

## When Simpler Is Better. Unicellular Green Algae for Discovering New Genes and Functions in Carbohydrate Metabolism

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Of all algae, those in the division chlorophyta (green algae) display the closest relationship to the vascular plants. Chlorophytes harbor a chloroplast that is considered to have originated from a single endosymbiotic event and contain the same types of photosynthetic pigments as land plants. At variance with other divisions of algae, starch is produced within chlorophyte plastids and displays a structure very similar to that described for vascular plants. Considerable variation is found in the organization and composition of chlorophyte cell walls. Some are characterized by the presence of a simple glycoprotein wall, whereas others synthesize elaborate walls with a polysaccharide composition like that of land plants. Algae often contain pulsatile vacuoles that in some cases allow growth in the absence of a normal cell wall structure, a useful feature for studying wall biology. Because of the presence of plastids and plant-like cell walls, unicellular chlorophytes may be considered true plant-like eukaryotic microorganisms. Their microbial nature permits the use of extremely powerful genetic techniques akin to those in yeast (*Saccharomyces cerevisiae*) for the dissection of plant pathways. In this scientific correspondence, we will emphasize the potential of using unicellular chlorophytes to understand plant pathways, including polysaccharide and cell wall metabolism. We hope to encourage plant biologists to consider these species because of the potential for rapid progress in understanding many basic plant pathways.

Among unicellular green algae, *Chlamydomonas reinhardtii* is by far the most studied system. Many recent reviews describing the speed and ease of *C. reinhardtii* genetics and molecular biology have appeared. In fact, within this issue of *Plant Physiology*, several detailed research articles depict the use of *C. reinhardtii* to study chloroplast biogenesis and cell motility. This alga grows rapidly in defined medium

both in liquid and on agar, and its sexual cycle can be as easily controlled as that of yeast. Single colonies grow within 5 d and crosses can be analyzed in less than a month. In addition, the nuclear genome can be efficiently transformed and gene replacement via homologous recombination in the chloroplast genome is a routine method. Unlike land plants, *C. reinhardtii* has the remarkable ability to dispense with photosynthesis and to use acetate as a carbon source; this has allowed the isolation of mutations in both nuclear and plastid genes that affect all possible aspects of chloroplast biogenesis. Similarly, the ease with which cell motility mutants can be isolated has allowed a thorough description of the structure, assembly, and function of flagella. These major achievements have overshadowed many aspects of plant metabolism for which *C. reinhardtii* could be an extremely useful model system such as commercially valuable pathways leading to carbohydrates, carotenoids, lipids, and secondary products, as well as other essential pathways.

### UNRAVELING STARCH METABOLISM

An excellent example of the power of unicellular algae is the use of *C. reinhardtii* to understand starch metabolism, which is resulting in the discovery of new functions even within enzymes that are well characterized. Such knowledge can guide rational efforts to manipulate starch composition for practical purposes (Slattery et al., 2000). Starch is an extremely valuable polymer both nutritionally and as an industrial raw material. It is stored in photosynthetically active leaf chloroplasts as transient starch and in seeds or tubers of economically important crops such as maize (*Zea mays*), rice (*Oryza sativa*), and potatoes (*Solanum tuberosum*) as storage starch. Starch is an insoluble crystalline granule composed of two polysaccharide fractions. Amylopectin is the predominant high- $M_r$  polymer in storage granules and contains an abundance of branched glucans having  $\alpha$ -1,6 linkages. Amylose is a lesser component of the gran-

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ule having relatively few branch points (Ball et al., 1998; Buleon et al., 1998). Storage starch has been studied extensively in higher plants particularly in maize, rice, pea (*Pisum sativum*), and potato, whereas mutants in leaf starch have been identified and studied in *Arabidopsis* (for example, see Casper, 1994; Zeeman et al., 1998; Yu et al., 2001). Such efforts have resulted in a relatively good description of the major biosynthetic enzymes in the pathway. It is unfortunate that this has also led to the impression among many scientists that little remains to be understood. Quite to the contrary, however, many aspects of starch metabolism remain poorly understood, including granule nucleation and assembly, regulation of synthesis and turnover, mechanisms of starch modification, and the contributions of the many enzyme isoforms to starch composition and crystalline structure.

*C. reinhardtii* produces starch granules that are similar to those in other plants morphologically as well as in composition and fine structure (Buleon et al., 1997). An extremely valuable feature of the alga is the ability to induce granule formation easily within several days by simple nutrient limitation as opposed to flowering plants where storage granule development requires seed set. Under nitrogen-limited conditions, algal colonies can be scored directly for starch composition by staining or biochemical methods. As mentioned, a key feature of *C. reinhardtii* is its microbial nature, which should permit large-scale screens that can be automated by the adoption of existing colony picking and screening robots. The ability to rapidly screen tens of thousands of colonies for mutants make algae an excellent complement to research in crop species where such efforts require significantly more time and labor.

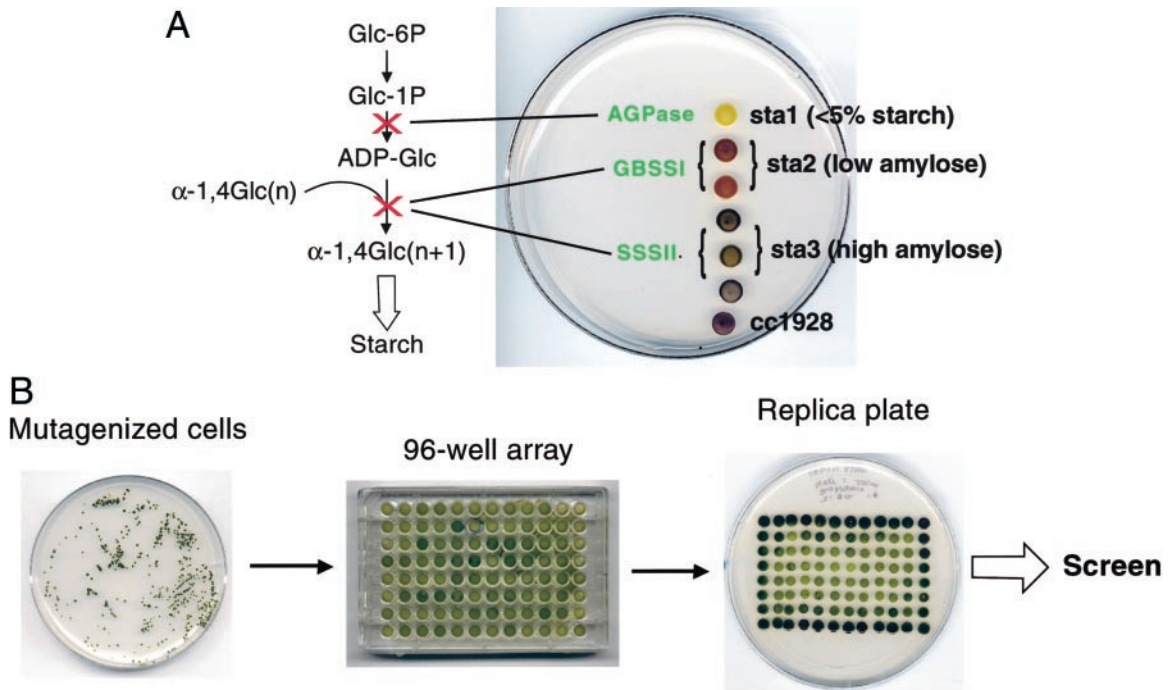
To date, 12 loci have been identified genetically in *C. reinhardtii* that are involved in starch biosynthesis using as mutagens UV, x-ray, and, in some cases, insertional disruption (Buleon et al., 1998). These loci define most of the components of the pathway known in land plants and orthologs can be readily identified by sequence comparisons, indicating the extremely high degree of conservation within the plant kingdom. Mutations at these loci were identified by a sensitive and straightforward iodine vapor screen for altered starch structure (Delrue et al., 1992). The method results in a variety of colors that are indicative of the length of the glucan chains and starch structure, and mutants are easily identified compared with wild-type cells that stain violet (Fig. 1A). In the examples presented, mutations in AG-Pase, granule-bound starch synthase I (GBSSI), and soluble starch synthase result in essentially no starch, low amylose, and high amylose, respectively. As an illustration to the general reader of the utility of *C. reinhardtii* in dissecting starch metabolism, we performed a modest screen of 5,600 methyl methanesulfonic acid mutants. We initiated the screen to estab-

lish methods that could be adapted for high-throughput screening of a large mutant collection in *C. reinhardtii* and to search for novel mutations using a chemical mutagen, which had not been tried previously in the alga to dissect the starch pathway.

Cells were mutagenized and grown in the light, then inoculated into microtiter plates (Fig. 1B). The cells were arrayed onto plates containing standard medium (stock plate) or onto medium without nitrogen to induce starch formation then stained by iodine vapor. Starch prepared from induced cultures of the mutants was examined for structure and relative amylose and amylopectin (Libessart et al., 1995). The spectral properties of the mutants, another indication of altered structure, were also measured. Several mutants displayed an apparent increase in amylose content; whereas other mutants could not be associated with known defects based upon starch analysis and other approaches such as isozyme assays, indicating the possibility of novel mutations. More informative in the short term are mutations within characterized genes, which provide comparative results. Therefore, we focused attention upon mutants defective in the well-characterized enzyme GBSSI, which is essential for the synthesis of amylose. Waxy starch from maize, a commercially valuable starch, is the result of natural mutations in GBSSI leading to reduced function and a high relative content of amylopectin due to a decrease in amylose biosynthesis. Three mutants were defective in GBSSI as indicated by the characteristic loss of amylose, altered spectral properties of the amylopectin fraction (Delrue et al., 1992), and isozyme analysis. It is interesting that one of the mutants retains granule-associated GBSSI protein, and subsequent mapping suggests the presence of at least one mutation within the protein structure that was not associated previously with loss of function. Detailed characterization is in progress, but here is an excellent example where an algal system may lead to the discovery of new regions necessary for function.

## NEW TOOLS

Algae such as *C. reinhardtii* offer many advantages, yet have lacked the coordinated development of resources aimed at sequencing the genome or producing genetic tools that are useful to the scientific community, such as large numbers of single nucleotide polymorphisms and insertion and expression tagged lines as are available in *Arabidopsis*. This is now changing with the development of genome-wide single nucleotide polymorphisms (Vysotskaia et al., 2001). To speed the discovery of novel components and functions in the starch pathway, we have constructed a collection of 50,000 insertion lines in *C. reinhardtii* using a vector that contains a gene essential for Arg biosynthesis as the selectable marker for insertion. The collection is in a genetic background containing the *sta2-1* mutation in the GBSSI gene



**Figure 1.** *C. reinhardtii* can be screened efficiently for mutations in starch. A, Distinct mutants define the starch pathway. Previously characterized mutants (Buleon et al., 1998) illustrate the color-based detection of mutants for altered starch (sta1, no color; sta2, red; sta3, olive; and cc1928 wild type, violet). Left, The starch biosynthetic pathway is depicted with intermediates indicated and addition of  $\alpha$ -linked Glc ( $n$ ) to the lengthening glucan chain ( $n + 1$ ). Mutations resulting in loss of enzyme function are indicated (X). Right, The affected enzymes are shown along with their respective mutant loci in *C. reinhardtii*. From top to bottom the loci are: sta1-1, sta2-1, sta2-2, sta3-1, sta3-2, and sta3-3, respectively. The sta2-1 mutation results in less than 5% (w/w) total starch. B, Mutagenized cells can be arrayed for efficient screening. Mutagenized cells are grown on standard medium for 7 d then inoculated into microtiter plates containing liquid medium and grown for an additional 5 d. Once in microtiter plates, cells are easily arrayed on solid medium to induce starch for screening. All steps indicated can be automated by the use of robots to increase the throughput.

(and Arg auxotrophy). Because the *sta2-1* allele causes the cells to stain red by iodine vapor, it will be possible to detect a broader range of mutations, such as those selectively defective for amylopectin synthesis, that cannot be detected easily in a wild-type background. Because disrupted genes will usually be linked to the selection marker, rapid cloning of affected genes should be possible. The results may aid in the discovery of useful genes that can be engineered into crops directly or provide valuable information about metabolism that can help focus our efforts in crop species. Although the collection was designed with starch metabolism in mind, many unrelated screens are possible for lipids or other valuable metabolites.

#### UNDERSTANDING THE CELL WALL

The plant cell wall is a complex and dynamic mosaic of three coextensive and interactive networks of cellulose/xyloglucan, pectin, and proteins (Showalter, 1993). Although significant inroads have been made recently in understanding the molecular details of cell wall synthesis (for example, see Faik et al., 2000), much of this research has focused on proteins

in the Hyp-rich glycoprotein (HRGP) superfamily. In the last decade, numerous HRGPs have been characterized at both the protein and DNA level. As a consequence, a lot is known about the structure and regulation of these proteins, but the precise function of any particular HRGP remains unclear. An alternative approach is to develop a model system for studying wall assembly using *C. reinhardtii*, whose walls are composed almost exclusively of HRGPs. In the walls that surround vegetative cells and gametes (Goodenough et al., 1986; Snell and Adair, 1990), the HRGPs are arranged in two major layers: an inner layer (W2) whose constituent HRGPs are insoluble and a contiguous outer crystalline layer (W6) of HRGPs that are soluble but salt extractable. The assembly of the vegetative wall can be induced by treating cells with a cell wall lytic enzyme produced by gametes (Claes, 1971; Kinoshita et al., 1992). Within several hours, the protoplasts secrete a new and insoluble wall.

Analysis of soluble enzyme activity combined with studies in which inhibitors that interfere with cross-linking are applied reveal that a peroxidase and a transglutaminase are involved in wall regeneration (Waffenschmidt et al., 1993, 1999). Because *C. rein-*

*hardtii* is haploid, mutations will produce a phenotype directly. Thus, mutagenesis and screening for defects in wall regeneration provide information about essential structural proteins or cross-linking enzymes as well as perception or signaling in response to stresses such as wall rupture. In contrast to vascular plants, most *C. reinhardtii* mutants defective in wall regeneration will remain viable due to the presence of pulsatile vacuoles, which obviates the need for tedious selection of conditional phenotypes. In fact, cell wall-defective colonies of *C. reinhardtii* can be distinguished easily by their mucoid morphology. Although *C. reinhardtii* is useful for understanding HRGPs, its wall lacks several components of vascular plants, including cellulose, xyloglucan, and pectin. However, other chlorophyte cell walls do possess elaborate vascular plant-like organization. The desmidiata are unicellular organisms with highly ornate walls organized in two symmetrical semi-cells. They synthesize cellulose through hexagonal arrays of rosettes and their walls contain pectins and arabinogalactan proteins. Several heterothallic species of desmidiata are available and conjugation has been mastered in the laboratory. Thus, desmidiata are a potentially useful system to study plant cell wall morphogenesis, although much work remains to develop them as genetic and molecular models equivalent in utility to *C. reinhardtii*.

## NEW MODELS

An important and perhaps overlooked aspect of unicellular green algae is the potential for reduced functional gene redundancy, which is displayed by higher plant genomes. Whereas *C. reinhardtii* displays a genome complexity approaching that of *Arabidopsis*, some recently characterized microalgae may have genomes simpler than that even of yeast. *Ostreococcus tauri*, a unicellular chlorophyte belonging to the prasinophyceae, defines the smallest eucaryote known to date (0.8  $\mu\text{m}$  in diameter) (Courties et al., 1994; Chrétiennot-Dinet et al., 1995). It also has one of the smallest genomes (10.2 Mbp; Courties et al., 1998). Yet *O. tauri*, recently identified as a picophytoplanktonic organism, displays all major features of chlorophytes and other plant cells. Picophytoplanktonic organisms were discovered only 2 decades ago, when it was realized that cell counts based on chlorophyll measurements from the surface of the open seas did not agree with the cell counts performed by classical techniques. It was then discovered that the seas contain tiny planktonic cells in abundance. Among these picophytoplanktonic organisms (between 0.3–3  $\mu\text{m}$  in diameter), phycologists found a great diversity of picoeukaryotes. Although axenic cultures of *O. tauri* are not yet available, other picochlorophytes appear to grow well both on defined solid media and in liquid cultures. Small size seems to offer a selective advantage

to oceanic planktonic species. To achieve such a small size, other picochlorophytes will likely have simplified genomes with reduced nDNA content. It is unlikely that *O. tauri* and its picoeukaryotic cousins, with roughly one-tenth the nDNA content of *Arabidopsis*, could maintain a level of "functional" redundancy equivalent to that of *C. reinhardtii* or *Arabidopsis*. This is particularly true given the need to maintain chloroplast, mitochondrion, and other major aspects of the eukaryotic way of life. It is more likely that these organisms will have streamlined pathways and will have reduced functional gene redundancy to core functions. Thus, a green *Escherichia coli* might very well be lurking out in the open seas, an organism that could turn out to greatly simplify functional studies of vascular plant pathways.

## A PROMISING FUTURE

We are just beginning to see the true value of algal species for the elucidation of important pathways in plants. Classical discoveries in chloroplast and flagella function in *C. reinhardtii* were only the beginning. Recent results in understanding carbohydrate metabolism indicate that chlorophytes like *C. reinhardtii* provide the efficiency and speed necessary to permit us to saturate mutationally basic pathways such as starch or cell wall synthesis more fully. This will lead to a new understanding of these and other important pathways. It will be essential ultimately to understand such pathways in vascular plants if we are to reap the practical benefits from our research. However, algae can serve as powerful microbial models for the rapid identification of novel genes and functional domains much as yeast has elucidated many core functions that are essential in animals. In fact, examples of the use of microbial genes for crop improvement are well known. To move forward requires a concerted effort to fully develop algae such as *C. reinhardtii* in terms of genome sequence and tools to further increase the speed of large genetic screens. We are developing tools such as insertion collections, and as we approach the completion of genome sequencing for a number of higher plant species, the opportunity to expand our list to include unicellular plants will present itself. As we have discussed, the identification of the picoeukaryotic algae such as *O. tauri* highlights the potential to develop models that avoid the functional gene redundancy found in vascular plants. Given the indications that their genomes will be quite small, sequencing should be relatively straightforward compared to the efforts applied in *Arabidopsis* and rice. The comparison of simple unicellular plant genomes to those in multicellular plants will in itself be quite illuminating.

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