT bulk and the Harris MUT bulk, respectively.

Detection of markers that uniquely segregated in either of the bulks was used as an approach to find sequences (markers) that are closely linked to *TBC1*.

## 3.2 Molecular markers:

### 3.2.1 AFLPs

Two different types of markers were tested for linkage to *TBC1*: AFLPs and SNPs. AFLPs are essentially PCR-amplified, small restriction-length polymorphisms. Their advantage seemed at the outset of this project to be that hundreds of distinct AFLPs can be resolved in a single lane of a denaturing polyacrylamide gel, making it easy to screen for rare AFLPs that are enriched in only one bulk and, therefore, linked to *TBC1*. The availability of the bulks and previously described primers (Table 5.) to generate an almost unlimited number of AFLPs (and the lack of the SNP markers described below) made this approach a logical step. The AFLP technique is based on the selective PCR amplification of restriction fragments from a restriction digests of total genomic DNA (Vos et al. 1995). Genomic DNA from the bulks, in this case, is digested with a pair of restriction enzymes one of which cuts frequently due to its 4 bp recognition sequence and another that is a rare cutter due to its 6 bp recognition sequence. Then, double-stranded adapters are ligated to the ends of these restriction fragments to generate template DNA fragments for amplification with primers that anneal to the adaptor sequences. The sequence of the adapters and the adjacent restriction site serve as primer binding sites for

subsequent amplification of the restriction fragments. In a second round of PCR, the primers have 1-3 specific "selective nucleotides" at their 3' ends, so that they can only amplify a subset of the restriction fragments with the adaptors. Only fragments in which the nucleotides flanking the restriction site are complementary to the selective nucleotides are amplified. This allows the amplification of a reasonable number of AFLPs for resolution on one gel lane (e.g. 100-150 AFLPs) and permits one to obtain multiple subsets of AFLPs from the same initial digests of the bulk genomic DNA by varying the selective bases on these primers in different reactions. Moreover AFLPs are randomly obtained and unlike restriction-fragment-length-polymorphisms, no knowledge of nucleotide sequence is required to obtain AFLPs.

The restriction fragments for amplification are generated by two restriction enzymes, a rare cutter and a frequent cutter. This causes generating of small fragments that amplify well and are nicely resolved on denaturing gels. It reduces the number of amplified fragments and the number of selective nucleotides needed for selective amplification. Different combinations of enzymes and primers give flexibility in tuning the number of fragments that are amplified.

Bulks were digested with the pairs of restriction enzymes indicated in the legend of Figure 1. The adaptors were described previously by Vos et al. (1995) and their sequences are given in Table 3. AFLPs were generated from the digests of bulk DNA using primers listed in Table 5. As expected, and seen in Figure 1, this approach yielded a large number of AFLPs for each combination of restriction enzyme and primers. Also as expected, most AFLPs were amplified to similar levels from both bulks revealing that these are unlinked to *TBC1*. One difficulty that was encountered was that only certain

primers combinations (see Materials and Methods) resulted in a few identifiable bulk-specific bands observed on denaturing polyacrylamide gels.

Silver-staining was used to visualize the AFLPs as opposed to $^{32}$P-labeling and autoradiography. Although the latter approach has a better sensitivity, silver staining allows DNA fragments to be visualized directly on the gel, which is essential for their successful and specific isolation. Furthermore silver-staining is a much faster and easier method and avoids all the complications surrounding the use of radioisotopes.

Four unique bands (Figure 1.) were identified in the following bulks:

130bp in Harris WT amplified with primers MseGA, BamC

125bp in Harris MUT amplified with primers MseGA, BamC

140bp in S1D2 WT amplified with primers MseGA, BamT

150bp in HarrisWT amplified with primers MseGA, BamG

Other bulk-specific AFLPs were observed when other primers and enzymes were used, but in most cases they were too close to other unlinked AFLPs and the risk of contamination during excision was apparent or attempts to specifically amplify them in a third round from the isolated samples were unsuccessful.

After isolation, the four PCR amplified fragments were verified by amplification in a third round of PCR, followed by resolution on both agarose gels and on a denaturing polyacrylamide gel next to the complex set of AFLPs from which they had been derived. All four isolated AFLPs in Table 9 comigrated with the original bulk-specific AFLPs and thus, they were subjected to further analyses.

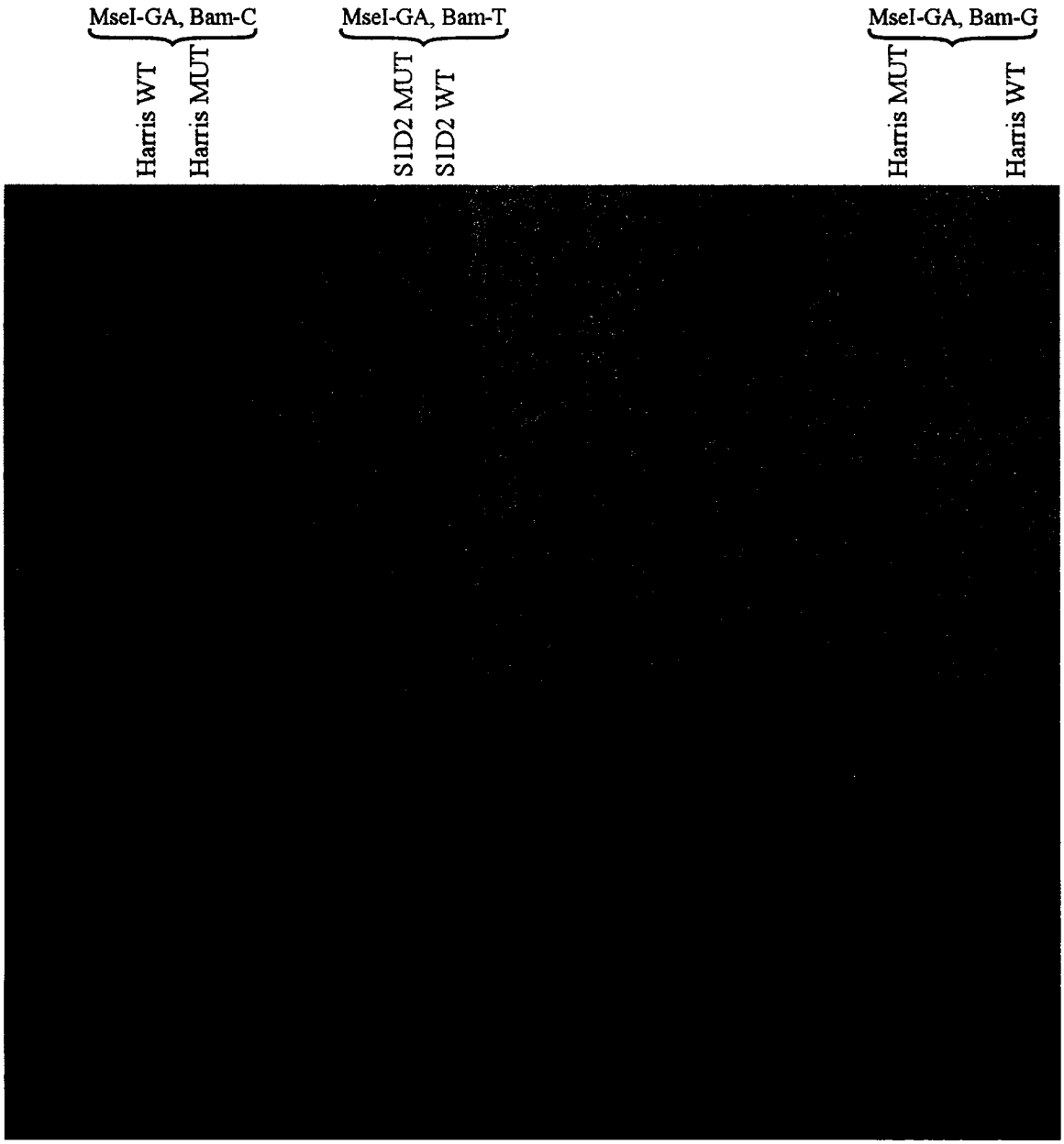



**Figure 1. Silver-stained polyacrylamide gel**

Pooled DNA was digested with restriction enzymes *MseI* and *BamHI* as well as with *PstI* and *MseI*. The primers and bulks used to generate the AFLPs are indicated on top of the gel. In each set of bulks an analogous range of AFLPs are present. Four unique AFLPs were identified, isolated and sequenced - marked with numbered asterisks.

| | Sequence | scaff old |
|---|---|---|
| 1. | CGGGAACCCGGGTNNCTATTATCGTTACCGGATCGAAGGGCGGGGGGCGGN ACAGGCCCTCGGGNATCGGCCTCGGGNACAGGGCCTCGNTCCCACCGGGNA TCGGTACGGCAGTCAAANNTTNNTN | 43 |
| 2. | CGGGGAACCGCGGTNGCTATTATCGTTACCGGATCGAAGGGCGGGGGGCGG NACAGGCCTCGGGNATCGGCCTCGGGNACAGGGCCTCGNTCCCGCCGGGNA TCGGTACGCCAGNTCAAA | 43 |
| 3. | NGNNCGTNCCCAAGCACACTAGCCCTNGGCCCGTGTAACCCGGTGCAGGGG CGTGACTCACATGCGTGCAACTGCACGTCCGCTGTATGGTCCAAATCTGCAT GGCCAAGGNATCGGTACGCAGTCAA | 20 |
| 4. | ANCGCCGCTATAANGATACTAGACCTAATAAAGTATAATTGGCGNTGCAGAT ATAAATTCTCATTCTACGCATAATCATAAAAGCAACAATGCGNTGNTTGNAC TGATCGCTGGGNTTCTTTCGGNATCGGTACGCAGNTCAAAC | 146 |
| 4.1 | NCNCCGCGACGTTCAACACGCATTGTTGCTTTTATGATTATGCGCTAGNAAT GAGNAATTTATACTGCACGCCAATTATACTTTATTAGGTCTAGNTATCCTTTA TAGCGGCAGGGACATTCTTACTCAGGGACTCATCAA | 146 |

**Table 9. Sequences of the identified unique AFLPs.** Sequences 1,2 and 3 are the 130 bp band amplified with primers MseGA, BamC from Harris WT bulk, the 125 bp band amplified with primers MseGA, BamC from Harris MUT bulk and the 140 bp fragment from the S1D2 WT bulk using primers MseGA, BamT; Figure 1. bands 1, 2, 3 resp. Sequences 4 and 4.1 are determined for the 150 bp band amplified with primers MseGA, BamG from HarrisWT bulk using the forward and the reverse primers resp. for the sequencing PCR (Figure.1 band 4). Matching them to the *C. reinhardtii* genome showed that they aligned to sequences from scaffolds 43, 20 and 146 resp.

The purified polymorphic fragments (see Materials and Methods) were sequenced (Table 9.). Two of them have identical sequences although as seen on Figure 1 (bands 1 and 2) there seemed to be a small but clear difference between their sizes. The program Blast (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=chlre2&advanced=1) was used to search for the genomic region(s) from which these AFLP were derived. Encouragingly, all four were derived from Linkage Group VI, thereby delimiting the location of *TBC1* to this linkage group (Figure 2.). The sequences of these AFLPs were identified in scaffolds: 20 (1086106 bp.), 43 (652989 bp.), 146 (262186 bp.) (JGI Chlamydomonas reinhardtii browser v.2.0). The fact that the sizes of these scaffolds are c.a. 1Mb, and only one AFLP was identified per scaffold, showed that although linked, these markers could not be as close to the *TBC1* locus as expected. Based on the 120 progeny in each bulk, bulk-specific AFLPs were expected to be within a few recombination units of *TBC1*, which should correspond to 100-300 kb (Kathir et al. 2003). Since verifying the exact positions of those scaffolds on the linkage group was not possible at that time (the scaffolds have since been assembled in Version 3.0) their locations on Linkage Group VI were determined by locating known markers in these scaffolds. The data published by the Silflow-Lefebvre laboratory at the University of Minnesota (http://www.chlamy.org/BAC/LG6.htm) on the Chlamydomonas database website showed that among the markers mapped to linkage group VI, several sequenced genes were mapped to the scaffolds identified by the AFLPs: TPX (accession AF312025) was located in scaffold 20, S813 (accession X53574) in scaffold 43, and ICL and CABI-1 (accession U18765 and X65119 resp.) in scaffold 146 (reviewed by Katrhir, 2003).

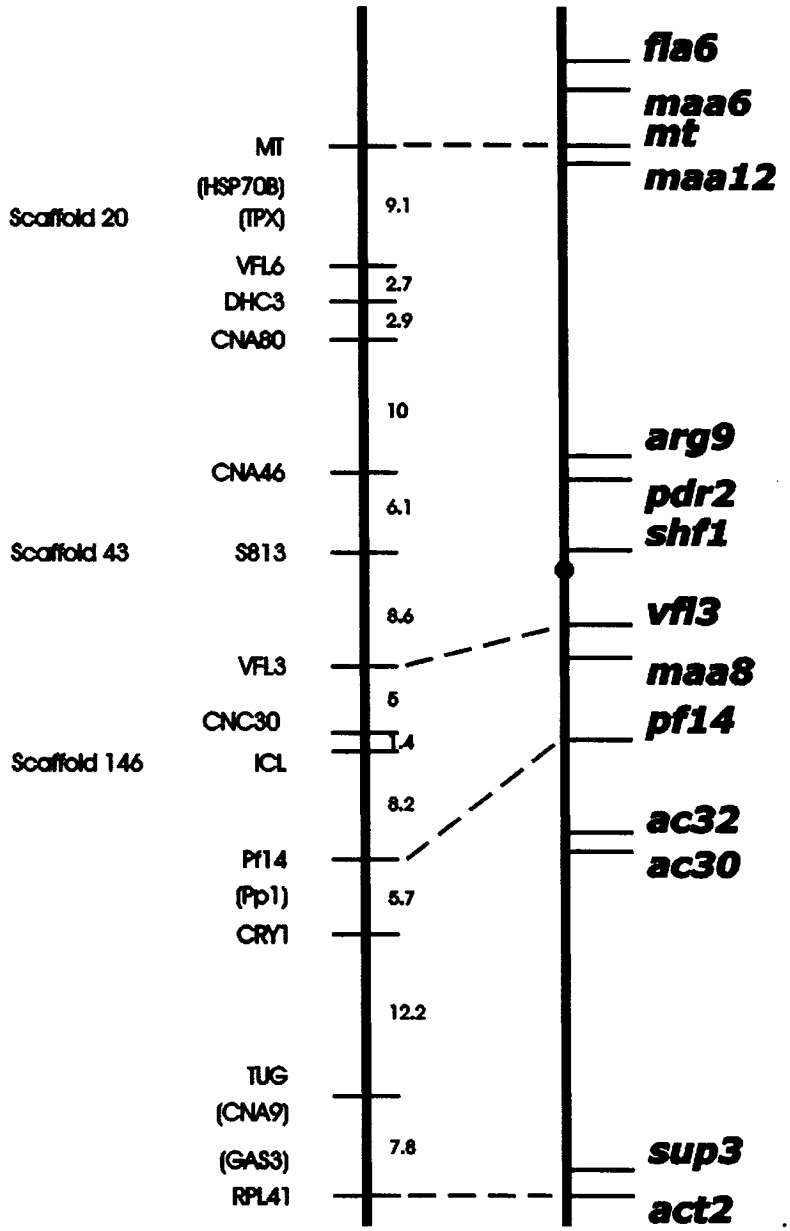



**Figure 2. Genetic map of linkage group VI (By Pete Lefebvre, university of Minnesota).** Known single nucleotide polymorphism (SNP) markers are indicated on the left and genetic markers are shown on the right. The distances between each pair of molecular markers is given in map units and the position of the centromere is shown as a circle. The scaffolds to which the unique AFLP sequences were matched are positioned relative to known markers on linkage group VI that they cover.

As shown on the left of Figure 2 these established positions of these scaffolds placed them throughout the linkage group, and thus additional work was required to narrow down the region with *TBC1*.

These results significantly narrowed down the position of *TBC1* to a single linkage group, but they also suggested that the AFLP procedure was not a very precise approach of detecting markers tightly linked to the segregating trait using BSA. There was also the problem of having weak band intensities after silver staining the denaturing gels and the risk of getting a high background if trying to get more prominent bands.

### 3.2.2 A PCR assay detected weak linkage beteen the mating-type (*MT*) locus and *TBC1*

The available genetic and molecular map of linkage group VI (Figure 2.) were searched for known and easily scorable markers that could be compared in the existing bulks and which could be used for a more precise mapping of *TBC1*. As seen in Figure 2 the *MT* locus is located at the distal end of the left arm of Linkage Group VI. It consists of three domains which have genes that are specific to the *mt+* or *mt-* loci (Ferris and Goodenough 1994).

Mating type in *C. reinhardtii* can be diagnosed using a PCR assay that amplifies a fragment from *mt+* or *mt-* specific genes. Using PCR, one set of primers amplifies a unique fragment only from the *mt-* locus and another set amplifies a fragment of a distinct size only from the *mt+* locus (Zamora et al. 2004). If the *MT locus* is linked to *TBC1* one of these bands should be preferentially amplified from each bulk and in each case it should be the fragment derived from the *mt* locus of the parental strain, i.e. *mt-*

should be enriched in the wild-type bulk and from the $mt^+$ locus in the *tbc1* mutant bulk. Thus, the diagnostic PCR assay for MT (Zamora et al. 2004) was performed using template DNA from bulks S1D2 WT and S1D2 MUT (Materials and Methods, 2.6). Bulked DNA was carefully quantified to ensure that equal amounts of template were used in these PCR reactions. The ratio of the amplified product from each bulk was an approximate measure of the initial ratio of the amplified fragments in the template.

In order to avoid very high intensities of the abundant PCR products, and make the assay more quantitative, serial dilutions were made from the amplified products and equal volumes of each dilution were resolved and visualized on an agarose gel. Comparisons of the intensities of the PCR products derived from the two bulks shown in lanes 1 – 6 and 7 – 12 in Figure 3 revealed that the level of the 620 bp amplified fragment was higher than the 520 bp. fragment in S1D2 wild-type bulk and that the level of the 520 bp product was higher than the 620 bp fragment in the PCR products from the S1D2 mutant bulk. This slight predominance of one fragment of the products amplified from each bulk and the fact that different polymorphic states were more abundant in the compared bulks, suggested that *MT* locus is weakly linked to *TBC1* and that the BSA approach is at least somewhat reliable. In order to get an objective measurement of this linkage, SynGene GeneTools - Version 3.00.00 (SynGene Laboratories) was used to measure the fluorescence intensity peaks and their values for those amplified fragments from each bulk (Figure 4.).

The ratio of the peaks corresponding to the *mt*+ and *mt*- PCR products in each PCR sample, reflecting the ratio of the actual *mt* specific sequences in each bulk, showed that although the *mt* locus is linked to *TBC1* this linkage is not very tight. Calculation of

the ratio of those peaks showed that the distance between *MT* and *TBC1* is approximately 40 map units. This suggested that *TBC1* is probably on the right arm of this linkage group.

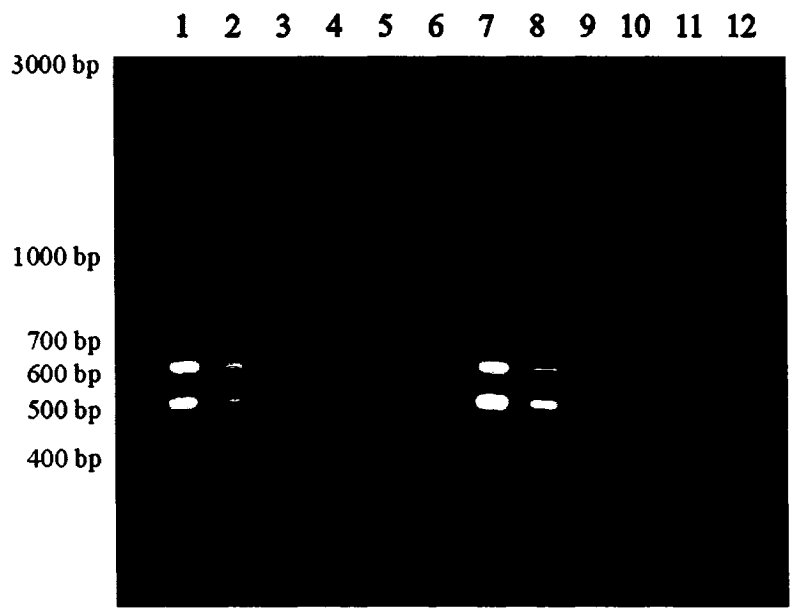
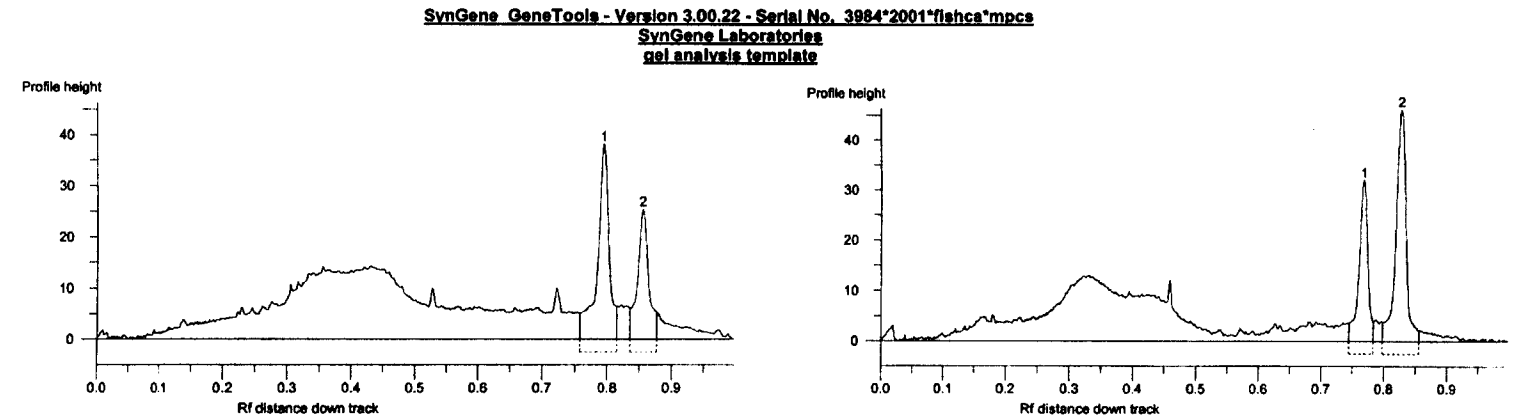



**Figure 3. Mating type PCR conducted on bulks S1D2 WT and S1D2 MUT.**

Lanes 1 – 6 have $2^{-1}$ – $2^{-6}$ diluted PCR product amplified from S1D2 WT bulk and lanes 7 – 12 have $2^{-1}$ – $2^{-6}$ diluted PCR product amplified from S1D2 MUT bulk resp. Equal volumes of each dilution were loaded. 100 bp. Ladder plus was used to verify products' sizes.




| Track 2 | | | | | | |
|---|---|---|---|---|---|---|
| Number | Mol. weight | Height | Raw vol. | ug/uL | % Raw vol. | Rf |
| 1 | 0.00 | 38.170 | 23630.66 | 0.80 | 61.311 | 0.7944 |
| 2 | 0.00 | 25.353 | 14911.77 | 0.50 | 38.689 | 0.8556 |

| Track 7 | | | | | | |
|---|---|---|---|---|---|---|
| Number | Mol. weight | Height | Raw vol. | ug/uL | % Raw vol. | Rf |
| 1 | 0.00 | 32.109 | 15276.14 | 0.60 | 37.555 | 0.7667 |
| 2 | 0.00 | 46.339 | 25400.89 | 1.00 | 62.445 | 0.8259 |

**Figgure 4. Measuring the fluorescence intensity of the MT PCR products amplified from each bulk.**

The values measured in lane 1 of figure 4 are shown on the left side indicated by the software as Track 2 (since the DNA ladder was used as Track 1) and the values measured from lane 7 on figure 4 are shown on the right indicated as track 7 (lane 6 having no detectable signal was no taken into account by the software). The original height measure was used to determine the ratio of the two products in each bulk and relate it to linkage to *TBC1*. In both cases peak 1 represents the 520 bp. fragment and peak 2 the 620 bp. fragment resp.

### 3.2.3 Single nucleotide polymorphisms (SNPs)

Since the other available molecular markers throughout Linkage Group VI were either single nucleotide polymorphisms (SNP) or mutant alleles, an approach to combine SNPs with BSA was developed and it showed to be a valuable method for mapping genes known by mutations. This approach has higher accuracy than mapping with respect to AFLPs using BSA. SNPs are chromosomal positions where a single nucleotide differs between two strains. They can be identified by amplification of a fragment with flanking sequences and are PCR based markers derived from comparisons of laboratory strains and the polymorphic strain S1D2. They are convenient since only a single nucleotide difference between both parents is required, and on average when comparing S1D2 and a laboratory strain 2.7 base substitutions are detected in every 100 bp of genomic sequence (Kathir et al, 2003).

Six SNP markers on Linkage Group VI were chosen and their relative levels in the wild-type and *tbc1* bulks were compared (see Materials and Methods). For each SNP marker a first round of PCR was conducted using sets of primers (Table 6., PCR primers) that amplified a several hundred bp genomic fragment containing each specific SNP (Materials and Methods, 2.7.2). S1D2 wild type and mutant bulks were used as template. Verification of the products of these PCR products on agarose gels confirmed they were of the expected sizes (data not shown). A second round of PCR was conducted using the purified product of the primary PCR as a template and only one primer that flanks the SNP (Materials and Methods, 2.7.3). The PCR mix had all four ddNTPs and each was labeled with a different fluorescent dye. The reaction extends the primer by only one nucleotide and inserts the ddNTP corresponding to the base for the particular SNP. The

purified product of the secondary PCR was analyzed on an ABI 310 Capillary DNA Sequencer and the amount of each base was reflected by the height of the peak in the graphical display of the products.

The genotyping results (Figure 5.) were visualized by Genotyper® Software v 3.7 which measured and provided numeric values for the peak heights for each of the two possible bases of the SNPs. As shown on Figure 5, for each of the markers two signals were obtained, each one was derived from one of the two bases of a particular SNP. Comparing these signal intensities for each SNP in the products of the wild type and mutant bulks showed that the ratio of the signals was inversed, consistent with the linkage of the markers to *TBC1*. Numeric values of signal parameters are also displayed. Peak heights are correlated to the amount of labeled nucleotides detected; hence they represent the abundance of each PCR product which in turn is dependent on the relative levels of the initial SNP templates. Peak color represented the nucleotide that was added to the primer. Red indicates a T, and green an A, black – C and blue – G. Comparing these with the SNP in the parental strains (Table 6.) (The Chlamydomonas Database, http://www.chlamy.org/chlamydb.html) showed that the SNP transmitted by the wild-type or *tbc1* parent was predominant in the corresponding bulk. Furthermore the specificity of the amplification from the bulks was confirmed by performing separate genotyping using genomic DNA from the parental strains (S1D2 and F34.4) as template. The signal peaks for those SNPs in each parent (Figure 6.) showed only one peak, which corresponded to the predicted nucleotide. Comparison of the results in Figure 5 and Figure 6 revealed that for each SNP both peaks (one from each parent) were present in the bulks. This confirmed the specificity of the SNP analyses in conjunction with BSA.

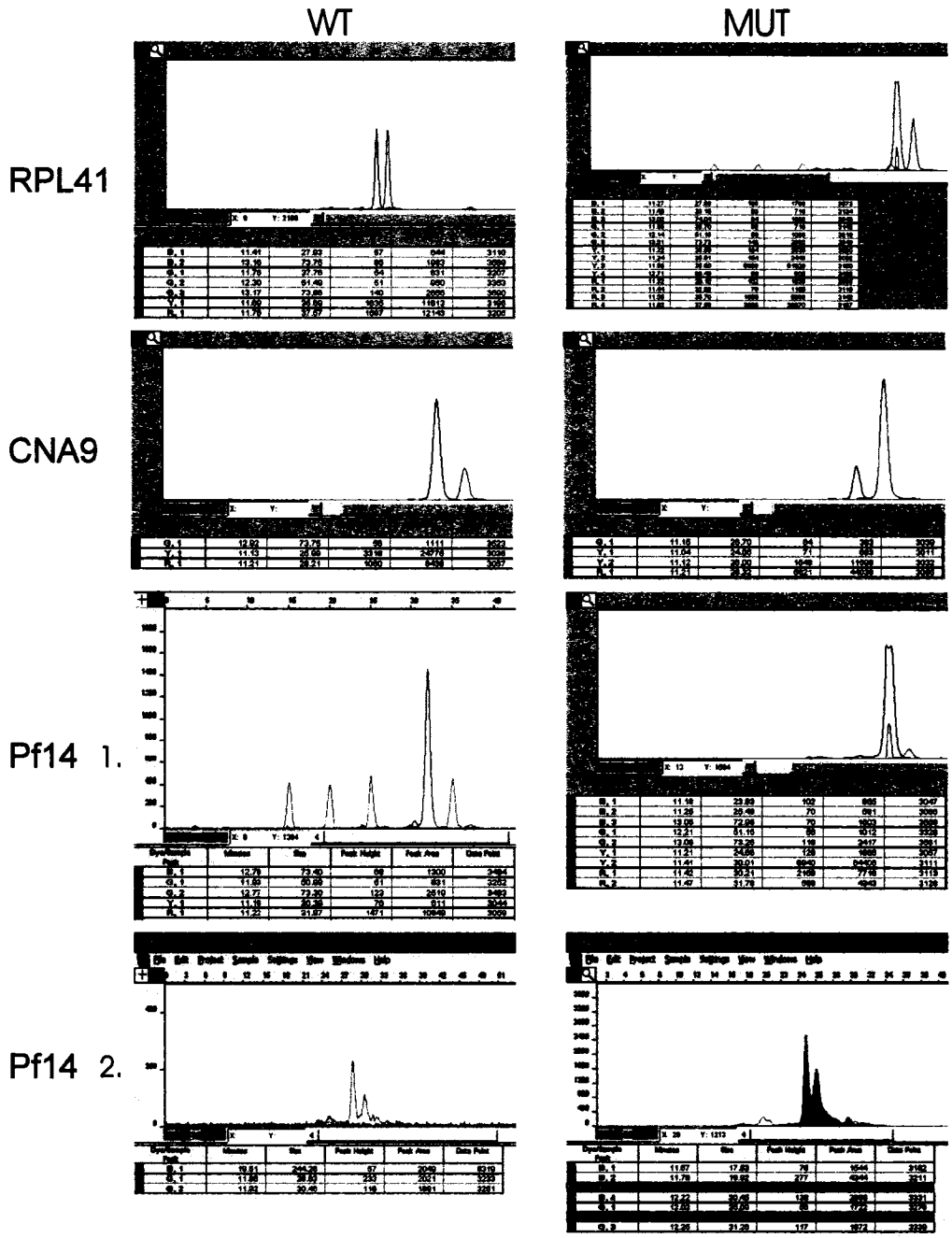



**Figure 5, panel a. SNP genotyping of linkage group VI.** (legend continues on the next page) The color of each peak corresponds to the labeled nucleotide integrated at the position of the SNP: red – T, black – C, green – A, blue – G. The orange peaks in some cases are signals from the size marker. For each SNP marker the signals from the S1D2 wild type bulk is shown on the left and the one from the mutant bulk on the right. PF14 1. was detected using the reverse SNP flank primer and 2. using the forward one, CNC30 1.

detected using the forward flank and CNC30 2. the reverse one. For all the other ones except S813 the SNP forward flank primer was used

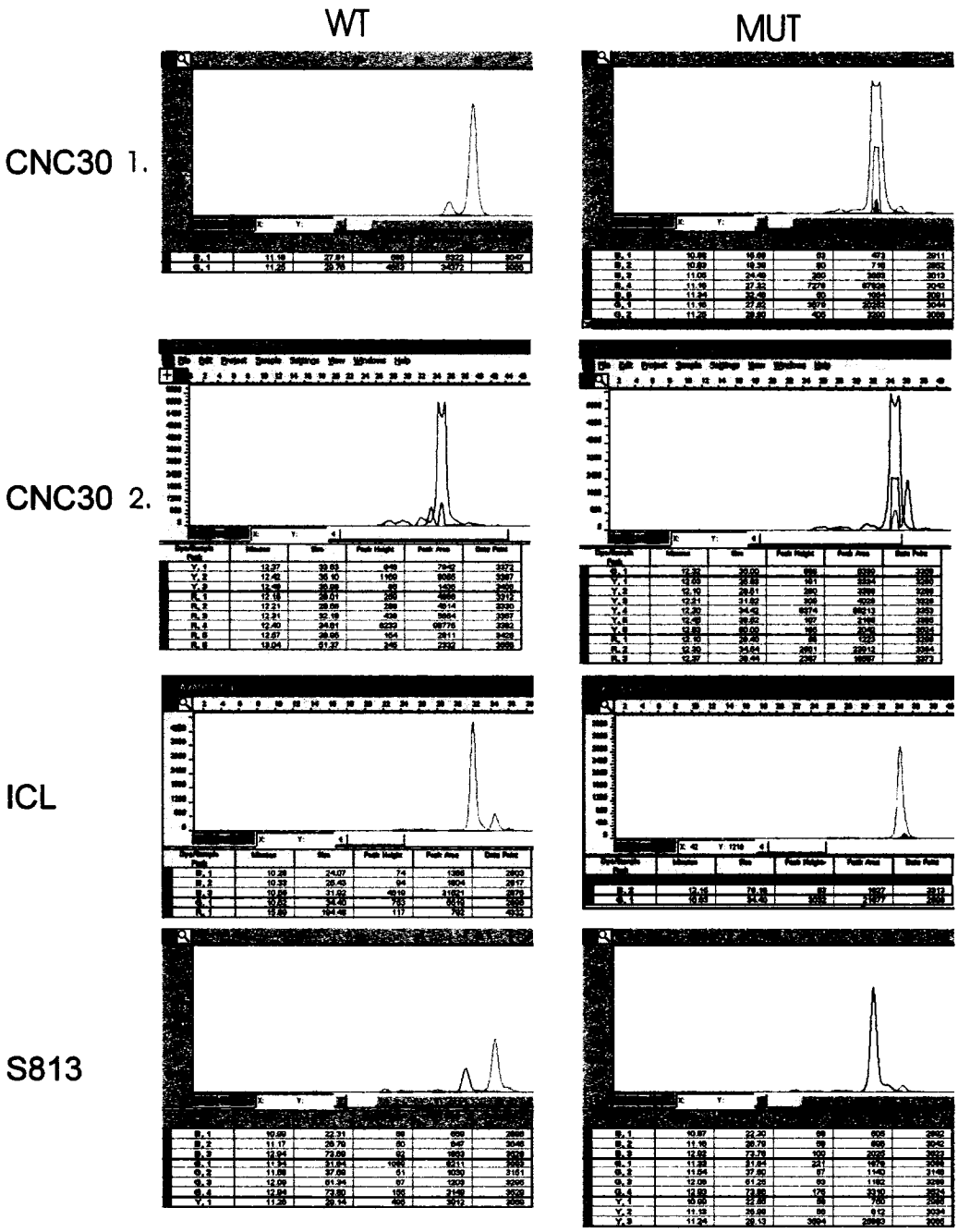



**Figure 5, panel b. SNP genotyping of linkage group VI.** Legend is the same as Figure 5, panel a.

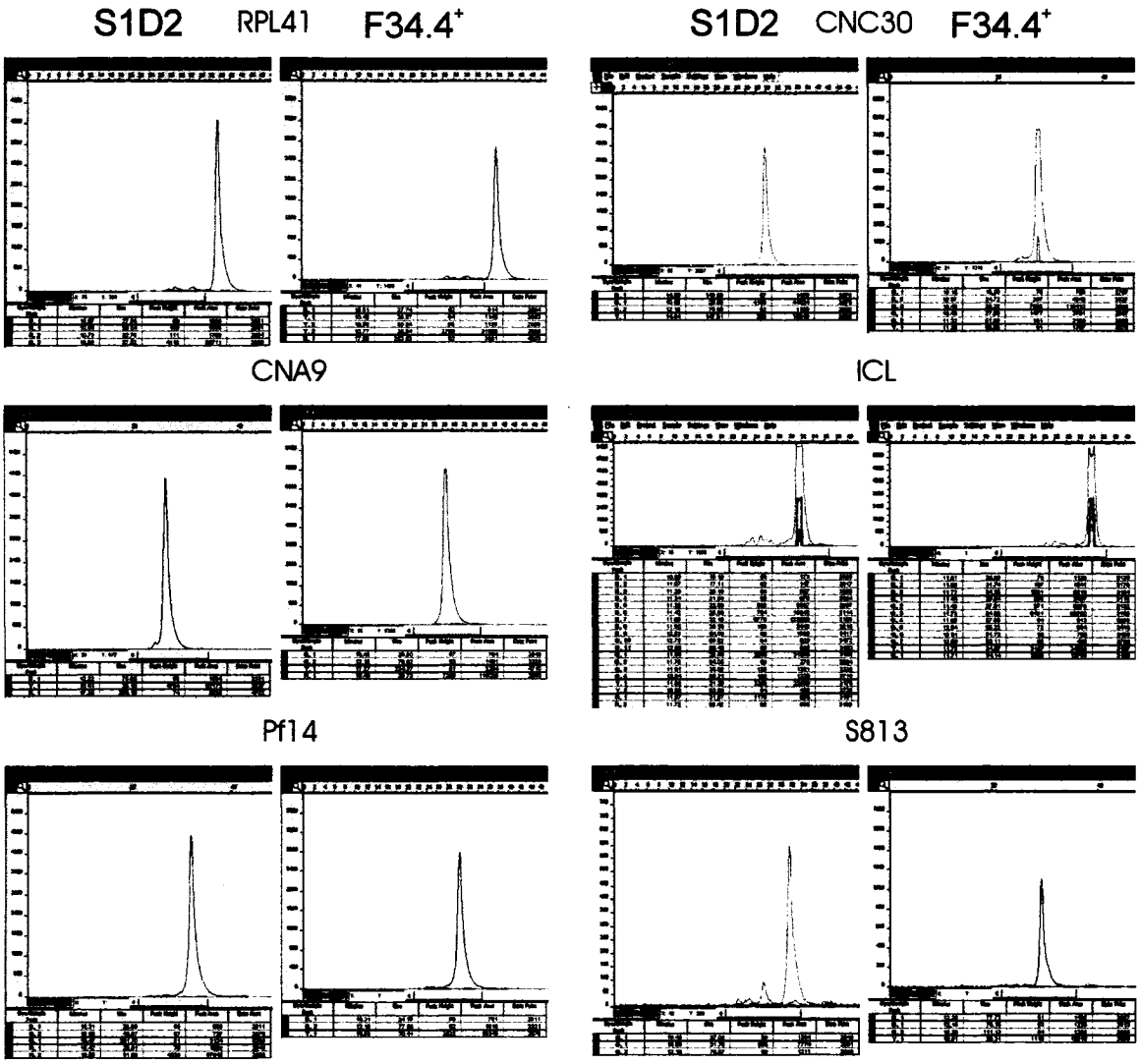



**Figure 6.Genotyping performed using parental DNA.** For each SNP marker the signal detected from parent S1D2 is shown on the left and the one detected from F34.4+, on the right. Peak colors represent the same nucleotides as on figure 5.

Following the same rationale described in Section 2.2, the relative levels of the two polymorphic bases for each SNP in the bulks, determined from the ratio of the peak heights in Figures 5 and 6, were used to approximate recombination distances between each SNP marker and *TBC1*. Recombinants in each bulk had the SNP from the opposite parental strain. Thus, the frequency of the less abundant SNP in the products from each bulk relative to the sum of the levels of both SNPs, is a measure of recombination frequency. The percentage of recombination was calculated as the ratio of the smaller peak value to the sum of the values of both signals expressed in percentage. Thus the recombination distances between each SNP and *TBC1* allowed a rough approximation of the position of *TBC1* on Linkage Group VI. These results were aligned to the existing map of linkage group VI and a new map showing the relative position of *TBC1* was created (Figure 7).

Although the initial template added when using parental genomic DNA as template was the same in couple of reactions, the results shown on Figure 6 indicate that the value of the signal peak generated in one parent is always higher than the other. The red peaks were always higher than the black ones and the blue peaks were higher than the green ones. This was due to the difference in the fluorescence strength of the four different dyes attached to the ddNTPs. This resulted in inaccuracy when calculating the recombination distances between the SNP markers and *TBC1*. Due to this inaccuracy, the ratios of the signal strengths in each pair of bulks for each SNP marker in Figure 5 differed in their absolute values. This could be noticed on the left of Figure 7 where each marker produced two different distances calculated from the peaks in each bulk (wild-type or mutant) namely due to the described effect.

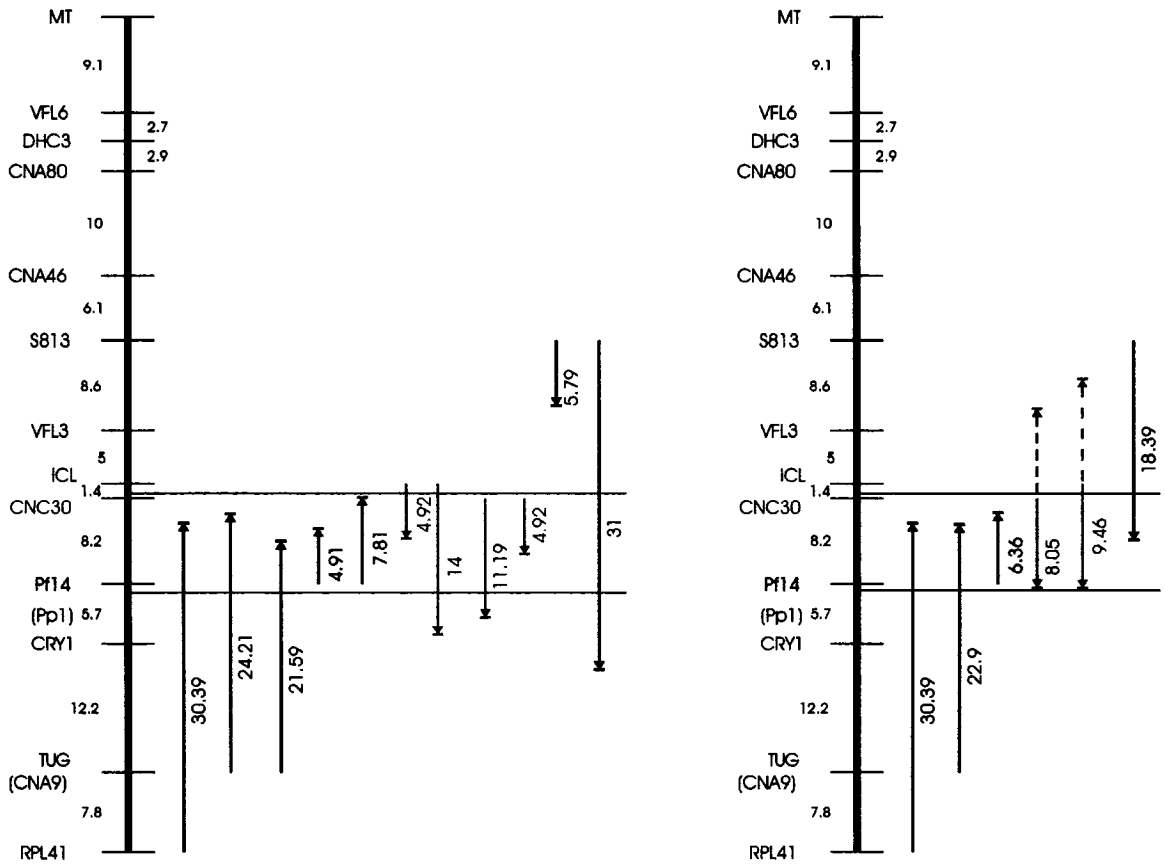



**Figure 7. Mapping of *TBC1* relative to the tested SNP markers.**

Arrows represent the calculated distances from each marker to TBC1 as numbers next to them indicate this distance in map units. On the left: two distances are calculated for each marker (except RPL41 see results), one from the values detected from the wild type and one from the mutant bulks. On the right: The average distances from both bulks for each marker were mapped. The region where most of the arrows collocate is marked with orange lines. Both possible orientations for SNPs CNC30 and ICL are indicated (solid and punctate arrows).

Calculations of genetic distances represented in Figure 7 were based entirely on recombination percentage without taking in account the double crossovers or the recombination interference because their impact is insignificant relative to the inaccuracy due to the different dye intensities.

Since calculations only produced the distance from each marker in map units, there was no way to know the direction along the linkage group in which *TBC1* should be mapped. Orientation relative to markers RPL41 and CNA9 was undisputable, because these markers are at the very end of the right arm of Linkage Group VI.

The RPL41 marker is approximately 31 map units away from *TBC1*. Only the distance determined by the wild type bulk was used to calculate it since the signal detected in the mutant bulk was off the scale of detection shown as a saturated black peak for RPL41 MUT on Figure 5.

The recombination distance between *TBC1* and *CNA9*, based on the average values obtained from the wild-type (24 map units) and mutant bulks (22 map units), is 23 map units. Those three distances fell in an approximately 8 map unit (761 kb.) region which was defined by SNP markers PF14 and CNC30.

The distance from the PF14 SNP marker suggested a very tight linkage. In the wild-type bulk the ratio of the SNP signal peaks gave a recombination distance of approximately 5 map units. The mutant bulk revealed a distance of less than 8 map units. The last distance is probably shorter since the peak representing the SNP inherited from the *tbc1* parent in the mutant bulk was above the limits of detection (Figure 5, PF14 1). Hence its actual value is higher. Of the two possible orientations of these calculated distances the one towards the centromere was consistent with the three previous

measurements, again pointing to the same region. Detecting the SNP state in the second PCR (using one primer) could be achieved by using either one of the primers flanking the SNP, which are called forward and reverse flank primers, respectively. As PF14 looked very promising when tested with one of those flank primers, the other SNP flank primer was used also. As in Figure 5, the result confirmed a definite very tight linkage of *TBC1* and PF14 2.

Mapping with SNP marker CNC30 also showed linkage to *TBC1* but not as tight as PF14. The signals measured in the wild-type bulk gave a distance of approximately 11 map units. In the *tbc1* bulk, as expected, the ratio of the signals was reversed and once again suggested a similarly tight linkage. However as the signal yielded by the SNP inherited by the *tbc1* parent was too strong and probably above saturation, calculations using its height could only suggest that the expected distance in this case would be less than 5 map units. The reverse SNP flank primer for this marker confirmed the tight linkage of this marker to *TBC1*, although the signals were too strong to be reliable for a precise determination of the distance (as shown on Figure 5, CNC30 2.).

Consistent with previous markers, the SNP ICL also produced results that pointed out the region between PF14 and CNC30 as the region with *TBC1*. The data obtained from the wild-type bulk was used to calculate a distance of 14 map units, while the one from the mutant bulk defined a distance of 5 map units, giving an average value of 9.5 map units.

When constructing the map in Figure 7 from these data the orientations of the distances between *TBC1* and markers PF14, CNC30 and ICL was presumed to be the one

most consistent with distances between *TBC1* and all the markers, however the other orientation, although unlikely, should not be excluded as a possibility.

Finally the SNP marker S813 is probably weakly linked to *TBC1* as the data from the wild-type bulk gave a distance of approximately 31 map units and the mutant bulk gave 6 map units. In all markers tested there was generally a difference between the peak ratios in both bulks, which as discussed above could be explained with the difference of the signal strength yielded by the two different fluorophores on the ddNTPs that were used to distinguish the bases of each SNP. As shown on the map in Figure 7 (on the left) this effect resulted in two different distances measured for each SNP. In order to simplify the mapping, the average values for the distances of the wild-type and mutant bulks for a marker were used and another map was generated (Figure 8, right). In both maps the arrows representing the distances from each SNP to the expected position of *TBC1* identified a region between by PF14 and CNC30 (outlined with orange lines on both maps on Figure 7)

While BSA proved to be a reliable approach to map SNP markers to the locus of interest, some difficulties were encountered. Concentrations loaded to the sequencing apparatus should be carefully titered to avoid signals above saturation that compromise determination of recombination frequencies.

This approach proved to be much more precise than the initial AFLP and BSA. Its resolution, although not as high as I expected, was sufficient to identify a region that could serve as a basis for identification of *TBC1* by complementation with BACs as described below.

### 3.2.4 Reversion of the peaks ratio between wild type and mutant bulks is observed only in markers linked to the segregating mutation

Since this is the first use of BSA in *C. reinhardtii*, and the subsequent approaches involving complementation were to be based on these results, it seemed at this point to be important to confirm that SNPs on other linkage groups do not test positive for linkage to *TBC1* using the methods described above to ensure that we were not obtaining misleading results from the SNPs on Linkage Group VI. Therefore, three SNP markers from linkage groups other than Linkage Group VI were tested for linkage to *TBC1* using the approach described above (Figure 8.). These SNPs were: CNA79, GP337 and GP350 mapped to linkage groups I, VIII and X respectively. SNP PCR1 products were confirmed to be of expected sizes (data not shown), and the SNP detected when using genomic DNA template from both F34.4 and S1D2 parents (Figure 8.) were also SNP specific (Table 6.)

As shown on Figure 8, the ratio of each of these SNPs was not significantly different between the wild-type and mutant bulks. Although in some of the cases signals were saturated, it is clear which one of each has higher peak height. These results clearly contrast the results obtained with the SNP markers on Linkage Group VI where cosegregation of these SNP and the parental *TBC1* allele was consistently observed. However, as mentioned above, the peak heights were not equal due to the difference in signal strength of the different fluorophores.

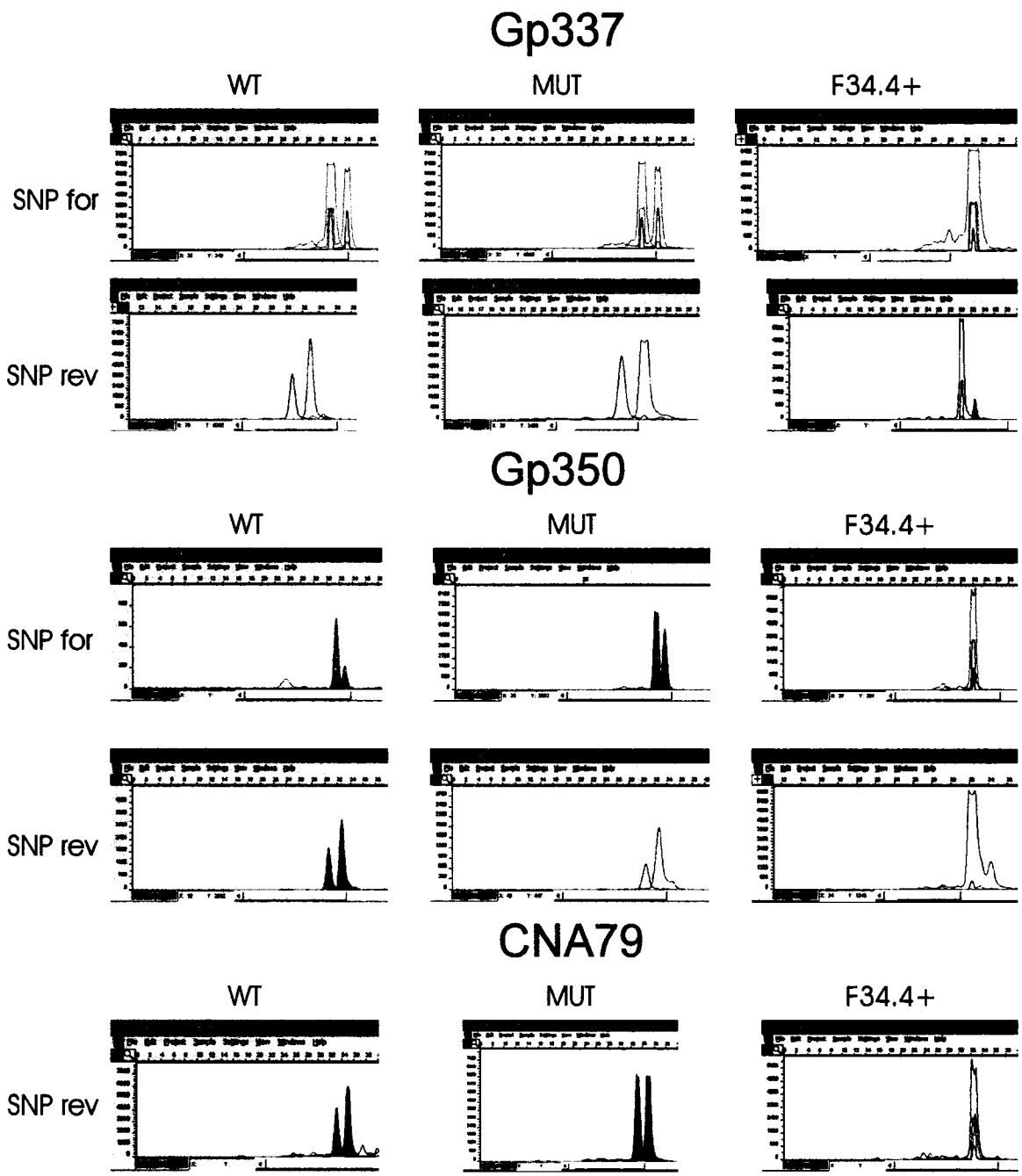



**Figure 8. Testing SNPs from other linkage groups.** For each SNP marker the readings from the wild type bulk, the mutant bulk and the F34.4+ parents (indicated as WT, MUT, F34.4+ resp.) using the forward and the reverse SNP flank primers (indicated as SNP for. and SNP rev.) are shown. CNA79 was detected using the reverse SNP flank only.

### 3.3 Localization of *TBC1* by complementation

At this point in the project, BSA had been fully exploited to localize *TBC1* to a 8 map unit or 761 Kb region of Linkage Group VI. However, there are more than 500 annotated genes described in this region on the *C. reinhardtii* JGI genome web site. To more precisely identify the genomic region with *TBC1*, I took advantage of the collection of recombinant BACs with known regions of *C. reinhardtii* genomic DNA, ranging in size from 22 – 190 kb. , with an average insert size of 75kb. These genomic BACs are now aligned with the genome sequence in Version 3 of the annotated genome. However, at this point of the project only the sequence of the ends of the genomic inserts in the BACs were available and provided in a searchable database. Thus, it was necessary to use Blast searches for Genomic BACs with at least one end in the region. By this approach, a minimal number of bacterial artificial chromosomes were identified that cover the region of linkage group VI where *TBC1* was predicted to be.

Since PF14 was the marker closest to *TBC1*, PF14 was used as a starting point to identify a region and BAC clones that cover it in order to test portions of it for complementation. Version v2.0 of the *C. reinhardtii* annotated genome on the JGI website was searched using BLAST (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=chlre2&advanced=1) for PF14, using the sequences of the primers that were used for SNP analysis at this locus (obtained from the online Genetics Center Database at http://www.chlamy.org). This approach identified a unique genomic sequence in scaffold 29 (869,210 bp). It was part of the sequence of Radial-spoke-protein 3 (*RSP3*), which extends from positions 794462-797152 relative to the start of scaffold

29. This was consistent with the fact that the PF14 is a mutant allele of *RSP3* (Piperno et al. 1981; Diener et al. 1993).

The same was done for the sequence flanking the marker CNC30 which was located at one end of the same scaffold in the region between positions 35543 and 35061. Thus a single scaffold was found that covered a region of linkage group VI between markers PF14 and CNC30 (Figure 9.).

The new version of the JGI Chlamydomonas BLAST tool (v 3.0) has a convenient feature for locating BACs within genomic DNA. At the time this step of the project was initiated, identifying BACs was an tedious process that required searching for sequences in scaffold 29 in the database with the sequences of the ends of genomic inserts of the BACs using BLAST. Since in most cases only one of the end sequences of the genomic DNA insert in a BAC was initially identified, due to the fact that most inserts extended beyond the query used, the other end had to be obtained from the BAC database (see Materials and Methods) and found in the genomic sequence in order to determine the entire region contained in a particular BAC. Twenty BACs were initially identified and eighteen more were obtained as soon as the new JGI Chlamydomonas BLAST v.3.0 was available. Twenty of these BACs found to cover the sequence of scaffold 29, were chosen (Table 7.). Their positions and the positions of the SNPs PF14 and CNC30 relative to the sequence of scaffold 29 are shown in the molecular map in Figure 9. Although this search was aimed at getting BACs with inserts that completely covered the region, i.e. with sufficient overlap to include intact potential genes at their junctions, for some junctions finding sufficient overlap was not possible because overlapping BAC were not in the collection.

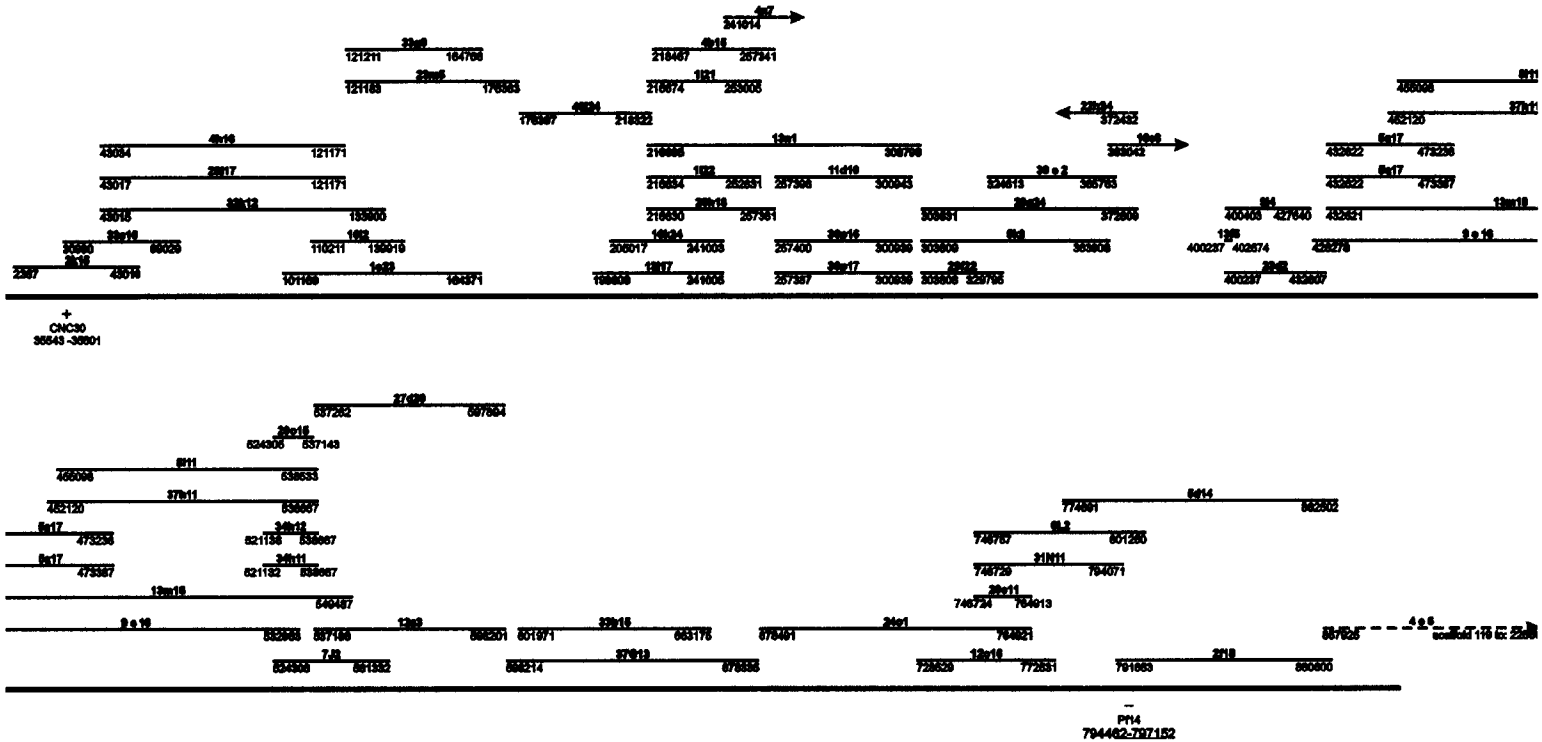



**Figure 9. Scaffold 29.** Scaffold sequence, 869210 bp (by data from JGI Chlamydomonas BLAST v2.0) presented as a straight black line. SNPs CNC30 and PF14 are shown under it with their relative positions on the scaffold sequence in base pairs. BACs found to cover this scaffold with their names and relative start and end positions in base pairs are depicted above it. BACs in red were used for complementation analysis. BACs having only one end sequenced are represented as that end's position relative to scaffold 29 with an arrow indicating their suggested orientation.

It should be noted that the in most recent release of the annotation of the *C. reinhardtii* genome (v3.0), the genomic BACs are annotated, their positions relative to the scaffolds are shown and they are graphically displayed. This made a subsequent search for BACs much faster and easier. The new version had also a better assembly of the scaffolds in terms of increasing their sequences. Scaffold 29 in v2.0 was renamed Scaffold 26 and its sequence was extended to 1626261 bp. Subsequent matching of the oligonucleotide primer sequences for the other SNP markers using v3.0 showed that scaffold 26 contained markers PF14, CNC30, CAB, ICL and VFL3. Thus the new scaffold 26 covered the region between markers PF14 – VFL3 (Figure 10.). Thus, the v3.0 and the extended sequences of the scaffolds allowed rapid identification and ordering an additional nineteen BAC clones and with the genomic sequence from markers PF14 to VFL3. These BACs are shown on Figure 11 with their relative positions on the scaffold sequence. The sequence of this scaffold, however, still had four large stretches of unidentified positions ("N"s) and no BAC covering these could be identified because their identification requires their precise sequence (as described above).

### 3.3.1. Correcting the genomic positions of markers ICL and CNC30

The verification of the markers on the actual sequence of this stretch of linkage group VI using the sequences of all SNP primers with v3.0 also showed that the genomic positions of markers ICL and CNC30 on the map prepared by Dr. Lefebvre (University of Minnesota) and published on the Chlamydomonas Database web site are actually inverted (Figure 2. and Figure 10.)

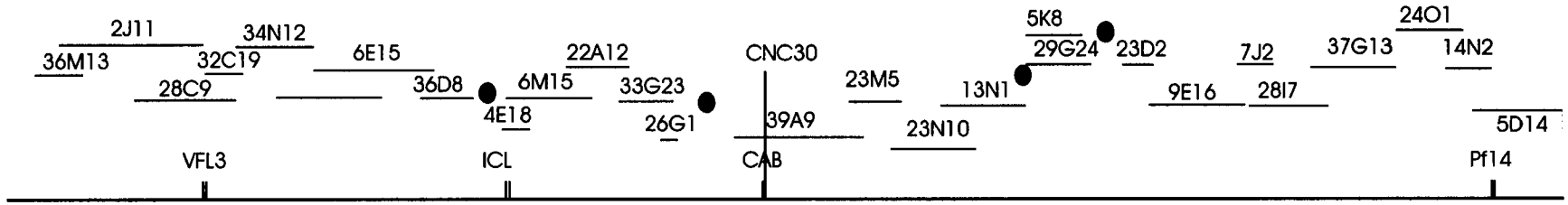





**Figure 10. Scaffold 26.** Scaffold sequence, 1620 Kb (by data from JGI Chlamydomonas BLAST v3.0) presented as a straight black line. SNP markers located on it are shown on the scaffold, their positions marked with red lines. Overlapping BACs covering the sequence of the scaffold are shown as green lines. Red dots indicate unsequenced regions not covered by any BAC.

### 3.3.2. Selecting for complementation of the *ac-* phenotype of *tbc1-F34*; optimization of efficiently transforming strains and transformation methodologies

These BACs (shown in red in Figure 9) were tested for the ability to complement the ac- phenotype produced by *tbc1-F34* following their introduction into a strain carrying this mutant allele by transformation. A previously reported protocol for high efficiency transformation by electroporation had to be optimized (See Materials and Methods, 2.9.1) due to its inefficiency with the available strains harboring *tbc1-F34* and electroporation efficiency was found to be largely dependant on the severity of the cell wall defect produced by the *CW15* mutation. *TBC1* mutant strains AS74, AS76, AS82 and AS86 (Table 1.) with intermediate severity of *CW15* were generated in this project and tested for high electroporation frequency. All of them transformed efficiently and AS76 was used in all subsequent electroporations as it regularly yielded 200 – 500 *arg+* transformants per electroporation. This is an important contribution to the research community because it reveals why most laboratories have been unable to achieve the high transformation frequencies described in the initial report of transformation of *C. reinhardtii* by electroporation.

*tbc1* mutants exhibit a PSII mutant phenotype and thus they cannot grow photoautotrophically. The *tbc1-F34* mutation also has a relatively high reversion rate of $10^{-5}$. Thus, one cannot select directly for ac+ transformants of *tbc1* mutants resulting from complementation by transformation of the wild-type gene. *Arg7* encodes argininosuccinate lyase, which carries out the final step in *de novo* arginine biosynthesis and it complements the arg7-8 mutation. This allele has never been observed to revert to wild-type and, therefore, it is used by many laboratories as a selectable marker gene for

transformation (Debuchy et al. 1989). In order to avoid false positive results due to the spontaneous reversion of *tbc1-F34*, a double selection was used using the strain that carries *tbc1-F34* and *arg7-8* (and *cw15*) as described in Materials and Methods. Thus, since the average number of arg+ transformants generated per electroporation is 200 – 500, spontaneous revertants of *tbc1-F34* should not be detected. Cells were cotransformed with each BAC and a plasmid containing and *ARG7* cDNA (Appendix 1.) and selected for arginine prototrophy and the *ac*+ phenotype, i.e complementation of both mutations.

### 3.3.3. Selection for complementation further narrowed the position of *TBC1* to a single BAC

The genomic DNA BAC clones were tested for *TBC1* by transformation of AS76 and selection complementation of its *ac*- phenotype. As described above, these transformants were also selected for complementation by the pT7arg7 plasmid of the *arg*- phenotype produced by *arg7-8*, which was co-transformed with each BAC. This eliminated the *ac*+ phenotypic revertants that arise spontaneously in strains carrying *tbc1-F34*; only one such colony should be detected in twenty transformations. After multiple rounds of transformation (see Appendix 1.), the BAC 28c9 (GJI sequence number PTQ10402) was found to consistently complement *tbc1-F34*. As this BAC contains a genomic DNA insert of 106 kb with 17 predicted genes (Figure 11.), additional work was required to identify *TBC1* therein.

Due to some difficulties during selection (Materials and Methods, 2.8.2) two approaches were used. The first involved an initial selection for transformants of the

ARG7 plasmid based on the complementation of the arginine auxotrophy and then a subsequent screening of hundreds of these transformants for the 10% of co-transformants that might be complemented by *TBC1* if it was present in the BAC. The second approach involved direct double selection on a minimal medium without arginine, thereby selecting for complementation of both phenotypes. The latter was found more reliable and, indeed, easier.

As a result BAC 28c9 emerged as the best candidate because in 5 (Appendix 1.) rounds of transformation and double selection it produced 4 – 10 colonies per transformation reaction. This frequency was expected based on previous reports that cotransformation frequency (i.e number of transformants that acquire and express two genes on different transforming DNA molecules) when using a plasmid and a large BAC clone is approximately 1 – 2 % (Kathir et al. 2003). Thus, considering that 100-500 *arg+* tranformants were obtained when only complementation of arg7-8 was selected, 2-10 cotransformants that also integrate the BAC DNA were expected. If a particular BAC has *TBC1*, then this percentage of the Arg+ transformants would be *ac+*. These colonies grew slowly but steadily on HSM without arginine and after being struck on fresh HSM plates formed viable colonies. No other BAC of the 39 tested yielded such results when simultaneously selecting for complementation of both mutant phenotypes.

The genomic DNA insert in BAC 28c9 is 106,957 bp. and it contains seventeen predicted protein-coding genes, based on the annotation of the *C. reinhardtii* genome in v3.0 (Figure 11 a.).

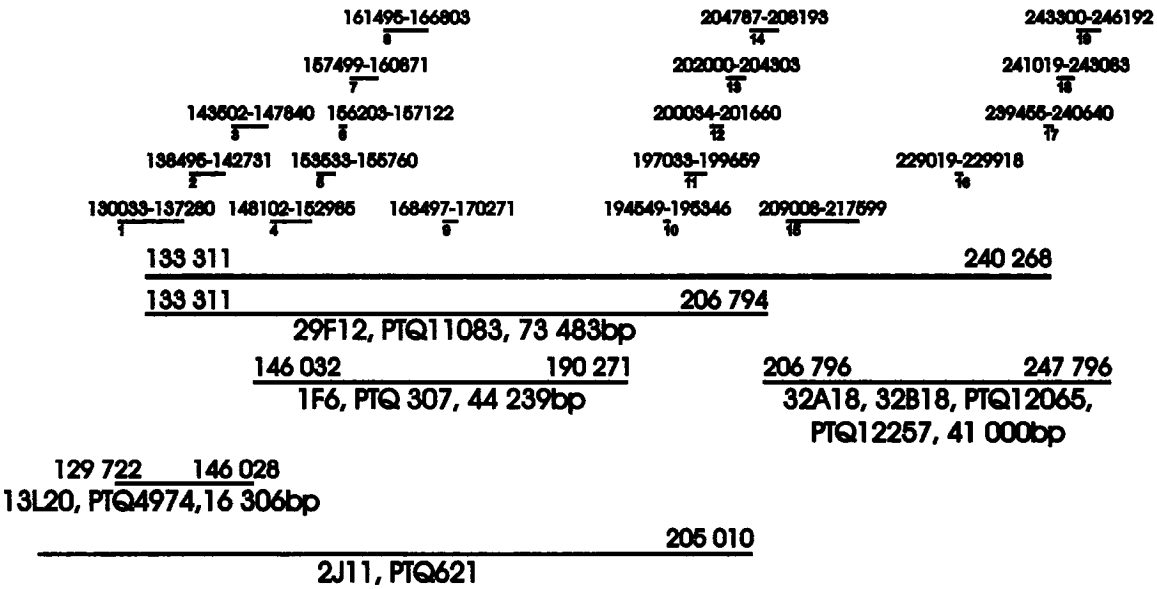



**Figure 11. BAC 28c9.** (legend continues on the next page), by JGI Chlamydomonas BLAST v3.0. BAC sequence represented as a continuous red line. The positions of the two ends of BAC 28c9 in bp. relative to the sequence of scaffold 26 (see Figure. 10) are indicated above the red line.

a. Predicted genes with their positions relative to 28c9 shown as numbered lines above the BAC. The location of the predicted start and stop codon for each gene model, relative to the scaffold 26 sequence in bp., are indicated above every gene. Three candidate genes were found by their putative structure/function. They are shown in green. Predicted genes are as follows:

1. Putative serine carboxypeptidase precursor[Gossypium hirsutum]
2. Putative serine carboxypeptidase precursor [Gossypium hirsutum]
3. Trans-splicing factor Raa3, chloroplast precursor [Chlamydomonas reinhardtii]
4. Hypothetical protein
5. Genomic DNA, chromosome 5, TAC clone:K11J9 [Arabidopsis thaliana]

6. No description

7. Tetratricopeptide repeat protein 2 [Drosophila melanogaster]

8. Putative transcriptional regulatory protein [Schizosaccharomyces pombe]

9. Cuticular collagen (CE17428) [Caenorhabditis elegans]

10. TMEM2; transmembrane protein 2 [Homo sapiens]

11. Putative methionine aminopeptidase [Arabidopsis thaliana]

12. C_600070 [Chlamydomonas reinhardtii]

13. Putative protein [Arabidopsis thaliana]

14. Variable flagellar number protein [Chlamydomonas reinhardtii].

15. CROL alpha (CG14938 protein) [Drosophila melanogaster]

16. PLEC1; plectin 1, intermediate filament binding protein [Homo sapiens]

17. EXO2_SCHPO Exonuclease II (Exo II) Putative 5-3 exonuclease domain

18. Cytochrome b-561 like protein

19. Hypothetical 28.6 kDa protein [Arabidopsis thaliana]

b. Other BACs covering portions of 28c9 are shown below its sequence. BACs are indicated with both their BAC clone names used in the BAC library and their JGI names. BAC sizes are given in bp. and the positions of their ends relative to the scaffold sequence in bp. are indicated above each BAC.

Three of these genes seemed to be candidates for *TBC1*, as the predicted functions of products or similarities with known chloroplast translation factors suggested a function in translational control (See Discussion). One has an amino acid sequence similarity to a chloroplast splicing factor. A second candidate encodes a predicted tetratricopeptide repeat protein. There is also an exonuclease II homolog with a putative 5'-3' exonuclease domain (Figure 11 a., genes No 3, 7, 17). The predicted sequence of the last gene was only partially present in 28c9,

The other predicted genes as well as non-coding sequence, according to JGI Chlamydomonas BLAST v3.0 sequence were also not excluded. Further analysis of BAC 28C9 showed that the sequence of the genome it covers could also be covered by several smaller BACs: 29F12, 1F6, 13L20, 32A18, 32B18 (Figure 11 b.). To further delimit the region with *TBC1*, these BAC clones were tested for their ability to complement *tbc1-F34* as described above.

After obtaining, preparing and cotransforming these BACs, two of them (32B18 and 32A18) were found to complement the PSII phenotype of *tbc1-F34* in several rounds of transformations (Figure 12). Both BACs cover the same genomic region of 41,000 bp and have a genomic DNA insert that partially overlaps BAC#28c9 in a 33.5 kb region. They also include the entire predicted Exonuclease II homolog. Analyzing its predicted sequence with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) predicted that the protein encoded by this gene has a chloroplast transit peptide. This along with its putative function (see Discussion) made it the most probable candidate for *TBC1* among all other genes in this 41 kb genomic region.

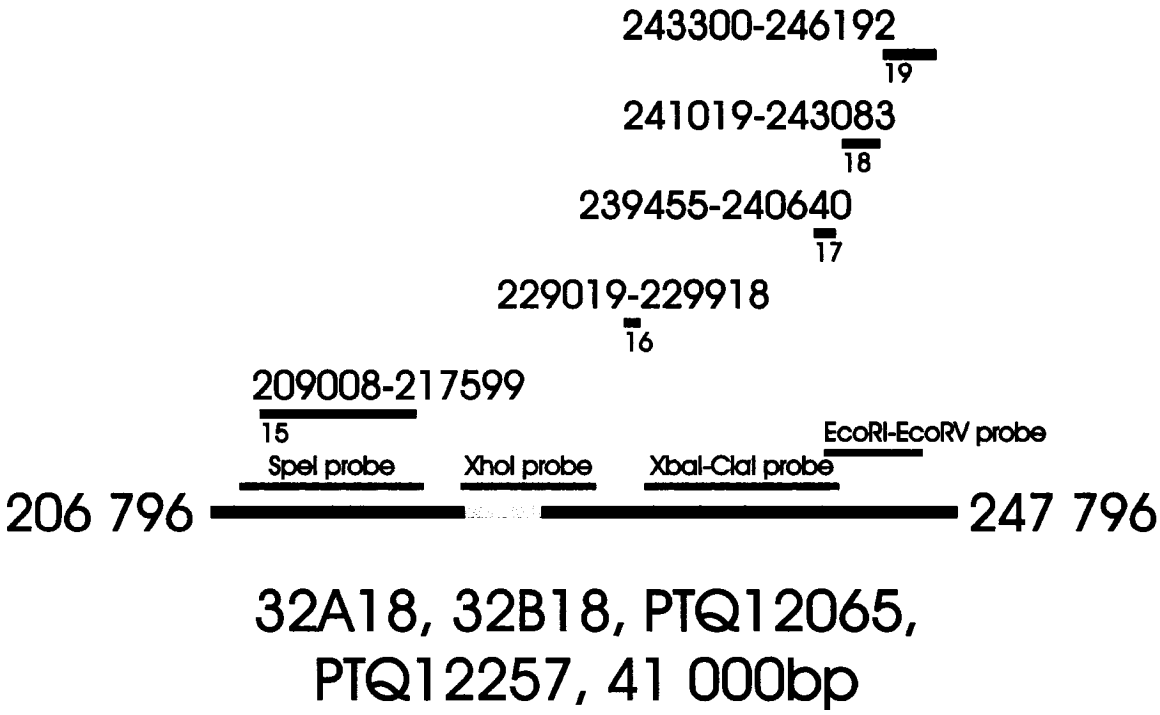



**Figure 12. BAC 32A18 and 32B18.** BAC sequence represented as a continuous black line. BAC ends' positions in bp. relative to the sequence of scaffold 26 (see Figure. 10) are indicated. Predicted genes covered by both 28c9 and 32A18/32B18 with their positions relative to 32A18 shown as numbered lines above the BAC. Predicted start and end positions of those genes relative to scaffold 26 sequence in bp. indicated above every gene. The gene numbers presented are as described in Figure. 12 b. Restriction fragments and the enzymes used to obtain them that cover different regions of 32A18 are indicated as green lines above 32A18 sequence. A 4.2 kb unsequenced gap in the genomic sequence covered by 32A18 is shown as a yellow region on the BAC.

To delimit further the region in the genomic DNA in BAC 32A18 with *TBC1*, subregions were excised as restriction fragments, purified and tested separately for the ability to complement the *tbc1-F34* as described above.

Four separate digests of restriction endonucleases *Spe*I, *Xho*I, *Xba*I and *Cla*I, *Eco*RI and *Eco*RV were performed in order to obtain these subfragments. As shown in Figure 12 these fragments cover different portions of the genomic region included in BAC 32A18. After having them isolated they are currently being used for further complementation tests.

### 3.4 Results of Southern hybridization suggest that the predicted exonuclease II ortholog in BAC 32A18 (and 28C9) (Figure 12) is *TBC1*

The high reversion frequency of the only mutant allele of *TBC1*, *tbc1-F34*, suggests that the mutation is a transposon insertion or a duplication, that can revert to wild-type by transposition or homologous recombination, respectively. Such lesions could be identified by genomic Southern blot analysis. With complementation, there is a slight risk that a mutant gene can be complemented by another gene because the two genes have overlapping functions. Thus, to confirm that *TBC1* is indeed within the region defined by positional mapping and complementation, Southern blot analyses were carried out to determine whether or not a genomic DNA rearrangement was present in a strain carrying *tbc1-F34* and, if so, whether this rearrangement is altered in a spontaneous revertant of a *tbc1-F34* strain. Thus, to detect any rearrangements in the genomic region covered by BAC 32A18 and more specifically the region encoding the putative ExoII orthologue, Southern analysis was performed. Genomic DNA was prepared from strains:

WZ120 (carrying *tbc1-F34*), $4C^+$ WT (wild type) and $F34.2A^+$ (a wild-type *tbc1* phenotypic revertant) and digested with *Pvu*II. The 10.7 kb *Cla*I-*Xba*I restriction fragment from 32A18 was radiolabeled with $^{32}P$ and used to probe a blot with the restricted genomic DNA from these strains (see Materials and Methods, 2.10.).

The preliminary results shown in Figure 13 revealed that there appears to be a change in this region in the strain carrying *tbc1-F34* that reverts to the pattern seen in the wild-type strain in the revertant strain. The results showed a prominent expected 3.5 kb fragment in wild-type and the *tbc1* revertant strain. A fragment was detected in the *tbc1-F34 strain* with a slightly larger size (of approximately 3.7 kb). A fragment of an unexpected size (approximately 3 kb) was also detected in the *tbc1* mutant strain (Figure 13). An expected 2.2 kb fragment was detected from the strain carrying *tbc1-F34* but not from the other two strains probably caused by weaker signals due to under loading of the DNAs from the wild-type and the revertant strains. Indeed upon visualizing the gel prior to the hybridization the lane loaded with restricted genomic DNA from the *tbc1* mutant strain was observed to be overloaded compared to the other two (data not shown). The fact that the other smaller expected fragments listed in the legend of Figure 13 were not detected could also be due to insufficient sensitivity caused by the under-loading of these lanes.

Southern hybridizations with various enzymes and probes deriving from different restriction fragments of 32A18 (Figure) as well as an EcoRI-EcoRV 23M5 fragment and a linearized 32A18 were done but due to background caused by unspecific hybridization abundant repeated sequences throughout in *C. reinhardtii* genome the results were not informative.

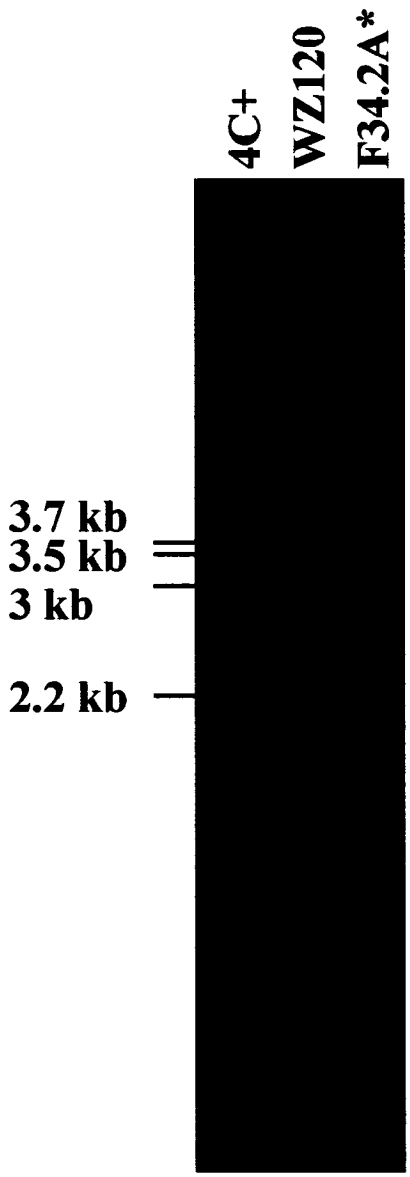



**Figure 13. Southern hybridization for rearrangements in *tbc1-F34* carrying strain.**

*Pvu*II digested genomic DNA from strains 4C+, WZ120 and F34.2A* (a *tbc1* revertant) was loaded in lanes 1 – 3 resp. and was probed with the 10.7 kb *Xba*I-*Cla*I fragment (Figure 12.). Restriction analysis of the genomic region covered by the probe showed that fragments of 3517, 2268, 1271, 840, 726, 563, 519, 511, 435, 289, 205, 139 bp were expected in the wild type strain. Detected fragments and their sizes are shown on the left. Two fragments of unexpected sizes (3.7 and 3 kb) are detected in strain WZ120.

# CHAPTER 4. DISCUSSION

This thesis explored the utility of BSA for the identification of nuclear genes in *C. reinhardtii* that have been identified only by genetic approaches. *TBC1* was identified within a 761 kb region of Linkage Group VI by recombination mapping with respect to known molecular markers in the first use of BSA in *C. reinhardtii*. This section describes the advantages and limitations of BSA in *C. reinhardtii* and proposes how future projects using this approach should be conducted. Also discussed is an initial characterization of the candidate gene for *TBC1* and how its product might function in the control of *psbC* translation.

## 4.1 The utility of BSA for the identification of nuclear genes in *C. reinhardtii*

As discussed in the Introduction, cloning by complementation is not possible for gene identification in *C. reinhardtii* in many cases. Genes that are identified only by mutant alleles that can not be identified by complementation using genomic libraries, because they produce no selectable phenotype, i.e. flagellar mutants or have a high spontaneous reversion rate. Mutant alleles that produce a dominant phenotype inherently cannot be complemented. Essential genes are poor targets for insertional mutagenesis and, therefore, are difficult to clone by gene tagging (see Introduction). Partial-loss-of-function mutations in essential genes, however, can be obtained by chemical or UV light mutagenesis. In these cases, map-based cloning may be a feasible approach. As previously described by Kathir et *al.* (2003), molecular markers mapped to a certain linkage group could be used for positional cloning after crossing the mutant strain to a

polymorphic lab strain and then scoring hundreds of progeny individually for each marker. However, this method of scoring is time consuming and expensive because hundreds of progeny are individually tested with labor intensive methods (given that PCR based markers are analyzed) for each molecular marker. Indeed, the simple step of pooling wild-type and mutant progeny into bulks reduces the number of samples tested from several hundred to two.

Introducing the BSA approach in *C. reinhardtii* will decrease the time, effort and the cost of the positional mapping, especially in the case of SNPs, which are the most abundant markers. This approach also avoids complications in data analysis that are associated with the current methods for positional cloning in *C. reinhardtii*. The mapping of *TBC1* was made possible with this technique and BSA was shown to be a useful approach for mutant alleles of genes that are desired to be cloned

The 761 kb region with *TBC1* that was identified by genetic mapping to molecular markers using BSA contained approximately 500 genes. This number of genes cannot practically be tested for a mutation by molecular methods. Thus, the identification of genes for which the only mutant alleles cannot be easily tested for complementation by transformed genomic DNA requires more precise methods of quantifying the levels of the molecular markers in the bulks are required to delimit a smaller region, i.e. with at most 5 -10 genes. More accurate methods might include mass spectrometry (Ross et al. 1998), pyrosequencing (Alderborn et al. 2000) or detection of SNP specific restriction sites.

However, as mentioned above, the low precision obtained in the mapping of *TBC1* with BSA did not delimit a practical number of genes for testing them individually for the *tbc1-F34* mutation by molecular methods (e.g., genomic southern blot analyses).

In some cases, when the nature of the biochemical function of a gene can be predicted from its mutant phenotype(s), (e.g., a specific auxotrophy, cytoskeletal defects, flagella defects). Thus, it may be possible to identify a candidate gene based on a review of numerous gene models within a large genomic region. But, in the region identified by the initial complementing BAC clone with *TBC1*, of the 17 predicted genes, three had features that suggest they could be *TBC1*. One had amino acid sequence similarity to a chloroplast mRNA splicing factor, another had TPR motifs, and the third is a potential orthologue of Exonuclease II. Any region identified by genetic mapping with the low precision fluorescence detection methods used in this study would presumably contain too many other possible candidates to test individually for a mutation.

Fortunately, the transformation of individual genomic BAC clones from this region of linkage group VI could be tested for the ability to complement the *ac-* phenotype produced by *tbc1-F34*. Recall that the frequency of cotransformation was too low for a practical large-scale screen of a genomic library. However, when individual BAC clones were used in each transformation, and transformants were selected for complementation of the arginine auxotrophy produced by *arg7-8* by a contransfromed plasmid containing an ARG7 cDNA expression cassette to exclude spontaneous *ac+* phenotypic revertants, it was possible to screen for the approximately 1% of these transformants that also integrate and express the *TBC1*, if it is present within the genomic DNA insert of that particular BAC. These complementation tests using BACs with genomic DNA inserts from the region of linkage group VI identified by recombination mapping showed that the smallest single BAC able to complement the *ac-* phenotype produced by *tbc1-F34* mutation is 41 kb (BAC number 32a18). The region of the genome

covered by the insert in this BAC contains five predicted genes (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Chlre3&advanced=1). Of these, an orthologue of Exonuclease II with a predicted ATP/GTP binding motif was considered as the most likely candidate for *TBC1* based on the known roles of exonuclease in translational control in the chloroplast of *C. reinhardtii* (as described below). None of the four other open reading frames were excluded. However other genes encode proteins with similarities to: plectin 1, an intermediate filament binding protein, a cytochrome b-561 like protein, and a hypothetical 28.6 kDa protein.

Moreover, this 41 kb genomic region contains a 4.2 kb region of undetermined sequence which could contain other genes, and possibly, *TBC1*. To identify the exact position of *TBC1* complementation tests with restriction fragments from the genomic DNA insert in this BAC are in progress.

### 4.1.1 AFLPs are not useful markers in BSA

In the course of this project two different molecular markers were tested for their reliability in the proposed approach. At the beginning the only available markers were AFLPs (Vos et al. 1995). Their choice was related to the fact that no insight on the sequence of the tested regions was necessary and that virtually an unlimited number of AFLP markers could be generated from a bulk. Yet mapping using AFLPs and BSA was very imprecise. The four unique AFLPs identified were derived from a 40 map unit region of linkage group VI, which corresponds to approximately 4 Mb. Extensive trouble-shooting was required at every step. The AFLP fragment must be isolated and sequenced, additional steps in which technical difficulties were encountered. Over nine

months was required to identify these AFLPs. Thus, the results of this project revealed that the identification of AFLPs linked to a gene of interest is too time-consuming, technically difficult, and imprecise to warrant the use of these markers in future positional cloning projects.

### 4.1.2 SNP Markers

Due to these difficulties encountered in the use of AFLP markers, other molecular markers had to be used. During the course of this thesis, a precise molecular map of *C. reinhardtii* genome was generated (Bowers et al. 2003; Rymarquis et al. 2005). Many of these markers are SNPs. SNPs were chosen due to their abundance; a known SNP marker was found on approximately every 10 map units on linkage group VI (Kathir et al. 2003) (http://www.chlamy.org/nuclear_maps.html). Additionally the online *C. reinhardtii* database provides information of the SNPs between the strains that were used to generate the bulks in this study; the highly polymorphic strain S1D2 and the laboratory strain from which most mutants have been derived (137C). In addition SNP detection is faster and easier compared to the generation, scoring and sequencing procedures associated with AFLP analysis (described above).

The use of SNP markers with BSA localized *TBC1* within a region of approximately 8 cM – 761 kb (Figure 7). This region was small enough to analyze by complementation, as described in Results. Comparisons of the ratios of the two SNPs in each bulk were used to determine genetic distance between the SNP marker and *TBC1* (see Results). This approach, however, provided no information about the direction of *TBC1* position relative to each SNP (Figure 7, punctate arrows). For some markers close

to the end of the right arm of linkage group VI, such as RPL41 and CNA9 there was only one possible orientation, however for SNPs close to *TBC1* (within PF14, CNC30 and ICL) the map distances had to be considered in the two possible directions.

It seemed logical to concentrate on the smallest region determined by considering all of the genetic distances between *TBC1* and these SNPs. Thus, initially the orientation shown by the solid arrows and the region between SNPs PF14 and CNC30 (Figure 7.) was considered. However, an initial survey of this region by complementation tests using genomic inserts in BACs that covered this region failed to identify one with *TBC1*. Upon testing the 750 kb more proximal region with additional set of genomic BACs, BACs #28c9 and #32a18 were identified as able to complement the ac- phenotype of *tbc1-F34*. The 33.5 kb region that is common to both of these BACs and presumed to contain *TBC1*, however, this was not precisely where I had predicted it to be based on the distances of *TBC1* and the SNP markers CNC30 and ICL SNP markers. Rather it is close to the SNP in VFL3. This suggests that for markers CNC30 and ICL the opposite orientation (Figure 7, punctate arrows) should have been considered. This exemplifies the impression obtained with mapping the locus with respect to SNPs using BSA.

Nevertheless the results obtained using SNPs identified a region that was small enough to start testing BACs for complementation. Apparently SNPs are much more reliable and accurate markers in *C. reinhardtii* than AFLPs.

More precise assays for the SNPs might improve the precision of mapping using BSA.Theoretically the accuracy should be much higher, thus it is possible that it was obstructed by the SNapShot$^{TM}$ method we used to score them. The different intensities of fluorescence of the different fluorophores on the ddNTPs (see 3.2.3) resulted in

deviations from the absolute values of the measured recombination ratios. Thus, it might be worth saving a few hundred individual progeny from the initial cross and as soon as the mapping with BSA and SNPs has reached a point where a certain region is identified, these could be tested individually for a precise individual mapping of one or two markers in the region. This should correct for the detection defect as only presence or absence of a SNP is detected in this case, and not their relative levels.

Since the error that affects the mapping accuracy is generated from the detection method it is a much better decision to use another unbiased approach for detecting SNP segregation in the bulks. It is expected that this will eliminate the accuracy problem and further improve the approach.

A much cheaper and relatively simpler technique that we are currently testing in order to improve the precision of the mapping in *C. reinhardtii* is amplifying a fragment containing the SNP from the bulks, then locating and test digesting a SNP specific restriction site, followed by measuring the ratios of fluorescence of the SNP specific restriction fragments visualized on an Ethidium Bromide stained gels. This approach should be less costly and relatively faster to perform, although it requires some initial analysis and verification of restriction sites affected by SNPs.

## 4.2 Suggestions for future map-based cloning projects involving BSA

From the perspectives provided by the results of this project, and established genetic and molecular procedures, combining BSA and the available SNP markers may prove to be a valuable approach for the identification of genes in *C. reinhardtii*. Therefore the following approach is proposed for future positional cloning projects. I

suggest that the use of BSA for map-based cloning of genes in *C. reinhardtii* begin by the identification of the linkage group with the gene of interest by testing a mutant allele for linkage to a SNP marker from each of the seventeen linkage groups. Primers for the analyses of SNPs throughout the genome are now available as a kit from the laboratory of David Stern (Cornell University, unpublished). This initial step would require less than one week of work and nominal costs. Once the linkage group with the locus is determined, then the same bulks could be used to map the locus with respect to c.a. five molecular markers distributed evenly across this linkage group. A subsequent round of mapping to an additional set of closely linked markers would be required to further delimit the region. Once a sufficiently narrow region is identified, a reasonable number of BACs could be identified and tested for complementation or the researcher could survey the annotated gene models in that region for potential candidates.

For genes indentified by mutant alleles that do not produce a phenotype that can be complemented or rapidly scored, it might also be useful to preserve c.a. 100-200 wild-type and mutant individual progeny from the initial cross of the mutant to SID2 for the more labor-intensive scoring of the frequency of the most tightly-linked SNP on either side of the locus of interest to obtain accurate recombination distances. In this case BSA could be used to identify a region of a particular linkage group and more precise mapping on individual progeny for just one or two markers.

## 4.3 A contribution to the transformation of *C. reinhardtii* by electroporation

Although high transform efficiencies by electroporation of *C. reinhardtii* have been reported, most researchers have been unable to achieve them. The basis for this has not been reported. Indeed, the initial complementation tests involving the genomic BAC clones in this project were unsuccessful because transformation frequencies with the standard approaches, including electroporation, were very inefficient. The individual testing each of the 39 genomic BAC clones for the ability to complement *tbc1-F34* required a reliable and efficient method of transformation. It is known that the cell wall defect produced by the *CW15* mutation improves electroporation efficiency (Brown et al. 1991; Shimogawara et al. 1998), possibly because the cell wall acts as a barrier to transforming DNA. However, I found that *CW15* mutant strains with a severe cell wall defect transform with low efficiency or not at all with electroporation.

By comparing the transformation efficiencies of several strains carrying *cw15*, I found an inverse correlation between the severity of the cell wall defect and transformation efficiency by electroporation as only strains with slight cell wall defects produced by *CW15* transformed efficiently (see 2.9.1). Cells with a wild-type cell wall and strains with a severe cell-wall defect did not transform. *CW15* mutants with slight cell wall deficiency transformed with drastically higher efficiency. The basis for the variable expressivity of this phenotype is unknown, although I presume it is related to the genetic background. Indeed, of several new mutant strains carrying *tbc1-F34*, *CW15* and *arg7-8* that I generated from several consecutive crosses, the strain (AS76) with the slightest cell wall defect transformed by electroporation with hightest efficiency; 100-500 transformants were obtained from each electroporation.

Although a severe cell wall defect increases the permeability of the cell wall to exogenous DNA, it probably also decreases the survivability of the cells during the high voltage potential to which they are exposed during this procedure. It is more likely that the cells with severe *cw15* phenotypes are killed during the electroporation rather than fail to recuperate after plating because an outgrowth in liquid medium or different types of embedding during plating on selective medium (Shimogawara, 1998) did not improve their electroporation efficiency. Furthermore cells when subjected to electroporation had low survivability even on non-selective medium. Therefore, strains with a slight CW15 phenotype have a cell wall that is sufficiently permeable to exogenous DNA but strong enough to protect cells from the harsh conditions imposed during electroporation. Thus, future projects that require high-efficiency transformation should generate and test multiple strains carrying the mutant allele of interest and CW15 to identify those with the least severe cell wall defect. The assay used to test this phenotype is described in the Materials and Methods.

## 4.4 *TBC1*

*TBC1* was localized to a 41 kb region of linkage group VI defined by the overlapping regions contained in two BAC clones that complement *tbc1-F34*. This region contains 5 predicted genes. My attention was focused on one of these genes, which encodes an Exonuclease II orthologue with a putative 5'-3' Exonuclease domain (Figure 11 a., genes No 3, 7, 17), because a substantial body of evidence points to a role of such an activity in translational activation in the chloroplast of *C. reinhardtii*. Although most of this gene is present at one end of the genomic DNA insert in BACs 28c9, 372 bp at the 5' end are

missing, corresponding to a 39 nt. region of its 516 nt. coding sequence. This could account for the low frequency of *ac+* complemented transformants that were obtained with this genomic BAC clone (Appendix 1). This protein, unlike the other ones on BAC 32a18 (see 4.1) was predicted to have a transit peptide at its N terminus, consistent with it having a function in the chloroplast (see Results)

A current model proposes that chloroplast mRNAs encoding certain core subunits of PSII and the cytochrome b6/f complex in *C. reinhardtii* require 5' terminal processing by a 5'-3' exonuclease for their stabilization and translation (Monde et al. 2000). These include the mRNAs of the multicistronic mRNA psbB/psbT/psbH (Adam and Loppes 1998) and the monocistronic mRNAs of *psbD* (Nickelsen et al. 1999), *rbcL*, *psbA* (Bruick and Mayfield 1998), and *petD* (Drager et al. 1999). These chloroplast mRNAs are present in two forms which differ at their 5' termini; a minor form has a 5' terminal extension relative to the major form. Furthermore some previous studies suggest that the shorter form could be translation competent, unlike the longer one (Gillham et al. 1994; Bruick and Mayfield 1998). For each mRNA, a nuclear gene product is required for the accumulation of the major form, but not the minor longer form. Other proteins with proposed roles in translational activation interact only with the short mature form (Nickelsen et al. 1994; Nickelsen et al. 1999; Monde et al. 2000). This led to the proposal of a model in which nucleolytic processing of the 5' terminal extension on the precursor mRNA is required to generate a stable and translatable major "mature" mRNA. These examples are reviewed in detail below.

Processing of the 5' UTR of the *rbcL* mRNA in barley was found to be related to translation initiation. This mRNA has two forms that differ in the lengths of their 5'

UTRs; one has 59 nt and the other 94 nt upstream from the initiation codon. In those two forms the longer 5' UTR is less translated despite similar levels of accumulation of the two forms (Reinbothe et al. 1993). Thus 5' processing of this mRNA was proposed to be involved in the activation of its translation. It was noted that the sequences in the longer "unprocessed" *rbcL* mRNA are complementary to a sequence at the 3' end. Base-pairing of these sequences was proposed to block the binding of the small ribosome subunit to a predicted SD-like sequence in the 5' UTR and thereby repress translation initiation. In barley, treatment with the hormone methyljasmonate increases the size of *rbcL* 5' UTR, alters the ratio of these *rbcL* mRNA forms and affects translation of the mRNA (Reinbothe et al. 1993). In these experiments it was observed that unlike the short form, the longer *rbcL* transcript is not associated with polyribosomes, which suggested it is translation incompetent.

In *C. reinhardtii* exonuclease processing of the 5' UTR of the *rbcL* mRNA was demonstrated to be a part of the translation control mechanism because changes in the translation of *rbcL* in response to light occur simultaneously with changes in length of the 5' UTR, possibly through processing. A lower level of *rbcL* translation is associated with an increase of the longer mRNA form (Shapira et al. 1997).

Similarly, in *C. reinhardtii* the *psbB/psbT/psbH* mRNA has a minor form with additional sequences at its 5' end (Vaistij et al., 2000). This form has been proposed to be a precursor which is processed to form the major "mature" form with the shorter 5' UTR. A nuclear mutation *mbb1* results in no detectable short form of *psbB* mRNA and it abolishes expression of *psbB* (Vaistij et al. 2000).

The *psbD* mRNA is also present in two forms that differ in the length of the 5' UTR: one is 74 nt and the other 47 nt (Nickelsen et al. 1994). These authors proposed that the shorter form is generated from the longer precursor by ribonucleolytic processing and that this processing is a requirement for translation of the mRNA (Nickelsen et al. 1999). It was observed that a 47 kD protein interacts only with the longer form but not with the translation competent shorter one (Nickelsen et al. 1994; Nickelsen et al. 1999). Mutation of the nuclear *NAC2* locus completely abolish expression of *psbD* because the short form of this mRNA does not accumulate and the longer form, although it accumulates, probably is not translated (Nickelsen et al. 1999). Similar, to *mbb1*, *nac2* nuclear mutation results in elimination of the short form only, which despite of the wild-type levels of the longer transcript completely abolishes its translation (Nickelsen et al. 1999).

The most conclusive evidence of RNA processing via endo- and exonuclease activity at the 5' end of a chloroplast mRNA was obtained by studies of the expression of *petD*, which encodes subunit IV of the cytochrome $b_6$/f complex (Sakamoto et al. 1994; Drager et al. 1999). Endonucleolytic processing of dicistronic *petA-petD* and processing of the resulting monocistronic *petD* mRNAs generates mature mRNAs that are translated (Sakamoto et al. 1994). *Mdc1* is a nuclear gene that is required for the 5'-3' exonucleolitic processing and accumulation of mionocistronic *petD*. It also has a role in *petD* translation (Drager et al. 1999). A model for *petD* translation was hypothesized in which the processing of its mRNA occurs in two steps, during the first step an endonuclease cleavage at position -20 occurs and then an exonuclease would process its 5' UTR to generate a mature message at -1. *Mcd1* is proposed to arrest this processing

activity to prevent complete degradation of the mRNA. In the *mcd1* mutant the entire mRNA is degraded (Drager et al. 1999). This model was supported by the finding that introduction of a poly-G stretch into the *petD* 5' UTR resulted in an increase in the level of the minor form, but not in a restoration of the *petD* expression in the *mcd1* mutant (Drager et al. 1999). Since poly-G is known to block 5' to 3' exonuclease activities, this result is consistent with the requirement for such an activity in the processing and translation activation of this mRNA and the proposed role of *MCD1* in arresting this activity at a certain position on the *petD* 5' UTR.

The *psbA* mRNA in *C. reinhardtii* also has two detectable forms with different 5' UTR lengths and only the shorter one is translated. The mature *psbA* mRNA has been proposed to be derived from a precursor with a longer 5' UTR. The longer form has a 90 nt 5' UTR while that of the major form is 36 nt. Ribonucleolytic processing of the longer one is presumed to be required to produce the shorter major form, which is translated (Erickson et al. 1984; Nickelsen et al. 1994). Other forms with different 5' UTRs were also suggested to exist (Bruick and Mayfield 1998). It was proposed that nuclear mutations causing a defect in translation of the *psbA* mRNA might affect this processing as in these mutants increased levels of the longer mRNA form were observed (Bruick and Mayfield 1998). This suggests that only the shorter message form is translation competent. A correlation between *psbA* 5'UTR processing and association with ribosomes was observed and data showed that the longer form is not associated with ribosomes (Bruick and Mayfield 1998). Furthermore the suggested cleavage site is within a stem-loop structure in *psbA* 5'UTR which was demonstrated to function as a target site for nucleus-encoded factors involved in translation activation by light (Danon

and Mayfield 1991). A model has been proposed in which these protein factors could function in the targeting or docking of *psbA* to the site of translation where cleavage would occur along with the dissociation of those factors and a translation competent *psbA* messenger would be produced (Rochaix 1996).

Ribonucleolytic processing also generates the 3' ends of mature chloroplast mRNAs, as in bacteria (Monde et al. 2000). Primary transcripts include 3' terminal extensions. In spinach chloroplasts, an endoribonucleolytic cleavage is generated at a specific site and the free 3' end generated is digested by an exoribonuclease until it is blocked by a stem-loop structure, which defines the 3' end of the mRNA (Stern and Gruissem 1987; Stern et al. 1989). This processing probably is required for translation because the chloroplast polyribosomes were found to translate the processed forms but not the unprocessed ones (Rott et al. 1998).

Other possible roles of exoribonucleolytic and endoribonucleolytic activities involve the cleavage of polycistronic mRNAs to produce translated monocistronic messages. Processing of the *psbB-petD* dicistronic mRNA by exoribonucleolytic and endoribonucleolytic activities in maize is important for the translation of the mature monocistronic *petD* (Barkan et al. 1994). Similarly, ribonucleolytic processing of the dicistronic *psaC-ndhD* mRNA in tobacco chloroplasts is required for its translation (Hirose and Sugiura 1997).

All those examples suggest that ribonuclease activities carry out the processing of chloroplast mRNAs to generate mature 5' UTRs and 3' UTRs that can interact with translation factors and ribosomes and promote their translation in chloroplasts of both *C. reinhardtii* and vascular plants. Although the models described above still have not been

rigorously tested, the predicted 5'-3' Exonuclease orthologue as a possible *TBC1* candidate is intriguing because it might identify the first exonuclease involved in these processes.

The cloning and identification of *TBC1* and the exact molecular mechanisms of its function will shed more light on the many different factors involved in the regulation of the expression of chloroplast genomes. Specifically, if *TBC1* does encode an exonuclease II, it would be possible to determine whether this protein is in the chloroplast and interacts with, and processes the *psbC* mRNA. Assays with the recombinant protein would allow experiments to test whether it has specificity for the 5' UTR of the *psbC* mRNA, or also to other mRNA that have been proposed to be processed to activate their translation and ensure their stability, as described above. It would also be of interest to determine whether other mutations in the same region of linkage group VI and that produce acetate-requiring phenotypes are alleles of *TBC1*. Characterization of their phenotypes might reveal additional functions of this gene product in chloroplast gene expression.

It should be noted that the hypothesis that *TBC1* encodes a 5' to 3' exonuclease that processes *psbC* mRNA predicts that this mRNA should have a minor form of this mRNA with a longer 5' UTR and possibly an abberant length in the *tbc1* mutant. An extensive series of primer extension experiments detected products that could correspond to such a longer minor form, however, the same positions were not mapped with primers that hybridize to different locations of the 5' UTR (Munir Rahim, personal communication). However, the primer extension products that identified the minor forms of the mRNAs of *psbD* and *psbB* were extremely rare. Thus, background bands in

the primer extension reactions on the *psbC* mRNA may have prevented the detection of the form with the 5' terminal extension. Also, this model predicts that a deficiency for the RNA exonuclease that processes a precursor mRNA to its mature form should result in destabilization of the mRNA. Yet, the *tbc1-F34* mutation does not affect the level of the *psbC* mRNA (Rochaix et al. 1989, Zerges and Rochaix 1994). This would be reconciled if this mutation only partially diminishes the activity of such an exonuclease of if the processing by this nuclease has a distinct function in translational control of *psbC*. Characterization of the molecular lesion of *tbc1-F34* and the level of the gene product expressed from this allele will be required to address this possibility.

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**Appendix 1. List of electroporations.**

Electroporations were done using 0.5 - 1 µg plasmid pT7-ARG7 (see Materials and Methods) and 1 - 2 µg of BAC DNA. BAC clones used for transformations are shown in the column indicated as DNA/BAC. Transformations with pT7-ARG7 only were also performed as positive controls (or negative) for the electroporations. Transformed cells were selected either initially for complementation of *arg7* (on TAP-R) and then consecutively selected for complementation of the *ac-* phenotype caused by *tbc1* (on HSM-R) or directly selected on HSM-R for both. Colonies were usually detectable within 20 days from transformation. For an unknown reason contamination (indicated with asterisks above the number of colonies of phenotypic revertants) temporary rescued *tbc1* mutant cells from their *ac-* phenotype. Those colonies however died shortly after transferring to HSM-R medium.

| Transformation and selection | | | | | |
|---|---|---|---|---|---|
| transformation date | DNA/BAC | selection | No colonies | picked to HSM-R | colonies |
| 11.07.2005 | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |
| | 13j7 | TAP-R | 0 | | |

| | | | | | |
|---|---|---|---|---|---|
| | 7j2 | TAP-R | 0 | | |
| | 32k12 | TAP-R | 0 | | |
| | pArg7,8 | TAP-R | 0 | | |
| | pT7 | TAP-R | 0 | | |
| | 33c16 | TAP-R | 8 | 8 | 0 |
| | 16c8 | TAP-R | 0 | | |
| | 23d2 | TAP-R | 0 | 10 | |
| 13.07.2005 | 16c8 | TAP-R | 13 | 13 | 0 |
| | 16c8 | TAP-R | 10 | 10 | 0 |
| | 37g13 | TAP-R | 12 | 21 | 0 |
| | 37g13 | TAP-R | 10 | 6 | |
| | 37g13 | TAP-R | 21 | 21 | 0 |
| | 29g24 | TAP-R | 5 | 6 | 0 |
| | 29g24 | TAP-R | 12 | | 0 |
| | 1o23 | TAP-R | 5 (+6 later) | 12 | 0 |
| | 40j24 | TAP-R | 6 | 10 | 0 |
| | 40j24 | TAP-R | 5 | | |
| 15.07.2005 | 16c8 | TAP-R | > 400 | 100 | 0 |
| | 16c8 | TAP-R | > 400 | 55 | 0 |
| | 37g18 | TAP-R | > 400 | 300 | 0 |
| | 5d14 | | > 400 | 200 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 6l2 | TAP-R | > 400 | 200 | 0 |
| | 24o1 | TAP-R | > 400 | 200 | 0 |
| | 12a3 | TAP-R | > 400 | 200 | 0 |
| | 4n7 | TAP-R | > 400 | 200 | 0 |
| | 13j7 | TAP-R | > 400 | 200 | 0 |
| | 23m5 | TAP-R | > 400 | 200 | 0 |
| | 40j24 | TAP-R | > 400 | 200 | 0 |
| | 7j2 | TAP-R | > 400 | 100 | 0 |
| | 13n1 | TAP-R | > 400 | 200 | 0 |
| | 9 e 16 | TAP-R | > 400 | 200 | 0 |
| | 29g24 | TAP-R | > 400 | 200 | 0 |
| | 32k12 | TAP-R | > 400 | 200 | 0 |
| | 23d2 | TAP-R | > 400 | 114 | 0 |
| | 1o23 | TAP-R | > 400 | 200 | 0 |
| | 2k15 | TAP-R | > 400 | 200 | 0 |
| | 33c16 | TAP-R | > 400 | 200 | 0 |
| | 4 e 6 | TAP-R | > 400 | 200 | 0 |
| | ARG7 only | | | | |
| 18.07.2005 | 13j7 | TAP-R | 1 | 1 | 0 |
| | 37g13 | TAP-R | 1 | 1 | 0 |
| | 33c16 | TAP-R | 1 | 1 | 0 |
| | 16c8 | TAP-R | 1 | 1 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 13j7 | TAP-R | 4 | 4 | 0 |
| | 1o23 | TAP-R | 4 | 5 | 0 |
| | 32k11 | TAP-R | 5 | 5 | 0 |
| | 32k12 | TAP-R | 3 | 3 | 0 |
| | ARG7 only | TAP-R | 0 | | |
| 19.07.2005 | 5d14 | TAP-R | 0 | | |
| | 5d14 | TAP-R | 0 | | |
| | 2k15 | TAP-R | 0 | | |
| | 3n1 | TAP-R | 4 | 4 | |
| | 5d14 | TAP-R | 17 | 17 | |
| | 2k15 | TAP-R | 3 | 3 | |
| | 5d14 | TAP-R | 11 | 11 | |
| | 9 e 16 | TAP-R | 6 | 6 | 0 |
| | 9 e 16 | TAP-R | 5 | 5 | 0 |
| | 23m5 | TAP-R | 16 | 16 | |
| | 4n7 | TAP-R | 0 | | |
| | 4n7 | TAP-R | 0 | | |
| | 23m5 | TAP-R | 12 | 12 | |
| | 6l2 | TAP-R | 14 | 14 | |
| | 2k15 | TAP-R | 7 | 7 | |
| | 13n1 | TAP-R | 5 | 5 | 0 |
| | 6l2 | TAP-R | 3 | 3 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 2k15 | TAP-R | 3 | 3 | 0 |
| | 13n1 | TAP-R | 4* | | |
| | 29g24 | TAP-R | 56 | 56 | 0 |
| | 37g13 | TAP-R | 25 | 25 | 0 |
| | 5d14 | TAP-R | 26 | 23 | 0 |
| | 5d14 | TAP-R | 27 | 11 | 0 |
| 20.07.2005 | All BACs Table 8. 1 | HSM-R | all 0 | | |
| 21.07.2005 | All BACs Table 8. 1 | HSM-R | all 0 | | |
| 22.07.2005 | All BACs Table 8. 1 | HSM-R | all 0 | 4 | |
| 25.07.2005 | 32k12 | TAP-R | 23 | 20 | 0 |
| | 23d2 | TAP-R | 15 | 15 | 0 |
| | 12a3 | TAP-R | 17 | 17 | 0 |
| | 23m5 | TAP-R | 30 | 26 | 1 |
| | 13n1 | TAP-R | 86 | 79 | 1 |
| | 24o1 | TAP-R | 7* | 8 | 0 |
| | 37g13 | TAP-R | 21* | 24 | 0 |
| | 13j7 | TAP-R | 10* | 12 | 0 |
| | 4 e 6 | TAP-R | 124 | 100 | 0 |
| | 33c16 | TAP-R | 36 | 31 | 0 |
| | 6l2 | TAP-R | 104 | 100 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 2k15 | TAP-R | 39 | 26 | 0 |
| | 29g24 | TAP-R | 86 | 80 | 0 |
| | 40j24 | TAP-R | 81 | 80 | 0 |
| | 7j2 | TAP-R | 137 | 108 | 0 |
| | 5d14 | TAP-R | 5 | 4 | 0 |
| | 4n7 | TAP-R | 1* | | |
| | 37g13 | TAP-R | 21* | 17 | 0 |
| | 16c8 | TAP-R | 0* | | |
| 26.07.2005 | All BACs Table 8. 1 | HSM-R | all 0* | | |
| 28.07.2005 | All BACs Table 8. 1 | HSM-R | 0 | | |
| 02.08.2005 | ARG7 only | TAP-R | 140 | | |
| | All BACs Table 8. 1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 4* | 4 | 0 |
| 04.08.2005 | 29g24 | TAP-R | 280 | 100 | 0 |
| | 33c16 | TAP-R | 138 | 100 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 23d2 | TAP-R | 20 | 20 | 0 |
| | 7j2 | TAP-R | 157 | 100 | 1 |
| | 4 e 6 | TAP-R | 0 | | |
| | 6l2 | TAP-R | 58 | 52 | 0 |
| | 2k15 | TAP-R | 4 | 4 | 0 |
| | 13n1 | TAP-R | 432 | 100 | 0 |
| | 32k12 | TAP-R | 184 | 100 | 0 |
| | 9 e 16 | TAP-R | 191 | 100 | 0 |
| | 5d14 | TAP-R | 320 | 100 | 0 |
| | 23m5 | TAP-R | 41 | 21 | 2 |
| | 1o23 | TAP-R | 9 | 9 | |
| | 12a3 | TAP-R | 503 | 88 | 0 |
| | 37g13 | TAP-R | 5 | 5 | |
| | 16c8 | TAP-R | 0 | | |
| | 40j24 | TAP-R | 435 | 100 | 0 |
| | 13j7 | TAP-R | 40 | 30 | 2 |
| | 33c16 | TAP-R | 64 | 50 | 0 |
| | 24o1 | TAP-R | 83 | 53 | 0 |
| | ARG7 only | TAP-R | 135 | 24 | 0 |
| 08.08.2005 | All BACs<br>Table 8. 1 | HSM-R | 0 | | |

| | | | | | |
|---|---|---|---|---|---|
| | ARG7 only | HSM-R | 0 | | |
| 16.08.2005 | ARG7 only | HSM-R | 380 | | |
| | All BACs<br>Table 8. 1 | HSM-R | 0 | | |
| 17.08.2005 | 23m5 | TAP-R | 380 | 80 | 1 |
| | 23m5 | TAP-R | 100 | 80 | 6 |
| | 32k12 | TAP-R | 100 | 100 | 0 |
| | 29g24 | TAP-R | 386 | | |
| | 23m5 | TAP-R | 7 | 5 | 0 |
| | 13j7 | TAP-R | 345 | 100 | 0 |
| | 6l2 | TAP-R | 230 | 100 | 0 |
| | 40j24 | TAP-R | 332 | 30 | 0 |
| | 40j24 | TAP-R | as above | 4 | 0 |
| | 40j24 | TAP-R | as above | 100 | 0 |
| | 9 e 16 | TAP-R | 170 | 100 | 0 |
| | 4n7 | TAP-R | 36 | | |
| | 24o1 | TAP-R | 214 | 100 | 0 |
| | 33c16, 4e6 | TAP-R | 48 | | |
| | 5d14 | TAP-R | 212 | 100 | 0 |
| | 32k12 | TAP-R | 280 | 100 | 0 |
| | 16c8 | TAP-R | 0 * | | |

| | | | | | |
|---|---|---|---|---|---|
| | 23d2 | TAP-R | 180 | 100 | 1 |
| | 1o23 | TAP-R | 30 | 20 | 0 |
| | 2k15 | TAP-R | 70 | 50 | 2 |
| | 7j2 | TAP-R | 210 | 100 | 1 |
| | 37g13 | TAP-R | 240 | 100 | 0 |
| | 12a3 | TAP-R | 400 | 100 | 0 |
| | 13n1 | TAP-R | 492 | | |
| | 13n1 | TAP-R | | 27 | 1 |
| 19.08.2005 | 13n1 | TAP-R | 210 | 100 | 2 |
| | 23m5 | TAP-R | 180 | 43 | 0 |
| | 23m5 | TAP-R | 80 | 100 | 0 |
| | 23m6 | TAP-R | | 64 | 0 |
| | 23d2 | TAP-R | 100 | 100 | 0 |
| | 4n7 | TAP-R | 238 | 100 | 0 |
| | 33c16 | TAP-R | 240 | 100 | 0 |
| | 16c8 | TAP-R | 220 | 100 | 0 |
| 23.08.2005 | 40j24 | HSM-R | 0 | | |
| | 19g14 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| 24.08.2005 | 13n1 | TAP-R | 18 | 18 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 23d2 | TAP-R | 2 | | |
| | 33c16 | TAP-R | 18 * | | |
| | 1o23 | TAP-R | 3 | 2 | 0 |
| | 32k12 | TAP-R | 24 | | |
| | 33c16 | TAP-R | 6 | | |
| | 12a3 | TAP-R | 6 | | |
| | 33c16 | TAP-R | 17 | | |
| | 16c8 | TAP-R | 0 | | |
| | 9 e 16 | TAP-R | 0 | | |
| | 33c16 | TAP-R | 1 | | |
| | 4n7 | TAP-R | 15 | | |
| | 33c16 | TAP-R | 2 | | |
| | 33c16 | TAP-R | 4 | | |
| | 40j24 | TAP-R | 12 | | |
| | 33c16 | TAP-R | 7 | 6 | 0 |
| 30.08.2005 | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 400 | | |
| | ARG7 only | TAP-R | 560 | | |
| | 13n1 | TAP-R | 154 | 38 | 18 |

| | | | | | |
|---|---|---|---|---|---|
| 30.08.2005 | 13n1 | TAP-R | 1 | | |
| | 33c16 | TAP-R | 2 | | |
| | 23m5 | TAP-R | 5 | | |
| | 9 e 16 | TAP-R | 1 | | |
| | 23d2 | TAP-R | 1 | | |
| | 1o23 | TAP-R | 1 | | |
| | 40j24 | TAP-R | 36 | | |
| | 32k12 | TAP-R | 31 | | |
| 02.09.2005 | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 10* | 10 | 0 |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 13* | 13 | 0 |
| | 23m5 | HSM-R | 7* | 7 | 0 |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 11* | 11 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 23m5 | HSM-R | 6* | 6 | 0 |
| | 23m5 | HSM-R | 0 | | |
| 02.09.2005 | ARG7 only | TAP-R | 0 | | |
| | 7j2 | TAP-R | 60 | 55 | 0 |
| | 13n1 | TAP-R | 21 | 18 | 0 |
| | 40j24 | TAP-R | 14 | | |
| | 23m5 | TAP-R | 4 | | |
| | 40j24 | TAP-R | 36 | | |
| | 13n1 | TAP-R | 16 | | |
| | 23m5 | TAP-R | 2 | | |
| | 13n1 | TAP-R | 12 | 2 | 0 |
| | 23m5 | TAP-R | 6 | 6 | 0 |
| | 23m5 | TAP-R | 3 | | |
| | 23m5 EcoRV | TAP-R | 13 | | |
| | 23m5 EcoRI-HpaI | TAP-R | 6 | | |
| | 23m5 EcorI-EcorV | TAP-R | 13 | | |
| | 23m 5 EcoRV | TAP-R | 21 | 20 | 1 |
| 08.09.2005 | ARG7 only | TAP-R | 7only baad transformation | | |

| | | | | | |
|---|---|---|---|---|---|
| 20.09.2005 | ARG7 only | TAP-R | 0 | | |
| | 7j2 | TAP-R | 88 | 34 | |
| | 13n1 | TAP-R | 25 | 20 | 0 |
| | 40j24 | TAP-R | 14 | | |
| | 23m5 | TAP-R | 4 | | |
| | 40j24 | TAP-R | 37 | 4 | |
| | 13n1 | TAP-R | 16 | 4 | |
| | 13n1 | TAP-R | 20 | | |
| | 23m5 | TAP-R | 14 | 17 | 0 |
| | 23m5 | TAP-R | 3 | | |
| | EcorI - 23m5 | TAP-R | 13 | | |
| | EcorI-HpaI - 23m5 | TAP-R | 6 | 4 | 0 |
| | EcorI-EcorV - 23m4 | TAP-R | 35 | 14 | 0 |
| | EcorI-EcorV - 23m5 | TAP-R | 13 | 9 | 0 |
| | 23m5 | TAP-R | 12 | 10 | 0 |
| | 23m5 | TAP-R | 14 | 14-18 | 0 |
| | EcoRV - 23m5 | TAP-R | 53 | 27 | 0 |
| | EcorI - 23m5 | TAP-R | 10 | | |
| | 7j2 | | 27 | | |

| | | | | | |
|---|---|---|---|---|---|
| 03.10.2005 | EcoRI+EcoRV (23m5) | TAP-R | 160 | 25 | 0 |
| | | | | 50 | 0 |
| | | | | 71 | 0 |
| | EcoRI+BglII (23m5) | TAP-R | 268 | 88 | 0 |
| | | | | 68 | 0 |
| | | | | 90 | 0 |
| | EcoRI+HpaI (23m5) | TAP-R | 166 | | |
| | EcoRV (23m5) | TAP-R | 360 | | |
| | EcoRV (23m5) | TAP-R | 104 | | |
| | EcoRI-HpaI (23m5) | TAP-R | 48 * | | |
| | 4 e 6 | TAP-R | 108 | 100 | 0 |
| | 4 e 6 | TAP-R | 81 | | |
| | 4 e 6 | TAP-R | 93 | | |
| | 13n1 | TAP-R | 65 | 100 | 0 |
| | 13n1 | TAP-R | 121 | 105 | 0 |
| | 7j2 | TAP-R | 123 | 120 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 7j2 | TAP-R | 109 | 100 | 1 |
| | 23m5 | TAP-R | 63 | 63 | 0 |
| | 40j24 | TAP-R | 140 | 100 | 0 |
| | 40j24 | TAP-R | 97 | 97 | 0 |
| 05.10.2005 | 23m5 | TAP-R | 0 | | |
| | 23m5 | TAP-R | 17 | | |
| | 23m5 | TAP-R | 12 | 53 | 0 |
| | 23m5 | TAP-R | 1 | | |
| | 23m5 | TAP-R | 11 | | |
| | 23m5 | TAP-R | 12 | | |
| | 7j2 | TAP-R | 3 | | |
| | 7j2 | TAP-R | 3 | | |
| | 7j2 | TAP-R | 6 | | |
| | 7j2 | TAP-R | 12 | 12 | 0 |
| | 4 e 6 | TAP-R | 5 | 10 | 0 |
| | 4 e 6 | TAP-R | 1 | | |
| | 4 e 6 | TAP-R | 0 | | |
| | 4 e 6 | TAP-R | 1 | | |
| | 4 e 6 | TAP-R | 6 | | |
| | 13n1 | TAP-R | 5 | 21 | 0 |
| | 13n1 | TAP-R | 10 | | |
| | 13n1 | TAP-R | 11 | | |

| | | | | | |
|---|---|---|---|---|---|
| | 13n1 | TAP-R | 10 | | |
| | 20j24 | TAP-R | 1 | | |
| | 40j24 | TAP-R | 6 | 8 | 0 |
| | pT7-Arg7 only | TAP-R | 2 | | |
| | pT7-Arg7 only | TAP-R | 2 | | |
| 07.10.2005 | EcorI - EcorV (23m5) | TAP-R | 420 | 100 | 0 |
| | | | | 60 | 0 |
| | BglII - EcorI (23m5) | TAP-R | 400 | 60 | 0 |
| | Bglii - EcorI (23m5) | TAP-R | 81 | 40 | 0 |
| | EcorV (23m5) | TAP-R | 94 | 52 | 0 |
| | EcorI - HpaI (23m5) | TAP-R | 260 | 100 | 0 |
| | EcorI - HpaI (23m5) | TAP-R | 180 | 100 | 0 |
| | | | | 100 | 0 |
| | | | | 90 | 0 |
| | 23m5 | TAP-R | 220 | | |
| | 23m5 | TAP-R | 60 | 48 | 0 |

| | | | | | |
|---|---|---|---|---|---|
| | 23m5 | TAP-R | 63 | | |
| | 23m5 | TAP-R | 140 | 100 | 0 |
| | 40j24 | TAP-R | 77 | 55 | 0 |
| | 40j24 | TAP-R | 83 | 100 | 0 |
| | 40j24 | TAP-R | 218 | 130 | 0 |
| | 40j24 | TAP-R | 395 | | |
| | 13n1 | TAP-R | 122 | 100 | 0 |
| | 13n1 | TAP-R | 140 | | |
| | 13n1 | TAP-R | 147 | | |
| | 4 e 6 | TAP-R | 124 | | |
| | 7j2 | TAP-R | 150 | 22 | 0 |
| | EcoRI-BglII (23m5) | TAP-R | 400 | 55 | 0 |
| | EcoRI (23m5) | TAP-R | 260 | 75 | 0 |
| 11.10.05 | 13n1 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 40j24 | HSM-R | 0 | | |
| | EcoRI+HpaI (23m5) | HSM-R | 0 | | |
| | EcoRI- | HSM-R | 0 | | |

| | | | | | |
|---|---|---|---|---|---|
| | EcoRV(23m5) | | | | |
| | EcoRI-EcoRV(23m5) | HSM-R | 0 | | |
| | EcoRV(23m5) | HSM-R | 0 | | |
| | EcoRI-BglII(23m5) | HSM-R | 0 | | |
| | XbaI (23m5), EcoRI(23m) | HSM-R | 0 | | |
| | 7j2 | TAP-R | 109 | 109 | 1 |
| 14.10.05 | XbaI (23m5) | TAP-R | 0 | | |
| | XbaI (23m5) | TAP-R | 0 | | |
| | XbaI (23m5) | TAP-R | 0 | | |
| | XbaI (23m5) | TAP-R | 0 | | |
| | XbaI (23m5) | TAP-R | 120 | | |
| | XbaI (23m5) | TAP-R | 180 | | |
| | XbaI (23m5) | TAP-R | 210 | 200 | 0 |
| | XbaI (23m5) | TAP-R | 105 | 100 | 0 |
| | XbaI (23m5) | TAP-R | 137 | | |
| | XbaI (23m5) | TAP-R | 156 | | |
| | XbaI (23m5) | TAP-R | 198 | | |
| | XbaI (23m5) | TAP-R | 110 | | |
| | XbaI (23m5) | TAP-R | 181 | | |

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|---|---|---|---|---|---|
| | XbaI (23m5) | TAP-R | 31 | | |
| | XbaI (23m5) | TAP-R | 64 | | |
| | XbaI (23m5) | TAP-R | 2 | | |
| | XbaI (23m5) | TAP-R | 178 | | |
| 18.10.05 | 23m5 | TAP-R | 14 | 33 | 0 |
| | 23m5 | TAP-R | 21 | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 1 | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 51 | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 121 | 100 | 2 |
| | EcoRI-HpaI (23m5) | TAP-R | 46 | 20 | 0 |
| | EcoRI-HpaI (23m5) | TAP-R | 63 | 45 | 0 |
| | EcoRI-HpaI (23m5) | TAP-R | 46 | | |
| | XbaI (23m5) | TAP-R | 138 | 132 | 1 |
| | XbaI (23m5) | TAP-R | 81 | 28 | 0 |
| | 7j2 | TAP-R | 48 | 60 | 1 |
| | 7j3 | TAP-R | 58 | | |

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|---|---|---|---|---|---|
| | 4 e 6 | TAP-R | 18 | 10 | 0 |
| | 13n1 | TAP-R | 63 | | |
| | 13n1 | TAP-R | 57 | 20 | 0 |
| 17.10.2005 | XbaI (23m5) | TAP-R | 0* | | |
| | XbaI (23m5) | TAP-R | 0* | | |
| | XbaI (23m5) | TAP-R | 0* | | |
| | XbaI (23m5) | TAP-R | 480 | 30 | 0 |
| | XbaI (23m5) | TAP-R | 30* | | |
| | XbaI (23m5) | TAP-R | 50* | | |
| | 7j2 | TAP-R | 350 | 100 | 0 |
| | 7j2 | TAP-R | 180 | | |
| | EcoRV (23m5) | TAP-R | 21 | | |
| | EcoRV (23m5) | TAP-R | 2 * | | |
| | EcoRV (23m5) | TAP-R | 0 * | | |
| | EcoRV (23m5) | TAP-R | 0 * | | |
| | EcoRV (23m5) | TAP-R | 0 * | | |
| | EcoRV | TAP-R | 0 * | | |

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|---|---|---|---|---|---|
| | (23m5) | | | | |
| | EcoRV (23m5) | TAP-R | 0 * | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 3 * | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 0 * | | |
| | EcoRI-HpaI (23m5) | TAP-R | 6 * | | |
| | ARG7 only | TAP-R | 8 * | | |
| 27.10.05 | 13n1 | TAP-R | | 100 | 0 |
| | 7j2 | TAP-R | | 52 | 0 |
| | 4 e 6 | TAP-R | | 100 | 0 |
| 31.10.05 | BamHI-AflII (23m5) | TAP-R | 204 | | |
| | BamHI-AflII (23m5) | TAP-R | 96 | | |
| | BamHI-AflII (23m5) | TAP-R | 0 * | | |
| | BamHI-AflII (23m5) | TAP-R | 224 | | |

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|---|---|---|---|---|---|
| | BamHI-AflII (23m5) | TAP-R | 128 | | |
| | BamHI-AflII (23m5) | TAP-R | 160 | | |
| 28.11.05 | 7j2 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 4n7 | HSM-R | 0 | | |
| | 24o1 | HSM-R | 0 | | |
| | 2k15 | HSM-R | 0 | | |
| | 1o23 | HSM-R | 0 | | |
| | 33c16 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 9c16 | HSM-R | 0 | | |
| | 12a3 | HSM-R | 0 | | |
| | 23d2 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 6l2 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 32k12 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 40j24 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| 22.11.05 | 12a3 | HSM-R | 0 | | |
| | 40j24 | HSM-R | 0 | | |
| | 40j24 | HSM-R | 0 | | |
| | 32k12 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |
| | 4n7 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 32k12 | HSM-R | 0 | | |
| | 1o23 | HSM-R | 0 | | |
| | 33c16 | HSM-R | 0 | | |
| | 4n7 | HSM-R | 0 | | |
| | 24o1 | HSM-R | 0 | | |
| | 23d2 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 1o23 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 13j7 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 9 e 16 | HSM-R | 0 | | |
| | 9 3 16 | HSM-R | 3* | | |
| | 24o1 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 2k15 | HSM-R | 0 | | |
| | 6l2 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| | 23d2 | HSM-R | 0 | | |
| | 1o23 | TAP-R | 17 | | |
| | 33c16 | HSM-R | 0 | | |
| | 16c8, | TAP-R | 145 | | |
| | ARG7 only | TAP-R | 142 | | |
| | no plasmid | HSM+R | 0 | | |
| 29.11.05 | ARG7 only | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 24o1 | HSM-R | 0 | | |
| | 40j24 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 23m5 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| | 9 e 16 | HSM-R | 0 | | |
| | 32k12 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |
| | 33c16 | HSM-R | 0 | | |
| | 12a3 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 6l2 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 32k12 | HSM-R | 0 | | |
| | 24o1 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 9 e 16 | HSM-R | 0 | | |
| | 12a3 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| | 33c16 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 40j24 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 13n1 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 6l2 | HSM-R | 3* | | |
| 07.12.05 | 7j2 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 16c8 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | XbaI (23m5) | HSM-R | 0 | | |
| | XbaI (23m5) | HSM-R | 0 | | |
| | XbaI (23m5) | HSM-R | 0 | | |
| | XbaI (23m5) | HSM-R | 0 | | |
| | XbaI (23m5) | HSM-R | 0 | | |
| | EcoRI-HpaI | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | (23m5) | | | | |
| | EcoRI-HpaI (23m5) | HSM-R | 0 | | |
| | EcoRI-HpaI (23m5) | HSM-R | 0 | | |
| | ARG7 only | TAP-R | 98 | | |
| 12.12.05 | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 13j7 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |

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|---|---|---|---|---|---|
| 12.2005 | 16c8, | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 5d14 | HSM-R | 0 | | |
| | 2k15 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 9 e 16 | HSM-R | 0 | | |
| | 33c16 | HSM-R | 0 | | |
| | 24o1 | HSM-R | 0 | | |
| | 4n7 | HSM-R | 0 | | |
| | 1o23 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 37g13 | HSM-R | 0 | | |
| | 4 e 6 | HSM-R | 0 | | |
| | 7j2 | HSM-R | 0 | | |
| | 4n7 | HSM-R | 0 | | |
| | 1o23 | HSM-R | 0 | | |
| | 6l2 | HSM-R | 0 | | |
| | 23d2 | HSM-R | 0 | | |
| | 9 e 16 | HSM-R | 0 | | |
| | 23m5 | HSM-R | 0 | | |
| | 29g24 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 4 e 6 | HSM-R | 0 | | |
| | 12a3 | HSM-R | 0 | | |
| | 13n1 | HSM-R | 0 | | |
| 16.01.06 | EcoRI-EcoRV (23m5) | TAP-R | 49 | 49 | 0 |
| | EcoRI-EcoRV (23m5) | TAP-R | 51 | 51 | 0 |
| | EcoRI-EcoRV (23m5) | HSM-R | 0 | | |
| | ARG7 only | TAP-R | 69 | | |
| | ARG7 only | TAP-R | 52 | | |
| | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |
| 27.01.06 | EcoRI-EcoRV (23m5) | HSM-R | 0 | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 240 | 140 | 0 |
| | EcoRI-EcoRV (23m5) | TAP-R | 61 | | |
| | EcoRI-EcoRV (23m5) | TAP-R | 0 | | |

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|---|---|---|---|---|---|
| | 23m5 | TAP-R | 62 | 100 | 0 |
| | 23m5 | TAP-R | 29 | | |
| | 23m5 | TAP-R | 53 | | |
| | ARG7 only | TAP-R | 242 | | |
| | ARG7 only | TAP-R | 75 | | |
| | ARG7 only | TAP-R | 101 | | |
| | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | HSM-R | 0 | | |
| 06.03.06 | 33g9 | HSM-R | 0 | | |
| | 4 e 18 | HSM-R | 0 | | |
| | 5k8 | HSM-R | 0 | | |
| | 6m5 | HSM-R | 0 | | |
| | 36m13 | HSM-R | 0 | | |
| | 34n12 | HSM-R | 0 | | |
| | 22a12 | HSM-R | 0 | | |
| | 36d8 | HSM-R | 0 | | |
| | 2j11 | HSM-R | 0 | | |
| | 26g1 | HSM-R | 0 | | |
| | 2j11 | HSM-R | 0 | | |
| | 6 e 15 | HSM-R | 0 | | |
| | 33g23 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 28l7 | HSM-R | 0 | | |
| | 4 e 18 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 14n2 | HSM-R | 0 | | |
| | 39a9 | HSM-R | 0 | | |
| | 23n10 | HSM-R | 0 | | |
| | 24j11 | HSM-R | 0 | | |
| | 28c9 | HSM-R | ~10 | 5 | 5 |
| | 28c9 | HSM-R | 5 | 4 | 4 |
| | 28c9 | HSM-R | 9 | 6 | 6 |
| | 28c9 | HSM-R | 4 | 4 | |
| 13.03.06 | ARG7 only | TAP-R | 38 | | |
| | ARG7 only | TAP-R | 0 | | |
| 20.03.06 | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 3* | | |
| | 33g23+34N12 | HSM-R | 3* | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 1* | | |
| | 39a9+23n10 | HSM-R | 40* | | |
| | 39a9+23n10 | HSM-R | 35* | | |
| | 39a9+23n10 | HSM-R | 36* | | |
| | 39a9+23n10 | HSM-R | 20* | | |
| | 34n12 | HSM-R | 0 | | |
| | 33g24+34n12 | HSM-R | 0 | | |
| | 33g24+34n12 | HSM-R | 0 | | |
| | 33g23+34N12 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 33g23+34N12 | HSM-R | 0 | | |
| | 39a9+23n10 | HSM-R | 0 | | |
| | ARG7 only | TAP-R | 180 | | |
| | ARG7 only | TAP-R | 92 | | |
| 28.03.06 | ARG7 only | TAP-R | 167 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 7 | | |
| 03.04.06 | ARG7 only | HSM-R | 0 | | |
| | ARG7 only | HSM-R | 20* | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 2 | | |
| | 28l7 | HSM-R | 0 | | |
| 10.04.06 | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 2j11 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 2j11 | HSM-R | 0 | | |
| | 2j11 | HSM-R | 0 | | |
| | 14n2 | HSM-R | 0 | | |
| | 14n2 | HSM-R | 0 | | |
| | 6 e 15 | HSM-R | 0 | | |
| | 6 e 15 | HSM-R | 0 | | |
| | 39a9 | HSM-R | 0 | | |
| | 36d8 | HSM-R | 0 | | |
| | 33g9 | HSM-R | 0 | | |
| | 33g9 | HSM-R | 0 | | |
| | 5k8 | HSM-R | 0 | | |
| | 5k8 | HSM-R | 0 | | |
| | 5k8 | HSM-R | 0 | | |
| | ARG7 only | TAP-R | 135 | | |
| | ARG7 only | TAP-R | 82 | | |
| | ARG7 only | HSM-R | 0 | | |
| | ARG7 only | HSM-R | 4* | | |
| 12.04.06 | 33g23 | HSM-R | 0 | | |
| | 14n2 | HSM-R | 0 | | |
| | 4 e 18 | HSM-R | 0 | | |
| | 26g1 | HSM-R | 5* | | |

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|---|---|---|---|---|---|
| 18.04.06 | ARG7 only | TAP-R | 0 | | |
| | ARG7 only | TAP-R | 0 | | |
| | 5x 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 6* | | |
| 20.04.06 | ARG7 only | TAP-R | 600 | | |
| | ARG7 only | TAP-R | 780 | | |
| | ARG7 only | TAP-R | 560 | | |
| | ARG7 only | HSM-R | 0 | | |
| | 29f12 | HSM-R | 0 | | |
| | 1f6 | HSM-R | 0 | | |
| | 13l2 | HSM-R | 0 | | |
| | 29f12 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 0 | | |
| | 28c9 | HSM-R | 3 | | |
| | 13l2 | HSM-R | 5* | | |
| | 13l2 | HSM-R | 8* | | |
| | 1f6 | HSM-R | 0 | | |
| | 1f6 | HSM-R | 0 | | |
| 21.04.06 | ARG7 only | TAP-R | 320 | | |
| | ARG7 only | HSM-R | 2 - 3* | | |
| | 13l20 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 29f12 | HSM-R | 0 | | |
| | 1f6 | HSM-R | 0 | | |
| | 1f6 | HSM-R | 0 | | |
| 26.04.06 -1 | ARG7 only | TAP-R | 82 | | |
| | 1312 (4ul) | HSM-R | 10* | | |
| | 1f6 (4ul) | HSM-R | 15* | | |
| | 1f6 (4ul) | HSM-R | 12* | | |
| | 1f6 (4ul) | HSM-R | 18* | | |
| | 29f12 | HSM-R | 7* | | |
| | 29f12 | HSM-R | 25* | | |
| 26.04.06 -2 | ARG7 only | TAP-R | 608 | | |
| | 1312 | HSM-R | 0 | | |
| | 1f6 (4ul) | HSM-R | 15* | | |
| 08.05.06 | ARG7 only | TAP-R | 99 | | |
| | ARG7 only | TAP-R | 86 | | |
| | ARG7 only | HSM-R | 0 | | |
| | 32b18 | HSM-R | 0 | | |
| | 32b18 | HSM-R | 0 | | |
| | 32b18 | HSM-R | 4 | | |
| | 32b18 | HSM-R | 0 | | |

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|---|---|---|---|---|---|
| | 32b18 | HSM-R | 3 | | |
| | 32b18 | HSM-R | 0 | | |
| | 32b18 | HSM-R | 2 | | |
| 09.05.06 | ARG7 only | TAP-R | 237 | | |
| | 32a18 | HSM-R | 5 | | |
| | 32a18 | HSM-R | 3 | | |
| | 32a18 | HSM-R | 2 | | |
| | 32a18 | HSM-R | 0 | | |