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Seeking the Ultimate and Proximate Causes of *Volvox* Multicellularity and Cellular Differentiation[1]

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SYNOPSIS. ***Volvox*** **and its relatives provide an exceptional model for integrative studies of the evolution of multicellularity and cellular differentiation. The volvocine algae range in complexity from unicellular *Chlamydomonas* through several colonial genera with a single cell type, to multicellular *Volvox* with its germ–soma division of labor. Within the monophyletic family Volvocaceae, several species of *Volvox* have evolved independently in different lineages, the ultimate cause presumably being the advantage that large size and cellular differentiation provide in competing for limiting resources such as phosphorous. The proximate causes of this type of evolutionary transition are being studied in *V. carteri*. All volvocine algae except *Volvox* exhibit biphasic development: cells grow during a motile, biflagellate phase, then they lose motility and divide repeatedly during the reproductive phase. In *V. carteri* three kinds of genes transform this ancestral biphasic program into a dichotomous one that generates non-motile reproductive cells and biflagellate somatic cells with no reproductive potential: first the *gls* genes act in early embryos to cause asymmetric division and production of large–small sister-cell pairs; then *lag* genes act in the large cells to repress the biflagellate half of the ancestral program, while *reg*A acts in the small cells to repress the reproductive half of the program. Molecular-genetic analysis of these genes is progressing, as will be illustrated with *reg*A, which encodes a transcription factor that acts in somatic cells to repress nuclear genes encoding chloroplast proteins. Repression of chloroplast biogenesis prevents these obligately photoautotrophic cells from growing, and since they cannot grow, they cannot reproduce.**

INTRODUCTION

Multicellularity has evolved repeatedly in the 3.5 billion-year history of life on earth (Kirk, 1998; Bonner, 1998). In most cases, however, this transition occurred so long ago, and/or under such obscure circumstances, that we can do little more than speculate what the last unicellular ancestor of that multicellular group might have been, or what sort of intermediate stages may have existed along the pathway leading from unicellularity to full-blown multicellularity accompanied by cellular division of labor.

The green flagellates known as ''the volvocine algae'' constitute a happy exception to this general rule. This group, which includes *Chlamydomonas* plus 9 genera and 40 species that have classically been grouped in the family Volvocaceae, spans the full range of size and complexity from unicellular *Chlamydomonas* to multicellular *Volvox* with its complete germ–soma division of labor (which is illustrated in Fig. 1). Hundreds of isolates of volvocine algae from around the world are currently in culture and available for detailed study. Molecular phylogenetic analyses based on both nuclear and chloroplast genes (Coleman, 1999; Nozaki *et al.*, 1999, 2000) indicates that the Volvocaceae constitute a coherent monophyletic group whose common unicellular ancestor was closely related to modern *Chlamydomonas reinhardtii* (Coleman and Mai, 1997). Indeed, molecular evidence indicates that *C. reinhardtii* is much more closely related to the volvocaceans than it is to most other species of *Chlamydomonas* (Buchheim and Chapman, 1991; Buchheim *et al.*, 1994, 1996; Larson *et al.*, 1992; Coleman and Mai, 1997; Pröschold *et al.*, 2001). Indeed, it has been estimated that *C. reinhardtii* and *Volvox carteri* shared a common ancestor less than 75 MYA (Rausch *et al.*, 1989), which means that the volvocaceans have been radiating less than 1/10 as long as members of the major multicellular lineages have (Fig. 2). Thus it seems likely that the volvocine algae may retain in their genomes more evidence of the pathway that they followed from unicellularity to multicellularity than members of the major multicellular groups do. For all these reasons, they appear to provide an unrivalled model system for exploring the evolution of multicellularity and cytodifferentiation.

In the spirit of ''integrative biology,'' I will discuss studies of the phylogeny, ecology, ontogeny, developmental genetics and molecular genetics of selected volvocaceans, and will conclude by mentioning two intriguing aspects of *Volvox* molecular evolution that remain be addressed in the future.

VOLVOCACEAN PHYLOGENY

For decades it has been fashionable for textbooks to arrange a few of the volvocine algae in order of increasing size (as in Fig. 3), and then to suggest—unencumbered by empirical evidence—that this was probably how the group evolved: as a simple, linear progression in size and complexity. Molecular-phylogenetic studies reveal, however, that the actual history of the group has been both more complex and more interesting than this simplistic scenario suggests, because although the family Volvocaceae is monophyletic, few if any of the taxa within it are!

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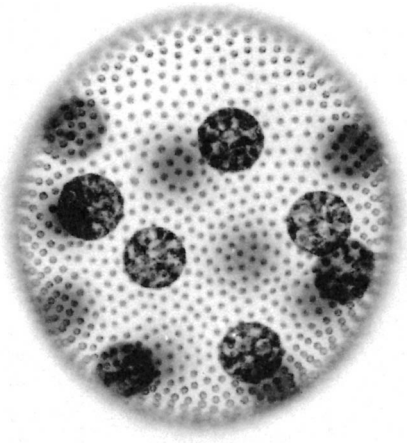

FIG. 1. A *Volvox carteri* spheroid (=individual). Each spheroid consists of >2,000 small, biflagellate somatic cells near the surface and ~16 large gonidia (asexual reproductive cells) beneath the surface of a transparent sphere of extracellular matrix. The gonidia act like stem cells, dividing to produce juvenile spheroids with the same complement of gonidia and somatic cells as are present in an adult. Meanwhile, the somatic cells provide the spheroid with motility, but then they undergo programmed death when they have fulfilled their function.

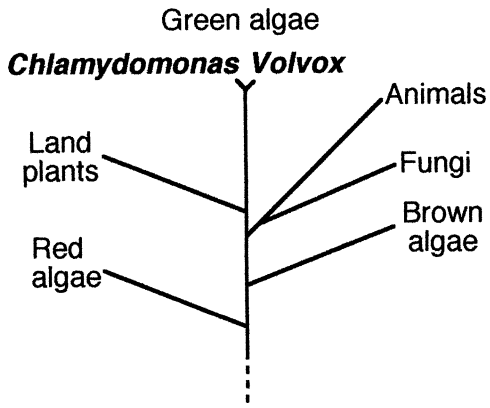

FIG. 2. The phylogenetic branching order of six groups of multicellular eukaryotes. The volvocaceans (including *Volvox*) shared a common unicellular ancestor far more recently than members of any of the other major multicellular groups did.

Several studies have concluded that the genus *Volvox* is polyphyletic (Larson *et al.*, 1992; Nozaki *et al.*, 1995, 1999, 2000; Coleman, 1999). Indeed, a recent study (H. Nozaki, personal communication, 2001) places 11 species of *Volvox* in five separate lineages (Fig. 4). This sort of result was not unanticipated. Although all of the ~18 recognized species of *Volvox* share the diagnostic features of large size and a germ–soma division of labor, developmental and morphological differences among the species have led to repeated suggestions that the genus must be polyphyletic. In a series of papers published 75 to 80 years ago, Shaw argued that only a few of these species should be retained in the genus *Volvox*, while the rest should be distributed to four new genera (reviewed in Kirk, 1998). (It should be noted in passing, however, that only two of the five *Volvox* lineages shown in Fig. 4 correspond to groups that Shaw proposed.)

Molecular-phylogenetic studies also indicate that neither the genus *Eudorina* nor the genus *Pleodorina* is monophyletic (Nozaki *et al.*, 1995, 1997, 1999, 2000; Angeler *et al.*, 1999; Coleman, 1999; also see Fig. 4). Beyond that, it has been shown clearly that the morphological species *Eudorina elegans* consists of several distinct clades (Nozaki *et al.*, 1997; Angeler *et al.*, 1999) that correspond closely to the ''syngens'' (reproductively isolated groups) of *E. elegans* that were defined by Goldstein (1964). Similarly, *Gonium pectorale* and *Pandorina morum* both consist of distinct syngens that can be distinguished at the molecular level (Coleman *et al.*, 1994; Fabry *et al.*, 1999; Schagerl *et al.*, 1999).

In short, it now appears that many volvocacean taxonomic categories identify grades of organizational complexity, rather than clades of organisms related by recent common descent. As a result, the volvocalean family tree now appears to be much bushier than was previously imagined, and it is clear that several branches of this ''family bush'' carry species of *Volvox* that have evolved independently—probably from rather similar-looking (but not necessarily closely related) ancestors. The evidence that multiple species of *Volvox* have evolved independently during the relatively brief period since the volvocaceans began their radiation leads to two interesting speculations: (i) the ecological factors favoring large volvocaceans with a germ-soma division of labor must have been powerful, and (ii) the number of genetic changes required to derive such organisms from smaller, simpler ancestors must have been rather modest. This encourages the belief that it may be possible to decipher both the ultimate and the proximate factors underlying the evolution of *Volvox*, and it stimulates interest in both the ecological conditions under which volvocaceans live, and the genetic features that distinguish *Volvox* from smaller relatives that lack a germ-soma division of labor.

VOLVOCACEAN ECOLOGY

Although a few volvocaceans inhabit fairly deep eutrophic lakes, most of them are found in small, shallow bodies of water in temperate zones around the world. They particularly favor ephemeral puddles, pools and ponds on agricultural lands, where the water tends to be nutrient rich (reviewed in Kirk, 1998). Where one volvocacean species is found, often several are. They typically appear in late spring or early summer, rapidly produce large populations through asexual reproduction, and then—as conditions become less favorable—they engage in sex and form dormant, resistant zygospores that sink to the bottom and await the return of favorable conditions in the following year.

In such environments the volvocaceans always must compete with one another and with other types of algae for mineral elements that are abundant in early summer, but disappear quickly. Although algal growth

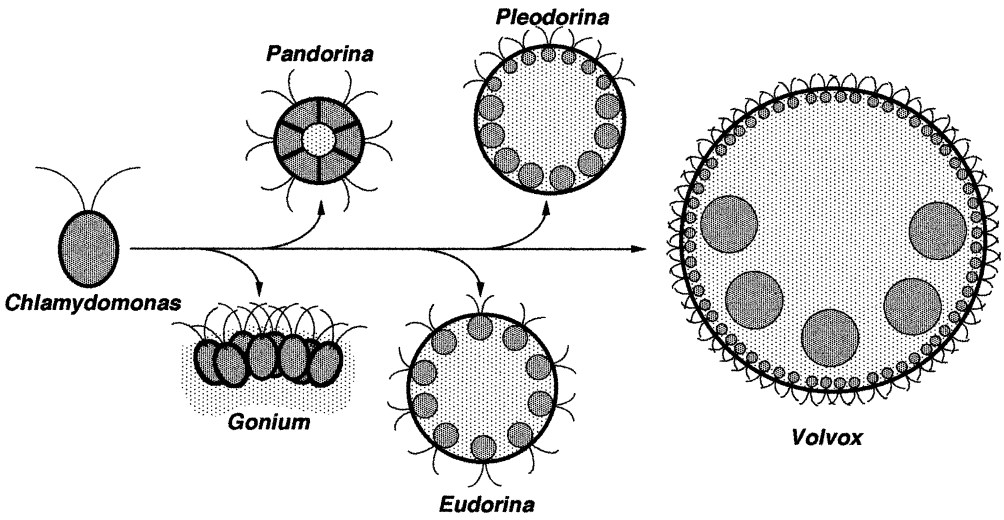

FIG. 3. A diagrammatic representation of a common textbook suggestion: that volvocine evolution involved a simple linear progression in size and complexity. From left to right there is a progressive increase in cell number, organismic size, ratio of extracellular matrix to cell volume, and the tendency to produce sterile somatic cells—with all of these trends culminating in *Volvox*. While this serves as a rough first approximation of the history of the group, it is obviously a gross oversimplification, as explained in the text.

is most commonly limited by the availability of phosphate in such environments, it is often impossible to detect any free phosphates in a pond in which algae are growing (Owens and Esias, 1976). This is because the algae hoard phosphate whenever it is available, and store it for future use in a variety of chemical forms, such as organic phosphates and polyphosphate (Hebeler *et al.*, 1992). It has been shown repeatedly that it is the size of such internal phosphate stores, and not the amount of free phosphate in the water, that limits algal growth (*e.g.*, Rhee, 1973).

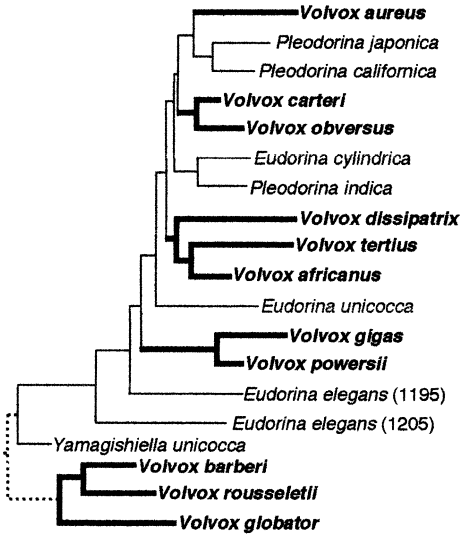

FIG. 4. A neighbor-joining tree based on 1128 bp of the *rbc*L gene (the chloroplast gene encoding the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase). Adapted with permission from a personal communication by H. Nozaki (2001). The dashed line connecting the clade at the bottom to the rest of the tree is to indicate that I have taken the liberty of adding this clade to the tree, based on the topology that has been given for this portion of the tree in three previous reports (Nozaki *et al.*, 1997, 1999, 2000).

Graham Bell (1985) reviewed such data on algal phosphorous metabolism, and concluded that when phosphate is abundantly available volvocaceans take it up and store it far more efficiently than unicells do. Moreover, because this effect is size-dependent, *Volvox* is particularly proficient as a scavenger of phosphorous, taking it up ten times more efficiently than smaller volvocaceans, and a thousand times more efficiently than unicellular algae. Bell hypothesized that these size-dependent differences result from the ability of volvocaceans to store phosphorous not only intracellularly, in the same ways that unicells do, but also as phosphorylated components of the extracellular matrix (ECM), which increases in relative abundance with increasing cell number (see Fig. 3). Accordingly, he mused that ''paradoxically, one of the most important structures of the volvocacean colony may be space in the middle'' (Bell, 1985).

As a test of this hypothesis, Bell and his student Vasso Koufopanou analyzed phytoplankton-abundance data that had been obtained by others in controlled field studies of experimental ponds supplemented with different amounts of fertilizer (Koufopanou and Bell, 1993). Results of these field studies were highly consistent with Bell's hypothesis: As the amount of fertilizer was increased, the total algal biomass increased, of course, but the important point was that the volvocine algae increased in abundance *relative to all other algae combined*! Moreover, the larger the volvocaceans, the greater was their increase in relative abundance as nutrient levels were increased.

Although further tests of Bell's hypothesis would be most welcome, the available data clearly supported his hypothesis that the larger the volvocacean, the more efficiently it competes for limiting nutrients.

But what about the selective advantage, if any, of germ-soma differentiation? In his ''source-sink hy-

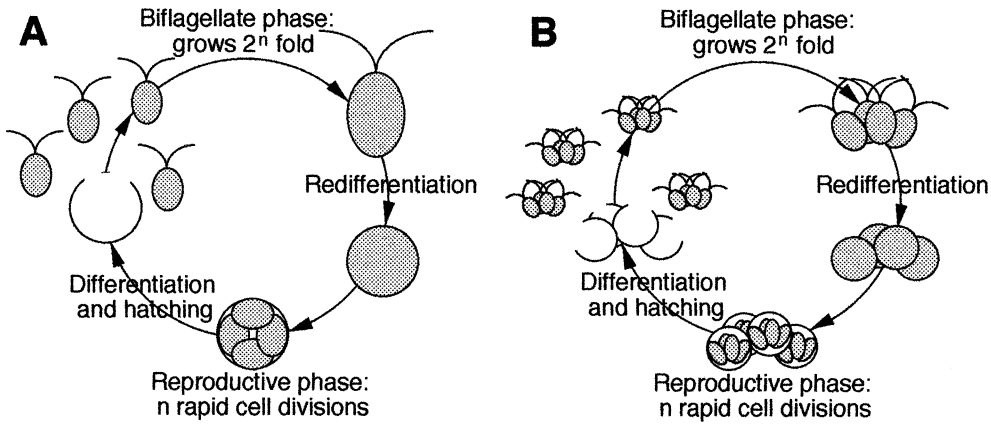

FIG. 5. Diagrammatic representation of the asexual reproductive cycles of (A) *Chlamydomonas*, and (B) *Gonium*. See text for details.

pothesis,'' Bell (1985) postulated that although somatic cells are sterile, they contribute greatly to the organism's fitness by serving as a *source* of nutrients that the gonidia—the *sink*—use to increase their rates of growth and reproduction. Koufopanou and Bell (1993) tested the source-sink hypothesis by comparing the growth rate of *V. carteri* gonidia in intact spheroids to the growth rate of gonidia in sibling spheroids that had merely been broken open mechanically (so that soluble nutrients presumably could not be sequestered in the internal ECM). They found that in nutrient-rich medium the gonidia of intact spheroids grew more than 100-fold faster than the gonidia of broken spheroids. They also established that this increase in fitness was more than adequate to compensate for the amount of cellular material that was devoted to making sterile somatic cells in every generation. To phrase it differently, they concluded that by pumping nutrients into the ECM to feed the gonidia, *V. carteri* somatic cells elevate the reproductive performance of the organism more than enough to provide a powerful selective advantage, despite the fact that they themselves have no reproductive potential whatsoever.

VOLVOCACEAN ONTOGENY

The principal mode of reproduction in all green flagellates is asexual (*i.e.*, mitotic proliferation). The primary function of green-algal sexual cycles is not reproduction, but the formation of dormant, over-wintering zygotes.

The cell-division cycle of asexually reproducing green flagellates is very different from the more familiar cycles seen in most other organisms. As illustrated in Figure 5, instead of merely doubling in size and then undergoing binary fission, volvocine cells typically grow $2^{\mathbf{n}}$-fold (where **n** may have any value from 2 to 15, depending on the species and the conditions), and then they eventually undergo ''multiple fission,'' dividing rapidly **n** times (in the absence of further growth) to produce $2^{\mathbf{n}}$ daughter cells.

In volvocine algae that have a single cell type, this unusual pattern of growth and division results in what may be called a *biphasic* developmental program, in which all cells first go through a motile ''biflagellate phase'' (during which they grow), and then enter a non-motile ''reproductive phase,'' during which they cease growing and execute **n** rounds of cell division in rapid succession (Fig. 5). Once the reproductive phase has been completed, the daughter cells differentiate as biflagellate cells, escape from the mother-cell wall, and swim away.

Asexual reproduction in a small volvocacean, such as *Gonium*, follows a biphasic pattern of development very similar to that seen in unicells like *Chlamydomonas*. The major difference is that whereas *Chlamydomonas* sister cells separate at the end of cell division and behave as independent units (Fig. 5A), in *Gonium* and other small volvocaceans the sister cells remain attached to one another and behave as an integrated multicellular unit (Fig. 5B).

In marked contrast to the foregoing, in *Volvox* the ancestral biphasic pattern of development has been transformed into a dichotomous pattern, in which most of the cells undergo terminal differentiation as motile biflagellate cells, while a few of the cells differentiate as non-motile cells specialized for growth and reproduction (Fig. 1). This dichotomy becomes visible earliest during the development of *Volvox carteri*, in which the two cell types are set apart by a stereotyped set of asymmetric divisions mid-way through embryonic development (reviewed in Kirk [2001] and Kirk and Nishii [2001]). At the end of embryogenesis, the two cell types of a *V. carteri* embryo are about 30-fold different in volume, and it has been shown that it is this difference in size, and not any difference in cytoplasmic quality, that triggers the program of differential gene expression that results in large cells developing as gonidia and small cells developing as somatic cells (Kirk *et al.*, 1993). The genes playing major roles in this program of dichotomous differentiation have been the object of considerable study.

DEVELOPMENTAL GENETICS OF *VOLVOX CARTERI*

Volvox carteri, like all related green flagellates, is haploid in all active phases of the life cycle, which greatly facilitates isolation of mutants. Of the many

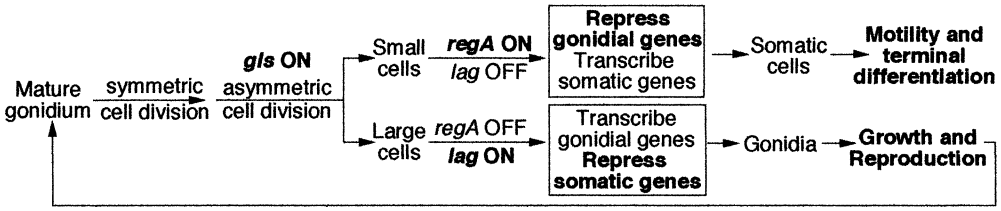

FIG. 6. A working hypothesis regarding the way that three types of genes (*gls, regA* and *lag*) are involved in programming germ–soma differentiation in *Volvox carteri*. See text for details.

types of *V. carteri* mutants that have been described (Kirk, 1998), the three categories of particular interest here are ones in which the germ–soma dichotomy is abrogated in one or both cell types.

In Reg (somatic regenerator) mutants, early development and the differentiation of the two cell types appears to proceed normally, and somatic cells become biflagellate and execute normal motility, phototaxis, and chemotaxis. About a day later, however, Reg somatic cells reveal their mutant nature when they turn darker green, and then begin to grow as they resorb their flagella, their eyespots, and other somatic-cell features, and redifferentiate as gonidia that will divide to produce progeny of similar phenotype. All Reg mutants analyzed have lesions in a gene called *regA*. Mutations of *regA* cause somatic cells to revert to the ancestral ''first biflagellate, then reproductive,'' biphasic pattern of development. Therefore, conventional genetic logic leads to the conclusion that the normal function of the *regA* gene is to suppress the reproductive phase of the ancestral developmental program in the small cells of the somatic lineage.

Lag (late gonidia) mutants have a complementary phenotype: in Lag mutants it is the large cells produced by asymmetric division that develop first as biflagellate somatic cells (albeit larger than normal ones) before redifferentiating as gonidia. We conclude that the normal function of the *lag* genes (of which there are four or five that act in a common pathway) is to suppress the somatic phase of the ancestral developmental program, thereby causing the cells to enter the reproductive phase directly.

Only a single size and type of cells are produced in the third category of mutants to be discussed here. These are the Gls (gonidialess) mutants, in which symmetric cell divisions of the embryo occur quite normally, but there are no asymmetric divisions, and thus no cells are produced that are large enough to activate the gonidial pathway of development. There are at least two (and probably several) genes that can mutate to produce a Gls phenotype. Presumably the products of these *gls* genes interact to shift the division plane from the center of the cell to one side in a predicable subset of dividing embryonic cells. The absence of gonidia would be fatal, of course, on a wild-type background; hence Gls mutants are isolated on a Reg background, in which somatic cells redifferentiate and assume the reproductive functions of gonidia. Gls/Reg double mutants resemble larger versions of colonial volvocaceans such as *Eudorina* (Fig. 3), in the sense that they have only one kind of cells, all of which follow the ancestral ''first biflagellate, then reproductive'' program of development.

The way in which it is believed that the *gls, regA*, and *lag* functions participate in programming dichotomous germ–soma differentiation in *V. carteri* is diagrammed in Figure 6. Molecular analysis of this program is now in progress.

MOLECULAR-GENETIC ANALYSIS OF *regA* ACTION

An inducible transposon that jumps so well that it was named *Jordan* (Miller *et al.*, 1993) has been used to tag and recover the *regA* gene (Kirk *et al.*, 1999), the *glsA* gene (Miller and Kirk, 1999) and other developmentally important genes of *V. carteri* (D. Kirk, S. Miller, I. Nishii, unpublished). Several such genes are being subjected to detailed functional analysis at this time, but owing to space constraints, only the *regA* studies will be reviewed here.

The *regA* gene comprises seven introns and eight exons, and encodes a somatic-cell-specific nuclear protein, RegA, with characteristic features of a transcriptional repressor (Kirk *et al.*, 1999). Cell-type-specific expression of *regA* is controlled entirely by *cis*-regulatory elements that are located within its introns: enhancers in introns 3 and 5 are both required for expression of *regA* in somatic cells, and a ''silencer'' in intron 7 is required to prevent *regA* expression in gonidia (Stark *et al.*, 2001).

If RegA is a transcriptional repressor that suppresses reproductive development, what are its target genes? Several years ago 18 genes with expression patterns that made them candidate targets of RegA regulation were identified by differential cDNA cloning (Tam and Kirk, 1991*a*, *b*). These genes are all expressed at high levels in wild-type gonidia and in the somatic cells of Reg mutants, but not in wild-type somatic cells. Sequencing led to the wholly unexpected finding that all 16 of these genes that encode recognizable proteins fall into a single class: nuclear genes encoding essential chloroplast proteins (Meissner *et al.*, 1999). Their products are involved in virtually every important aspect of chloroplast function, including light harvesting, photolysis of water, electron transport, ATP generation, the dark reactions, chloroplast protein synthesis, and so forth.

How can this unanticipated finding be rationalized? *V. carteri* is an obligate photoautotroph in which

growth of cells is photosynthesis limited. Each somatic cell inherits a tiny piece (<0.04%) of the chloroplast of the gonidium from which it was derived during embryogenesis. But if unable to produce more chloroplast materials, somatic cells obviously could not grow significantly. And if unable to grow, they surely could not reproduce. What better way to lock cells into a terminally differentiated state?

It remains to be seen whether the other genes involved in programming *V. carteri* germ–soma differentiation will have equally intelligible mechanisms of action.

TWO KINDS OF INTRIGUING NEW QUESTIONS

The results summarized in the preceding section raise two particularly interesting questions.

The first question is generic, in the sense that it could be asked with respect to many kinds of evolutionary novelties: What are the sources of genes (such as *regA* and *lag* in the case of *V. carteri*) whose products execute functions for which there are no known precedents in the organism's ancestors? Are they variants of old genes that played quite different roles in the ancestors, and that have been co-opted and modified to play entirely new roles in the descendants? Or have they been cobbled together more recently from unrelated bits and pieces of DNA? Efforts to address this question are underway with respect to *regA*, so conceivably an answer might be forthcoming in the not-too-distant future.

The second question is more specific to *Volvox*, and will be much more difficult to address. Specifically: Have other species of *Volvox* that evolved independently of *V. carteri* used a similar molecular mechanism to establish their germ–soma division of labor, or have different lineages reached similar evolutionary endpoints by different routes? Demonstration that similar molecular mechanisms are used for cytodifferentiation in different *Volvox* lineages would imply that there was a covert preadaptation for germ–soma differentiation in the ancestral volvocacean genome that was uncovered by natural selection more than once. On the other hand, demonstration that different mechanisms of cytodifferentiation are involved in different *Volvox* lineages (which I am guessing is much more likely) would imply that the selective advantage of germ–soma differentiation was so strong, and the developmental changes that it required were so modest, that different routes to the same endpoint could be selected for readily in different lineages. On first glance, it seems unlikely that this important issue will be resolved very soon, because not a single genetic study of any species of *Volvox* other than *V. carteri* has ever been reported as of this date. On the other hand, given the rapid rate at which molecular genetics is changing. . . .who dares predict what may become possible in a few years?

ACKNOWLEDGMENTS

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