Effects of mutations at the two processing sites of the precursor for the small subunit of ribulose-bisphosphate carboxylase in *Chlamydomonas reinhardtii*

Cédric INVERNIZZI, Jonathan IMHOF, Gabriela BURKARD¹, Katharina SCHMID and Arminio BOSCHETTI²

Departement für Chemie und Biochemie, Universität Bern, Freiestrasse 3, CH-3012 Bern, Switzerland

The role of the two processing sites in the precursor of the small subunit (SS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSS) of *Chlamydomonas reinhardtii* was studied by introducing mutations at the cleavage sites for the stromal processing peptidases SPP-1 and SPP-2, which hydrolyse wild-type pSS (20.6 kDa) to an intermediate-sized product iSS (18.3 kDa) and to the mature SS (16.3 kDa), respectively. The mutations introduced into cDNA resulted in exchange of (a) two amino acids flanking processing site 1, or (b) one or (c) both amino acids flanking processing site 2. Mutation (a) prevented pSS from being processed at site 1 but not from cleavage at site 2. Mutation (c) abolished the action of SPP-2 but not SPP-1. When pSS with mutation (c) was imported into isolated chloroplasts, iSS accumulated while SS formation was abolished. However, mature SS was produced even in the absence of iSS synthesis (mutation a). Import of pSS bearing mutation (b), which only

partially inhibited processing at the SPP-2 site, slowed the rate of SS formation down whereas iSS and some slightly smaller derivatives accumulated. These experiments suggested that in *Chlamydomonas* processing of pSS can occur in two steps, whereby the first step is facultative. The same three mutations were studied *in vivo* after transformation of SS-deficient *C. reinhardtii* T60-3 with mutated genomic DNA. Growth and photosynthesis was as in control transformants, except for the slower-growing transformants (mutation c) where no mature SS was immuno-detected. However, pSS fragments with molecular masses between those of iSS and SS were present even in the ribulose-1,5-bisphosphate carboxylase/oxygenase holoenzyme.

Key words: chloroplast, protein import, stromal processing peptidase, transformation.

INTRODUCTION

Proteins to be imported into chloroplasts are synthesized as precursor proteins containing an N-terminal extension called a pre-sequence or transit peptide, which is sufficient for import of precursor proteins into the stroma [1–5]. After translocation of precursor proteins across the double chloroplast envelope membrane the transit peptide is cut off by a stromal processing peptidase (SPP), resulting in a smaller molecule, e.g. a mature stromal protein. Particularly in higher plants, almost all precursor proteins are imported through the same translocation apparatus in one step [6,7] and the presence of one ‘general stromal processing peptidase’ has been reported [8,9]. It is not obvious how this enzyme can recognize the different precursor proteins as substrates and can specifically cut them at their individual processing sites, especially since sequence similarity between the amino acid sequences of different transit peptides is very low [10].

In contrast to higher plants, *Chlamydomonas* chloroplasts contain various SPPs, which can be separated from each other by chromatography [11,12]. *In vitro*, specifically one or a small group of precursor proteins are processed by each enzyme. The protein fractions containing single enzyme activities showed no cross-reactivity. For the precursor of the small subunit (pSS) of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO; E.C. 4.1.1.39), two different specific processing enzymes, SPP-1 and SPP-2, were found, specific for processing sites 1 and 2,

respectively, of pSS from *Chlamydomonas reinhardtii*. *In vitro*, a stromal protein fraction enriched in SPP-2 cleaved pSS (20.6 kDa) at the N-terminal side of Met-46 (processing site 2), thereby producing the ‘mature’ protein small subunit (SS; 16.3 kDa), whereas SPP-1 cleaved pSS N-terminally of Met-25 in the middle of the pre-sequence (processing site 1), producing the intermediate-sized form iSS (18.3 kDa) [13].

iSS could also be detected after import of heavily labelled pSS into isolated chloroplasts [14]. By fractionation of such chloroplasts into supernatant and membranes, SS and iSS were found to be soluble proteins while pSS remained bound to the membrane fraction. Furthermore, treatment of chloroplasts after pSS import both with thermolysin, which degrades proteins outside the outer envelope membrane, and with trypsin, acting additionally in the intermembrane space, revealed that iSS but not SS was located in the intermembrane space of the chloroplast envelope [14]. These findings raised the question about the physiological role of iSS and the enzyme SPP-1. If in *Chlamydomonas* iSS is not simply the product of an unknown by-pass reaction but indeed an intermediate, formed during processing of pSS, then a two-step mechanism for pSS import can be hypothesized; the iSS would be formed in the intermembrane space and only after the second translocation would mature SS be produced in the stroma by SPP-2. Such a hypothesis contradicts the postulated mechanism for higher plants according to which import across the double envelope membrane occurs in one step at contact sites

Abbreviations used: HSM, high-salt medium; RFLP, restriction fragment length polymorphism; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAP, shrimp alkaline phosphatase; LS, large subunit of RuBisCO; SS, small subunit of RuBisCO; SSHis, SS with a hexahistidyl tail; iSS, intermediate-sized pSS; iSSHs, iSS with a hexahistidyl tail; pSS, precursor of SS; pSSHs, pSS with a hexahistidyl tail; SPP, stromal processing peptidase; SPP-1, SPP-2, SPPs specific for processing sites 1 or 2 of pSS from *C. reinhardtii*.

¹ Present address: Universität Zürich, Institut für Medizinische Virologie, Gloriastrasse 30, CH-8028 Zürich, Switzerland.

² To whom correspondence should be addressed, at Römerstrasse 1, CH-3047 Bremgarten, Switzerland (e-mail arminio.boschetti@bluewin.ch).

between the two membranes [15]. To test whether both processing sites 1 and 2 are essential for pSS import and for fitness or viability of the cells, they were mutated to abolish cleavage by the corresponding SPPs.

EXPERIMENTAL

Plasmids, organisms and cultivation

C. reinhardtii T60-3, a strain deficient in both *rbcS* genes and lacking a normal cell wall, was a kind gift from Dr R. J. Spreitzer [16]. *C. reinhardtii* cw-15 mt+ (CC-1615 from the Chlamydomonas Genetics Centre, Duke University, Durham, NC, U.S.A.), containing the wild-type *rbcS* genes and described in the following as the wild-type strain, was used as a control strain in most experiments, except for the isolation experiments of the RuBisCO holoenzyme, where *C. reinhardtii* *arg*₂ mt+ (no. 137c from The Culture Centre of Algae and Protozoa, Cambridge, U.K.) served as a control. Wild-type strains and transformants produced in this study were cultivated phototrophically in high-salt medium (HSM) [17] or mixotrophically in Tris/acetate/phosphate medium [18] supplemented with 2 g · l⁻¹ sodium acetate, or, for measuring growth curves and O₂ evolution, in HSM also containing 2 g · l⁻¹ sodium acetate. The cultures were incubated in Erlenmeyer flasks at 25 °C on a shaker under continuous illumination at either high light intensity (80 µE · m⁻² · s⁻¹ photosynthetic active radiation from 400 to 750 nm; strong light) or low light intensity (4–8 µE · m⁻² · s⁻¹).

The plasmids pSSpSP64 and pSSHispSP64, containing the cDNA of *rbcS*-2 and used for *in vitro* synthesis of pSS and pSS with a hexahistidyl tail (pSSHisp), were described previously [19], as were the plasmids used for *in vitro* synthesis of iSS and iSS with a hexahistidyl tail (iSSHisp) [12]. The plasmids pRbcS-2 and pRbcS-1, encoding the genomic *rbcS* genes, were described and put at our disposal by Dr R. J. Spreitzer [16].

DNA analysis

Handling of plasmids and plasmid DNA, restriction enzyme analysis, agarose gel electrophoresis followed by ethidium bromide staining, and transformation of *Escherichia coli* were carried out according to Sambrook et al. [20] or according to the instructions of the enzyme producer. All restriction enzymes originated from New England Biolabs (Hitchin, Herts., U.K.) except for *Eco*RI, *Hind*III, *Nsi*I, *Sal*I (Roche Diagnostics, Zurich, Switzerland) and *Pvu*II (Promega, Madison, WI, U.S.A.). Total cellular DNA was isolated from 300 mg of pelleted and frozen *Chlamydomonas* cells by solubilization in a thermomixer at 55 °C and 1000 rev./min for 5 h in 430 µl of extraction buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA and 1 % SDS), 50 µl of guanidinium chloride (5 M) and 20 µl of proteinase K (20 mg/ml). After 15 min of centrifugation at 12000 g the supernatant was mixed with 1 ml of Wizard resin (Promega) and the DNA was isolated according to the manufacturer's protocol.

Amplification and characterization of wild-type and mutated *rbcS* genes

To distinguish between *rbcS*-1 and *rbcS*-2 the PCR protocol and the primers described by Khrebtukova and Spreitzer [16] were used. In order to amplify the transit sequence of *rbcS*-2, the PCR was run using the primers 5'-GATGTTTGATGGGGTATT-TGAGCA-3' and 5'-GATTCCTGACTTGGGCACTGTTC-3' (2 mM MgCl₂; 3 min at 96 °C followed by 50 cycles of 90 s at 92 °C, 120 s at 62 °C and 180 s at 74 °C, followed by 180 s at

Table 1 Mutations introduced at the processing sites of pSS and *rbcS*-2

The mutations refer to pSS coded by genomic DNA as well as cDNA.

Processing enzyme	Wild-type sequence	Mutant sequence	Designation of the mutation
SPP-1	Pro-24/Met-25 CGCCCCATGGCC	→ Ile-24/Trp-25 CG CATATG GGCC	1-2aa
SPP-2	Met-46 AACCAGATGATG	→ His-46 AACCAG CATATG	2-1aa
SPP-2	Gln-45/Met-46 AACCAGATGATG	→ Ile-45/Trp-46 A ACATATG GATG	2-2aa

74 °C). We received *Taq* polymerase either from Promega or ABgene (Epsom, Surrey, U.K.). Mutated processing sites were verified by *Nde*I restriction analysis. To prepare sufficient DNA for sequencing, the PCR products were ligated into a pGEM-T_Easy vector (Promega) and amplified in *E. coli* XL-1 Blue.

The initial steps for introducing the mutations listed in Table 1, all generating the *Nde*I restriction site CATATG, into the pSS gene on cDNA in pSSHispSP64, followed the two-level PCR strategy of Mikaelian and Sergeant [21]. However, the very high GC content of *rbcS*-2 DNA, especially in the region to be mutated, caused stable secondary structures which hampered PCR and led to deletions.

Mutation 2-1aa

The first step consisted of two PCRs, both with *Pvu*II-linearized pSSHispSP64 as a template, one with primer M containing the mutation (5'-CCGGGGTCCAGACC**CATATG**CTGTTGG-3'; with the mutation underlined) and primer 1 (5'-CCGGGGT-GCGGAACGGCGTTACCAGAAA-3'), the other with primer 3 (5'-CTGCCGAAGCGGATGGCCGACTCGTTGG-3') and mismatch primer 2 (5'-**AAATGCAGCTGTCGTTAGAACGC**-GGTACAAT-3'). Conditions for PCR: 1.5 mM MgCl₂, 0.35 % DMSO; 3 min at 96 °C, 35 cycles of 1 min at 94 °C, 1.5 min at 60 °C and 3 min at 73 °C, followed by 3 min at 72 °C. The PCR products of 446 bp and 404 bp were isolated from agarose gels, and used in a mixture (0.7 fmol/50 µl each) as a template in the second level of PCR primed with 0.5 µM primer 1 and primer 3 (1.5 mM MgCl₂; 3 min at 95 °C, 40 cycles of 0.6 min at 95 °C, 1.5 min at 60 °C and 3 min at 72 °C, followed by 3 min at 72 °C). The broad band of 500–550 bp on agarose gel was double-digested with *Bam*HI/*Pst*I. After inactivation of the restriction enzymes at 80 °C the mixture was directly used for ligation of the presumptive 270 bp fragment to the shrimp alkaline phosphatase (SAP)-treated 3285 bp fragment of similarly digested pSSHisp-SP64 and transformation into *E. coli* XL-1 Blue. Sequencing of those plasmids containing the new *Nde*I restriction site demonstrated the introduction of the correct mutation, although also of deletions making *in vitro* translation to pSSHisp impossible. Therefore, a 75 bp DNA fragment (*Psh*AI/*Nco*I) containing the correct mutation was ligated into a SAP-treated 3580 bp fragment (*Psh*AI/*Nco*I) from pSSHispSP64. The resulting plasmid was sequenced and pSSHisp containing mutation 2-1aa could be synthesized *in vitro*.

Mutation 2-2aa

A similar procedure as for 2-1aa was used with primer M containing mutation 2-2aa: 5'-CCGGGGTCCAGACC**CATATG**TGTTG-3'. After the two-step PCR the selected and amplified plasmid contained the correct mutation. An in-frame

deletion nearby, however, lead to a truncated protein upon *in vitro* translation. From this plasmid a *Bam*HI/*Bgl*II fragment of 145 bp containing the mutation was cut out and ligated to a SAP-treated mixture of the largest fragments (around 3511) obtained by extensive and partial digestion of pSSHispSP64 with *Bam*HI and *Bgl*II, respectively. As *Bgl*II produces no enzyme-specific though sequence-specific sticky ends, ligation of this mixture with the mutated 145 bp fragment should result in formation of the expected specific plasmid. Indeed, after cloning in *E. coli* XL-1 Blue, sequencing of the final plasmid confirmed the presence of the mutation 2-2aa but also showed a substitution (G → A) at nucleic acid position 199, causing an amino acid exchange Val-52 → Ile in the *in vitro*-synthesized pSSHisp.

Mutation 1-2aa

Two long oligonucleotides, overlapping at the mutation site, were synthesized. One contained the *Sfi*I restriction site (5'-GGCCGTGGCCCGCCCGGCCGCTCCAGCGTGCGCA-TATGGGCCGCGCTGAAGCCCGCCGTCAA-3'; 63 bases), the other the *Psh*AI site (5'-GTTGTTGACCGGGGTCCAGACCATCATCTGTTGGCCTGAGCCGGGGCAGCCACGGGGCGGCGCTTGACGGCGGCTTCAGCGCGGCCCA-TATGC-3'; 95 bases). These oligonucleotides were annealed and double strands formed (annealing, 1.5 mM MgCl₂, 0.5 μM oligonucleotides, no polymerase; 5 min at 99 °C, then reduced 1 °C/min to 96 °C; elongation, addition of polymerase and 1 min at 96 °C followed by 5 min at 20 °C). The product of 127 bp was isolated from the agarose gel, digested with *Sfi*I/*Psh*AI, and ligated to the SAP-treated 3550 bp restriction fragment (*Sfi*I/*Psh*AI) from pSSHispSP64. Sequencing confirmed the presence of the desired mutation 1-2aa but also of a substitution (A → G) at nucleic acid position 193 causing an amino acid exchange Thr-50 → Ala in the *in vitro*-synthesized pSSHisp.

Mutagenesis at processing sites of pSS on genomic DNA

The plasmid pRbcS-2 of 10.5 kb contains in addition to the pUC19 vector and the genomic *rbcS*-2 gene two unknown sequences of 0.5 and 5.0 kb. First, the 5.0 kb sequence was eliminated by digestion with *Nsi*I. Then, a 5.7 kb fragment was treated with *Eco*RI (and *Sca*I to prevent formation of two fragments of similar size) and a 3.1 kb fragment containing the *rbcS*-2 gene was recovered. It was ligated to a SAP-treated 2643 bp fragment obtained from pUC19 (*Eco*RI/*Pst*I), generating the shortened plasmid pRbcS-2(5.7 kb) used for mutation.

Mutations 1-2aa and 2-1aa

The plasmids pSSHispSP64 bearing the appropriate mutations on cDNA (see above) and pRbcS-2(5.7 kb) were cut with *Psh*AI and *Sfi*I. The 102 bp fragment from the cDNA was ligated to the large, SAP-treated fragment from pRbcS-2(5.7 kb) and amplified in *E. coli* XL-1 Blue.

Mutation 2-2aa

Since in the 2-2aa-mutated pSSHispSP64 the *Psh*AI restriction site was damaged, two large overlapping oligonucleotides, 5'-GAACGTGGCCCGCCCGGCCGCTCCAGCGTGCGCC-CATGGCCGCGCTGAAGCCCGCCGTCAAAGCCCGCC-CCCGTGG-3' (77 bases) and 5'-GTTGTTGACCGGGGTCC-AGACCATCCATATGTTGGCCTGAGCCGGGGCAGCC-ACGGGGGCGGCGCTTGACGGCGGGCTTC-3' (79 bases) were annealed. 3'-Elongation resulted in a 127 bp product in

which, however, the *Sfi*I site was destroyed (annealing, 1.5 mM MgCl₂, 0.5 μM oligonucleotides, no polymerase; 5 min at 99 °C, then 1 °C/min to 96 °C; elongation, addition of polymerase and 1 min at 96 °C followed by 1 °C/min to 90 °C and 10 min at 90 °C). Therefore, the product was double-digested with *Nco*I and *Psh*AI, ligated to the SAP-treated 3576 bp fragment (*Nco*I/*Psh*AI) obtained from pSSHispSP64, and amplified in *E. coli* XL-1 Blue. From the resulting plasmid the *Psh*AI/*Sfi*I fragment was cut out and introduced into pRbcS-2(5.7 kb) as above.

Radiolabelling of pSS, its import and processing in isolated chloroplasts

Intact chloroplasts from synchronous cultures of *Chlamydomonas* were isolated according to the procedure of Mendiola-Morgenthaler et al. [22], modified by Su and Boschetti [11]. For the synthesis of radiolabelled pSS and pSSHisp, linearized plasmids pSSpSP64 or pSSHispSP64 were transcribed *in vitro* and translated in a wheatgerm system in the presence of [³⁵S]methionine [19]. Conditions for chloroplast import were as described by Su et al. [23].

Preparation of SPP-1 and SPP-2, and processing *in vitro*

Protein fractions enriched in SPP-1 or SPP-2 were prepared from the stroma of *Chlamydomonas* chloroplasts according to Su and Boschetti [11] and used for *in vitro* processing as described by Rüfenacht and Boschetti [12]. Additionally, plain stroma was used instead of enriched SPP-2.

Electrophoresis of proteins and Western blotting

Samples solubilized with 2 % SDS and 3 % 2-mercaptoethanol for 1 min at 95 °C were centrifuged and the supernatant analysed by SDS/PAGE according to Laemmli [24], using either homogeneous 15 % polyacrylamide (Mini Protean II; Bio-Rad) or 12–18 % gradient gels (Protean II; Bio-Rad). Protein blots on nitrocellulose were immunostained using polyclonal antisera raised in rabbits against purified SS or LS (the large subunit of RuBisCO) isolated from *Chlamydomonas*, followed by the Chemiluminescent Protein Detection System (Bio-Rad) for staining. Radioactive bands were detected and quantified by Phosphor-Imager (Molecular Dynamics) and ImageQuant software.

Transformation of *Chlamydomonas* T60-3

The general procedure of Kindle [25] was followed. In detail, T60-3 cells grown in TAH (Tris/acetate/phosphate medium, 2 g/l sodium acetate and 0.4 g/l yeast extract) at 4 μE · m⁻² · s⁻¹ to a density of (1–2) × 10⁶ cells/l, were pelleted for 5 min at 4000 g and re-suspended in TAH to a concentration of 2 × 10⁸ cells/l. Of this cell suspension, 0.3 ml were added to 0.3 g of sterile glass beads (diameter, 500 μm; Sigma catalogue no. G-8772), and inserted together with 2–3 μg of transforming DNA into a small glass tube. After vortexing (Vortex Gene-2) for 15 s at maximal speed, the suspension was transferred to 10 ml of TAH and the cells were cultivated in low light for 1 day to allow for expression of the mutant phenotype. The pelleted cells were re-suspended in 0.5 ml of HSM, of which 0.25 ml were mixed with 3.5 ml of soft agar held at 42 °C (0.5 % agar in TAH or HSM) and immediately poured on to 2 % agar plates containing the corresponding media. The TAH plates were incubated as controls in low light, while the HSM plates were held in strong light. The first colonies of transformants complemented with the wild-type phenotype could be picked after 2 weeks. The clones were additionally screened for photoautotrophic growth and for the newly introduced *Nde*I restriction site in the transit sequence of *rbcS*-2.

Quantification of photosynthetic O₂ evolution

Light-dependent O₂ evolution was determined as described by Su et al. [23]. Light intensity was measured with a Quantum-Spectrometer QSM-2500 (Spectroscania GmbH, Herrsching, Germany) from 400 to 750 nm over the range 0–5000 μE · m⁻² · s⁻¹. Chlorophyll content was determined according to Vernon [26].

Isolation of the RuBisCO holoenzyme

Pelleted, frozen cells grown phototrophically under high light intensity were suspended in buffer A (500 mM KCl, 25 mM MgCl₂, 50 mM Tris/HCl, pH 7.6, and 14 mM 2-mercaptoethanol) at a chlorophyll *a* + *b* concentration of 3 mg/ml. To reduce viscosity, 4 ml were incubated twice during 20 min at 37 °C with 2 μl of DNase I (10 units/ml), disrupted in a French press at 16000 p.s.i. (110400 kPa) and centrifuged (20 min, 4 °C, 38000 g). The post-mitochondrial supernatant (3.5 ml) was brought to 5 % Triton-X-100 and 5 mM free Mg²⁺ by addition of 0.7 ml of 25 % Triton and 0.4 ml of 0.2 M K-EDTA and kept for 1 min at 30 °C. Then 0.5–1 ml were layered in an exponential sucrose gradient (10–35 % in 500 mM KCl, 5 mM MgCl₂ and 50 mM Tris/HCl, pH 7.6) and ultracentrifuged (Beckman SW41; 40000 rev./min, 4 °C, 4 h). The tube was punctured at the bottom and fractions of 0.5 ml were collected. They were analysed on Western blots by immunostaining with anti-SS and anti-LS polyclonal antisera and chemiluminescent protein detection (Bio-Rad). Fractions enriched in RuBisCO holoenzyme (about 550 kDa) were colourless and located between the small ribosomal subunits and the gradient/top layer boundary.

RESULTS

Amino acid exchange at the two processing sites of pSS

To verify an eventual two-step processing of pSS in *Chlamydomonas*, experiments with modified pSS bearing mutations at the processing sites were performed *in vitro* and *in organello*, i.e. by

Figure 2 Import and processing of mutated pSSHis in isolated chloroplasts

After import of [³⁵S]Met-labelled wild-type pSSHis (wt) and pSSHis containing the indicated mutations into isolated *Chlamydomonas* chloroplasts, they were re-isolated through Percoll, solubilized and subjected to SDS/PAGE and autoradiography. The radioactive bands were quantified and the ratios of iSS or SS to pSS were calculated relative to the wild-type ratios, which were taken as 1.

import of radiolabelled pSS into isolated chloroplasts. By exchange of amino acids flanking the cleavage sites for the enzymes SPP-1 and SPP-2 processing was expected to become inefficient or abolished. For the *in vitro* synthesis of these modified precursor proteins the nucleotide mutations listed in Table 1 were placed into a pSP64 plasmid containing *rbcS-2* cDNA coding for pSSHis. The new *Nde*I restriction site introduced simultaneously with the mutations at the modified cleavage sites facilitated screening of mutated DNA.

In vitro processing of mutated pSS

The mutated cDNAs were subjected to *in vitro* transcription and translation to produce mutated precursor proteins pSSHis. These

Figure 1 In vitro processing of mutated pSSHis proteins by SPP-1 and SPP-2

In vitro-synthesized [³⁵S]Met-labelled wild-type pSSHis (wt) and pSSHis containing the indicated mutations were incubated with and without partially purified SPP-1 or SPP-2, then subjected to SDS/PAGE and autoradiography. The molecular masses indicated refer to the His-tagged proteins and are 0.7 kDa higher than those of the native proteins; a difference hardly detectable by this kind of SDS/PAGE. The radioactive bands were quantified (PhosphorImager and ImageQuant software) and the ratios of iSS or SS to pSS were calculated relative to the wild-type ratios, which were taken as 1 by definition.

Figure 3 RFLP analysis of the 628 bp fragment coding for the transit peptide sequence from plasmid and cellular DNA of transformants

The PCR products obtained from the indicated template DNA using the primers specific for *rbcS*-2 transit peptide (see the Experimental section) were digested with *Nde*I and the mixture analysed on two agarose gels with ethidium bromide staining. The templates were in: (A) lanes 1, 2, cellular DNA from wild-type (wt) and T60-3 *Chlamydomonas* cells; lanes 3–5, cellular DNA from T60-3 cells transformed with the full-length pRbcS-2, pRbcS-1 or the shortened pRbcS-2(5.7 kb) plasmids; lanes 6–10, plasmid DNA from the full-length pRbcS-1, pRbcS-2 and from pRbcS-2(5.7 kb) containing the indicated mutations. (B) Lane 1, cellular DNA from T60-3 transformant with pRbcS-2(5.7 kb); lanes 2–10, three different clones from each of the transformations with pRbcS-2(5.7 kb) containing the indicated mutations; lane 11, cellular DNA from the wild type.

modified pSSHs species were differently processed *in vitro* using stromal fractions enriched in peptidases SPP-1 or SPP-2 (Figure 1). In control experiments the wild-type pSSHs was cleaved as expected by SPP-2 to SS with a hexahistidyl tail (SSHs) and by SPP-1 to the intermediate iSSHs. The appearance of a doublet upon SPP-1 digestion in place of a single iSSHs band might be due to high salt concentration in the SPP-1 fraction, which for this experiment was isolated by ion-exchange chromatography; when isolated by gel filtration such doublets were not visible [13]. The exchange of two amino acids in processing site 1 (1-2aa) did not have much effect on cleavage in processing site 2 but abolished processing by SPP-1. Replacement of only one amino acid in processing site 2 (2-1aa) had no appreciable effect on SPP-1 or SPP-2, while replacement of two amino acids (2-2aa) inhibited the action of SPP-2 but not of SPP-1. These results indicated that *in vitro* the two enzymes act independently, each one recognizing only its own processing site.

***In organello* import and processing of mutated pSS**

To study whether the same cleavage pattern also occurred *in organello*, i.e. during protein import into isolated chloroplasts, radiolabelled mutated pSSHs was incubated for 20 min with isolated chloroplasts under import conditions. Figure 2 shows that all three mutated, radiolabelled pSSHs species could be imported into isolated chloroplasts, but that the processing patterns were different. Whereas import of wild-type pSSHs

resulted mainly in formation of SSHs accompanied by a faint band of iSSHs, the mutant pSSHs containing two altered amino acids in processing site 1 (1-2aa) was only processed to SSHs, without apparent iSSHs formation. When two amino acids in processing site 2 were altered (2-2aa) iSSHs accumulated, but no SSHs was produced, whereas the exchange of only one amino acid in processing site 2 resulted in the formation of iSSHs and SSHs. However, in comparison with wild-type pSSHs, the amount of iSSHs was very high, as confirmed by quantification of the radioactive bands (Figure 2).

Obviously, processing to the intermediate-sized or the mature protein were independent from each other and were carried out by two different peptidases. However, blocking of processing at site 2 (Figure 2, 2-2aa), as well as just slowing down the rate of SSHs production (Figure 2, 2-1aa) resulted in increased formation of iSSHs. Nevertheless, SSHs could also be formed regardless of the presence of iSSHs (Figure 2, 1-2aa). These findings do not rule out that some variant of a two-step processing mechanism might exist in *Chlamydomonas* chloroplasts. However, it would not be an obligate, but rather a facultative event which may have a supporting function in the import process.

In higher plants it was shown that a thylakoid lumen protein with a mutation conferring reduced SPP processing efficiency was nevertheless imported into the thylakoid lumen at a rate comparable with that of the wild-type protein and was cut by a membrane-bound protease to the mature protein [27]. Furthermore, intermediate-sized fragments of mutated pSS, which upon import into isolated pea chloroplasts could not be processed

Figure 4 Growth curves for transformants in liquid cultures in high light intensity and in the presence of acetate (mixotrophic)

Chlorophyll *a* + *b* content in the culture was measured. (A) (controls), T60-3 transformed with wild-type *rbcS*-1 or *rbcS*-2 genes in the original full-length plasmids or the shortened plasmid pRbcS-2(5.7 kb). (B–D) Five, three and 10 clones from three transformations of T60-3 with mutated plasmids pRbcS-2(5.7 kb) containing the indicated mutations. For comparison, the growth curve of a transformant with the wild-type gene was included (thick line, ■).

correctly, were found to be incorporated into the RuBisCO holoenzyme [28]. That means that processing by SPP is not an obligate step in the overall import process. A similar scenario might also be true for the import process of pSS into chloroplasts of *C. reinhardtii*.

Complementation of SS-deficient *Chlamydomonas* by transformation with mutant *rbcS*-2

As the above experiments do not refute the possibility of a two-step import process of pSS into chloroplasts of *C. reinhardtii*, and processing of the precursor to the intermediate iSS seems to be only a facultative step, it has to be investigated whether formation of iSS has any supporting function in the overall import process. To this end Dr R. J. Spreitzer provided us kindly with the *Chlamydomonas* strain T60-3 which is deficient in SS formation due to a knocking-out of both *rbcS*-1 and *rbcS*-2 genes. The strain cannot photosynthesize but grows well on acetate. Transformation with the plasmids pRbcS-1 or pRbcS-2, containing the genomic DNA for *rbcS*-1 or *rbcS*-2, resulted in phenotypical wild types, i.e. photosynthetically competent transformants [16]. This complementation could not be achieved with plasmids bearing the cDNA of *rbcS*-1 or *rbcS*-2. Therefore, the three mutations of *rbcS*-2 on cDNA used so far for *in vitro* protein synthesis were introduced into modified genomic pRbcS-2 which was shortened by excision of a non-essential sequence of 5 kb. The resulting pRbcS-2(5.7 kb) was still able to complement T60-3.

Chlamydomonas strain T60-3 was transformed with these mutated and with the wild-type pSS genes in pRbcS-2(5.7 kb). Incubation of the transformation mixture on agar plates containing no acetate resulted in all cases in growth of phototrophic,

Figure 5 Light-dependent O_2 evolution of transformants cultivated under different growth conditions

Of each transformation with mutated *rbcS*-2 genes (1-2aa, 2-1aa, 2-2aa) three arbitrarily selected clones were grown together with a wild-type transformant (RbcS2) under the indicated growth conditions. O_2 evolution was based on chlorophyll *a* + *b* content and measured over the spectral range 400–750 nm (photosynthetic active radiation).

green colonies. Colonies obtained after transformation with plasmids containing the 1-2aa or 2-1aa mutations grew fast and comparably with those with the wild-type gene, while for mutation 2-2aa, tiny, autotrophic colonies could be observed only after about 4 weeks. From the 1-2aa transformation five clones, from 2-1aa three, and from 2-2aa 10 clones were selected and further studied.

Table 2 Chlorophyll content of transformed cells grown under different conditions

In parentheses in the first column are the number of determinations (*n*), of transformations with different wild-type plasmids (strains), and of different clones from one transformation (clones). In the other columns means $\pm$ S.D. are shown.

Strain	Chlorophyll <i>a</i> + <i>b</i> /cell ($\mu\text{g}/10^6$ cells)		
	Strong light (phototrophic)	Low light (phototrophic)	Low light (mixotrophic)
T60-3 (2 strains)	—	—	1.76 $\pm$ 0.14
T60-3 transformed with wild-type <i>rbcS</i> -2 (4 strains)	1.11 $\pm$ 0.23	3.35 $\pm$ 0.52	1.50 $\pm$ 0.27
1-2aa (5 clones)	1.04 $\pm$ 0.06	4.12 $\pm$ 0.62	1.55 $\pm$ 0.21
2-1aa (3 clones)	0.91 $\pm$ 0.08	3.92 $\pm$ 0.42	1.54 $\pm$ 0.26
2-2aa (9 clones)	1.08 $\pm$ 0.19	3.79 $\pm$ 0.45	1.61 $\pm$ 0.13

To verify that in the transformants an *rbcS*-2 gene with the correct mutation had indeed been inserted, rather than the *rbcS*-1 gene having been restored, DNA was isolated from each clone and tested first for the presence or absence of *rbcS*-1 or *rbcS*-2

genes. Using the primers according to Khrebtukova and Spreitzer [16], which are specific for either *rbcS*-1 or *rbcS*-2, PCR analysis revealed the presence of *rbcS*-2 in all clones, but not of *rbcS*-1 (results not shown). Second, restriction fragment length polymorphism (RFLP) analysis of the amplified transit peptide (using the PCR primers described in the Experimental section) demonstrated the presence of a new *Nde*I restriction site in all mutant transformants but not in the wild-type transformants. In all clones this new restriction site was found at the expected position, as illustrated in Figure 3 by representative analysis of three arbitrarily chosen clones from each transformation. When DNA isolated from a wild-type strain, but not from T60-3, was used as a template, a PCR product of 628 bp was obtained that was not cleavable by *Nde*I. The same PCR product without the *Nde*I site was formed with DNA isolated from T60-3 transformed with the control plasmids containing wild-type *rbcS*-2 [RbcS2, RbcS2(5.7 kb)], but not when transformed with the *rbcS*-1 gene (RbcS1). When mutated plasmid DNA was subjected to PCR and *Nde*I digestion, the expected RFLP patterns were observed. The same RFLP patterns were obtained with DNA isolated from all clones of all transformations with mutated genes (1-2aa, 2-1aa, 2-2aa). A mutation in processing site 2 (2-1aa and 2-2aa) led to two fragments of identical length (314 bp), while with the

Figure 6 In vivo processing of mutated pSS

Three arbitrarily chosen clones of each of the three T60-3 transformations with the mutant *rbcS*-2 genes 1-2aa, 2-1aa and 2-2aa, one clone of both transformations with wild-type gene [RbcS2, RbcS2(5.7 kb)], and the untransformed T60-3 as well as a wild-type strain of *Chlamydomonas* were cultivated under the indicated growth conditions. Cells were solubilized and the *rbcS*-2 gene products analysed on Western blots by immunostaining using polyclonal antiserum against SS from *Chlamydomonas*. All samples contained the same amount of chlorophyll *a* + *b*, i.e. 7.5 $\mu\text{g}/\text{lane}$. For size comparison [^{35}S]Met-labelled, *in vitro*-synthesized pSSHs (21.3 kDa), iSSHs (19.9 kDa) and SS (16.3 kDa) were run on the same gels (autoradiograms). Under the electrophoretic conditions used the molecular mass difference between wild-type and His-tagged molecules was too small to significantly influence the position of the protein bands.

Figure 7 Processing products of SS in RuBisCO holoenzyme of transformants

Mutant transformants (1-2aa, 2-1aa, 2-2aa), the wild-type transformant [RbcS2(5.7 kb)] and a wild-type strain were cultivated phototrophically in strong light without acetate. Cells were broken, the supernatant treated with 5% Triton X-100 and centrifuged into a sucrose gradient. Aliquots of the gradient fractions containing the highest concentration of RuBisCO holoenzyme were analysed by SDS/PAGE and Western blotting. Loading was adjusted to comparable amounts of LS. The upper part of the nitrocellulose with the LS band was immunostained with anti-LS serum, while the lower part containing the SS bands was stained with anti-SS serum. For comparison, the whole cell extract of a 2-2aa clone (cells) was run on the same gel and the locations of pSS, iSS and SS are shown.

mutation in processing site 1 (1-2aa) two fragments of 376 and 252 bp were produced. This demonstrated the presence of the expected mutated, and the absence of the wild-type, *rbcS-2* gene. Third, from one clone of each transformation the DNA sequence of the PCR product was determined. All sequences contained the expected mutations but were otherwise identical to the wild-type sequence (results not shown).

***In vivo* effects of the mutated pSS on growth and O₂ evolution in transformed *Chlamydomonas* cells**

In this study the selection criterion for transformed clones was the restoration of photosynthetic growth of the SS-deficient strain T60-3 by introducing differently mutated *rbcS-2* genes. Based on the experiments with *in vitro*-synthesized mutated pSS described above one would also expect that the three different mutations would differently impair photosynthetic capacity and cell growth. Therefore, growth rates and O₂ evolution of the mutated transformants were compared with control transformants with wild-type *rbcS-2*.

While T60-3 is not able to survive in strong light even in the presence of acetate (results not shown), it grows well when transformed with plasmids containing the *rbcS-1* or *rbcS-2* genes or with the shortened plasmid pRbcS-2(5.7 kb) (Figure 4A). The growth rates of these control transformants and of the five and three transformants containing mutations 1-2aa (Figure 4B) and 2-1aa (Figure 4C), respectively, were almost identical. Duplication time was 9–10 h. Obviously, when sufficiently provided with energy, i.e. with high light intensity and acetate, inhibition of processing by SPP-1 at site 1, or reduced SS formation by SPP-2 at processing site 2, did not influence growth. However, the growth rates of all 10 transformants containing mutation 2-2aa, which *in vitro* and *in organello* inhibited the formation of mature SS, were inferior to the rates of wild-type transformants, and duplication times of up to 17 h were observed (Figure 4D).

The photosynthetic capacity of cells grown under different conditions was measured by their O₂ evolution as a function of

light intensity (Figure 5). Three clones of each transformation were randomly selected for these studies. Regardless of the type of mutation, the O₂ evolution in most clones was raised at comparable rates with increasing light intensity, differing only according to growth conditions. Since in Figure 5 O₂ evolution is based on chlorophyll content, this dependency upon growth conditions at least partially reflects the well-known relation between chlorophyll content and growth conditions (Table 2). At saturating light intensity, O₂ evolution varied considerably between clones but showed no correlation with the different mutations. This might reflect differences in the efficiency of pSS expression depending on the insertion site of the gene into the genome. The only exception was found in cells containing the 2-2aa mutation and grown without acetate at low light intensity, i.e. with a limited energy supply. In such cells O₂ evolution at saturating light was much lower and less variable than in transformants bearing the 1-2aa or 2-1aa mutations, although chlorophyll content per cell number was comparable (Table 2).

Presence of SS, iSS and pSS in transformed cells

It was rather surprising that from all transformations, including those with the mutation 2-2aa, clones could be selected which were phenotypically complemented with phototrophy. Therefore, it was interesting to study the processing of these mutated pSSs *in vivo* under different growth conditions. Since in whole cells proteins cannot be specifically radiolabelled, pSS and its fragments were immunologically detected on Western blots using a polyclonal antiserum directed against SS of *Chlamydomonas* and luminescence staining (Figure 6). In the marker lanes small immunologically undetectable amounts of ³⁵S-labelled, *in vitro*-synthesized pSSHs, iSSHs and SS were mixed with solubilized cells and run simultaneously on the same gel.

In control cells, including a wild-type strain and two clones obtained by transformation with pRbcS-2 or with the shortened plasmid pRbcS-2(5.7 kb), only mature SS, but neither pSS nor iSS, was immunologically detectable under all growth conditions. In untransformed T60-3 cells, which grew only at low light intensity in the presence of acetate, no SS was found.

As expected, in transformants containing the mutation 1-2aa, exclusively SS was found under all cultivation conditions. In case of mutation at processing site 2, which is the correct processing site for the mature protein, interesting differences in pSS processing were found. In cells grown under conditions where energy supply was not limiting, i.e. in the presence of acetate or at high light intensity, mutation 2-1aa resulted both in the formation of SS and in accumulation of more than one pSS fragment with a molecular mass between that of SS and iSS. No iSS or pSS could be detected. When energy supply was limiting, SS was still formed, although the pSS fragments were highly reduced. In cells containing mutation 2-2aa, even when grown phototrophically, neither SS nor pSS could be detected by the method used. Instead, iSS accumulated, especially in strong light or in the presence of acetate. Additionally, pSS fragments similar to those in 2-1aa mutants were formed. Their amount and size distribution, however, varied considerably. At limiting energy supply the amount of low-mass pSS fragments increased and in some cases iSS was missing.

Composition of the RuBisCO holoenzyme from transformants

The absence of SS in phototrophically grown 2-2aa cells together with the accumulation of pSS fragments of slightly higher molecular mass than SS raised the question whether in the mutants the RuBisCO holoenzyme was formed and whether

therein SS could eventually be replaced by such pSS fragments. To address this problem, protein fractions enriched in RuBisCO holoenzyme were isolated from cells grown phototrophically at high light intensity by centrifugation in a sucrose gradient and analysed immunologically (Figure 7). Analysing wild-type cells or clones from T60-3 transformed with pRbcS-2(5.7 kb) by Western blot revealed the presence of SS as well as LS at the expected positions in a non-coloured fraction below the sucrose/sample interface and above the small ribosomal subunits. The analogous fractions from the analysis of 1-2aa transformants contained SS and LS in about the same ratio as the controls. The RuBisCO holoenzyme of the 2-1aa transformant contained less SS. In addition, small amounts of pSS derivatives of lower molecular mass than iSS were present. In the RuBisCO holoenzyme of 2-2aa transformants neither SS nor iSS were observed, but two different pSS derivatives in various amounts could be detected depending on the clone. The absence of iSS demonstrated that this intermediate was not incorporated into RuBisCO, although it accumulated in the extract from whole cells (Figure 7, right-most lane).

DISCUSSION

The presence of the processing enzyme SPP-1 in *Chlamydomonas* cleaving the precursor protein pSS to an intermediate-sized iSS, as well as the *in vivo* and *in organello* discovery of measurable amounts of iSS, raised the question about a two-step processing mechanism for pSS. Using pSS with genetically modified processing sites, the role of iSS formation in processing of pSS was studied *in vitro* with isolated processing enzymes, *in organello* by import into isolated chloroplasts and *in vivo* in transformed *Chlamydomonas* cells.

The *in vitro* processing experiments showed that exchange of two amino acids flanking processing sites 1 or 2 (Table 1; 1-2aa, 2-2aa) abolished cleavage by the respective processing enzymes SPP-1 and SPP-2, while exchange of only one amino acid in processing site 2 (Table 1; 2-1aa) did not inhibit processing by SPP-2.

Similar results were obtained from *in organello* processing experiments: pSS containing mutation 1-2aa was processed to SS with no formation of iSS or other byproducts. When containing mutation 2-2aa, no SS was formed, though iSS and a slightly smaller degradation product accumulated. As expected, mutation 2-1aa did not block processing of pSS by SPP-2, however, in addition to SS, iSS was also formed. It seemed that processing at site 2 was slowed down so that SPP-1 had sufficient time to work at processing site 1 to form iSS. These results suggest that SS is usually formed regardless of the presence of iSS, implying that no two-step processing mechanism needs to be postulated. However, in the case of inhibited recognition of processing site 2, the formation of iSS will dominate. This will allow iSS or iSS derivatives to replace mature SS in the holoenzyme and thereby re-establish, to some extent, the formation of active RuBisCO.

Introduction of the mutated *rbcS*-2 genes into T60-3 cells, which are unable to form wild-type RuBisCO due to inactivation of both wild-type *rbcS* genes, allowed us to study this hypothesis. Transformation of T60-3 cells with each of the three mutated genes led to phototrophic clones containing *rbcS*-2 genes with the desired mutations. Even in transformants with mutation 2-2aa, for which the *in vitro* and *in organello* experiments excluded correct processing of pSS by SPP-2, photosynthesis was restored.

Phenotypically, i.e. with respect to growth and photosynthesis, transformants with mutation 1-2aa and 2-1aa were almost identical to wild-type cells. In contrast, clones with mutation 2-

2aa showed lower and variable growth rates. The variation in the growth rates of these transformants may be related to random insertion and hence different location of the gene in the nuclear genome and to concomitant different expression efficiency. Furthermore, cells containing the 2-2aa mutation and growing at limited energy supply showed drastically reduced photosynthetic efficiency. Under such growth conditions, where protein synthesis, in particular synthesis of pSS, is restricted to low level, the slow formation of small iSS fragments able to restore RuBisCO activity will become the limiting factor for photosynthesis. This interpretation implies that cells containing the 2-2aa mutation, i.e. with inhibited processing of pSS to SS, are, at least in part, able to restore the formation of active RuBisCO by integration of some intermediate-sized fragments of pSS into the holoenzyme.

Biochemical analysis demonstrated that, as in wild-type transformants, only SS and no other cross-reacting protein could be detected by immunological methods in transformants containing the mutation 1-2aa. In transformants containing the mutation 2-1aa at least two additional protein bands with molecular masses between those of iSS and SS were present, but no iSS could be detected. Obviously, iSS, which was formed in intact chloroplasts, as shown by the *in organello* experiments, was further degraded to shorter fragments, which accumulated during the time of cell growth. The relative amounts of the low-molecular-mass intermediate forms were highest in cells grown heterotrophically in light, and rather low when energy supply during growth was limiting. In transformants containing mutation 2-2aa, no SS could be detected under any of the growth conditions, demonstrating also that *in vivo* cleavage at the mutated processing site 2 was not possible. Instead, iSS was present and the above-mentioned smaller intermediate forms accumulated in variable amounts depending on the clone and on growth conditions. Limiting energy supply during growth led to decreased production of the relative production of iSS and to an increased production of the smallest intermediate.

Since the transformants containing mutation 2-2aa grow phototrophically in spite of the absence of SS, it was interesting to study the protein composition of their RuBisCO. As expected, in soluble protein fractions enriched in the holoenzyme, no SS was found. In addition, iSS was also missing. However, low-molecular-mass intermediates were present together with LS, the amount of which was also reduced as compared with wild-type or 1-2aa transformants. Small amounts of low-molecular-mass intermediates were also detectable in the holoenzyme of 2-1aa transformants. These results suggest that smaller fragments of pSS slightly larger than SS may indeed substitute SS in RuBisCO to restore some enzymic activity and hence phototrophic growth. However, the enzymes involved in shortening iSS to molecular species which can be assembled with LS to form active RuBisCO are not known.

We thank Dr R. J. Spreitzer for the generous donation of strains and plasmids containing the *rbcS* genes, Dr Q. X. Su for helpful advice and Miss Michèle Stark for kindly helping with the English.

REFERENCES

- Cline, K. and Henry, R. (1996) Import and routing of nucleus-encoded chloroplast proteins. *Annu. Rev. Cell Dev. Biol.* **12**, 1–26
- Soll, J. and Tien, R. (1998) Protein translocation into and across the chloroplastic envelope membranes. *Plant Mol. Biol.* **38**, 191–207
- Chen, X. J. and Schnell, D. J. (1999) Protein import into chloroplasts. *Trends Plant Sci.* **9**, 222–227
- Keegstra, K. and Froelich, J. E. (1999) Protein import into chloroplasts. *Curr. Opin. Plant Biol.* **2**, 471–476
- May, T. and Soll, J. (1999) Chloroplast precursor protein translocon. *FEBS Lett.* **452**, 52–56

- 6 Perry, S., Buvinger, B. W., Bennett, J. and Keegstra, K. (1991) Synthetic analogues of a transit peptide inhibit binding or translocation of chloroplastic precursor proteins. *J. Biol. Chem.* **266**, 11882–11889
- 7 Row, P. E. and Gray, J. C. (2001) Chloroplast precursor proteins compete to form early import intermediates in isolated pea chloroplasts. *J. Exp. Bot.* **52**, 47–56
- 8 VanderVere, P. S., Bennett, T. M., Oblong, J. E. and Lamppa, G. K. (1996) A chloroplast processing enzyme involved in precursor maturation shares a zinc-binding motif with a recently recognized family of metalloendopeptidases. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7177–7181
- 9 Richter, S. and Lamppa, G. K. (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7463–7468
- 10 Von Heijne, G., Steppuhn, J. and Herrmann, R. G. (1989) Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* **180**, 535–545
- 11 Su, Q. X. and Boschetti, A. (1994) Substrate- and species-specific processing enzymes for chloroplast precursor proteins. *Biochem. J.* **300**, 787–792
- 12 Rüfenacht, A. and Boschetti, A. (2000) Chloroplasts of the green alga *Chlamydomonas reinhardtii* possess at least four distinct stromal proteases. *Photosynthesis Res.* **63**, 249–258
- 13 Su, Q. X. and Boschetti, A. (1993) Partial purification and properties of enzymes involved in the processing of a chloroplast import protein from *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* **217**, 1039–1047
- 14 Su, Q. X., Schumann, P., Schild, C. and Boschetti, A. (1999) A processing intermediate of a stromal chloroplast import protein in *Chlamydomonas*. *Biochem. J.* **344**, 391–395
- 15 Schnell, D. J. and Blobel, G. (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J. Cell Biol.* **120**, 103–115
- 16 Khrebtukova, I. and Spreitzer, R. J. (1996) Elimination of the *Chlamydomonas* gene family that encodes the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13689–13693
- 17 Sueoka, N. (1960) Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* **46**, 83–88
- 18 Gorman, D. S. and Levine, R. P. (1965) Cytochrome *f* and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1665–1669
- 19 Rothen, R., Thiess, M., Schumann, P. and Boschetti, A. (1997) Import inhibition of poly(His) containing chloroplast precursor proteins by Ni^{2+} ions. *FEBS Lett.* **403**, 15–18
- 20 Sambrook, J., Fritsch, I. and Maniatis, T. (1987) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor
- 21 Mikaelian, I. and Sergeant, A. (1992) A general and fast method to generate multiple site directed mutations. *Nucleic Acids Res.* **20**, 376
- 22 Mendiola-Morgenthaler, L., Leu, S. and Boschetti, A. (1985) Isolation of biochemically active chloroplasts from *Chlamydomonas*. *Plant Sci.* **38**, 33–39
- 23 Su, Q. X., Schild, C., Schumann, P. and Boschetti, A. (2001) Varying competence for protein import into chloroplasts during the cell cycle in *Chlamydomonas*. *Eur. J. Biochem.* **268**, 2315–2321
- 24 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- 25 Kindle, K. H. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1228–1232
- 26 Vernon, L. P. (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. *Anal. Chem.* **32**, 1144–1150
- 27 Bassham, D. C., Creighton, A. M., Karnauchov, I., Herrmann, R. G., Klosgen, R. B. and Robinson, C. (1994) Mutations at the stromal processing site of a thylakoid lumen protein precursor affect the rate of processing but not the fidelity. *J. Biol. Chem.* **269**, 16062–16066
- 28 Ostrem, J. A., Ramage, R. T., Bohnert, H. J. and Wassmann, C. C. (1989) Deletion of the carboxyl-terminal portion of the transit peptide affects processing but not import or assembly of the small subunit of ribulose-1,5-bisphosphate carboxylase. *J. Biol. Chem.* **264**, 3662–3665

Received 6 March 2002/29 May 2002; accepted 5 June 2002

Published as BJ Immediate Publication 5 June 2002, DOI 10.1042/BJ20020378