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RESEARCH ARTICLE

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The transcriptional program of synchronous gametogenesis in *Chlamydomonas reinhardtii*

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Abstract Cells of *Chlamydomonas reinhardtii* undergo gametogenesis to produce sexually competent gametes under nitrogen-starved conditions. By using a synchronized system for gametogenesis of early G_1 cells, several previously identified marker genes and 18 novel nitrogen-starved gametogenesis (*NSG*) genes isolated by macroarray analysis were placed into at least three temporal classes of expression. Early genes are induced transiently in the first 2 h after transfer to nitrogen-free medium. Middle genes are strongly induced between 3 h and 4 h after nitrogen removal, a time corresponding to the acquisition of mating competency, suggesting their involvement in the gamete program. Late genes are induced between 5 h and 8 h after nitrogen removal, a time after the completion of gametic differentiation, suggesting that they are not directly involved in the formation of sexually competent gametes. All of the 18 *NSG* genes examined are induced in both mating-type plus and minus gametes and about two-thirds of the genes are also expressed in the mitotic cell cycle, especially at S/M phases.

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Introduction

Nitrogen starvation triggers the formation of specialized cell types in many organisms. In lower eukaryotes such as yeasts (Davey 1998; Kassi et al. 2003), *Aspergillus* (Skromne et al. 1995), and *Neurospora* (Nelson and Metzenberg 1992), nitrogen deprivation in the growth medium induces the morphogenesis of asexual or sexual spores. Higher plants respond to a reduced availability of nitrogen with changes in their developmental pattern, including the root architecture and early flowering (Zhang and Forde 2000).

In the unicellular green alga *Chlamydomonas reinhardtii*, removal of a utilizable nitrogen source, especially ammonium ions (Matsuda et al. 1992), from the growth medium induces gametic differentiation and the resulting mating-type plus (mt^+) and minus (mt^-) gametes undergo mating, when they are mixed, to form zygotes (Sager and Granick 1954). Some laboratory strains of *C. reinhardtii* also require light for this gametic differentiation (Beck and Haring 1996; Saito et al. 1998).

When nitrogen is depleted, the vegetative cells undergo two critical programs (Beck and Haring 1996; Goodenough 1991). First, they acclimate to nitrogen starvation through a variety of metabolic changes, including the synthesis of nitrogen-scavenging enzymes (Quesada and Fernández 1994; Vallon et al. 1993), the degradation and renewal of ribosomes (Martin et al. 1976; Siersma and Chiang 1971), and a decrease in photosynthetic activity (Bulté and Wollman 1992). Second, cells express a gamete program that produces cells competent for mating. Changes that occur during this gametogenesis program include the formation of mating-type specific agglutinins responsible for cell–cell recognition and adhesion (Adair et al. 1982; Saito and

Matsuda 1984), increased synthesis of a matrix metalloprotease (gametolysin) and its activation enzyme responsible for removal of gametic cell walls (Kinoshita et al. 1992; Matsuda 1998; Snell et al. 1989), and formation of mating structures (which in mt^+ gametes contain the gamete membrane protein FUS1) responsible for protoplasmic fusion (Ferris et al. 1996; Friedmann et al. 1968; Goodenough et al. 1982; Misamore et al. 2003).

The transcriptional activation of a number of genes by nitrogen starvation has been analyzed (Beck and Haring 1996). The *NIA1*, *NRT2;1* (Quesada and Fernández 1994), *NIT2* (Schnell and Lefebvre 1993), and *NCG2* (Merchán et al. 2001) genes are involved in the adaptation program and they are induced quite rapidly after transfer to nitrogen-free (−N) medium. The *FUS1* (Ferris et al. 1996), *MID* (Ferris and Goodenough 1997), *MTA1*, and *MTA2* (Ferris et al. 2001, 2002) genes are involved in the gamete program, although their temporal pattern of gene expression during gametogenesis has not yet been analyzed. What types of regulatory networks of transcription exist among the genes for the adaptation program and the gamete program are still unknown.

Gene expression during gametogenesis in *Chlamydomonas* has been analyzed using a heterogeneous population of cells (Merchán et al. 2001; Rodriguez et al. 1999; von Gromoff and Beck 1993). However, in order to investigate the transcriptional networks of gametogenesis, a large and rather homogeneous population of cells is required, as in the case of the study on the transcriptional program of sporulation using a synchronized population of budding yeast (Chu et al. 1998). We found previously that only vegetative cells in the early G_1 phase can directly undergo gametogenesis in −N medium (Matsuda et al. 1990). In the present work, we synchronized the vegetative cells by exposing them to alternating periods of light and darkness (Harris 1989) and the resulting early G_1 cells were transferred to −N medium to analyze gene expression during synchronized gametogenesis. Besides using genes which have been identified previously (see above) as probes, we used 18 novel gametogenesis genes designated nitrogen-starved

Table 1 Newly identified genes, *NSG*, expressed during nitrogen-starved gametogenesis

Gene	EST clone[a] (accession no.)	Expression stage		Genomic analysis		
		Gametogenesis	Cell cycle	Annotation	Position[b]	Gene model[b]
NSG1	LCL088c09[c] (AV631066)	Middle	S/M	Unknown protein	17:267783	C_170123
NSG2	MXL026a03 (BP094629)	Middle	S/M	Putative ribonucleotide reductase, small subunit (*Nicotiana tabacum*)	63:90901	C_630047
NSG3	LC031b07 (AV621095)	Middle	None	Putative reverse transcriptase (*Volvox carteri*)	247:53619	C_2470009
NSG4	HC080b12 (AV637979)	Middle	G_1, S/M	Actin (*C. reinhardtii*)	131:206864	C_1310009
NSG5	MXL091f12[c] (BP098333)	Middle	S/M	Putative ribonucleotide reductase, large subunit (*N. tabacum*)	74:378174	C_740021
NSG6	LCL011c01[c] (AV626555)	Middle	None	Unknown protein	12:1126182	C_120113
NSG7	HCL040a11[c] (AV641777)	Middle	None	Unknown protein[d]	54:456862	C_540078
NSG8	LC097c03 (AV625740)	Middle	G_1, S/M	Putative 26S proteasome AAA-ATPase subunit RPT4 (*Arabidopsis thaliana*)	17:79323	C_170176
NSG9	LCL095h05 (AV631538)	Middle	G_1, S/M	Putative 26S proteasome AAA-ATPase subunit RPT2 (*A. thaliana*)	45:499587	C_450105
NSG10	LC056c08 (AV622899)	Middle	S/M	Putative dUTP pyrophosphatase (*Oryza sativa*)	684:2938	C_6840001
NSG11	HC058e05[c] (AV636361)	Middle	G_1, S/M	Unknown protein[d]	42:35133	C_420026
NSG12	LC020h10 (AV620353)	Middle	G_1, S/M	Putative DEAD box RNA helicase (*A. thaliana*)	1:1453292	C_10233
NSG13	CL75b10[c] (AV397052)	Late	None	Unknown protein[d]	45:382920	C_450029
NSG14	MXL038a10 (BP095208)	Middle	G_1, S/M	Putative valosin-containing protein (*A. thaliana*)	27:400863	C_270130
NSG15	MXL090d10 (BP098263)	Middle	Weak	Putative clathrin heavy chain (*A. thaliana*)	2:2017182	C_20111
NSG16	CM012h05 (AV387175)	Middle	S/M	Putative cyclin-dependent kinase regulatory subunit (*O. sativa*)	86:412495	–
NSG17	LCL019e08[c] (AV626997)	Early	None	Unknown protein[d]	24:180176	C_240006
NSG18	MXL052c11 (BP096082)	Late	Weak	Glutamine synthetase, cytosolic isozyme (*C. reinhardtii*)	2:586643	C_20337

[a]EST clone numbers of the Kazusa DNA Research Institute
[b]The scaffold number, the starting base position of EST clone and the gene model in the JGI *Chlamydomonas* genome database ver. 2 are indicated.
[c]The entire cDNA sequences were determined in this paper and have been deposited in the Genbank/EMBL/DDBJ database (accession no. AB167472 to AB167478).
[d]Annotated from the entire ORF sequence as a putative protein with ankyrin repeats (NSG7), ADF domain (NSG11), Arm repeats (NSG13), and bHLH domain (NSG17), respectively (see Fig. 7).

gametogenesis (*NSG*; Table 1), isolated by expressed sequence tag (EST)-based macroarray analysis.

Materials and methods

Cells and culture conditions

C. reinhardtii strains 11-32b (mt^+) and C-9 (mt^-) were used (Kubo et al. 2002). The 11-32b mt^+ strain is known to be suitable for synchronous culture (Schlösser 1981, 1984) and for synchronous gametogenesis (Matsuda et al. 1990). Cells were synchronized in minimal (M) salt medium containing 3.7 mM ammonium nitrate (Sager and Granick 1953) under a cycle of 12 h light and 12 h dark (Matsuda et al. 1995). Synchronized vegetative cells at the early G_1 stage (i.e., cells at the beginning of the light period, L-0 cells) were harvested and resuspended either in nitrogen-containing (+N) M medium to culture vegetatively at 25°C under continuous illumination or in nitrogen-free (−N) M medium to induce gametes (Matsuda et al. 1990). Gametic differentiation of cells was monitored by mixing them in equal numbers with tester gametes of the opposite mating type and incubating at 25°C for 30 min. The mating efficiency (the cell fusion) was calculated after counting the proportion of biflagellated and quadriflagellated cells (Matsuda et al. 1978).

Identification of genes for nitrogen-starved gametogenesis using cDNA macroarray analyses

A *Chlamydomonas* cDNA macroarray was constructed from Kazusa EST libraries representing cells grown under 25 different conditions, including gametogenesis and zygote formation (Asamizu et al. 1999, 2004; Miura et al. 2004). A total of 10,368 PCR-amplified EST clones were spotted in duplicate on Biodyne-A Nylon membranes. Then, [^{32}P]-labeled targets were prepared from two mRNA pools derived from +N (target A) and −N (target B) cell mixtures, respectively. The L-0 cells of both mating-types were cultured separately in either +N or −N medium at 1.2×10^7 cells/ml at 25°C in the light. Aliquots (20 ml) were taken at 1 h intervals until 8 h incubation, the cells being harvested quickly by centrifugation and then frozen in liquid nitrogen. They were then mixed together and total RNA was prepared from the +N and −N cell mixtures by the methods described by Kubo et al. (2001). Polyadenylated [poly(A)+] RNA was isolated using a PolyATtract mRNA isolation system (Promega). The poly(A)+ mRNAs were labeled by incorporation of [^{32}P]dCTP during first-strand cDNA synthesis and labeled cDNA products were purified with a CHROMA Spin-200 column (Clontech). Hybridization of macroarray filters with the labeled targets was carried out using an ExpressHyb hybridization solution (Clontech) at 68°C for 12–16 h (Sambrook and Russell 2001). After incubation and washing, the membranes were exposed to an imaging plate (Fuji Film) for detection. Quantification of signals was carried out using a FLA-2000 high-resolution scanner (Fuji Film) and ArrayVision software (Amersham). From two independent experiments, 102 ESTs were primarily isolated, whose average expression ratio of target B (−N) to target A (+N) exceeded 2.5-fold. To select genes that are expressed specifically upon a shift to −N medium, we generated contigs, assayed Northern blotting of the isolated EST clones, and identified 18 novel genes whose expression ratio of −N/+N exceeded 10-fold (cf. Fig. 3). These were designated as *NSG1–NSG18* (Table 1).

Northern blot analyses

Total RNA (10 μg RNA per lane) was analyzed by Northern blotting (Sambrook and Russell 2001). The *NSG* gene probes for the hybridizations were prepared by a Gene Images random-prime labeling module (Amersham) using cDNA clones (Table 1) as the templates. The primer pairs used for PCR were T3-kobe (5′-CGCAATTAACCCTCACTAAAGGGA-AC-3′) and M13-20-kobe (5′-GACGTTGTAAAA-CGACGG- CCAGT-3′). The following fragments of previously identified genes were used to prepare probes for hybridization: (1) *NIA1* (Fernández et al. 1989), the 0.35-kb 3′UTR region (positions 2,921–3,265, GenBank accession no. AF203033), (2) *NRT2;1* (Quesada et al. 1994), the 0.69-kb 3′UTR region (positions 2,143–2,832, accession no. Z25438), (3) *NCG2* (Pozuelo et al. 2000), the 2.05-kb ORF plus 3′UTR regions (positions 189–2,239, accession no. AF195795), (4) *FUS1* (Ferris et al. 1996), the 0.53-kb 3′UTR region (positions 4,022–4,545, accession no. U49864), (5) *GAS28* (Rodriguez et al. 1999), the 0.37-kb 3′UTR region (positions 1,695–2,065, accession no. AF015883), and (6) *MTA1* and *MTA2* (Ferris et al. 2002), the 0.55-kb 5′UTR plus ORF regions of *MTA1* (positions 2,778–3,629, accession no. AF417571). The *L27a* gene, encoding a *Chlamydomonas* 60S ribosomal protein, was used as a standard because of its relatively constant level of expression during gametogenesis and the cell cycle. The probe was generated by PCR using *L27a* cDNA (accession no. AV640287). The expression profiles of each gene were shown in a tabular form using Cluster and TreeView (Eisen et al. 1998).

cDNA sequencing

The cDNA clones were subcloned into pBluescript II SK− (Strategene) and their sequence determined using a Thermo Sequence cycle sequencing kit (Amersham) with T3 and T7 primers (Nisshinbo) and a LiC-4200 DNA sequencer (Li-Cor).

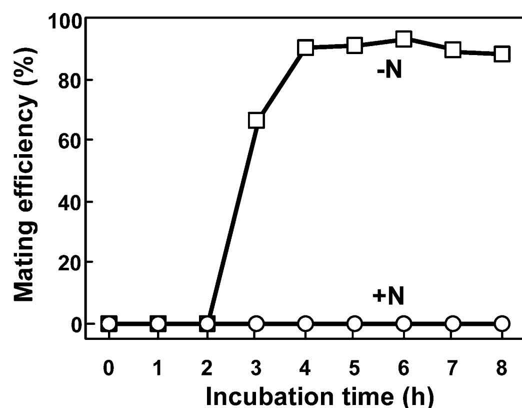


Fig. 1 Time-course of synchronized gametogenesis of early G_1 cells in −N medium. Synchronously grown mt^+ cells (11-32b) at the beginning of the light period (L-0 cells) were transferred to −N or +N medium at 1.2×10^7 cells/ml and incubated for 8 h in the light. At the times indicated, these cells were mixed with tester mt^- (C-9) gametes in equal numbers and the mating efficiency determined after 30 min incubation

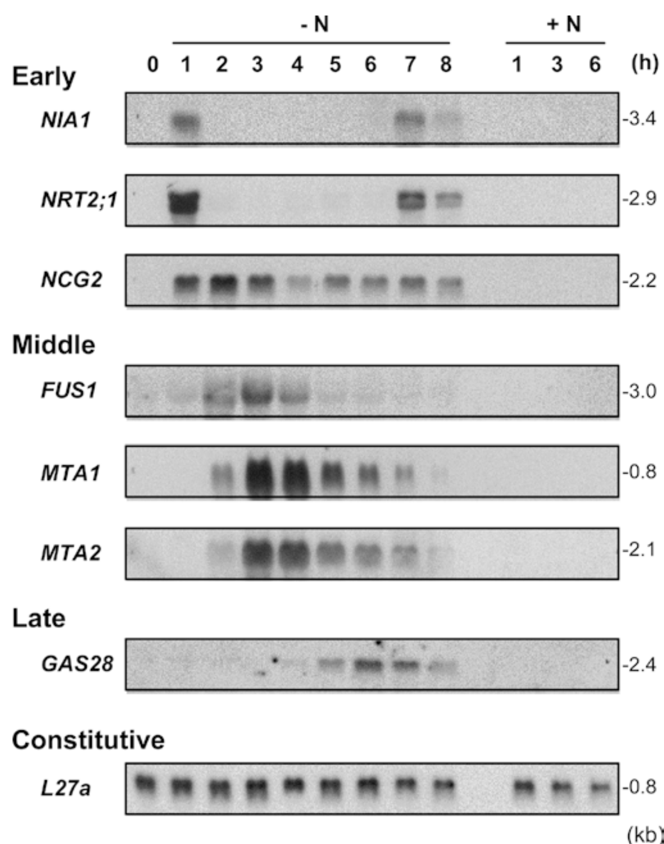


Fig. 2 Temporal classification of gene expression during synchronized gametogenesis using previously identified marker genes *NIA1*, *NRT2;1*, *NCG2*, *FUS1*, *MTA1*, *MTA2*, and *GAS28*. Cell samples were taken from the synchronous culture transferred into −N or +N medium (see Fig. 1) at the indicated time-points. Total RNA (10 µg/lane) was used for Northern blot analysis. Probes are described in the Materials and methods. The size of the RNA is indicated on the *right* in kilobases. The *L27a* gene (encoding ribosomal protein L27a) was used as a loading control

Results

Expression profiles of previously identified marker genes during synchronized gametogenesis

Using the *Chlamydomonas* synchronized system for gametogenesis (Fig. 1), we first examined the expression profiles of several marker genes (*NIA1*, *NRT2;1*, *NCG2*, *FUS1*, *MTA1*, *MTA2*, *GAS28*) which had been identified previously.

The *NIA1*, *NRT2;1*, and *NCG2* genes encode proteins for acclimation to nitrogen starvation and are induced rapidly and transiently after transfer to −N medium (Fernández et al. 1989; Merchán et al. 2001; Quesada and Fernández 1994). Our time-course study also showed (Fig. 2) that the transcripts of these genes were detectable 1 h after transfer to −N medium, then dropped sharply after 2 h and accumulated again, although slightly, after 7 h incubation. In +N medium containing ammonium nitrate, expression of these genes was undetectable during 6 h incubation (Fig. 2). It has been reported that ammonium and nitrate repress the expression of *NIA1*, *NRT2;1*, and *NCG2* genes (Fernández et al. 1989; Pozuelo et al. 2000).

The *FUS1* gene is unique to the mt^+ chromosome (Ferris and Goodenough 1994) and encodes a sex recognition protein called fringe (Ferris et al. 1996). In our gametogenetic system, the levels of *FUS1* mRNA increased markedly at around 3 h incubation (Fig. 2), the time corresponding to when cells were just developing into gametes (Fig. 1). The transcripts for the two other gamete program genes, *MTA1* and *MTA2* (Ferris et al. 2001, 2002), were also accumulated transiently between 3 h and 4 h.

The *GAS28* gene is expressed in the late phase of gametogenesis and is assumed to encode a hydroxyproline-rich glycoprotein of the zygotic cell wall (Rodriguez et al. 1999; von Gromoff and Beck 1993). We found that the steady-state levels of *GAS28* mRNA increase between 6 h and 7 h (Fig. 2), the time corresponding to when cells have already become fully competent gametes (Fig. 1).

The above results indicate that gametogenesis in *Chlamydomonas* is characterized by the sequential expression of at least three sets of genes—early, middle, and late (Fig. 2). Early genes such as *NIA1*, *NRT2;1*, and *NCG2* are induced in the first 2 h after transfer to −N medium. Middle genes such as *FUS1*, *MTA1*, and *MTA2* are induced between 3 h and 4 h, paralleling the acquisition of mating competency. Late genes like *GAS28* are induced between 5 h and 8 h, corresponding to the period after completion of gametic differentiation.

Expression profiles of novel *NSG* genes during synchronized gametogenesis

Figure 3 shows the steady-state levels of mRNAs for novel *NSG* genes, isolated by EST-based macroarray

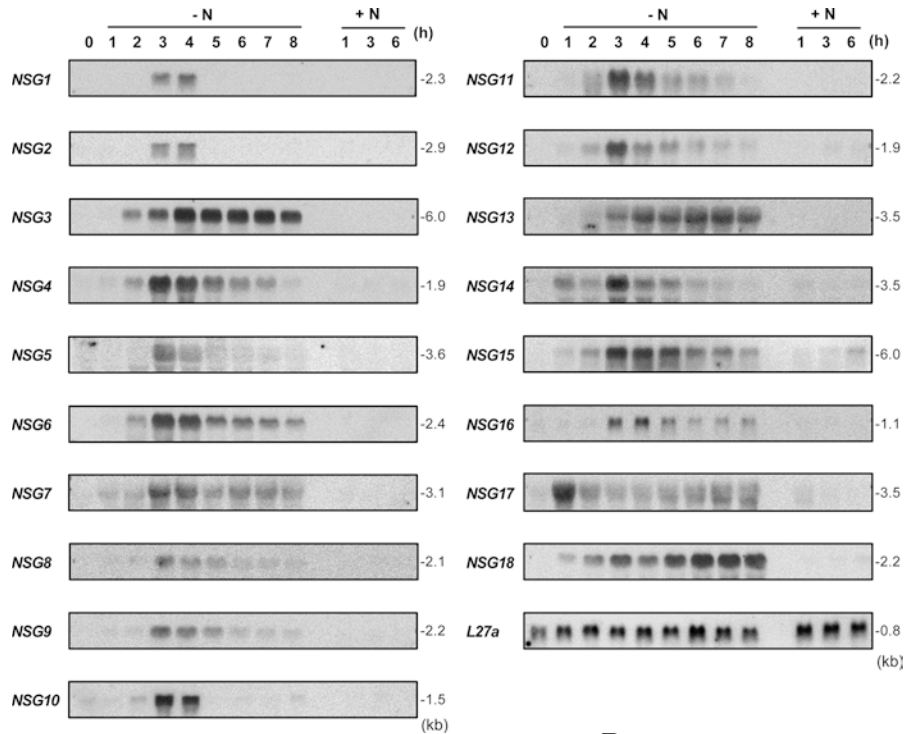


Fig. 3 Expression of newly isolated *NSG* genes during synchronized gametogenesis. Northern blot analysis was performed as in Fig. 2. Probes used are full-length cDNAs

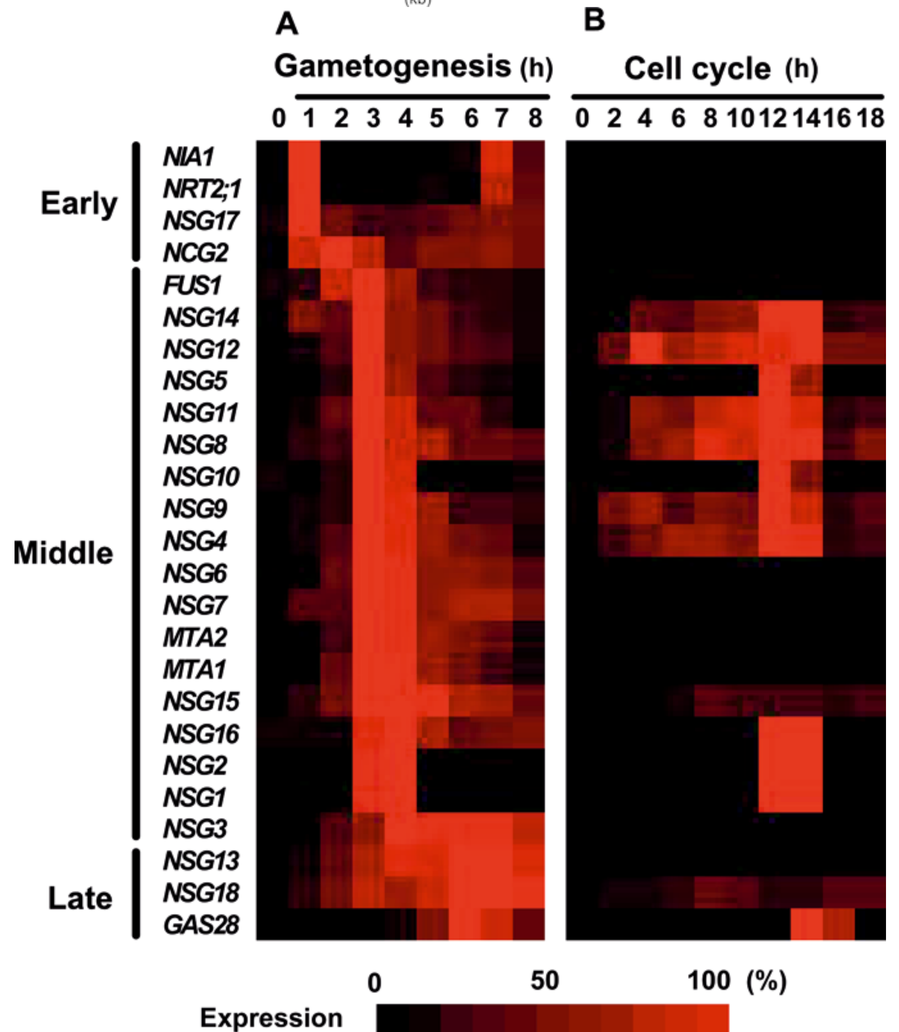


Fig. 4 The global pattern of gene expression during **a** gametogenesis and **b** mitotic cell cycle. Data shown in Figs. 2, 3, 6 are graphically displayed with color to represent the relative changes in the expression of each gene. Increases in mRNA levels are shown as shades of red. The genes that showed the most significant expression during gametogenesis are ordered so that genes with similar expression are grouped into three temporal stages: early, middle, and late (see Expression profiles of previously identified marker genes during synchronized gametogenesis, above)

analysis, during synchronized gametogenesis. Similar to previously identified genes described above (Fig. 2), all of the 18 *NSG* genes showed a strong expression in cells placed under −N conditions, whereas no or very weak expression was observed in cells incubated under +N conditions for at least 6 h (Fig. 3). In −N cells, many *NSG* genes exhibited transient accumulation of mRNAs, but some genes such as *NSG3*, *NSG13* and *NSG18* showed sustained accumulation through the rest of the time-course.

To order genes on the basis of the time of the most significant accumulation during gametogenesis, the measured changes in mRNA levels were displayed in a graphical format (Fig. 4a). The *NSG* genes were then grouped according to similarities in their accumulation patterns to those of previously identified marker genes, which can be defined by three temporal classes. Class 1, the early genes: as with *NIA1* and *NRT2;1*, *NSG17* was induced transiently within 1 h after transfer to −N medium. Class 2, the middle genes: 14 *NSG* genes (*NSG1*, *NSG2*, *NSG4*–*NSG12*, *NSG14*–*NSG16*) followed the accumulation patterns of mRNAs characterized by *FUS1*, *MTA1*, and *MTA2* genes. They were accumulated markedly and transiently between 3 h and 4 h after transfer to −N medium. In contrast, *NSG3* exhibited a middle–late pattern of induction. The mRNA was accumulated significantly after 4 h incubation and the accumulation was sustained through the rest of the time-course. Class 3, the late genes: two *NSG*

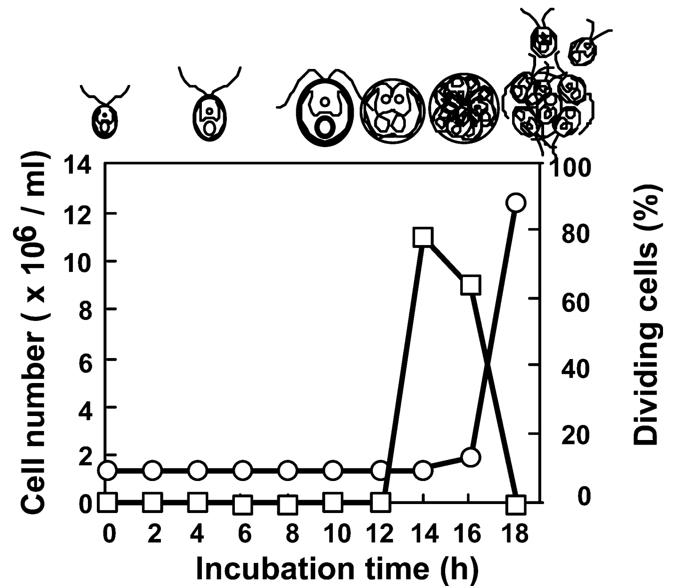
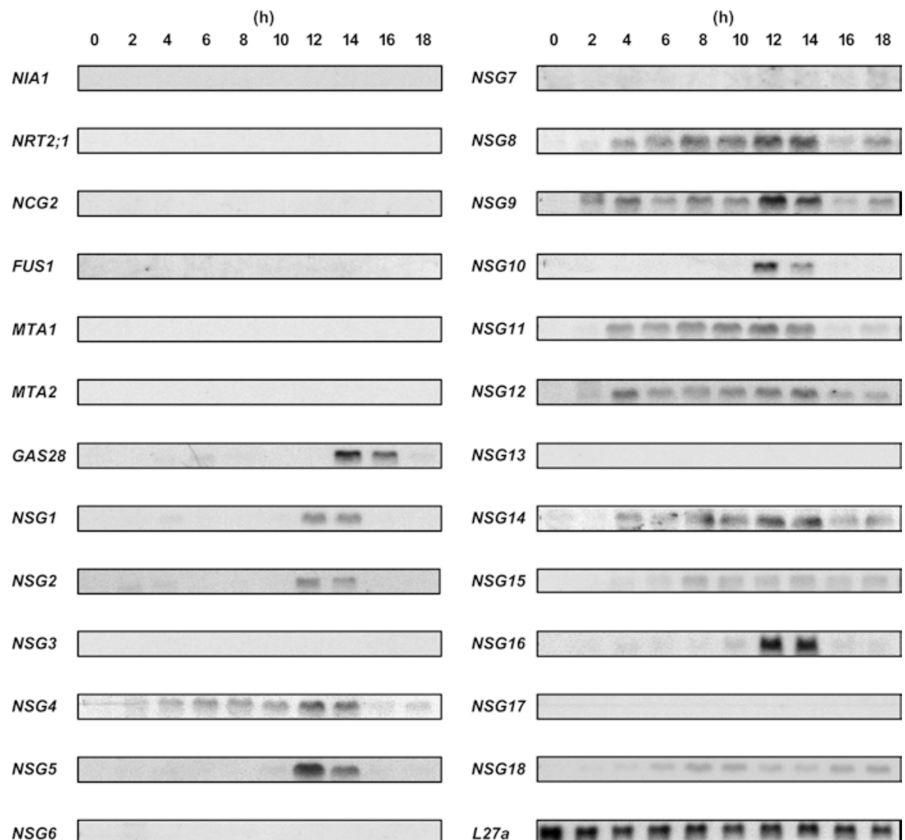


Fig. 5 Time-course of synchronous growth and division of early G_1 cells in +N medium. Synchronously grown mt^+ cells at the beginning of the light period (L-0 cells) were transferred to M medium at a density of 1.6×10^6 cells/ml and incubated for 18 h in the light. Note that the cell density was about 8-fold less than that in Fig. 1, so that the cells could proceed through one round of the mitotic cell cycle with no apparent limitation of nutrients. *Open circles* Cell number, *open squares* dividing cells (%)

Fig. 6 Expression of marker genes and *NSG* genes during the mitotic cell cycle. Cell samples were taken at the time-points indicated in Fig. 5 and used for Northern blot analysis



genes (*NSG13*, *NSG18*) followed a pattern of induction and accumulation characterized by *GAS28*, detectable strongly between 6 h and 7 h after transfer to −N medium.

Expression-specificity of *NSG* genes

We found that all of the *NSG* genes examined are also expressed during gametogenesis of mt^- cells (data not shown), with similar temporal patterns to mt^+ cells (Fig. 3). It was suggested, therefore, that *NSG1–NSG18* are transcribed as a common process for gametic differentiation between the two mating-type cells and not as a sex-limited or sex-specific process for differentiation (Goodenough et al. 1995).

To further evaluate the expression-specificity of the *NSG* genes, their mRNA levels were examined during the mitotic cell cycle under +N conditions. As shown in Fig. 5, early G_1 cells in synchronous culture grow during the first 12 h, undergo S and M phases between 12 h and 16 h, and liberate daughter cells after 18 h. Total RNA was isolated from cells collected every 2 h throughout the 18-h light period and used for Northern blot analysis, using previously identified genes and *NSG* genes as probes (Fig. 6). Figure 4b displays the global patterns of accumulation of transcripts during the mitotic cell cycle, where each gene is ordered according to the three temporal classes of mRNA accumulation during gametogenesis (Fig. 4a).

None of the early genes (*NIA1*, *NRT2;1*, *NCG2*, *NSG17*) showed significant expression during the mitotic cell cycle. Among the middle genes, no significant expression was observed in *FUS1*, *MTA1*, *MTA2*, *NSG3*, *NSG6*, and *NSG7*. In contrast, *NSG4*, *NSG8*, *NSG9*, *NSG11*, *NSG12*, and *NSG14* mRNAs accumulated weakly at middle/late G_1 phase (4–10 h) and significantly at S/M phase (12–14 h). *NSG1*, *NSG2*, *NSG5*, *NSG10*, and *NSG16* appeared to be induced restrictively during S/M phase. *NSG15* was accumulated weakly through G_1 and S/M phases. Among the late genes, *NSG13* mRNA was not detected at all throughout the cell cycle. In contrast, *GAS28*, which has been reported to be a gametogenesis-specific gene (Rodriguez et al. 1999; von Gromoff and Beck 1993), was transcribed during M phase (14–16 h). The *NSG18* transcript was accumulated weakly through G_1 and S/M phases.

Table 1 summarizes the transcriptional specificity of *NSG* genes during gametogenesis and the mitotic cell cycle.

Functional features of *NSG* genes

To annotate polypeptides encoded by *NSG* genes, we performed a BLAST analysis (Altschul et al. 1997) of each cDNA (largely 5′ end sequence) against the GenBank database and a genome analysis against the JGI *Chlamydomonas* genome database (Grossman et al. 2003; www.jgi.doe.gov/chlamy). All *NSG* genes localize to different genomic loci without linking to each other or to the mating-type locus (Table 1). Thirteen out of 18 *NSG* genes were annotated proteins with a BLAST *e* value less than $6.00\times e^{-25}$, while one early gene (*NSG17*), four middle genes (*NSG1*, *NSG6*, *NSG7*, *NSG11*), and one late gene (*NSG13*) showed no matches with any entry in the databases (Table 1).

Several middle genes encode putative polypeptides related to nucleotide/protein metabolism: a small subunit (*NSG2*) and a large subunit (*NSG5*) of ribonucleotide reductase, the RPT4 (*NSG8*) and RPT2 (*NSG9*) subunits of the 26S proteasome triple-A ATPase, a dUTP pyrophosphatase (*NSG10*), a DEAD box RNA helicase (*NSG12*), a valosin-containing protein (*NSG14*), and a clathrin heavy chain (*NSG15*). Putative regulatory proteins are a reverse transcriptase homologue (*NSG3*) and a cyclin-dependent kinase regulatory subunit (*NSG16*). *NSG4* encodes a *Chlamydomonas* actin, which functions in elongating a fertilization tubule from the mt^+ gamete during mating (Sugase et al. 1996). A late gene, *NSG18*, encodes a *Chlamydomonas* glutamine synthetase1 (Chen and Silflow 1996), suggesting its involvement in the assimilation of alternative nitrogen sources.

Since we could not annotate proteins for six *NSG* genes by a database search using the partial nucleotide sequence (Table 1), their full-length cDNA clones were isolated and the entire nucleotide sequences determined. As diagrammed in Fig. 7, *NSG1*, *NSG6*, *NSG7*, *NSG11*,

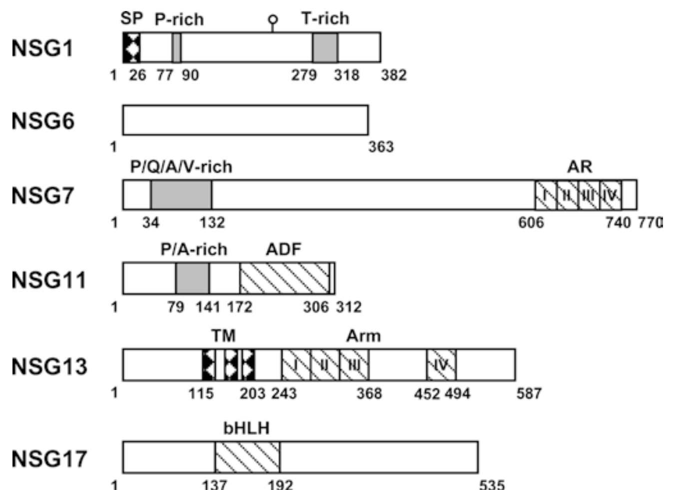


Fig. 7 Predicted structural features of the NSG1, NSG6, NSG7, NSG11, NSG13, and NSG17 proteins deduced from the full-length cDNA sequences. For NSG1, a predicted signal peptide (*SP*), a P-rich region, a T-rich region, and a potential N-linked glycosylation site (marked by a *circle*) are indicated. For NSG7, a P/Q/A/V-rich region and four ankyrin repeats (*AR*, marked I–IV) are indicated. For NSG11, a P/A-rich region and a putative actin-depolymerizing factor (*ADF*) domain are indicated. For NSG13, three helical transmembrane (*TM*) regions and four armadillo (*Arm*) repeats (marked I–IV) are indicated. For NSG17, a basic helix-loop-helix (*bHLH*) domain is indicated

NSG13, and *NSG17* were found to code for polypeptides of 382 (M_r 41,357), 363 (M_r 38,799), 770 (M_r 82,886), 312 (M_r 33,235), 587 (M_r 57,582), and 533 (M_r 53,834) amino acids, respectively. A search of the genome database revealed that the six *NSG* genes have 6, 7, 21, 6, 13, and 2 introns, respectively. The ORFs were then analyzed using Pfam (Bateman et al. 2002), SMART (Schultz et al. 1998), SOSUI (Hirokawa et al. 1998), PredictProtein (Rost and Liu 2003), and PREDATOR (Frishman and Argos 1997) programs.

NSG1 contains a predicted signal peptide (positions 1–26), as expected for a secreted protein (von Heijne 1985), a P-rich region (positions 77–90), and a T-rich region (positions 279–318; Fig. 7). No significant homology was found by a database search. NSG6 was characterized as a soluble protein with totally unknown function (Fig. 7). NSG7 contains a P/Q/A/V-rich region (positions 34–132) and four presumptive ankyrin repeats (positions 606–638, 641–673, 674–706, 708–740) at the C-terminus (Fig. 7). NSG11 has a P/A-rich region (positions 79–141) and a C-terminal domain (positions 172–306) which is highly homologous to an actin-depolymerizing factor (ADF; Fig. 7). Comparison with other members of the ADF/cofilin family demonstrated an amino acid identity of 36–45% and similarity of 60–70%, with the highest identity to *Acanthamoeba* actophorin (Quirk et al. 1993). Furthermore, the deduced secondary structure and a putative phosphorylation site (Ser-172) within the ADF domain of NSG11 are highly conserved among the ADF/cofilin family. NSG13 is predicted to contain three helical transmembrane regions (positions 115–136, 156–178, 182–203) and four armadillo/beta-catenin (Arm) repeats (positions 243–285, 286–326, 327–368, 452–494) toward the C-terminus (Fig. 7). Finally, *NSG17* codes for a putative transcription factor with a basic helix-loop-helix (bHLH) DNA-binding domain (positions 137–192; Fig. 7). The bHLH domain showed highest identity (54%) to that of a putative DNA-binding protein of *Arabidopsis thaliana* (accession no. NP_177064).

Discussion

Utilizing a synchronized system, important information on the temporal program of gene expression during gametic differentiation was obtained. Using several previously identified marker genes and 18 newly identified *NSG* genes, we determined their temporal patterns of induction and classified all of them into three temporal classes (Table 1). Since genes with related functions appear to be induced in similar patterns, we speculate about possible roles for the *NSG* genes, based on their temporal association with the marker genes of known functions. All *NSG* genes that are described in this report may be "autosomal" (Goodenough et al. 1995), since they are expressed in gametes of both mating-types in response to nitrogen starvation.

Properties of an early *NSG* gene

NSG17 was classified as an early gene that followed the expression pattern of *NIA1* and *NRT2;1* during gametogenesis with no apparent expression during the vegetative cell cycle (Fig. 4, Table 1). This gene codes for a putative transcription factor with a bHLH domain similar to those that bind to DNA and exert a determinative influence in various developmental pathways of eukaryotes (Morgenstern and Atchley 1999). In the fission yeast, the $esc1^+$ gene is induced early in response to nitrogen starvation and codes for a putative transcription factor with a bHLH domain that promotes sexual differentiation of *Schizosaccharomyces pombe* (Benton et al. 1993). It is of interest to examine whether NSG17 also promotes the early stage of sexual differentiation in *C. reinhardtii*.

Properties of middle *NSG* genes

Fifteen *NSG* genes were identified as middle genes (Fig. 4, Table 1) and considered to be of great importance in generating the gametic phenotypes. One of the genes, *NSG4*, encodes actin (Sugase et al. 1996). It is known in *C. reinhardtii* that actin polymerization accompanies elongation of the fertilization tubule from the mt^+ gametes during mating with mt^- gametes (Detmers et al. 1983, 1985). The *Chlamydomonas* genome has only a single actin-encoding gene (Sugase et al. 1996) and its mutation, originally marked by the *ida5* mutation (Kato et al. 1993), causes slow swimming due to a lack of functional flagellar inner-arm dynein and also causes deficient growth of the fertilization tubule during mating (Kato-Minoura et al. 1997). The cytoplasmic actin displays dynamic reorganization during mitosis and cytokinesis in the cell cycle (Harper et al. 1992). Not surprisingly, the actin-encoding gene, *NSG4*, is expressed throughout the *Chlamydomonas* life cycle, as clearly seen by its expression both during the middle stage of gametogenesis and also during the S/M phase of the vegetative cell cycle (Fig. 4). It is of interest that a putative ADF gene, *NSG11* (Fig. 7), follows the accumulation patterns of *NSG4* in both gametic and vegetative stages (Fig. 4). ADF/cofilins are a family of actin-binding proteins in all eukaryotic cells and have the ability to remodel the cytoskeleton by severing and depolymerizing actin filaments (Maciver and Hussey 2002). Detmers et al. (1983) observed a rapid breakdown of the fertilization tubule cytoskeleton in the zygote after gametic cell fusion is completed, suggesting a possible role of NSG11 in actin remodeling after cell fusion. The ADF domain of NSG11 most closely resembles the *Acantamoeba* actopholin and higher plant ADF/cofilins, although NSG11 contains a unique, long N-terminal extension (170 amino acids) with unknown function (Fig. 7).

It is known that specific changes occur in the nucleotide/protein synthesis machinery during *Chlamydomonas* gametogenesis (Bulté and Bennoun 1990; Martin

et al. 1976; Picard-Bennoun and Bennoun 1985; Siersma and Chiang 1971). Changes at the morphological level during gametogenesis have also been examined by Martin and Goodenough (1975), who observed the modification of ER and the appearance of new Golgi-derived vesicles (named gametic vesicles) prior to the appearance of mating structures in the mt^+ and mt^- gametes. The functions of many middle genes—*NSG2* and *NSG5* encoding two subunits of ribonucleotide reductase (Jordan and Reichard 1998), *NSG8* and *NSG9* encoding two subunits of 26S proteasome (Voges et al. 1999), *NSG10* encoding a dUTP pyrophosphatase (Mol et al. 1996), *NSG12* encoding a DEAD box RNA helicase (Aubourg et al. 1999), *NSG14* encoding a valosin-containing protein/p97/Cdc48p (Latterich et al. 1995; Ye et al. 2001), and *NSG15* encoding a clathrin heavy chain (Brodsky et al. 2001)—suggest they play a role in the specific changes that occur in the protein-synthesizing machinery during gametogenesis. *Chlamydomonas* cells acclimate to diverse environmental stresses, including nitrogen starvation, by the specific expression of a number of genes encoding polypeptides involved in proteolysis (Shrager et al. 2003).

NSG16 may encode a cell cycle regulatory protein, since the deduced amino acid sequence shows high homology with a subunit of cyclin-dependent kinases such as Cks1 and Suc1 in yeast (Harper 2001). We do not know at present the functional roles of *NSG16* in gametes. However, since this gene and also other middle genes such as *NSG 1*, *NSG2*, *NSG4*, *NSG5*, *NSG8*, *NSG9*, *NSG10*, *NSG11*, *NSG12*, and *NSG14* are transcribed mainly during the S/M phase in the cell cycle (Fig. 6), they all may play pivotal roles both in asexual and sexual stages of the *Chlamydomonas* life cycle.

In contrast, three middle genes, *NSG 3*, *NSG6*, and *NSG7* appear to be specifically expressed under −N conditions, since no transcripts were detected in the vegetative cell cycle (Figs. 4, 6). The deduced amino acid sequence of *NSG3* showed the highest homology to a reverse transcriptase from the *copia*/*ty1*-like retrotransposon named *Lusen*, from a related alga, *V. carteri* (Lindauer and Schmitt, unpublished data). The *copia*-like retrotransposons are present universally among higher and lower plants, insects, fungi, and protists (Lindauer et al. 1993; Voytas et al. 1992; Xiong and Eickbush 1990). Only a small number of plant retrotransposable elements have been shown to be active; and this activity seems to be restricted to unusual stress situations called "genomic shock" (Grandbastein 1992; McClintock 1984). It is therefore likely that *NSG3*, which is restrictively expressed under −N conditions, may be involved in facilitating genomic restructuring and, hence, adaptation to extreme environmental conditions (McClintock 1984). *NSG6* and *NSG7* encode novel proteins that are expressed only in gametes. Furthermore, the deduced amino acid sequence of a full-length cDNA clone of *NSG7* contains four tandem ankyrin repeats proximal to the C-terminus (Fig. 7). Ankyrin repeats are 33-residue motifs involved in protein–protein interactions (Sedwick and Smerdon 1999). In this alga, the *ZYS3* gene encodes a protein containing ankyrin repeats and is expressed only in the young zygotes (Kuriyama et al. 1999). Thus, these two proteins with ankyrin repeats may function as developmental regulators at different steps in the *Chlamydomonas* sexual cycle. A BLAST search of the *Chlamydomonas* genome database using the nucleotide sequences of *NSG6* and *NSG7* indicated that genes for each protein were present in the database and that ESTs for the sequences were present in libraries enriched for gamete and zygote genes and also in libraries from carbon-stressed cells. It is therefore possible that these genes could be related to stress rather than sexual development.

Properties of late *NSG* genes

Rodriguez et al. (1999) showed that *GAS28* is expressed in the late phase of gametogenesis and hypothesized that this transcript is stored until activated for translation as a constituent of the zygote cell wall. Similarly, one of the late genes, *NSG13*, that encodes a putative transmembrane protein containing Arm repeats and is expressed only in gametes might be related to the zygotic stage of sexual cell cycle. Arm repeat domains are ubiquitous among eukaryotes and mediate specific protein–protein interactions for diverse functions such as cell–cell adhesion and intracellular signalling (Coates 2003; Peifer et al. 1994). In this alga, an Arm repeat protein, PF16, is required for cellular motility brought about by the flagellum (Smith and Lefebvre 1996, 2000).

NSG18 encodes a glutamine synthetase (Chen and Silflow 1996) and may be induced for acclimation of cells to a long-term starvation of nitrogen. Quesada and Fernández (1994) reported that nitrate assimilation genes such as *NIA1* and *NRT2;1* are transcribed transiently at the early stage and again at the late stage of gametogenesis (see also Fig. 2), while *NRT2;2*, a gene sharing sequence similarity with *NRT2;1*, is only accumulated after a long incubation period in −N medium.

The three sets of *NSG* genes reported here that are expressed at distinct points during synchronized gametogenesis of *C. reinhardtii* may be essential tools for defining their regulatory networks of gene expression, by analyzing their expression in mutants for gametic differentiation like *dif* (Saito and Matsuda 1991).

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