technologies to evaluate how microbial communities function by, for example, developing gene chips with millions of diagnostic probes.

Determination of microbial-community physiology must be linked with analysis of soil physicochemical states—facilitating analysis of soil emissions or carbon sequestration in soils. Improved methods of soil carbon analyses need to be developed and made available in high-throughput format. Current methods are extremely laborious. Improving analytical methods for analyzing composition of plant cell walls, described below, also may facilitate analysis of soil carbon composition.

Technical Milestones

Within 5 years

- Determine the effects of corn-stover and other crop-residue removal on soil productivity in each major agroecosystem of the United States.
- Use existing study sites where stover has been removed for 5 or more years to initiate comprehensive analysis of microbial communities (and soil carbon and mineral content) in underlying soils, in comparison with land where stover has not been removed.
- Establish long-term study sites for future sustainability studies on prospective energy crops in lands in all major agroecosystems.
- Pursue long-term contracts with private or public providers. The European *Miscanthus* Productivity Network, supported by the European Commission, provides a useful model. Sequence analysis of DNA extracted from soils should be carried out to survey diversity (Handelsman et al. 1998). Sequence analysis should be used to develop diagnostic methods for examining the dynamics of soil microbial composition and abundance.

Within 10 years

- Conduct additional studies with varying harvest rates and management practices, including cropping systems and analyses of microbial communities and of carbon levels in the soils.
- Perform comprehensive ecological system analyses of soils as a living medium.
- Include not only microorganisms present but also how their complex interactions contribute to their net function as a community. Markers of optimally functioning microbial communities should be developed to enable identification of sustainability requirements for soils in each major agroecosystem. In addition to its value to the energy-production function of croplands, this research should enhance the sustainability of food production.

Within 15 years

- Formulate management guidelines on the amount of biomass that can be removed in each major U.S. agroecosystem.

Model Systems for Energy Crops

Application of model systems toward the study of both basic and applied problems in plant biology has become routine and can quickly bring the techniques of 21st Century systems biology to bear on the complex problems associated with domesticating energy crops (see sidebar, Translational Research: The Path from Discovery to Applications, p. 72). Researchers using the model dicot *Arabidopsis thaliana* have made tremendous strides in understanding areas of plant biology ranging from nutrient uptake and metabolism to plant-pathogen interactions. Unfortunately, as an annual dicot, *Arabidopsis* is not an optimal model to study questions unique to potential woody and grassy perennial energy crops (e.g., wood formation in trees or cell-wall composition in grasses). Despite its sequenced genome and genetic resources, rice also is not an ideal model for grassy perennial energy crops because it is a specialized semiaquatic tropical grass. In addition, its large size, long generation time, and demanding growth requirements make experiments expensive. Using *Brachypodium* and *Populus* as model systems would provide researchers working to domesticate energy crops with some of the most powerful tools developed by the highly successful *Arabidopsis* community. These model systems would help identify genes controlling traits relevant to energy-crop productivity and quality, including such global processes as cell-wall biosynthesis, nutrient uptake, carbon flux, and plant architecture. The models can be used for rapid testing of strategies to improve the usefulness of grasses and trees as energy crops. Such tools would allow scientists to use both forward and reverse genetic approaches and modern molecular genetic methods to identify genes controlling traits relevant to the design of superior energy crops. This is important because of the problems (i.e., difficult transformation, large size, long generation time, and self-incompatibility) associated with working directly with energy crops.

Brachypodium distachyon is a small temperate grass with all attributes needed to be a modern model organism, including simple growth requirements, fast generation time, small stature, small genome size, and self-fertility (Draper et al. 2001). *Brachypodium* also is transformed readily by *Agrobacterium* (Vogel et al. 2006) or biolistics (Christiansen et al. 2005), thus facilitating many biotechnological applications. *Brachypodium* is now scheduled to be sequenced by the DOE Joint Genome Institute. The *Populus* genome has been sequenced (Brunner, Busov, and Strauss 2004; Tuskan, DiFazio, and Teichmann 2004), assembled, and annotated through investments from DOE, NSF, and a large international consortium (Tuskan et al., in press). This resource is available to researchers working on a dedicated woody crop for biofuels and biomass energy applications. Despite these advances, many molecular tools and resources currently not available would greatly enhance the discovery and use of genes and gene families related to energy traits and the development of poplar as an energy crop (see sidebar, Enhancing Poplar Traits for Energy Applications, p. 62). As with other model organisms, research on poplar would be facilitated by organism-specific tools such as full-genome DNA

Translational Research: The Path from Discovery to Applications

Model organisms play an important role in both basic and applied research. The most highly developed plant model is *Arabidopsis thaliana*, a small plant in the mustard family (Fig. 4. Growth Rate Modification, p. 65). It was adopted as a model in the early 1980s because of its rapid life cycle, simple genetics, small genome (125 Mb), easy transformability, and traits typical of flowering plants in most respects. Genome sequencing was completed in 2000 by an international consortium, and about 13,000 researchers worldwide currently use the plant for studies of plant biology.

The *Arabidopsis* community has developed a suite of very powerful experimental resources that include several hundred thousand sequenced insertion mutations available through stock centers, a full-genome DNA chip that can be used to measure the expression of most genes, a high-density polymorphism map, and a heavily curated database (arabidopsis.org) that provides access to genomic information. These and other resources have resulted in an explosion of fundamental discoveries about all aspects of plant growth and development. In addition, several companies, founded to exploit knowledge gleaned from *Arabidopsis,* have translated the new information into such crop species as corn, soybean, and canola (see Fig. A. Drought Resistance in Canola Caused by Directed Modification of a Single Gene, below). Plant-improvement goals that were unattainable by applied research on crop plants have been realized by translating basic research on model species to crops.

Much accumulated knowledge about crop species and model organisms will be applicable to improving species suited for use as dedicated biomass crops. However, some traits of interest in that regard have not been a priority in crop research and are poorly understood. For instance, knowledge about cell-wall structure, function, and synthesis is very underdeveloped and will need extensive research. One important species suited for use as a biofuel is poplar, a model woody species that has a relatively small genome. Poplar was sequenced recently at the DOE Joint Genome Institute (see sidebar, Enhancing Poplar Traits for Energy Applications, p. 62).

The DNA sequence of many poplar genes is similar to that of corresponding *Arabidopsis* genes. This comparability will help link mechanistic knowledge about these species to a holistic understanding of common processes. Poplar is relatively easy to transform, which greatly facilitates experimental tests of theories about protein and gene function. Most important, poplar has several important traits, such as a long life cycle and the formation of wood, that cannot be studied in herbaceous plants.

Brachypodium distachyon is another potentially important model for such highly productive grasses as switchgrass and *Miscanthus*. Interest in *Brachypodium* arises because its very small genome has a DNA content about 2.5 times larger than that of *Arabidopsis*. Additionally, its simple growth requirements, small stature, self-fertility, and ready transformability make it well suited to become a model organism. Because *Arabidopsis* is distantly related to and differs from grasses in a number of important respects (e.g., cell-wall composition), *Brachypodium* could become a powerful new model for cell and molecular biological studies of grasses. A high priority in facilitating the development of *Brachypodium* is sequencing its genome.

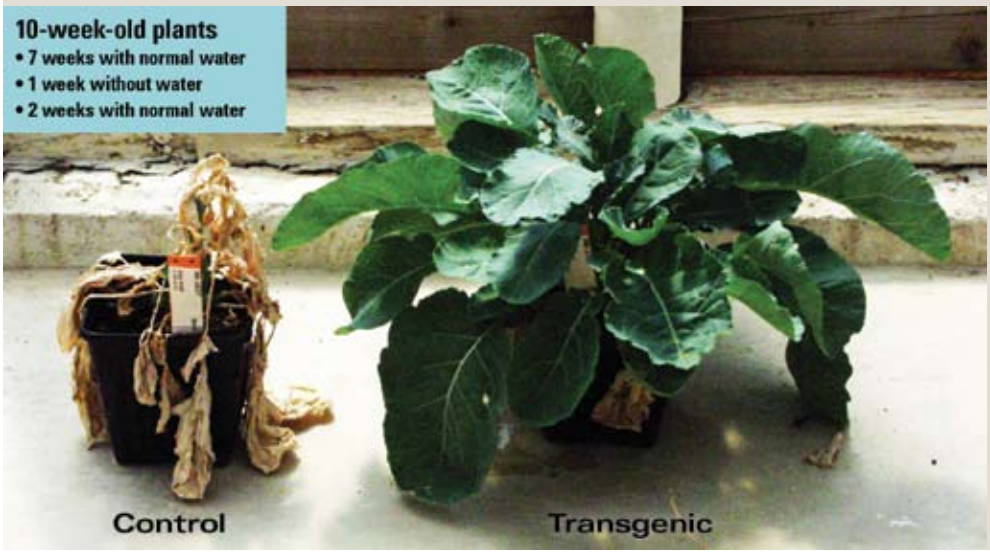

Fig. A. Drought Resistance in Canola Caused by Directed Modification of a Single Gene. [Photo ©2003 Monsanto Company. Used with Permission.]

chips, sequence-indexed mutations, facile transformation methods, and specialized libraries for methods including two-hybrid screens.

Brachypodium and *Populus* must be accelerated into powerful model systems by first sequencing *Brachypodium* and then creating extensive EST databases (i.e., 1 to 2 million per species) from diverse (including full-length) cDNA libraries for both organisms (Sterky et al. 2004). *Agrobacterium*-mediated transformation of both *Brachypodium* and *Populus* has been developed but must be optimized for high-throughput approaches to gene discovery. High-throughput capacity for transforming diverse genotypes would enable forward and reverse genetic approaches by generating collections of sequence-indexed insertional mutants, including but not limited to activation tagging (Groover et al. 2004, Busov et al. 2005), and by allowing targeted silencing or overexpression of large numbers of genes. Transposon-based methods for generating insertional mutants also should be developed. High-throughput resequencing capacities should be used for identifying molecular markers (e.g., SNPs) associated with biomass and bioenergy traits. Similarly, phenotyping (transcript and protein profiling) capacities that identify genotypes associated with relevant cell-wall composition or agronomic traits would be useful.

Using this suite of tools, the function of all *Brachypodium* and *Populus* genes will be evaluated for their contribution to biomass energy-relevant traits. Genes deemed relevant will be used to create advanced genotypes in energy crops. Public organism-specific databases should be created and curated in parallel with advanced experimental capabilities for these organisms.

To set research priorities, the DOE Office of Science's GTL program is interacting with the conversion program managed through EERE's Office of the Biomass Program and other interested parties (e.g., USDA, NSF, and private industry).

Technical Milestones

Within 5 years

- Complete *Brachypodium* genome sequencing, plus EST sequencing and molecular-marker identification for both *Brachypodium* and *Populus*.
- Optimize transformation methods for diverse genotypes of *Brachypodium* and *Populus,* and generate sequence-indexed insertion mutant populations of >100,000 events for both organisms.
- Develop tools for transcriptional and protein profiling and carry out first-stage profiling of key tissues (e.g., cambial development in *Populus*).
- Develop organism-specific databases around annotated genome sequences.

Within 10 years

- Identify genes relevant to biomass production (e.g., those that control cell-wall composition, nutrient uptake, carbon partitioning, flowering, stress tolerance, and disease resistance) using forward and reverse

genetic screens and natural populations based on high-throughput phenotyping [e.g., use Fourier transform infrared spectroscopy (called FTIR) or other techniques that can be automated to identify genotypes with altered cell-wall composition and advanced image analysis to examine plant architecture].

- Develop and test strategies using these genes to improve energy crops in *Brachypodium* and *Populus*.

Within 15 years

- In breeding programs, use genes and strategies shown to affect biomass production and quality in *Brachypodium* and *Populus* to improve energy crops, either by transgenic approaches or by marker-assisted selection of naturally occurring variability in orthologous genes.
- Transfer improved energy crops and genetic information to the conversion program managed through EERE and the Office of the Biomass Program and to other interested parties (e.g., USDA, NSF, and private industry).

The Role of GTL Capabilities for Systems Biology

Capabilities of GTL (within DOE's Office of Biological and Environmental Research) will support or enable the attainment of lignocellulosic characteristics (see Lignocellulosic Biomass Characteristics chapter, p. 39) and feedstock objectives in numerous ways. Potential contributions of each of these, whether distributed or consolidated in a facility, are described below.

Protein Production Capabilities

Most enzymes of interest in cell-wall biosynthesis are thought to be membrane associated and, therefore, difficult to purify and characterize by conventional methods. Based on preliminary experiments with membrane-localized glycosyltransferases, a potentially powerful tool in characterizing the function of all plant glycosyltransferases is to express them in suitable hosts such as insect cells, in which endogenous activities will not interfere with assays. This capability could undertake the high-throughput expression of all glycosyltransferases and other cell wall–active enzymes (e.g., peroxidases and laccases) from higher plants (e.g., *Arabidopsis*, *Populus, Brachypodium,* or rice) or specific biomass species in one or more suitable surrogate hosts. Such expression systems will greatly facilitate function identification of enzymes that catalyze synthesis of cell walls. Heterologously expressed proteins would be tested for their substrate specificity and catalytic activity and also will be used as a resource for generating antibodies and tags.

Antibody resources would have multiple applications. A collection of fluorescent- or epitope-tagged proteins in a plant such as *Arabidopsis* or *Brachypodium* also would be very useful for a variety of reasons. First, the tagged proteins could be used to recover complexes for assay by mass spectrometric and imaging methods. Fluorescent tags could be utilized for cellular localization or collocation [e.g., confocal microscopy or fluorescence

resonance energy transfer (called FRET)]. Additionally, if certain proteins are not active in a surrogate, the tagged version in a plant host might be used to identify function by in vitro assays of affinity-purified proteins. Developing such a resource would require a high-throughput transformation capability that also would be useful for generating insertion mutants in *Brachypodium* and *Populus*.

Molecular Machines Capabilities

Cellulose is synthesized by an intricate multienzyme complex in the plasma membrane, and matrix polysaccharides probably are synthesized by multienzyme complexes in the Golgi. Understanding the function of these complexes and their interactions with metabolic pathways that produce sugar nucleotides in the cytosol will be important for understanding the control of polysaccharide biosynthesis. In addition, cellulose synthase interacts with the cytoskeleton to control the orientation of cellulose fibrils and possibly influence fibril length. Many questions about this and related processes will benefit from the application of new technologies for imaging and manipulating protein complexes. Since most or all such complexes are membrane bound, specialized methods must be developed to work with the complexes. Also, because plant cell walls are intricate mechanical assemblies, tools developed for investigating protein machines may be used to gain new insights into assembly and function of the large polysaccharide complexes that comprise cell walls.

Proteomic Capabilities

These capabilities will facilitate identification of proteins that make up complexes in such model plants as *Arabidopsis*, *Populus*, and other biomass crops. Since many complexes are membrane bound, innovative methods will be needed to purify membrane complexes using an array of capabilities. Also, because of poor correlation between mRNA and protein abundance, proteomic capabilities will enable documentation of the proteome of living cell types found in vascular and other tissues, including ray parenchyma and phloem, as a function of time and conditions. Proteomic analysis of plants with fully sequenced genomes will identify post-translationally modified proteins. Preliminary evidence indicates that some key proteins involved in cell-wall synthesis are regulated by modifications such as phosphorylation. Finally, comprehensive proteomic analysis coupled with imaging analysis is required to identify proteins located in such compartments as the cell wall, nuclear membrane, Golgi, and endoplasmic reticulum.

Cellular System Capabilities

These capabilities are envisioned to help investigators understand how complicated microbial communities in soils respond to various cropping regimes by developing baseline analyses of species composition and abundance in suitable experimental plots. Also needed are cost-effective tools (e.g., diagnostic DNA chips) for monitoring these and other plots over

long time periods and for integrating resulting data into a view of how biomass cropping alters soil ecology.

In addition to analyzing soil microbial communities, acquisition and analysis of transcriptomic and proteomic data from model and crop plants will be a powerful tool for assigning probable function to genes implicated in cell-wall synthesis. As a function for each enzyme emerges, the cellular systems capability through imaging and dynamical analyses will be used to develop a systems model to incorporate the wall's biophysical aspects with structural properties and knowledge of the functions of proteins involved in wall synthesis. This model will facilitate the rational development of feedstock species based on "design principles," in which the wall's chemical composition and structure are optimized while, at the same time, not compromising maximal plant productivity. The cellular systems' analytical and modeling resource and proteomic capabilities will carry out a systematic approach for identifying plant biomarkers to guide protein production and the generation of specific molecular tags utilizing protein production capabilities.

The long-term vision is to make available in situ images of living plant cell walls that show all key molecular processes occurring in real time. Studies will cover the full life cycle of cell-wall formation, maturation, transformation, dehydration, and processing into simple feedstocks for conversion into ethanol or production of other fuels and chemicals. The understanding obtained through research that uses such imaging is expected to result in quantitative, predictive modeling as a guide to developing advanced feedstocks and processing them into fuels. These tools will take advantage of various chemically specific imaging tags created with protein production resources.

DOE Joint Genome Institute

DOE JGI will sequence, assemble, and annotate the gene space or entire genomes of model and emerging bioenergy crops and will resequence additional cultivars or ecotypes for marker discovery. Identification of genes that control cell-wall polysaccharide synthesis or modifications in biomass species and the development of tools such as gene chips will depend on the availability of nucleotide sequences. Micro-RNAs are expected to play a role in expression control of many relevant genes, so DNA sequencing must be deep enough to identify them in these species. JGI also will sequence community genomes, including those of the rhizosphere associated with key proposed energy crops and trees.

Other Needed Capabilities

GTL capabilities as currently envisioned do not encompass all resources needed for development of energy crops. To accomplish objectives associated with the energy mission, the current vision should be expanded to include the three capabilities described below.

Transformation Services

Substantial capabilities will be needed to carry out transformation of model and applied species important to feedstock development on behalf of the R&D community and to warehouse genetic resources for these species.

Chemical Phenotyping Services

Feedstock improvement for increased yield and processing will require manipulation and assessment of multiple genes in candidate organisms or model systems. New tools are needed to detect, quantify, and compare changes in cell-wall composition and 3D architecture in primary and secondary walls during assembly and before and during processing. The tools would facilitate feedstock development for improved performance in biomass conversion. Detailed analysis of cell-wall composition by 2D nuclear magnetic resonance (NMR) imaging or other methods is beyond the scope of routine processing in most research laboratories. Capabilities for such analyses would broadly facilitate research.

Synthetic Carbohydrate Chemistry

Analyses of cell walls in their native state and as a function of treatment and processing require advanced synthetic carbohydrate chemistry capabilities to create standards and models. NMR and mass spectroscopy facilities will be necessary to support the synthetic chemistry work.

Other Biofuel Opportunities: Development of High-Productivity Biodiesel Crops

To maximize solar energy use and storage, an ideal biomass crop would carry out photosynthesis at the theoretical maximum every day of the year and would store fixed carbon in a directly useful and easily harvestable form, even without mineral nutrients. In carrying out this process, many plants accumulate large amounts of oils or waxes in such specialized storage tissues as seeds or mesocarp tissues. In some cases, oil accounts for more than half the dry weight of these tissues. Most oilseed species, however, are not as productive as others such as maize that store primarily starch rather than oil. In theory, obtaining higher yields of oil should be possible by genetically modifying already highly productive species (e.g., sugar beet, potato, and maize) so they will accumulate oils or waxes instead of carbohydrates in their storage and vegetative tissues.

Other plants produced by genetic modifications of developmental processes accumulate oil in their roots (Ogas et al. 1997), suggesting the feasibility of this strategy. Acetyl-CoA carboxylase is the first committed step in fatty-acid biosynthesis in potato tubers, which accumulate starch almost exclusively. Overexpression of this enzyme resulted in a fivefold increase in triacylglycerol accumulation and provided an example of oil deposition in a carbohydrate-storage tissue (Klaus et al. 2004). However,

Decoding the DNA of Soybean—A Source of Biodiesel

DOE and the U.S. Department of Agriculture will support genome sequencing of the soybean as the first project in an agreement to share resources and coordinate studies of plant and microbial genomics. The soybean, *Glycine max*, is the world's most valuable legume crop and the principal U.S. source of biodiesel, a renewable alternative fuel. Diesel engines inherently are more thermodynamically efficient than combustion engines. Biodiesel has the highest energy content of any alternative fuel and is significantly more environmentally friendly than comparable petroleum-based fuels, since it degrades rapidly in the environment. It also burns more cleanly than conventional fuels, releasing only half the pollutants and reducing the production of carcinogenic compounds by more than 80%. Sequencing will take place at the DOE Joint Genome Institute, supported by DOE's Office of Science. The soybean genome is about 1.1 billion base pairs in size, less than half the size of maize and human genomes.

understanding how to reprogram plant cells to store oil rather than carbohydrate is very challenging. It requires not only large-scale changes in the complement of expressed metabolic enzymes but also changes in cellular structure typically associated with cell identity.

Because recovery of oil from plants is technically simple and efficient, processing oil-accumulating plant material on the farm may be possible so mineral nutrients and soil adjuvants can be retained at the farm for return to the soil (see sidebar, Decoding the DNA of Soybean—A Source of Biodiesel, p. 77). In principle, oil could be recovered during harvest by inexpensive screw-press technology, greatly reducing transport and processing costs and enhancing sustainability.

The lipids that comprise most plant oils are highly reduced forms of carbon and therefore represent the most energy-dense plant-storage compounds. Derivatives such as biodiesel can be produced easily by inexpensive and efficient conversion of plant-derived oil to fatty acid methyl esters. Production of high-yielding, oil-accumulating plants could result in a new biofuel source that would reduce loss of carbon associated with fermenting sugars to ethanol as well as costs associated with converting lignocellulosic feedstocks to liquid fuels. The plant that most nearly meets this description is oil palm (see sidebar, Oil Palm, this page).

Oil Palm: An Important Biofuel Plant

Oil palm, *Elaeis guineensis* Jacq., is a tropical tree species that produces bunches of oil-rich fruit resembling avocados. The plants grow in lowlands of the humid tropics (15°N to 15°S), where rainfall of 1800 to 5000 mm is evenly distributed throughout the year. Oil palms mature slowly but, once established, yield as much as 10.6 tonnes of oil per hectare (ha) per year, although the average is less than half that amount. They begin to bear fruit after about 3 years and remain in use for some 25 years, so annual maintenance costs are low.

Palm oil from the mesocarp, which contains 45 to 55% oil, is similar in composition to soy oil. Oil from palm kernels, extracted from the endosperm, contains about 50% oil rich in medium-chain fatty acids and well suited for biodiesel applications. Malaysia is the major source of these oils, producing 13.4 billion pounds of mesocarp oil and 3.5 billion pounds of kernel oil from about 3.8 million ha. Plants are harvested by hand, but typical planting density is only 150 plants/ha, and labor costs are not a major factor in production. Because of the relatively straightforward conversion of palm oils to diesel and food uses, palm acreage in the tropics is expected to expand significantly. [Picture source: C. Somerville, Stanford University]

Some questions to be addressed by basic research:

- How is cell identity controlled? More specifically, what high-level, regulatory genetic controls program a cell to express genes involved in lipid synthesis and accumulation?
- What regulates carbon flux from photosynthetic-source leaves to storage organs?
- What regulates metabolic partitioning of carbon among proteins, starch, and oil production?
- What species are most promising for conversion to oil accumulation? For instance, is oil potato a better candidate than oil beet or very high oil maize, or should plant stems be engineered to deposit extremely thick wax-rich cuticles?
- What are the opportunities for developing new high-yielding oilseed species tailored to energy production by uncoupling oil accumulation from seed carbohydrate or protein accumulation?
- What is the impact of high lipid levels on plant cells? Most cellular mechanisms operate in aqueous environments.

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