Characterization of novel genes induced by sexual adhesion and gamete fusion and of their transcriptional regulation in *Chlamydomonas reinhardtii*

Takeaki Kubo 1,5, Jun Abe 2,6, Takeshi Oyamada 1, Makoto Ohnishi 1, Hideya Fukuzawa 3, Yoshihiro Matsuda 4 and Tatsuaki Saito 1,*

1 Department of Applied Science, Faculty of Science, Okayama University of Science, Okayama, 700-0005 Japan
2 Department of Molecular Science, Graduate School of Science and Technology, Kobe University, Nada-ku, Kobe, 657-8501 Japan
3 Division of Integral Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502 Japan
4 Department of Biology, Faculty of Science, Kobe University, Kobe, 657-8501 Japan

When mating type plus and minus gametes of *Chlamydomonas* are mixed, they agglutinate with each other via their flagella, fuse, then initiate the zygote formation program which includes synthesis of the zygote cell wall, fusion of nuclei and chloroplasts, and the digestion of chloroplast DNA from the minus parent. The mRNAs from gamete and zygote cells was isolated and hybridized to cDNA-macroarray filters both to identify new genes expressed during the mating reaction and the early zygote formation process and to analyze the gene expression programs that underlie these sexual processes. Twenty-one novel genes were identified in this screen, designated as EZY (early zygotic expressed) genes. The EZY genes included genes encoding proteins whose function is unknown, and genes encoding proteins that appear to be involved in processes such as cell wall synthesis, gene expression, intracellular trafficking or secretion, and vesicular transport in zygotic cells. All of the EZY genes were strongly induced within 1 h during the mating process, including early zygote formation. The transcriptional characteristics of EZY genes were analyzed by using the fusion-defective mutant *fus mt* 1. Among the EZY genes, 12 genes were not activated in fusion-defective conditions, suggesting that cell fusion is required for their expression. The remaining nine that were transcribed in fusion-defective *fus* matings were also inducible by cell wall removal in either vegetative or gametic cells, indicating that these genes were induced only indirectly by the cAMP signaling pathway initiated by flagellar agglutination as a result of mating-induced cell wall loss.

**Keywords:** Cell–cell recognition — Chlamydomonas reinhardtii — Transcriptional regulation — Zygote formation.

Abbreviations: CHX, cycloheximide; cpDNA, chloroplast DNA; DRP, dynamin-related protein; ECM, extracellular matrix; ER, endoplasmic reticulum; EST, expressed sequence tag; EZY, early zygotic expressed; miRNA, microRNA; ORF, open reading frame; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

**Introduction**

When sperm and egg interact with each other during the sexual reproduction process, both gametes undergo changes from the molecular to the physiological level to prepare for fusion, and then to adopt new diploid systems. Signals generated as a result of the interaction or fusion of gametes result in changes in the expression of mRNAs and in the translation and/or modification of proteins (Gilbert 1988). The interaction between gametes has been studied in detail in mammalian cells (Wassarman 1999). Pre-gametes of mammalian cells differentiate into eggs and sperm, and they interact with each other to form the fertilized egg that is already full of mRNA. Then in the early developmental process in the embryo, these mRNAs are translated into functional proteins that result in the successful fertilization becoming a normal individual (Gilbert 1988). In plants, the developmental pathways of the male and female gametes and their interactions during the fertilization process are being studied in many species, including the pollination process (Huang and Sheridan 1994, Heslop-Harrison et al. 1999, Faure et al. 2002). In flowering plants, a long-lasting period of interaction between the diploid pistil and haploid pollen tube is essential to complete the fusion of the sexual cells (Higashiyama et al. 1998, Higashiyama et al. 2001). In the heterothallic green alga *Closterium peracerosum*, the transcriptome profiles during sexual reproduction were obtained by cDNA-based microarray analysis (Sekimoto et al. 2006). However, the detailed gene expression programs underlying the interaction of male and female gametes have not been reported yet.

The unicellular biflagellated green algae *Chlamydomonas reinhardtii* is an excellent model system to dissect the...
sexual cell–cell recognition and diploid formation process morphologically and physiologically (Ferris et al. 1996, Pan et al. 2003, Ferris et al. 2005, Goodenough et al. 2007). *Chlamydomonas reinhardtii* is sexually heterothallic, with separate *mt* and *mt* strains. Cells of both mating types independently differentiate from vegetative cells to gametes under nitrogen-starved conditions (Matsuda et al. 1992). Three gene expression programs are generally recognized as a response to nitrogen depletion: a program to adapt to nitrogen starvation; a gamete differentiation program; and a zygote formation program, that are hierarchically regulated (Abe et al. 2004, Abe et al. 2005).

When *mt* and *mt* gametes are mixed, opposite sex gametes rapidly agglutinate due to the sexual agglutinins located on the surface of each flagellum (Ferris et al. 2005). This flagellar adhesion triggers transient elevation of cAMP levels inside the flagellar and cell body (Pasquale et al. 1987, Saito et al. 1993), which makes both of the gamete cells prepare for the cell fusion to become diploid zygotes, such as activation of the gamete lytic enzyme (gametolysin) to shed the cell wall of the gametes and activation of both mating structures (Kinoshita et al. 1992, Wilson et al. 1997, Matsuda and Kubo 2004). The cell wall-less gametes then fuse, starting at their mating structures followed by full cytoplasmic confluence. Cell fusion results in a shift from gamete-specific processes to zygote formation/maturation events including: digestion of minus chloroplast DNA (cpDNA), flagellar resorption, synthesis of the zygotic cell wall and fusion of nuclei. These cellular and molecular events specific to zygote cells occur within 7 h after both mating types of gametes are mixed (Harris 1989).

Some of the genes transcribed early in the zygote formation process have been identified by screening cDNA libraries from zygote cells (Ferris and Goodenough 1985, Wegener and Beck 1991, Uchida et al. 1993, Ferris et al. 2002). These genes are all transcribed at a very early stage (~1 h) in the zygote formation process and have been characterized as zygote-specific genes in previous extensive studies (Woessner and Goodenough 1989, Matters and Goodenough 1992, Woessner and Goodenough 1992, Armburst et al. 1993, Uchida et al. 1993, Kuriyama et al. 1999, Suzuki et al. 2000, Ferris et al. 2002). Although a number of zygote genes are characterized, it is unclear whether all zygote-specific genes have been identified. Furthermore, the zygote formation process would include not only zygote-specific genes, but also the genes that promoted the expression of zygotes more than that of vegetative cells or gametes. Kurvari et al. (1995) also identified genes activated by sexual adhesion when *mt* gamete cells were mixed with flagella isolated from *mt* gametes.

In order to isolate novel genes expressed in this early stage of the sexual process of this unicellular algae, and to analyze temporal gene expression programs, we used the cDNA-based-macroarray that contain 10,368 expressed sequence tags (ESTs) of *Chlamydomonas* (Asamizu et al. 1999, Asamizu et al. 2000) and screened it with probes derived from zygote cells produced using a synchronized cell culture system. We have identified 21 new genes that are up-regulated either in mating or in early zygote formation, and the transcriptional characteristics of these genes were also analyzed.

### Results

**Expression of new EZY genes during the mating reaction including early zygote formation**

We ascertained that four previously identified zygote genes (Class II, Class VI, Class VIII and Zys1A) were present among the Kazusa ESTs used to make the macroarray (Ferris and Goodenough 1985, Uchida et al. 1993). Since the Class VIII gene is present on the array, we used this gene as a reference gene in the course of the array analysis. The 92 EST clones were identified in a first round of screening as clones whose [Z]/[G] expression ratio was >3.0-fold. These candidates were re-screened by using them as probes on RNA blots with total RNA derived from vegetative cells, gametes and zygotes, and ultimately 21 genes (including Class VIII which had been added to the array as a positive control) were found to be transcriptionally up-regulated in zygotes (Table 1).

To assess the detailed transcriptional regulation systems in the early period of the sexual cycle, the steady-state levels of mRNA for these 21 genes previously identified as zygote-specific genes during the mating reaction and early zygote formation were examined (Fig. 1). In the early zygote formation process following an initial sexual adhesion, the transcription of all 21 genes was greatly induced within 60 min after gametes of both mating types were mixed. We found that mRNA of almost all of the EZY (early zygote expressed) genes, except EZY4 and EZY9, had increased within 15 min after gametes were mixed. Class II, Class IV and Zys1A genes identified as early genes in zygote development (Ferris and Goodenough 1985, Uchida et al. 1993) were also expressed within 15 min after gametes were mixed (Fig. 1). The accumulation of EZY4 and EZY9 mRNA was not apparent until the 1 h time point. This difference in the timing of transcription suggests that several gene expression programs from the mating reaction through to the early zygote formation process might exist.

**Functional features of EZY genes by annotation**

After the confirmation that EZY genes were transcriptionally up-regulated during the mating reaction and in early zygote cells, we determined the nucleotide sequence of
full-length cDNAs for all the EZY genes, then analyzed the functional features of the protein product predicted for each clone. The annotation of the proteins encoded by the EZY genes was performed by BLAST analysis of the full cDNA sequences on the JGI *Chlamydomonas* genome database version 3 (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) (Table 1).

Annotation of the 21 genes revealed that four groups of genes had related functions. The deduced gene products of three genes, EZY4 (UDP-glucose 4 epimerase), EZY11 (UDP-glucose:protein transglucosylase) and EZY12 (UDP-glucose/GDP-mannose dehydrogenase), were involved in sugar metabolism for synthesis/turnover of carbohydrate. EZY16 (cell wall protein pherophorin-C15) encodes a pherophorin, a component of the extracellular matrix (ECM) of algae and land plants (Hallmann, 2006). EZY8 (dynamin), EZY9 (ZYS3 protein) and EZY23 (secE/sec61 gamma protein) appear to be involved in intracellular trafficking, secretion or vesicular transport. EZY8 has structural features of dynamin-related proteins (DRPs), containing a GTP-binding domain and a dynamin signature. EZY9 has high similarity with the whole polypeptide

<table>
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<tr>
<th>Gene name</th>
<th>Kazusa EST clonea</th>
<th>Full-length cDNA accession No.</th>
<th>Putative function</th>
<th>Organism</th>
<th>Protein IDc</th>
<th>Positiond</th>
<th>Activation on fus mating</th>
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<td>185246</td>
<td>42:658586</td>
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</tr>
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</table>

**Table 1** Newly identified genes, EZYs, expressed in the mating reaction and early zygote formation

*a* EST clone numbers on the macroarray derived from the Kazusa DNA Research Institute (Asamizu et al. 2000, Asamizu et al. 2001).

*b* The full length-cDNA sequences of EZY3–EZY23 were determined in this study and have been deposited in the Genebank/EMBL/ DDBJ database.

*c* Protein ID in the JGI *Chlamydomonas* genome database ver.3.

*d* The scaffold number and the starting base position of the full-length cDNA in the JGI database are indicated.
sequence of Zys3, known as a zygote-specific protein in *C. reinhardtii* (Kuriyama et al. 1999). Zys3 is a postulated secretory protein with a signal peptide sequence and contains two segments of an ankyrin repeat and a WW domain in its amino acid sequence. EZY9 has a similar sized polypeptide sequence and also contains the same motifs in comparable regions of its sequence. EZY23 encodes SecE/Sec61 gamma protein, which plays a role in protein translocation through the endoplasmic reticulum (ER) in eukaryotes.

The EZY genes also include the genes involved in gene expression or chromosome partitioning. The C-terminal domain of EZY7 has a conserved domain of the stage V sporulation protein-S, SpoVS, identified as a regulatory protein that activates the genes involved in spore formation of the Gram-positive bacterium *Bacillus subtilis* or several bacteria and archa (Reneskov et al. 1995). The product of the EZY18 gene is a protein similar to Pbf-2 protein, which functions as a novel transcriptional regulator in higher plants (Desveaux et al. 2000). The EZY19 gene encodes a chloroplast REC1 protein, a eubacterial type of RecA homolog that functions in the chloroplast in *C. reinhardtii* (Nakazato et al. 2003).

We could not annotate proteins encoding in the four EZY genes EZY3, EZY5, EZY6 and EZY20 by BLAST analysis. The predicted open reading frames (ORFs) were then analyzed using prediction programs SignalP 3.0, Pfam, SMART, SOSUI, PredictProtein and PREDATOR (Frishman and Argos 1997, Hirokawa et al. 1998, Schultz et al. 1998, Bateman et al. 2002, Rost and Liu 2003, Bendtsen et al. 2004) (Table 2).

EZY3 and EZY20 were predicted to be expressed proteins of 162 amino acids (M_r 17,205 Da) and 382 amino acids (M_r 42,090 Da), respectively, and the JGI genome database revealed that the EZY3 gene has three introns and EZY20 has nine introns. The EZY20 polypeptide has proline-rich regions in its sequence (positions 42–68 and 275–295). The EZY3 polypeptide yielded no hits for typical structural features by database analysis. EZY5, encoding a polypeptide of 182 amino acids (M_r 20,420 Da), has three introns in its gene structure. EZY5 contains a possible signal peptide sequence in the N-terminal

![Fig. 1](image-url) The expression of newly identified EZY genes (EZY3–23) and previously identified zygote-specific genes (Class II, Class IV, Class V and Zys1A) in the early zygote development process, and the effect of gametic adhesion without cell fusion. The transcription of EZY genes and previously identified zygote-specific genes was analyzed in wild-type mt^+^/C2 mt^-^/C0 and fus mt^+^/C2 wild-type mt^-^ at 0 (G), 15, 30 and 60 min after gamete mixing. The percentage of zygote formation at each time point is indicated. Asterisks indicate the EZY genes whose transcription was not affected in fusion-defective mating as in wild-type mating. The L27a gene (encoding ribosomal protein L27a) was used as a loading control. The size of mRNA is indicated on the right in kb.
region (position 1–21) and a predicted transmembrane segment (position 112–136). This polypeptide also contains a predicted WW domain (position 52–82), thought to be involved in protein–protein interaction (Table 2).

In the analysis of EZY6, whose full-length cDNA is 1,511 bp, we failed to find an obvious ORF, although there were several short ORFs (encoding 18–67 amino acids) terminated by stop codons in all three frames of the sequence. In the JGI genome database, the predicted EZY6 gene has no introns and a 67 amino acid polypeptide sequence which has no hit with known motifs or proteins. Since we could detect the transcription of EZY6 in zygotes and after mixing of gametes, and its size was consistent with its nucleotide sequence (Fig. 1), EZY6 is the gene transcribed specifically in the mating reaction but its function is not known. Recently, Molnár et al. (2007) identified microRNAs (miRNAs) in *Chlamydomonas*, which guide post-transcriptional regulation by means of targeted RNA degradation and translational arrest. Interestingly, the EZY6 gene contains the short sequence (position 119–128 bp on cDNA) that is identical to the sequence of one of the miRNAs, CrsRNA679914 (Table 2). This sequence in the EZY6 gene suggests that transcripts of EZY6 might be a target for post-transcriptional regulation by miRNA during the zygote formation process.

From the analysis of the JGI genome database, all of the 21 EZY genes localize to different loci in the *Chlamydomonas* genome (Table 1). None of the EZY genes, except EZY8, was closely linked to the mating type locus. The EZY8 gene localizes 240 kbp from the *FUS1* gene in the MT plus locus (Ferris and Goodenough 1994), indicating that the gene is located in the T (telomere-proximal) domain of the MT plus locus (Patrick J. Ferris, personal communication) and therefore should be in a comparable position in the MT minus locus.

**Transcription of EZY genes in fusion-defective matings**

Inter-related steps or transcriptional cascades involved in sexual mating can be analyzed by utilizing mutants that are unable to complete the sexual process (Matsuda et al. 1978). First of all, in order to determine the transcriptional characteristics of EZY genes during the mating reaction and early zygote formation, the transcription of EZY genes was analyzed using the fusion-defective mutant, fus mt+ (Matsuda et al. 1978). The fus mt+ gamete and wild-type mt/C0 gamete can agglutinate each other, then lose the cell wall and activate the mating type structure normally, but they cannot undergo cell fusion since fus lacks the fringe protein on the tip of the mating type plus mating structure.

### Table 2 Predicted structural features of EZY3, EZY5, EZY6 and EZY20

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<th>Gene</th>
<th>Size of full-length cDNA (bp)</th>
<th>Deduced polypeptide (amino acids)</th>
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<td>EZY3</td>
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</tr>
<tr>
<td>EZY5</td>
<td>1,744</td>
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<tr>
<td>EZY6</td>
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<td>67</td>
</tr>
<tr>
<td>EZY20</td>
<td>2,456</td>
<td>382</td>
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</table>

aDeduced polypeptides of non-annotated EZY genes from full-length cDNA were listed.

bCDNA of EZY6 has the sequence thought to be the target region of miRNA, CrsRNA679914 (Molnár et al. 2007).
Transcription of the four previously identified genes and the EZY genes was tested by RNA blot analysis during fusion-defective mating with fus mt\(^+\) (Fig. 1). The mRNAs of five EZY genes (EZY3, 4, 7, 8 and 9), as well as the previously identified genes, were not transcribed or were barely detectable in the early stage of the mating process with fus. Furthermore, the mRNA levels of another six EZY genes (EZY10, 15, 17, 18, 19 and 22) remained the same as in gametes during fusion-defective mating and were not up-regulated to the levels seen in zygotes during wild-type matings. The data indicate that the transcription of these EZY genes is elicited by the transcriptional system activated by cell fusion of the gametes. Previously Ferris and Goodenough (1985) identified zygote-specific genes, including Class II, Class IV and Class V, and showed that these genes require cell fusion for their transcription under fusion-defective conditions (Fig. 5). The data presented here suggest that the transcriptional systems activated by cell fusion have an effect not only on the genes expressed specifically in zygotests but also on the genes expressed faintly in gametes and predominantly in zygotes.

On the other hand, the mRNAs of the other nine EZY genes (EZY5, 6, 11, 12, 13, 14, 16, 21 and 23) accumulated from an early stage (from 15–30 min) even in fusion-defective conditions, comparable with mating under normal fusion conditions in wild-type gametes (Fig. 1, panels with asterisks), indicating that cell fusion was not necessary for the transcription of these nine genes. These results suggest that these nine genes are subject to another transcriptional regulation that is independent of cell fusion.

**The transcriptional characteristics of EZY genes transcribed in fusion-defective mating**

In order to assess and clarify in detail the transcriptional characteristics of the EZY genes before and after cell fusion of gametes, nine EZY genes transcribed in fusion-defective mating conditions were examined. Although gametes cannot fuse in fus mt\(^+\) and wild-type mt\(^-\) mating, both mating types of cells strongly agglutinate via their flagellar surface as in normal mating between wild-type cells. This cell–cell adhesion by flagella, even in fusion-defective conditions with fus, generates a rapid rise in intracellular cAMP levels which stimulates such mating responses as the activation of the gamete lytic enzyme (gametolysin) which causes cell wall shedding, and construction or activation of mating structures in mt\(^+\) and mt\(^-\) cells as a prelude to cell fusion (Buchanan et al. 1989, Wilson et al. 1997, Matsuda and Kubo 2004).

Recently, Hoffmann and Beck (2005) showed that GAS genes (GAS28, GAS30 and GAS31) encode zygote cell wall constituents, expressed not only in gametogenesis and the early zygote formation process, but also in the protoplast state of vegetative cells after cell wall shedding with gametolysin. In the early stage of mating, the cell walls of both gametes are removed because agglutination causes cAMP elevation in the flagella and cell body, resulting in the activation and release of gametolysin, digesting the cell wall. Therefore, an explanation for the transcription of these nine EZY genes could be that it is simply a response to becoming a protoplast after cell wall shedding in either vegetative or gamete cells (Fig. 2). To answer this question, we performed a cell wall removal experiment for the nine EZY genes whose transcription was not elicited by cell fusion. Fig. 2 shows the transcription of these nine EZY genes when treated by gametolysin to create protoplast cells in both vegetative cells and gametes. A crude solution of gametolysin was added to wild-type vegetative cells or to gametes of the imp3 mutant, which is a signal-defective mutant that can strongly agglutinate but fails to generate the normal mating-triggered cAMP production (Goodenough et al. 1976, Saito et al. 1993). The levels of protoplast cells were monitored 30 and 90 min after gametolysin was added. All of the EZY genes transcribed in fusion-defective conditions were eventually accumulated in concert with cell wall shedding in both vegetative cells and the imp3 gametes (Fig. 2). These results suggest that transcription of these nine EZY genes in fusion-defective conditions is not a direct response to intracellular elevation of cAMP by sexual adhesion but a response to becoming a protoplast by removal of the cell wall as a result of the cAMP signal cascade.

**Expression profiles of novel EZY genes during zygote formation**

The transcriptional profiles of the other 12 EZY genes whose expression was elicited by cell fusion were examined during the process of zygote formation (Fig. 3). During the first 1–7 h of zygote development, we found that the expression of the seven genes fell into four patterns. The transcription of most of the EZY genes (EZY3, 15, 17, 18, 19, 20 and 22) was activated by 1 h after mating and the accumulation of mRNA was maintained during 7 h of the zygote formation process. EZY4, EZY7 and EZY10 were transcribed transiently from 1 to 4 h after mating and then gradually decreased. Transcripts of EZY8 were visible 1 h after mating and rapidly decreased. The EZY9 gene was expressed from 1 h after mating and its accumulation was gradually increased until 3–5 h.

In the early stage of the sexual process, EZY4 and EZY9 were visible only 1 h after gametes were mixed, while the other EZY genes were clearly transcribed from 15 min after mating (Fig. 1). This time lag of the transcription among EZY genes in the early sexual process suggests that a transcriptional cascade which is regulated by the zygote gene expression program exists in the process.
previously identified zygote-specific genes, the transcription of EZY genes isolated from array analysis showed expression in the early stage of zygote formation, and the transcriptional profiles were temporally varied during the mating reaction and zygote developmental process.

**Effect of protein synthesis inhibition on early zygote development**

To investigate the effect of protein synthesis inhibition on the expression of EZY genes, especially for those genes that were induced by cell fusion during the early zygote formation process, gametes of both mating types were incubated with 10 μg ml⁻¹ cycloheximide (CHX) for 1 h and then mixed with each other to mate. Total RNAs were isolated from the samples 1, 2 and 3 h after mixing gametes with CHX (Fig. 4). Since EZY4 and EZY9 were transcribed
from 1 h after mixing gametes and required cell fusion (Figs. 1, 3), the translation of some genes transcribed at the same stage as the other 10 genes may be necessary for EZY4 or EZY9 transcription.

**Discussion**

In the present study, we isolated and characterized new genes expressed during the mating reaction and early zygote development by screening the EST clones spotted on a macroarray membrane (Table 1). Using the synchronized mating system of *Chlamydomonas*, we demonstrated that the 21 novel genes were transcribed within 1 h of zygote formation. In addition, we showed that the EZY genes have unique transcriptional characters that depend on the stages of sexual adhesion and early zygote formation, and can be classified into distinct groups regulated by different transcriptional programs.

We have confirmed by Northern analysis that the 21 genes chosen from the array screening were transcribed during the mating process in *Chlamydomonas*. From their predicted amino acid sequences, we can summarize how these EZY genes may function during sexual adhesion and zygote development (Table 1, Fig. 5). In *Chlamydomonas*, the formation of zygotes rapidly triggers synthesis of a new zygote ECM whose major components are hydroxyproline-rich glycoproteins (Woessner and Goodenough 1989, Woessner and Goodenough 1992). This drastic change would require gene expression of the components of the zygotic cell wall and of the sugar metabolic pathways that are needed for synthesis of various glycoproteins. Three EZY genes, EZY4, EZY11 and EZY12, are involved in sugar metabolism, potentially needed for the synthesis of cell wall glycoproteins (Table 1), and EZY16 encodes pherophorin-C15, a known component of the ECM (Hallmann 2006). In addition, we showed that the transcription of EZY11, EZY12 and EZY16 was also induced by cell wall shedding in the haploid state of the cell (Fig. 2). Therefore, these EZY genes would be induced for cell wall synthesis not only in zygotes but also in vegetative cells or gametes responding to a protoplast condition of the cell (Fig. 5).

The EZY8 protein resembles the GTP-binding domain and dynamin signature of the DRP4 subfamily of the *Arabidopsis* DRP families (Hong et al. 2003). Dynamins are large GTPases that belong to a superfamily in eukaryotes that includes classical dynamins, dynamin-like proteins and Mx proteins (Praefcke and MacMahon 2004). These proteins are involved in the scission of a wide range of vesicles and organelles, and play a role in many processes including budding of transport vesicles, division of organelles and cytokinesis (Vallee and Shpetner 1990, Vallee 1992). DRP families in *Arabidopsis thaliana* have been shown to be present in different subcellular locations including the cell plate, plasma membrane, Golgi apparatus, vesicles, mitochondria and chloroplasts (Hong et al. 2003). In the *Chlamydomonas* zygote, events such as nuclear fusion, flagellar resorption and chloroplast fusion occur within 2–8 h after gamete fusion (Harris 1989). EZY8 protein might also have a crucial role as a dynamin-like protein involved in the budding and scission of secretory vesicles during the period of forming the new zygotic ECM.

Kuriyama et al. (1999) have shown that Zys3 localized in the ER, and speculated that Zys3 may function in changing ER morphologically or may be involved in ER systems for synthesis, sorting or transport of proteins to the Golgi apparatus. EZY9 and Zys3 are highly similar over their entire polypeptide sequence, including the possible signal peptide at the N-terminus (23 amino acids), the ankyrin repeats (position 55–122) and the WW domains (positions 164–189 and 286–309), suggesting that EZY9 has a function in zygote development similar to that of Zys3.
Three EZY genes have properties suggesting that they may be involved in gene expression systems or chromosome rearrangement in zygote. The C-terminal domain of EZY7 has high similarity to sporulation protein-S from various bacteria and archea that have a dormant stage in their cell cycle and form a thick ECM to resist harsh environments. Especially in *B. subtilis*, SpoVS interferes with sporulation at an early stage and seems to play a positive role in allowing cells to progress beyond stage V sporulation (Reneskov et al. 1995). Perhaps EZY7 may have a similar role to SpoVS, in regulating multi-stages not only in vegetative cells or gametes but also in the early stage of zygote formation.

EZY19 encodes REC1, the chloroplast recA recombination protein of *Chlamydomonas*, which is a eubacterial type of recA protein that localizes in the chloroplast in tobacco cells (Nakazato et al. 2003). In *Chlamydomonas* zygotes, the cpDNA contributed by the mt+ gamete disappears by 3–4 h into the zygote formation process (Harris 1989). Only cpDNAs from mt- are inherited in the fused chloroplast of the young zygote cell, known as uniparental inheritance. These dramatic changes in the situation of cpDNA may require the expression of REC1 for maintaining, repairing or homologous recombination of cpDNAs during early zygote development.

EZY18 is a homolog of Pbf-2, one of the transcriptional factors that regulate the expression of pathogenesis-related genes which expressed during the plant defense response in higher plants such as rice or tomato (Desveaux et al. 2000). Pbf-2 is also known as a single-stranded DNA-binding factor that binds with sequence specificity to the inverted repeat sequence. The mRNA of EZY18 was slightly transcribed in vegetative cells or gametes, but its accumulation was elicited in zygotes dependent on cell fusion, and then maintained for 7 h in zygote formation (Figs. 1, 3). EZY18 might play a role in a transcriptional cascade regulating the expression of late genes in zygote formation.

EZY21 encodes NSG14, identified as a gamete gene that is expressed in the middle stage (3–4 h) during gametogenesis after nitrogen depletion. This gene is also expressed in the S/M phase of the mitotic cell cycle (Abe et al. 2004). Since mRNA of the EZY21 gene is also elicited by the cell wall shedding state in vegetative cells or gametes (Fig. 3), this gene might belong to the group of genes whose transcription responds to environmental changes such as nitrogen starvation and osmotic changes in the cells (Abe et al. 2004, Hoffman and Beck 2005).

The transcription of EZY6 was induced by the cell wall shedding state of the cells (Fig. 3). Its ORFs encode polypeptides (18–67 amino acids in-frame) with no typical known motifs in EZY6 and a typically short cDNA with a length of 1,511 bp. The cDNA of EZY6 also contains a polyadenylation signal (TGTAAl) and poly(A) segments, and the size of the mRNA was consistent with mRNAs in gel analysis (Fig. 1), suggesting that EZY6 was transcribed normally as intact mRNA. EZY6 contains the target sequence of miRNA CrsRNA679914, suggesting that EZY6 might be subject to miRNA regulation (Molnár et al. 2007).

Under the fusion-defective condition of a fus mating, 12 EZY genes (EZY3, 4, 7–10, 15, 17–20 and 22) were not transcribed or were strikingly reduced in their transcription, meaning that the induction of these genes requires fusion of the two gametes (Fig. 1). We also confirmed that previously identified genes, Class II, Class IV, Class V and Zys1A, were not transcribed in non-fusing conditions in the synchronized culture system (Fig. 1). These data suggest that the 12 EZY genes were activated by the same regulation process after gamete cells fused. In addition, the transcription of 10 of these 12 EZY genes was not blocked by CHX (Figs. 4, 5), suggesting that their transcription did not require new protein synthesis during zygote development. On the other hand, the transcript of EZY4 and EZY9 which first appeared from 60 min after cell fusion (Fig. 1) failed to appear when CHX was present (Figs. 4, 5), indicating that new protein synthesis after cell fusion is required for their expression. The zygote-specific gene Class V is also CHX sensitive (Ferris and Goodenough 1987), although it was not expressed until 2 h and its transcriptional peak was at 3–4 h in zygotes. This delay of the transcription of the Class V gene compared with that of EZY4 and EZY9 suggests that these two EZY genes may belong to a transcriptional group of zygote-specific genes whose expression depends on transcription factor(s) synthesized earlier (1 h) in zygote development than the factors responsible for expression of Class V and its transcriptional group (2 h).

Ferris and Goodenough (1985) speculated that fusion-regulated induction of zygote gene transcription is activated by the mixing of pre-programmed cytoplasms of the mt+ and mt- gamete, i.e. mt+ and mt- gametes each produce mating type-specific regulatory protein(s) that immediately interact to turn on the transcription of zygote-specific genes following cell fusion. Recently, Kurvari et al. (1998) and Wilson et al. (1999) showed that GSP1, a homeodomain protein, is expressed only in mt- gametes, and proposed that it is involved in the regulation of gamete- and zygote-specific genes. Moreover, Zhao et al. (2001) reported that when GSP1 protein was ectopically expressed in mt- gametes, the cell expressed many zygote-specific genes even in a haploid state. Therefore, they proposed that GSP1 interacts with an mt- gamete-specific molecule, and the newly formed transcriptional regulatory complex activates zygote-specific gene expression (Fig. 5). Our newly characterized EZY genes have not been tested in...
the GSP1/mt\(^{-}\) gamete system, but the regulation of 12 EZY genes suggests that they would belong to the transcription group induced by cell fusion of gametes. It would be of interest to confirm the turning on/off of the expression of EZY genes in the mt\(^{-}\) gamete cells in which GSP1 protein was ectopically expressed.

We also determined that nine EZY genes (EZY5, 6, 11–14, 16, 21 and 23) are activated independently from cell fusion. Since these genes were transcribed in the period from sexual adhesion through the early zygote cell development process (Fig. 1), expression could be triggered by events occurring from the initial flagellar agglutination until just before cell fusion. As we demonstrate in Fig. 2, the shedding of the cell walls, not only of imp3 gametes but also of wild-type vegetative cells, induces the transcription of these nine EZY genes. These data indicate that the transcription of these nine genes is not directly triggered by the cAMP-dependent signaling pathway, but directly by the shedding of cell walls during flagellar agglutination (Fig. 5). Since the cAMP signaling pathway in sexual adhesion leads to the activation and release of gametolysin that makes gamete protoplasts, these nine EZY genes could be temporally activated when gamete cell walls were digested and detached from the plasma membrane prior to cell fusion. Hoffman and Beck (2005) also showed that the GAS genes are activated by cell wall removal in vegetative cells or gametes using gametolysin, as well as during the mating reaction. When the cell walls are shed by gametolysin during the sexual mating process, these EZY genes and the GAS genes may be regulated by the same transcriptional factors that respond to the protoplast state of Chlamydomonas cells.

**Materials and Methods**

**Cells and growth conditions**

*Chlamydomonas reinhardtii* strains 11-32b (mt\(^{-}\)) and 11-32a (mt\(^{+}\)), the non-fusing mutant *fus* (mt\(^{-}\)) and the signal-defective mutant *imp3* (mt\(^{+}\) and mt\(^{-}\)) were used in this work. All strains were cultured synchronously in minimal (M) salt medium under a cycle of 12 h light and 12 h dark as described (Matsuda et al. 1990, Matsuda et al. 1995, Abe et al. 2004). For the preparation of gametes, synchronized vegetative cells at the beginning of the light period (L-0 cells; G\(_{1}\) stage) were harvested and transferred into nitrogen-free (−N) M medium at 25\(\degree\)C with moderate shaking for 0, 15, 30, 60 min and 1–7 h before harvesting the samples. Gametic differentiation of the cells and the mating efficiency were monitored by mixing the gametes in equal numbers with tester gametes of the opposite mating type and incubating at 25\(\degree\)C for 30 min. The flagellar agglutinability was scored in a hemocytometer as − (no agglutination), + (<1/3 cells were agglutinated), ++ (1/3–2/3 cells were agglutinated) or +++ (>2/3 cells were agglutinated). The mating efficiency (extent of cell fusion) was determined after counting the proportion of biflagellated and quadriflagellated cells (Harris 1989).

For the protein synthesis inhibition experiments, CHX was added to a final concentration of 10\(\mu\)g ml\(^{-1}\) to both mating type gametes 30 min before mixing to form zygotes. The agglutinability and mating efficiency were monitored, and the subsequent zygotic events, such as fusion of two nuclei, resorption of flagella and formation of a zygote wall, were observed under the microscope. The disappearance of the cpDNA in the early zygote was also observed with 4,6-diamidino-2-phenylindole (DAPI) staining (Kuroiwa et al. 1982).

**Preparations of DNA macroarrays, probes, hybridization and identification of genes for the zygote-forming process**

*Chlamydomonas* cDNA macroarray was constructed from Kazusa EST libraries that represent 25 different culture conditions of the cells, including gametogenesis and zygote formation. The PCR products of 10,368 EST clones were spotted on six Biodyne-A nylon membranes per set, and one membrane can contain 1,728 cDNAs spotted in duplicate. Two 32P-labeled targets were prepared: one from an mRNA pool derived from zygotes (target A; \([Z]\)); the other from gametes (target B; \([G]\)). Both mating type plus and minus cells were cultured synchronously, and L-0 cells of both mating types were transferred to nitrogen-free medium at 1.2 \(\times\) 10\(^{7}\) cells ml\(^{-1}\) at 25\(\degree\)C under continuous light. After both mating types had formed gametes with agglutinability of +++, and a mating efficiency of >90\%, an aliquot from each mating type of the cells was harvested and frozen rapidly in liquid nitrogen. The remaining plus and minus gametes were mixed, and after 30 and 90 min the cells were harvested and frozen. The two gamete, and the two zygote frozen samples were mixed together and total RNA was prepared from the gamete and zygote cell mixtures by the method described in Kubo et al. (2001, 2002). The poly(A\(^{+}\)) RNAs were isolated using a PolyATract mRNA isolation system (Promega, Madison, WI, USA). The 32P-labeled target DNA samples were prepared from 1\(\mu\)g of poly(A\(^{+}\)) RNAs as described previously (Abe et al. 2004, Miura et al. 2004). The 32P-labeled targets were used for hybridizations of six macroarray filters with ExpressHyb Hybridization solution at 68\(\degree\)C for 12–16 h. The membranes were washed with 0.1\(\times\) SSC, 0.5% SDS and with 0.08\(\times\) SSC, 0.5% SDS. The membranes were exposed to an imaging plate (Fujifilm Co., Tokyo, Japan) to detect the radioactive images. Quantification of signals was carried out using a FLA-2000 high-resolution scanner (Fujifilm Co.) and ArrayVision software (Amersham, GE Healthcare Bio-sciences Co., Piscataway, NJ, USA). Two independent experiments were carried out during this work.

A total of 92 ESTs were isolated in a first round of screening as EST clones whose expression ratio of \([Z]/[G]\) exceeded 3.0-fold. To re-screen these candidates for genes transcribed specifically or up-regulated in the mating reaction and early zygote formation process, we used each of these isolated EST clones as probes for RNA blots with total RNA derived from vegetative cells, gametes and zygotes. Finally, 21 novel genes transcribed specifically or up-regulated in zygotes were identified. Since these genes started to be expressed within 1 h after gametes are mixed, we designated them EZY\(_{1}\) (early zygote expressed genes). Seven genes expressed specifically in zygotes were designated as EZY3–9, with their numbering following that of the early zygote genes EZY1 and EZY2 identified previously (Armbrust et al. 1993, Ferris et al. 2002). The other 14 genes, transcribed normally in vegetative and gamete cells but remarkably up-regulated in zygotes, were designated as EZY10–23.
cDNA sequencing and 5’ RACE methods

Seven EST clones designated as EZY genes (EZY3-EZY23) were subcloned into pBluescriptII SK−/KS− (Stratagene, La Jolla, CA, USA) and the sequences of the insert fragments were determined using a BigDye Terminator v.3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with T3 and T7 primers (Nishinbo, Tokyo, Japan) and an ABI PRISM 310 DNA sequencer (Perkin Elmer, Waltham, MA, USA). To obtain full-length cDNA sequence of each EZY gene, 5’ RACE (rapid amplification of cDNA ends) was carried out using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) according to the supplier’s protocol. Amplified fragments were ligated into pT7blue (Novagen, Darmstadt, Germany), and transformed into Escherichia coli JM109 (Sambrook et al. 1989). The inserted fragments were sequenced using the T7 primer.

Northern blot analysis

RNA blot analyses were performed as described previously (Kubo et al. 2001, Abe et al. 2004) using probes generated by PCR (Kubo et al. 2001, Abe et al. 2004) probes were PCR amplified from genomic DNA using primers corresponding to the 3’ end of each EST or the 5’ end of the probe sequence. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and cloned into the pBluescript II SK−/KS− vector using the pBluescript II SK−/KS− vector (Stratagene, La Jolla, CA, USA). The magnetic beads were washed with 70% ethanol and then the plasmids were eluted with distilled water. The purified plasmids were then mixed with T3 and T7 primers and the inserted fragments were ligated into pBluescript II SK−/KS− vector. The ligation mixture was used to transform Escherichia coli JM109 (Sambrook et al. 1989). The selected clones were isolated and the DNA inserts were sequenced using the T7 primer.

Removal of the cell wall from vegetative cells and gametes by gametolysin

Removal of the cell wall from vegetative cells and gametes (density 2 × 10^7 cells ml^−1) was used as a standard or control for the removal of cell walls monitored at 30 and 90 min after suspension. A 100 μl aliquot of cells after 30 and 90 min was heated at 60°C for 2 min in small glass tubes; the survival of cells was observed and counted under the microscope (Tamaki et al. 1981). Then the rates of cell wall removal were calculated, and the samples were harvested and frozen by liquid nitrogen for RNA isolation to perform Northern blot analysis.

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