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Knock-out of a putative transporter results in altered blue-light signalling in *Chlamydomonas*

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Summary

Nitrogen starvation and blue light are the two environmental cues that control sexual differentiation in *Chlamydomonas reinhardtii*. Insertional mutagenesis was applied to generate mutants that still require nitrogen starvation as the initiating signal for gametogenesis but were no longer dependent on irradiation. In one mutant analysed, sequences adjacent to the site of insertion were cloned and used for the isolation of a genomic clone that, upon transformation, could complement the mutant phenotype. The gene identified (*LRG6*) encodes two mRNAs that appear to be the products of differential splicing. The two putative gene products derived from these mRNAs differ in their C-terminal ends. Both predicted gene products exhibit multiple hydrophobic domains with α-helical secondary structure typical for integral membrane proteins. These proteins may form pores, and may function as transporters of as-yet unknown substrates. Since rendering the *LRG6* gene non-functional resulted in light-independence of gamete formation, it is suggested that this transporter may inhibit signal flux from the photoreceptor to target genes – either directly by its activity or indirectly by serving as a scaffold for signalling proteins. Shutting off this transporter may be required for the activation of signal flux in this pathway. This concept is supported by the observed reduction in *LRG6* mRNA levels during the first phase of gametic differentiation.

Keywords: blue-light signalling, constitutive activation, gametogenesis, transporter knock-out.

Introduction

Light is an environmental factor of particular importance for plants as it controls growth and development. In higher plants, light controls these processes by the combined action of multiple photoreceptor systems. Several classes of photoreceptors including UV- and blue-light receptors and red/far-red-light receptors mediate these responses to light. The receptors that respond to red/far-red light, the phytochromes, and their signalling pathways have been studied in considerable detail in higher plants (reviewed by Neff *et al.*, 2000). However, this family of photoreceptor genes appears to be absent in *Chlamydomonas* (Bonenberger *et al.*, 1994; unpublished observations).

Blue-light receptors have been characterized at the molecular level only in recent years. Two photoreceptors that respond to the blue region of the electromagnetic spectrum have been defined at the molecular level in higher plants and in *Chlamydomonas*, the cryptochromes and the phototropins (Ahmad, 1999; Batschauer, 1998; Briggs *et al.*, 2001; Christie and Briggs, 2001; Huang *et al.*, 2002; Small *et al.*, 1995).

The blue-light response best studied in *Chlamydomonas reinhardtii* is the light-requiring step in gametic differentiation. Two extrinsic signals are required for gamete formation: the first signal is nitrogen starvation that, when vegetative cells are incubated in the dark, results in the formation of pregametes not yet competent for mating. The second signal is blue light (Beck and Haring, 1996; Treier *et al.*, 1989). The action spectrum for this response indicates that the photoreceptor involved has properties typical for blue-light receptors that have been defined in higher plants and various lower eukaryotes (Weissig and Beck, 1991). Suggestive evidence for an essential role of phototropin in this process has recently been obtained by analysing strains with reduced levels of this photoreceptor (K. Huang and C.F.B., unpublished results).

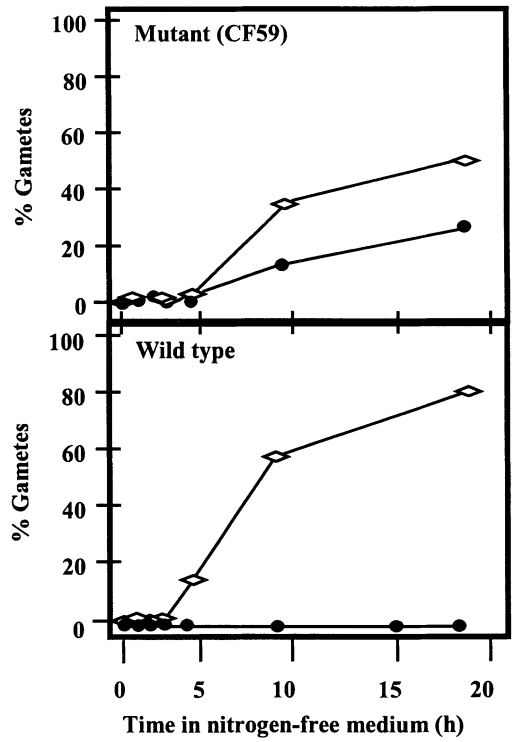

Figure 1. Kinetics of gametic differentiation of mutant strain CF59 and wild type.
Nitrogen was removed at time 0. Incubation was performed either with continuous irradiation (fluence rate 0.14 mmol m^{-2} sec^{-1} of white light) (◇) or in the dark (●). At the times indicated, cells were removed, mixed with an excess of sexually competent mating partner, and incubated in the dark. After 1 h cells were fixed by the addition of glutaraldehyde and the percentage of quadriflagellate cells was determined. From these data, the percentage of gametes was calculated as described by Beck and Acker (1991).

The downstream signalling pathway that mediates the blue-light-induced conversion of pregametes to gametes has been characterized in some detail at the genetic and biochemical level (Beck and Haring, 1996). Thus mutations in three linked loci named *LRG* (light regulation of gametogenesis) result in light independence of gamete formation (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). A different mutation affecting gene *LRG2* causes a partial defect in the blue-light signalling pathway as the mutant exhibits a delay and a reduced rate in pregamete-to-gamete conversion. The latter phenotype can be partially compensated by applying higher fluence rates (Buerkle *et al.*, 1993). A gene that suppresses the *lrg2* mutant phenotype in a gene dose-dependent manner has been identified (*LRG5*) and shown to encode a putative nuclear protein (Gloeckner and Beck, 1997).

Further information on this blue-light signal pathway has been obtained by applying pharmacological compounds known to affect specific reactions in signalling. These studies provide suggestive evidence for a role of a protein kinase C-like kinase, a tyrosine kinase, a phosphodiesterase, and cAMP in this pathway of blue-light-induced gamete formation (Pan *et al.*, 1996; Pan *et al.*, 1997).

Here we describe the knock-out of a gene that, due to differential splicing of its mRNA, may encode two membrane proteins. The amino acid sequence of these proteins suggests that they function as transporters. The phenotype of a knock-out mutation in this gene is light-independence of gamete formation. This mutant phenotype and the turning down of its mRNA levels in the early phase of gametogenesis suggest that this gene may play a role as a negative regulator in blue-light signalling.

Results

Identification of blue-light signalling mutants

In *C. reinhardtii*, blue light is required for the completion of gamete formation – the conversion of mating-incompetent pregametes into sexually active gametes (Beck and Haring, 1996; Treier *et al.*, 1989). One set of mutants previously identified exhibited light independence of pregamete-to-gamete conversion (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). The corresponding genes could not be isolated due to the lack of a selection system for transformants in which the defective genes were complemented. For the isolation of a new set of mutants, we employed insertion mutagenesis to physically mark genes that are involved in light signalling during gametogenesis. We used a linearized plasmid with the *ARG7* gene of *C. reinhardtii* [pARG 7,8ø3 (Gumpel and Purton, 1994)] as a mutagenic agent and an *arg7* mutant strain (CF30) as a recipient. This strain in addition harboured mutation *lrg2* that has previously been shown to cause reduced sensitivity towards blue light, resulting in a delay in pregamete-to-gamete conversion (Buerkle *et al.*, 1993). Presence of the *lrg2* mutation in mutant screens strongly reduced the number of false positives.

A total of 13 120 Arg^+ transformants were generated and screened for mutants with defects in gametogenesis. Besides 47 mutants unable to form gametes, 10 mutants were isolated that exhibited light independence of sexual differentiation. Back-crosses revealed that in six of these mutants the newly introduced *ARG7* gene was strictly coupled with the mutant phenotype – suggesting that the mutations were caused by insertional mutagenesis. In one case no viable progeny could be obtained, and in three cases the Arg^+ phenotype and the mutant phenotype did not segregate together (data not shown).

One mutant strain (CF59) was chosen for further studies. As judged by Southern blot analysis, this strain harboured a single insertion of the pARG7,8ø3 plasmid (data not shown). The physiological characterization revealed that this mutant, in contrast to wild type, may form mating-

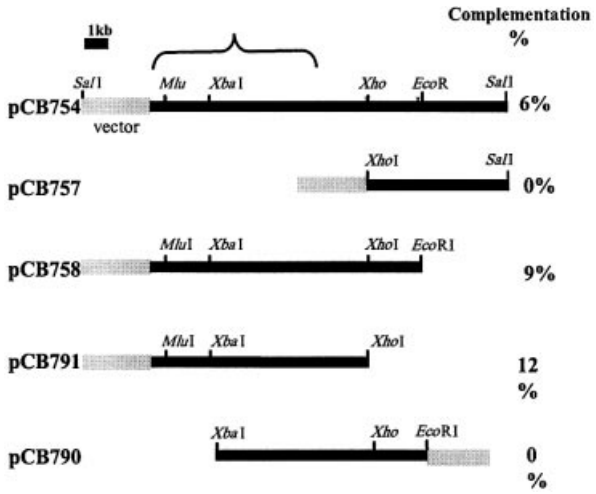

Figure 2. Complementation of mutant CF59 with plasmids harbouring genomic DNA.
A partial restriction map of the primary clone tested is shown. Deletion derivatives were generated by restriction of the DNA with the enzymes indicated. The percentage of complementation was calculated after analysing at least 88 independent transformants. The genomic region required for complementation is indicated by a bracket.

competent gametes in the dark when tested under standard conditions (Figure 1). Three to 5 h after removal of the nitrogen source and incubation in the light, an increase in the number of mating-competent cells could be observed for mutant CF59 as well as the wild-type strain. However, in the dark only the mutant was able to form mating-competent gametes (Figure 1). The differences in the kinetics of gamete formation between mutant and wild-type strains are caused by differences in genetic background (data not shown). The delay and somewhat reduced rate in gamete formation seen in the dark as compared to light conditions may be accounted for by a reduced supply of energy in the absence of light, as acetate serves as the sole source of energy under these conditions (Treier *et al.*, 1989). Although an analysis of the mutant for other potential defects was negative (data not shown), we cannot rule out that some blue-light-mediated effects are impaired. The principal effect of the mutation, though, is clearly on gametic differentiation.

We next tested whether the mutation in CF59 may affect one of the *LRG* loci (*LRG1*, *LRG3*, *LRG4*), mutations in which have previously been shown to result in light independence of gamete formation (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). Random progeny from crosses of CF59 with the three mutant strains was tested for light independence/light dependence of gamete formation. In case the mutation in CF59 was located in one of the previously defined *LRG* loci, we would have expected 100% of the progeny to show light-independent gamete formation. If they were unlinked we expected approximately 75% of the progeny to form gametes in the dark. We also tested the Arg phenotype of the progeny: Since the *lrg1*, *lrg3*, *lrg4* mutants were arginine autotrophic and CF59 harboured an *arg7* allele and, in addition (unlinked to this locus) the *ARG7* wild-type gene introduced with the transforming DNA, we expected about 12.5% of the progeny to exhibit an Arg^- phenotype. The results (Table 1) showed that 69–73%, but not 100%, of the progeny exhibited light-independent gamete formation. As expected, all these clones were Arg^+. This result indicates that the mutated gene in CF59 is unlinked to the loci previously identified (Gloeckner and Beck, 1995). This gene was named *LRG6*.

Table 1. Analysis of progeny from crosses of different mutants that exhibit light independence of gamete formation with mutant strain CF59

Strains crossed	Number of progeny tested	Phenotypes of progeny (%)[a]		
		Arg^+Lin^{-b}	Arg^+Lin^{+b}	Arg^-Lin^{+b}
lrg1 × CF59	88	73	15	12
lrg3 × CF59	88	71	18	11
lrg4 × CF59	88	69	18	13

[a]Progeny from random-spore analysis of different crosses were tested for light dependence/light independence of gametogenesis and the Arg phenotype.
[b]Lin^- indicates light independence of gamete formation; Lin^+, light dependence.

Cloning of the gene defective in the mutant and its analysis

Using the plasmid-rescue method (Wilson *et al.*, 1989), we isolated plasmids that harboured a fragment of the *C. reinhardtii* genomic DNA adjacent to the site of insertion. With this genomic DNA fragment as a probe, a λEMBL3 genomic library was screened and eight positive clones (with about 15.5 kb inserts) were isolated. These turned out to be identical.

To assay for complementation of the mutant phenotype by the genomic clones we employed co-transformation of a *nit1-305*, *lrg6* double mutant strain using two plasmids: one with the *NIT1* gene (for selection of growth on nitrate as a nitrogen source) and one plasmid with the genomic DNA. About 6% of the transformants that grew on nitrate were found also to be restored for their light dependence of gamete formation (Figure 2). Subsequently, four deletion derivatives were generated and tested for their ability to complement the mutant phenotype. A fragment of 9.3 kb flanking the vector sequences was sufficient to restore the wild-type phenotype to the mutant (Figure 2).

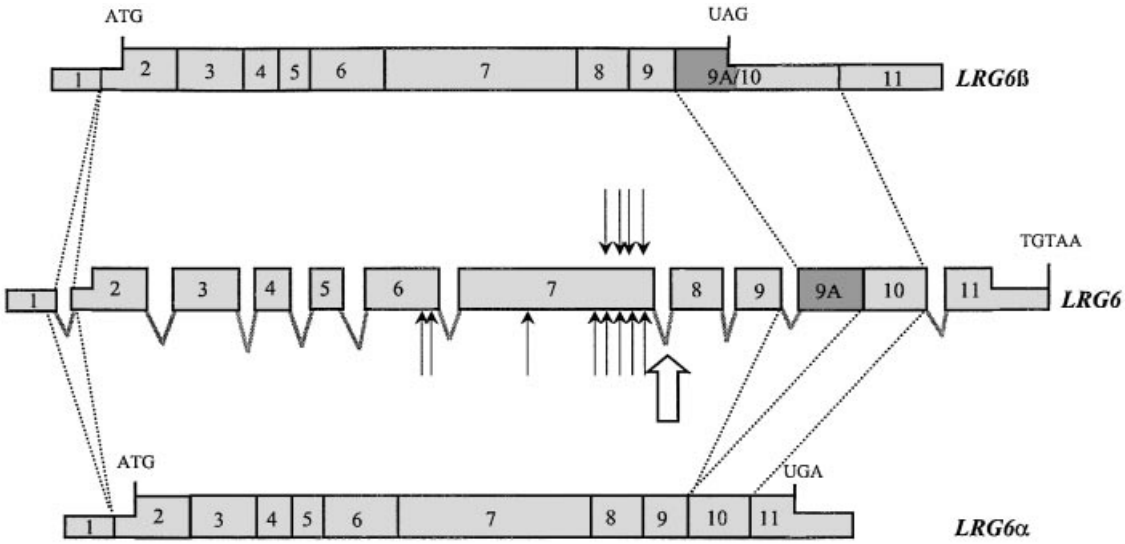

Figure 3. Structure of gene *LRG6* and of the two types of cDNAs derived from it.
In the centre, a schematic presentation of the exon–intron structure of *LRG6* is shown. Exons of the coding region (large boxes) and introns (black lines) are indicated. The 5′- and 3′-UTRs are shown as thin boxes. The shaded box labelled 9A represents an exon that arises by alternative splicing. Vertical arrows mark the positions of differences in the nucleotide sequence observed between strains CW15 *mt*– and 137c *mt*+. The large, open arrow indicates the site where the plasmid used for mutagenesis has inserted. The polyadenylation signal (TGTAA), typical for *C. reinhardtii,* is indicated.
LRG6α cDNA contains 11 exons, one of which is located within the 5′-UTR. Protein-encoding exons with start and stop signals (large open boxes) and the 5′- as well as 3′-UTRs (thin open boxes) are indicated. Dotted lines refer to splicing events.
LRG6β is the product of alternative splicing. The use of a different splice site within the intron flanked by exons 9 and 10 results in a new exon (9A/10) composed of intron sequences (9A) and exon 10.

By sequencing, one gene with 10 predicted protein-encoding exons was localized within the complementing DNA fragment (Accession No. AJ431665). Additional evidence that this region harbours gene *LRG6* was provided by sequencing of the *C. reinhardtii* DNA harboured by the rescued plasmid which showed that the insertion in mutant CF59 was in an intron near the centre of the predicted gene (Figure 3).

A cDNA library in λNM1149, generated from vegetative cells (J.-D. Rochaix, personal communication), was screened and four clones were isolated – one had an insert of 2.1 kb and three contained 3 kb inserts. By restriction analysis and sequencing from both ends, it was shown that two of the latter clones were identical; one of the 3 kb clones was shorter at the 5′ end by 300 bp.

Determination of the nucleotide sequence of the 2.1 kb cDNA clone and its comparison with the sequence of the genomic clone revealed near-identity with the structure of a gene predicted from the genomic DNA sequences. However, an additional intron was found within the 5′-UTR. The gene identified by the 2.1 kb cDNA clone was designated as *LRG6α* (Accession No. AJ431664) and its structure is shown in Figure 3. This gene comprises 11 exons, 10 of which are predicted to be protein-encoding. The 5′ UTR of the mRNA, disrupted by an intron, comprises 340 nucleotides. The sequence at the predicted start site of translation (CACCATGG) exhibits a high level of homology with consensus sequences for translation start sites in *C. reinhardtii* [(A/C)A(A/C)(A/C)ATGG/C] (Silflow, 1998). The 3′-UTR has a length of 405 nucleotides and harbours the polyadenylation signal TGTAA observed in the vast majority of *C. reinhardtii* protein-encoding genes. The coding region exhibits a GC content of 64.8%, a value typical for *C. reinhardtii* genes.

Sequence analyses of the 3 kb cDNA clones revealed an identity with the 2.1 kb clone up to and including exon 9 (Figure 3). Thereafter, sequences corresponding to the intron located between exons 9 and 10 were detected. As deduced from the cDNA sequence, the proximal splice site between exon 9 and the next intron was maintained. However, in this variant, an alternative splice site within the intron between exons 9 and 10 was used. This resulted in a new exon named 9A/10, which contains the residual sequences of the intron as well as those of exon 10. The distal splice site of exon 9A/10 is the same as that of exon 10. The resulting mRNA is 950 bp longer than that of *LRG6α*. This variant of the gene was named *LRG6β* (Accession No. AJ430531). At the splice site within the intron and the 5′ end of exon 9A/10 is the sequence CAG//G, which is compatible with the consensus sequence of *C. reinhardtii* splice sites [(C/A)(A/C)G//G] (Silflow, 1998). We suggest that *LRG6α* and *LRG6β* represent splice variants of gene *LRG6*.

A comparison of the sequences of the *LRG6α* and *LRG6β* cDNAs with those of the genomic clone revealed 12 differences, two within exon 6 and 10 clustered in exon 7

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LRG6α       MGSGARWDAAVLFLTRTVRMLAYGSTGVVLALFLSAVGLS  40
LRG6α       DREIGSLLTLTLLGDSAISLWVTRHADGLGRRACLAASCL  80
LRG6α       LMVLAGAVYGTVQHPSFALLLVAATVGVLSPSGNEVGPFM 120
LRG6α       ALEQAVLLELVPAAARTHVFAWYNLVGYAMTAIGALVAGH 160
LRG6α       ALTWAQAAYGITAVQGYRFIFLQYAASGAVLLLMFLLLTN 200
LRG6α       KVERKAKLPAPAPAPALQTRGDDEEAGLRAPLLPAAADED 240
LRG6αCW15                    L
LRG6α       APAAAAAAAAHPAAAAAAPSEPAAAPAAAPPVHPPPQAQP 280
LRG6αCW15                        P                M
LRG6α       AQPSSSGHSHHHQQQQQQQQPDPQQQRRGFLGLTPPTRSL 320
LRG6αCW15         R      M        Q
LRG6α       VLRLSALFSLDSLAGGLVTGTLLVYFFQTKYGVSTAYLGG 360
LRG6α       LLFGANMLAAVSALASGFVAARLGLINTMVFTHLPSNVLM 400
LRG6α       LLVPLMPSLESATAMVFARYSISQMDVAPRSAYVAGVVPA 440
LRG6ß       LLVPLMPSLESATAMVFARYSISQVANQTTPAGLNLRAGA
LRG6α       DERSVAMGVINIAKSLGAAFGPLITGWLAQQGLFAWAFYL 480
LRG6ß       ALKAAPRCPPPRGLARPLRHHRARRHCRWAQGRPCRRHHR
LRG6α       CGGGKIVYDLLLYFLFSHIHPQH                  503
LRG6ß       AQRRGRCCCRR                              491
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Figure 4. Deduced amino acid sequences of the *LRG6* gene products that may arise by differential splicing.
The deduced product of *LRG6α* from strain 137c *mt+* is shown. Indicated are the amino acid differences observed between strains 137c *mt+* and CW15 *mt–*. For the C-terminal end, the amino acid sequences predicted for both LRG6α and LRG6β (boxed) are shown.

Table 2 Nucleotide exchanges within exons 6 and 7 observed between the *LRG6* genes of *Chlamydomonas reinhardtii* strains CW15 *mt–* and 137c *mt+*

		Chlamydomonas strain		
		CW15 *mt–*		137c *mt+*
Exon	Position of exchange[a]	Codon	Amino acid	Codon
6	531	GTG	Val ↔ Val	GTC
	544	GGA	Gly ↔ Gly	GGC
	768	CTT	Leu ↔ Pro	CCT
	903	CCG	Pro ↔ Gln	CAG
	919	GGG	Gly ↔ Gly	GGC
	922	TTG	Leu ↔ Leu	CTG
7	949	ATG	Met ↔ Leu	CTG
	956	TTG	Leu ↔ Leu	CTG
	972	CGG	Arg ↔ Leu	CTG
	988	ATG	Met ↔ Leu	CTG
	1017	CAG	Gln ↔ Leu	CTG
	1023	GTA	Val ↔ Val	GTC

[a]Numbers refer to position of nucleotide exchange where 1 is the first nucleotide of the coding region of the cDNA.

(Figure 3). These differences have been confirmed by the sequencing of additional cDNA clones and that of PCR products derived from the corresponding genomic DNAs. The genetic background of the two *C. reinhardtii* strains from which the libraries were derived is supposed to be identical, but the strains have been separated for about 40 years (E. Harris, personal communication). As summarized in Table 2, six out of the 12 nucleotide differences have changes in the amino acid sequence as a consequence. The presence of nine nucleotide changes in a region of 120 bp suggests that this part of exon 7, in contrast to the rest of the gene where no differences could be detected, is a hot spot for mutations.

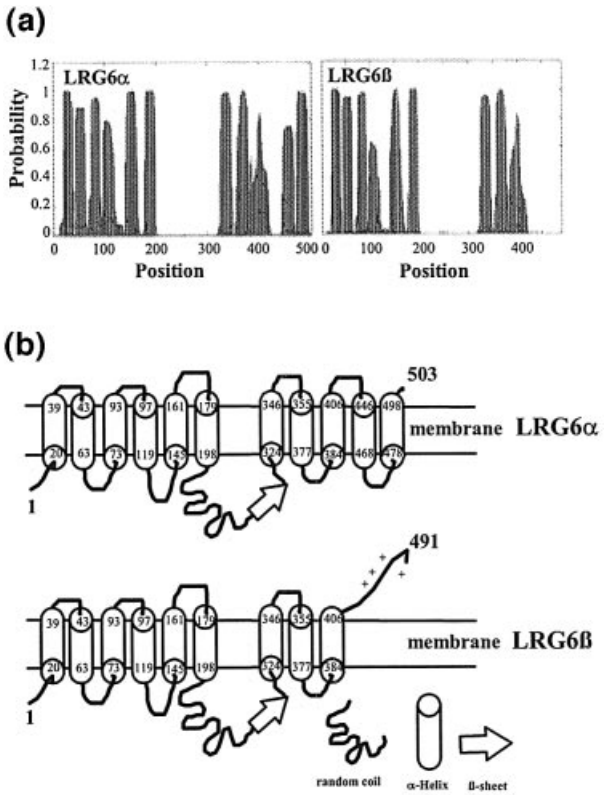

Figure 5. Analysis of the structure of the *LRG6* gene products.
(a) Prediction of transmembrane domains of the deduced gene products of *LRG6α* and *LRG6β*. The ordinate gives the probability that an amino acid sequence may form a transmembrane structure, calculated according to Krogh *et al.* (2001). On the abscissa, the positions within the amino acid sequences of the proteins are indicated.
(b) Topological model for *LRG6α* and *LRG6β* gene products with 11 and nine transmembrane helices, respectively, as predicted by the TMHMM method (Krogh *et al.*, 2001).

LRG6 gene product(s)

The deduced amino acid sequence of *LRG6α* is shown in Figure 4. The ORF of *LRG6α* predicts a protein of 503 amino acids. The ORF of *LRG6β* may encode a protein of 491 amino acids where the last 67 amino acids differ from that of the putative *LRG6α* product.

Analysis of the deduced *LRG6α* and *LRG6β* products for hydrophobicity/hydrophilicity revealed that both putative proteins may possess multiple hydrophobic domains with predicted α-helical secondary structure (Figure 5a). The number of predicted membrane-spanning domains varies with the prediction programs used. For the α version of the LRG6 protein, the membrane protein topology prediction method TMHMM (Krogh *et al.*, 2001) predicts 11 trans-

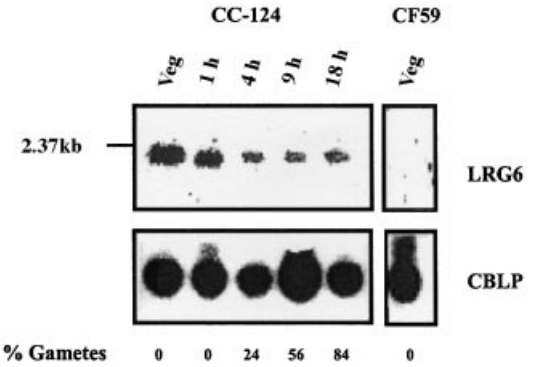

Figure 6. Analysis of *LRG6* expression in the wild-type strain (CC-124) and mutant CF59 at the RNA level in vegetative cells and during gametogenesis (in the wild type).
Poly(A)+ RNA was isolated from vegetative cells (Veg) and cells during gametic differentiation (indicated by hours for which cells were incubated without a nitrogen source with continuous illumination; fluence rate 0.14 mmol m^{-2} sec^{-1}). In each lane, ≈5 μg poly(A)+ RNA were separated by gel electrophoresis and, after blotting, probed with a cDNA fragment comprising exons 6 and 7 of *LRG6*. The percentage of gametes observed is indicated. For a loading control, blots were stripped and rehybridized with the cDNA of the *CBLB* gene (von Kampen *et al.*, 1994). Size of RNA marker indicated on left ordinate.

membrane helices, and for the β version of the protein, nine such domains. Yet the programs TMP RED (Hoffmann and Stoffel, 1993; http://www.ch.embnet.org/software/TMPRED_form.html) and SOSUI (Hirokawa *et al.*, 1998) predict 10 transmembrane helices for the α version, and nine or eight transmembrane helices for the β version, respectively. The central hydrophilic region is predicted to be located intracellularly in each of the models. Likewise, the C-terminus of the LRG6β protein by all programs is predicted extracellularly. As different predictions exist for the location of the N-termini of the α and β proteins and the C-terminus of the α protein, the true orientation and structure of these protein domains remain unclear.

The difference in predicted structure between the putative *LRG6α* and *LRG6β* gene products is caused by differential splicing by which the hydrophobic elements encoded by exons 10 and 11 of *LRG6α* are replaced by a stretch of hydrophilic amino acids in LRG6β. Thus, at the C-terminal end of the *LRG6β* product, 18 arginine residues are observed among 67 amino acids.

From these data we conclude that *LRG6* may encode integral membrane proteins with either 10 or 11 (*LRG6α*), or eight or nine (*LRG6β*), transmembrane helices. A model of these proteins as predicted by the TMHMM method (Krogh *et al.*, 2001) is shown in Figure 5(b). The reduced number of transmembrane helices of the LRG6β protein as compared to that of the LRG6α protein, and the pronounced hydrophilic character of its C-terminal end, may indicate different functions for the two *LRG6* gene products.

Expression of *LRG6*

To assay for the expression of *LRG6* by RNA-blot techniques, poly(A)+ RNA was isolated from vegetative cells and cells in various stages of gametogenesis. In vegetative cells of the wild-type strain CC-124, an RNA of about 2.1 kb was detected (Figure 6). After nitrogen removal and the initiation of gametogenesis, the abundance of this RNA decreased. Four hours after the initiation of gametic differentiation (the time point when gametes first appeared), the level of RNA had decreased to about 10% of that seen in vegetative cells, and remained at this low level for the rest of gametogenesis. In the mutant strain no RNA hybridizing with the exon probe could be detected. From these results we conclude that the mRNA detected by the probe is encoded by the *LRG6* gene.

Surprisingly, only an RNA of 2.1 kb was detected by RNA-blot analyses. A signal corresponding to an RNA of 3 kb was undetectable even after prolonged exposure of the film. Using RT–PCR and primers that bind to sequences within exons 9 and 9a, a fragment of about 200 bp could be amplified. Sequencing of this fragment revealed that it corresponded to an mRNA fragment of *LRG6β* (data not shown). We conclude that both spliced variants of the *LRG6* mRNA were present in the strain, although the mRNA of *LRG6α* appears to be much more abundant than that of *LRG6β*.

Expression of gamete-specific genes is altered in mutant *CF59*

Sexual differentiation in *C. reinhardtii* is accompanied by changes in gene-expression patterns (Treier and Beck, 1991). Several genes that are specifically upregulated during gametogenesis have been identified (von Gromoff and Beck, 1993; Rodriguez *et al.*, 1999). Some of these genes – *GAS28*, *GAS29* and *GLE* – are upregulated after approximately 9 h of nitrogen starvation. The increased expression of these genes is strictly light-dependent, as it was not observed in wild-type cells that were incubated in the dark (Figure 7; von Gromoff and Beck, 1993; Rodriguez *et al.*, 1999). In the mutant, expression of these genes was altered in two aspects. First, while only very low mRNA levels were observed in vegetative cells, nitrogen starvation of the mutant, in contrast to wild type, resulted in a rapid increase in mRNA levels – within 30 min. Second, expression of these genes has become light-independent (Figure 7). The fluctuations in mRNA levels seen have been observed before and were attributed to changes in gene expression in response to the progression in the programme of sexual differentiation (Merchán *et al.*, 2001). From these results, we conclude that knocking out *LRG6* results in the activation of the light-signalling pathway which mediates both the differentiation of pregametes to

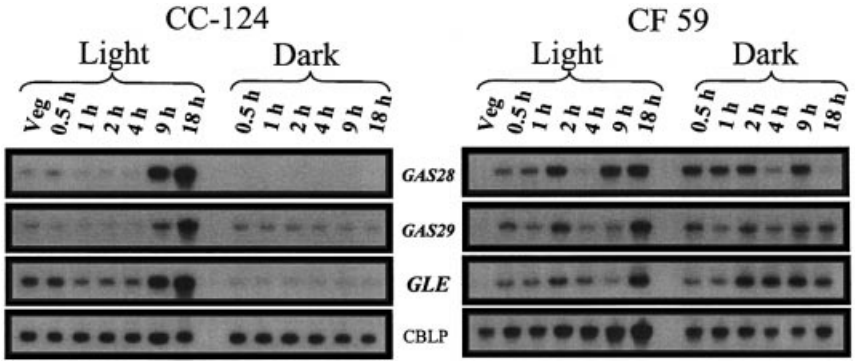

Figure 7. Expression of genes activated during gametic differentiation in the *lrg6* mutant and wild-type cells.
The kinetics of mRNA accumulation during gametogenesis in the light and in the dark are compared between wild type (CC-124) and mutant CF59. The nitrogen source was removed at time 0 and cells were incubated either with continuous illumination (fluence rate 60 μmol m^{-2} sec^{-1}) or in the dark. Samples for RNA isolation were removed at the times indicated. Northern blot hybridizations were performed as described in Experimental procedures. Hybridization with the CBLP probe was used as a loading control.

gametes and the upregulation of genes that are molecular markers for gametogenesis.

Discussion

Here we report on the characterization of a *C. reinhardtii* mutant in which a step in the sexual life cycle, normally requiring blue light, has become light-independent. Among more than 13 000 clones that carry an insertion, six mutants were recovered in which the phenotype light-independence of gamete formation was found to be linked to the inserted DNA. In four of these six mutants, genetic analyses indicated that different loci were affected (data not shown). The discovery of several mutants with the same phenotype that map to different loci suggests that the inactivation of multiple genes may result in a constitutive activation of the blue-light signalling pathway. This conclusion is supported by the previous identification of another three linked loci that, when mutated, result in light-independence of gametogenesis (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). Genetic analyses showed that *LRG6*, analysed here, is not identical with these three genes (Table 1).

The relatively high frequency of mutants with a light-independent, constitutive phenotype raises the question of what roles these genes may play in signalling. Their abundance and the observation of knock-out mutants with this phenotype suggest that they do not play essential roles, but rather may act as negative regulators in the signalling pathway by which blue light controls the conversion of pregametes into gametes. Individual steps in signal transduction (at least upstream of transcription factors) are controlled by counteracting positive and negative regulators. Loss-of-function mutations in a negative regulator would not result in a constitutive phenotype unless the signal transduction pathway had a basal flux passing through it in the absence of the stimulus. This flux was proposed to be a result of a basal level activity of the signalling intermediates (Bowler and Chua, 1994). Data supporting this concept for the blue-light signalling pathway controlling gamete formation in *C. reinhardtii* have been obtained. Thus a protein kinase C-like kinase has been proposed to play the role of a negative regulator, as inhibition of its activity by staurosporine resulted in light-independent, constitutive gamete formation. On the other hand, activation of this kinase by naphthalene sulfonamide derivatives and phorbol esters inhibited light-induced gamete formation (Pan *et al.*, 1996).

Characterization of gene *LRG6* revealed the existence of two cDNAs that varied in their lengths and in their sequences at the 3′-end. The two transcripts could plausibly be explained by the use of two alternative acceptor splice sites within the sequences that follow exon 9 (Figure 3). As the two variant mRNAs may encode proteins that differ at their C-terminal ends, we assayed various strains of *C. reinhardtii* for the size of their *LRG6* mRNAs, but always observed predominance of the smaller RNA (data not shown). We speculate that the quantitative distribution between the two mRNAs is determined by the growth conditions. Such an influence of growth conditions on the ratio between two mRNA species derived by differential splicing has been observed previously for the *C. reinhardtii* gene that encodes a plastidic GrpE homologue. Here the ratio between the two mRNAs is strongly influenced by temperature (Schroda *et al.*, 2001).

The putative *LRG6* gene products exhibit features typical for integral membrane proteins. For the N-terminal region, five to six hydrophobic amino acid sequence segments with α-helical structure are predicted. These are proposed to constitute transmembrane domains (Figure 5). This first set of transmembrane helices is followed by a hydrophilic domain of about 146 amino acids. In this domain, encoded

by exons 6 and 7, multiple exchanges in the DNA sequence have been observed when two different *C. reinhardtii* strains were compared. Indeed, there appears to be a hot spot for mutations: in the 120 bp at the 3′-end of exon 7, nine single nucleotide exchanges were noted (Table 2). Some of the changes in nucleotide sequence also manifest themselves in differences in the amino acid sequence (Figure 4). These differences do not affect the structure of the protein, although the region of amino acid exchanges extends from the central hydrophilic region into the adjacent transmembrane region. Since the two strains that exhibit these differences both require light for gamete formation (data not shown), we assume that both variant gene products are functional. The tolerance of multiple amino acid exchanges also suggests that at least part of the hydrophilic domain may not be essential for LRG6 function.

The C-terminal ends differ between the two splice variants termed LRG6α and LRG6β. For this region of the proteins, all programs used predict fewer transmembrane helices for LRG6β than for LRG6α (Figure 5). A striking feature of LRG6β is its very C-terminus that comprises 85 mostly hydrophilic amino acids, many of which are positively charged (Figure 4). Although evidence for a function of LRG6α and LRG6β is lacking, it is tempting to speculate that the differences between the postulated gene products manifest themselves in different functions of the proteins.

But what is the function of the LRG6 proteins? The predicted structure of these proteins suggests that they may form pores through membranes. However, analysis of their amino acid sequences and their comparison to sequences in the database provided no information on either potential substrates or the membrane systems of which these proteins may be a constituent. Alignments over the total length of LRG6α were possible only with a putative substrate/H^+-transporter from yeast (YJR124c) and a transmembrane protein from *Sinorhizobium meliloti* (NP_384858). These proteins have similarities of 57% to the *LRG6α* gene product and represent permeases of the major facilitator superfamily (COGO477). However, these permeases, in contrast to the predicted *LRG6* gene products, contain either 12 or 14 transmembrane domains (Pao *et al.*, 1998). We thus conclude that *LRG6* encodes a novel type of transporter whose specificity remains to be elucidated.

The knock-out mutation in *LRG6* not only resulted in light-independence of gamete formation, but also affected the expression of genes specifically or preferentially expressed during gametogenesis (von Gromoff and Beck, 1993). Expression of genes *GAS28*, *GAS29* and *GLE* is known to be induced by light in the late phase of gamete formation (von Gromoff and Beck, 1993; Rodriguez *et al.*, 1999). In the mutant we observed two differences. First, these late genes were upregulated during gametogenesis in the dark (Figure 7). These results indicate that *LRG6* is a regulator in a signalling pathway that controls the expression of late gamete-specific genes. The knock-out of *LRG6* apparently causes this pathway to become constitutively active. Second, increased expression of these genes could be observed already 30 min after a shift to nitrogen-free medium. This is in contrast to the situation in wild-type cells, where only incubation without a nitrogen source for at least 7 h resulted in competence for induction of these genes by light (Figure 7; von Gromoff and Beck, 1993; Rodriguez *et al.*, 1999).

The absence of expression of *GAS28*, *GAS29* and *GLE* in vegetative cells of mutant CF59 indicates that, in the presence of a nitrogen source, the signal pathway downstream from the LRG6 protein is blocked. Removal of the nitrogen source results in an elimination of this block in less than 30 min (Figure 7). Since, in contrast to mutant CF59, these genes were activated by irradiation in wild-type cells with a delay of several hours during gametogenesis, we speculate that the upstream signal pathway between photoreceptor and LRG6 protein is non-functional in the early phase of sexual differentiation. Signalling through this proximal section of the pathway may require the completion of the programme of differentiation induced by nitrogen starvation (Beck and Acker, 1991).

A defect in *LRG6* results in a constitutive phenotype. On a formal basis, this result may be interpreted by the generation of a constitutively active gene product. This possibility is rather unlikely as in the mutant, the gene was destroyed by an insertion close to its centre. Rather, we envision three mechanisms by which *LRG6* may control blue-light signalling. According to model 1, the *LRG6*-encoded proteins, besides being transporters, also serve as anchor(s) that recruit negative regulators of signal flux to the membrane. Loss of the membrane anchor perturbs the function of these regulators, resulting in a constitutive activity within the signal pathway. Such a situation has been described in yeast where, in the absence of a membrane anchor, signalling proteins are not functionally assembled (Aronsheim *et al.*, 1994). In the wild type, activation of signal flux by blue light may override the effect of the negative regulators. In model 2, one variant of the LRG6 proteins is proposed to serve as a sensor of an as-yet unknown substrate, while the second protein may function as a transporter. This sensor, in the absence of blue light, inhibits signalling; it is proposed to be inactivated by blue-light treatment. A transporter whose primary function is the sensing of ammonium ions has been identified in *Saccharomyces cerevisiae* (Lorenz and Heitman, 1998). Model 3 envisions that the *LRG6*-encoded transporters are integral components of the signalling pathway. By the transport of as-yet unknown substrates, signal flux is blocked. Blue-light treatment inhibits trans-

porter activity, and this in turn activates signal flux. The lack of transporter activity in the mutant turns on the signal pathway in a constitutive manner. Models 2 and 3 are in agreement with the observed downregulation of *LRG6* expression in the early phase of gametogenesis (Figure 6).

Two types of events that involve the transport of ions have been described to follow blue-light irradiation: a depolarization of the plasma membrane (Spalding, 2000), and an increase in cytosolic calcium (Baum *et al.*, 1999). We consider a role of *LRG6* in these two processes as rather unlikely, as mutants disturbed in these transport events are expected to be defective rather than being constitutive in blue-light signalling. However, evidence has accumulated for additional transporters that appear to be involved in light signalling. Thus a mutant defective in gene *DET3* develops morphologically as a light-grown plant, even when it is kept in the dark (Schumacher *et al.*, 1999). This gene of *A. thaliana* was shown to encode the subunit of a vacuolar H^+-ATPase. Another example is an ABC transporter that, as shown by over- and under-expression studies, modulates the light regulation of hypocotyl elongation (Sidler *et al.*, 1998) and a plastidic ABC transporter of *A. thaliana* with specificity for porphyrins has a function in modulating phytochrome signal transduction. A mutant defective in this transporter exhibits reduced responsiveness preferentially towards continuous far-red light (Møller *et al.*, 2001).

These examples illustrate that transporters appear to be constituents of light-signalling pathways, although their roles remain to be determined. The easy-to-score phenotype of *Chlamydomonas* mutants defective in *LRG6* offers a basis for the elucidation of this transporter's function in the regulation of signal flux.

Experimental procedures

Strains and culture conditions

Chlamydomonas reinhardtii wild-type strain CC-124 (*mt*–) was obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). Strain *nit1-305* (Sosa *et al.*, 1978) was provided by E. Fernández (University of Cordoba, Spain). Strain CF30 (*lrg2, arg7-3, mt*–), used for insertional mutagenesis, was the product of a cross between strain 105 (*arg7-3, mt*+) obtained from R. Loppes (University of Liège, Belgium), and an *lrg2* strain (Gloeckner and Beck, 1997). *Chlamydomonas* cells were grown either in liquid or on solid (supplemented with 1.5% agar) Tris-acetate-phosphate (TAP) media (Harris, 1989) at 23°C in the dark or under continuous irradiation with white light (fluence rate 0.14 mmol m^{-2} sec^{-1}) provided by fluorescent tubes (Osram L 36 W/25, Munich, Germany). Flasks with liquid cultures were incubated on a rotary shaker. Nit^+ transformants were selected on plates with TAP medium in which KNO_3 (4 mM) was substituted for NH_4Cl. In addition, the agar was washed with distilled water in order to remove ammonium ions.

Gametogenesis, screening for mutants and genetic analyses

Gametes were generated by resuspending vegetative cells grown on plates in nitrogen-free TAP medium (Harris, 1989) at a density of 1–2 $\times$ 10^7 cells ml^{-1}, and incubated under continuous light for 16–24 h. For the generation of pregametes, liquid cultures of the vegetative cells were centrifuged and resuspended in nitrogen-free TAP medium at a density of 1 $\times$ 10^7 cells ml^{-1} and incubated for 18 h in the dark (for this purpose cultures were wrapped and placed in a light-proof black box). Gametes were obtained from pregametes by exposing them to light for 2 h. The percentage of gametes was assayed by mixing the cells to be tested with a threefold excess of mature gametes of opposite mating type, as described previously (Beck and Acker, 1991).

To assay large numbers of clones for gamete formation, we employed protocols similar to those described previously (Buerkle *et al.*, 1993; Gloeckner and Beck, 1995). In order to screen for mutants generated by insertional mutagenesis and altered in gamete formation, gametogenesis was performed in 96-well microtitre plates. After suspension of individual clones in nitrogen-free medium in the wells and an initial incubation in the dark for 18 h, gametes of opposite mating type were added. After an additional incubation for 18 h in the dark, the plates were screened for the presence or absence of zygote pellicles using a stereomicroscope. Transformants that showed zygote pellicles in the dark were analysed in more detail.

For genetic analyses, crosses were performed using standard procedures (Harris, 1989).

Nuclear transformation of C. reinhardtii

Chlamydomonas reinhardtii nuclear transformation was performed using the glass bead method (Kindle, 1990). The cell wall of the recipient strains was removed by autolysin treatment prior to transformation. Immediately after vortexing with glass beads in the presence of DNA, cells were spread onto selection plates without arginine for selection of Arg^+ transformants, or plates containing nitrate instead of ammonium for selection of Nit^+ transformants. Plates were incubated at 23°C in the light (about 30 μmol m^{-2} sec^{-1}) and transformants were recovered after 1 week. For insertional mutagenesis, 100 ng of plasmid pARG7,8ø3 (Gumpel and Purton, 1994), harbouring the *ARG7* gene with a fragment of phage øX174 inserted into one intron, linearized with *Eco*RI, was employed for the transformation of 1 $\times$ 10^8 cells. For transformation of the nit1-305 derivatives, plasmid pMN24, harbouring the *NIT1* gene (Fernández *et al.*, 1989), was employed. In co-transformation experiments with pMN24 and clones harbouring *C. reinhardtii* genomic DNA, mass ratios of 1 : 1, 1 : 2, and 1 : 4 of pMN24 to the co-transformed DNA were employed.

Plasmid rescue

The protocol of Wilson *et al.* (1989) was employed with some modifications. Total genomic DNA of insertion mutant CF59 was digested with *Nar*I. Hybridization with a probe, derived from pARG7,8ø3 that flanks the insertion site, revealed at fragment of 3.5 kb. This fragment was predicted to contain *C. reinhardtii* genomic DNA from the site of insertion and the *ori* as well as the *bla* gene of pARG7,8ø3. In wild-type DNA, using this DNA as a probe, a 1.4 kb *Nar*I fragment was detected. *Nar*I-digested genomic DNA of CF59 was separated by gel electrophoresis and

the DNA corresponding to a size of 3.5 kb was extracted. This DNA was ligated and used to transform *Escherichia coli* strain DH5α. Transformants were recovered and tested for the presence of the *C. reinhardtii* genomic DNA flanking the insertion.

Library screening

A library of genomic *C. reinhardtti* DNA in phage lambda EMBL3 (Goldschmidt-Clermont, 1986), kindly provided by M. Goldschmidt-Clermont, was screened using as a probe the *C. reinhardtii* genomic DNA fragment isolated by plasmid rescue. Preparation of filter replicas, phage lysis and DNA blotting were carried out as described (Benton and Davies, 1977). Hybridization was performed as described previously (von Gromoff *et al.*, 1989). For isolation of cDNA clones, we screened a cDNA library generated from vegetatively growing cells of *C. reinhardtii* in λNM1149 (kindly provided by J.-D. Rochaix). As a probe, an RT–PCR fragment of ≈350 bp was employed that was generated using primers 2U and 4L see below).

DNA extraction, Southern blot hybridization, RNA extraction, Northern blot hybridization and DNA sequencing

DNA and RNA isolation, prehybridization, hybridization and signal detection were carried out as described (von Gromoff *et al.*, 1989). Sequencing was carried out by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using the ALF DNA Analysing system (Amersham Biosciences, Freiburg, Germany). Analyses of DNA sequences and of putative ORFs and restriction sites were performed with THE BCM SEARCH LAUNCHER and the EXPASY TOOLS programs.

RT–PCR for generation of LRG6 *cDNA fragments*

Total RNA from vegetative cells (CC-124) and from cells during gametogenesis at various time points (von Gromoff *et al.*, 1989) was pooled and used as a template for reverse transcription with specific antisense primer 10L (5′-GCTTTCAACCCTGTCTCA-3′) and Superscript II reverse transcriptase (Gibco BRL, Invitrogen GmbH, Karlsruhe, Germany) at 45°C. One μl aliquots of the reverse transcription mixture were used in PCR reactions. Primer combinations employed were: forward 2U (5′-GCTCGCTGCTG-ACTCTGA-3′) and reverse 4L (5′-GTACCAGCTCCGACAG-3′; and forward 6U (5′-GCT CCGGGCACCACTA-3′) and reverse 7L (5′-CACCATGGTGTTGATGAG-3′). To detect *LRG6α*-specific DNA, the primer combination forward 8U (5′-TCCAACGTGCTGATGC-3′) and reverse 9L (5′-CACACGCACACCTGCTGA-3′) was used. For PCR we used the following protocol: 1.5 min at 95°C, 3 min at 85°C (hot start) followed by 40 cycles of 1 min at 95°C, 1 min at 55–70°C, 1 min at 72°C.

To confirm the alternatively spliced mRNA of *LRG6β*, total RNA of vegetative cells was used as a template for reverse transcription. The same protocols as above were used. The forward primer for PCR was 8U (5′-TCCAACGTGCTGATGC-3′) and the reverse primer 9AL (5′-TGTTCGGAGCAGAGG-3′). These primers detected only *LRG6β* cDNA, as these sequence should not be present in *LRG6α* mRNA.

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