BOX 2-1
Nature's Nylons

Common orb-weaving spiders spin as many as seven different types of silk fiber, each critical to the spider's survival. The fibers are made of proteins that are high-molecular-weight linear polymers exhibiting a broad range of mechanical and physical properties. Some silks are strong, tough framework filaments that support the web. Others are elastic filaments that absorb the kinetic energy of insects striking the web. Accessory filaments are produced that wrap captured prey or provide cocoon material. Silk fiber production imposes high energy and material demands on arachnids. Consequently, spiders have evolved to produce protein fibers that are highly efficient structures and one of the best high-performance natural materials.

Spiders use dragline filaments to control their movement in the wind and to form the primary load-bearing framework of the orb web. The combined strength and toughness of dragline filaments are unmatched by any existing man-made fiber: they have a tensile strength two to three times greater than steel and an elongation-to-break ratio approaching 30 percent. Dragline filaments offer an attractive benchmark for next-generation materials because of their exemplary physical and mechanical properties and also because they are processed at ambient temperatures from aqueous media.

The fibers in dragline silk are made up of glycine- and alanine-rich linear proteins containing oligopeptide units that function like the hard and soft segment units of conventional man-made polymers. The unique properties and processibility of dragline silk proteins result from the length and distribution of the protein segments as well as the amino acid sequence of each segment. Recombinant DNA technology has now made possible the development of bioengineered analogs that theoretically will perform as well or better than natural dragline filaments.

Several academic, government, and industrial laboratories in the United States are currently conducting research along these lines. The initial focus has been expression of protein polymers in microbial hosts, resulting in the successful production of several structural proteins in experimental quantities. Researchers also have prepared several fibers and films from de novo designs produced via fermentation. Early experience suggests that the economics of protein polymer production will need to improve before high-volume production and commercialization of these polymers become possible. Additional challenges will be determining how the constituent protein structures and biological spinning apparatus influence the transition from aqueous solution to insoluble fiber and finding ways to mimic the effects of spider anatomy and physiology at an industrial scale.

SOURCE: Tirrell (1996).

the structural properties of zein by genetic engineering to produce novel characteristics.

Plant Oils

Many crops can serve as sources of plant oils; currently soybeans account for 75 percent of the vegetable oil produced in the United States.

Soybean crops are a major target of plant oil research. Approximately 305 million pounds of soybean oil were used in nonfood applications such as livestock feed and the manufacture of resins, plastics, paints, inks, and soaps in 1996 (ERS, 1997b). Fatty acids derived from soybean oil are being converted into surfactants, emulsifiers, and alkyd resins for paints. Soybean oil can be chemically transesterified to produce biodiesel (methyl esters). In the future biodegradable lubricants may be produced from soybean oil; genetically modified soybean varieties hold the promise of yielding lubricant products that outperform petroleum-based lubricants (ERS, 1997b). Additional oilseed crops, some yielding oils with unusual properties, could be grown in the United States (e.g., petroselenic acid from coriander oil) (see Box 2-2). Over the near term the volume of oil produced for such uses will remain small relative to petrochemical sources.

Fermentable Sugars

Fermentable sugars are by far the largest feedstock that might support a biobased chemicals industry in the United States. A wide range of fermentable sugars can be found in crops and wastes from agriculture and silviculture. Major feedstocks include corn, wheat, sorghum, potato, sugarbeet, and sugarcane; other sources include potato-processing residues, sugarbeet and cane molasses, and apple pomace (Polman, 1994). Sugars can be produced directly or derived from polysaccharides (such as cellulose and starch) and then, via microbial fermentation, used to produce a wide range of commodity and specialty chemicals. Existing commercial fermentations primarily utilize glucose (6-carbon sugar) to produce ethanol, acetic acid, amino acids, antibiotics, and other chemicals. Over the long term new sources of glucose will be required to meet the demands of a biobased industry. Growth of a biobased chemicals industry will depend on production of cellulose-rich crops, including those currently under production (e.g., corn and alfalfa) and others that presently are not grown commercially (e.g., switchgrass and hybrid poplar).

Significant increases in glucose reserves are available from lignocellulosic substances found in most plants, crop residues, and waste paper. Cellulose can be hydrolyzed by acid to glucose, although much of the glucose is destroyed during this process. The second most abundant sugar, found in hardwood and agricultural residues, is xylose derived from xylan hemicelluloses. Xylose is relatively easily recovered by acid or enzymatic hydrolysis but can be fermented to ethanol only by a few naturally occurring organisms or recombinant microbes. The practical sugar yield from lignocellulosics would increase significantly if commercial fermentations could utilize xylose (a 5-carbon sugar or pentose) as well as glucose (a 6-carbon sugar or hexose). Novel genetically engi-

BOX 2-2
Evaluating Alternative Crop Sources of Petroselenic Acid

Coriander is grown primarily for its use as a spice, but it may have potential industrial uses. The plant's seeds are 17 to 20 percent triglyceride, 80 to 90 percent of which is (esterfied) petroselenic acid (an 18-carbon fatty acid with a single unsaturated bond at C-6). Oxidative cleavage of the petroselenic acid's double bond yields adipic acid, a 6-carbon dicarboxylic acid used in the synthetic polymers or nylon industry, and lauric acid, a 12-carbon fatty acid used in the soaps and detergent industry. Also, the derivation (hydrolysis) of the petroselenic acid ester from the seed triacylglyceride yields glycerol, another chemical with industrial utility. All of the carbon atoms in the seed oil can thus be used with no intrinsic processing losses.

Coriander seed yields vary greatly (100 to 2,000 kilograms per hectare in Europe and India). Experimental yields in Europe have been as high as 2,500 kilograms per hectare. Production of coriander for industrial purposes would become an attractive area for research and commercialization if high yields can be maintained over large areas and if the processing chemistries for industrial feedstocks are made practicable on a greater scale.

The desirability of developing coriander as a crop source of petroselenic acid needs to be weighed against other potential biological sources, however. Genetic engineering may one day produce a comparable source of this fatty acid in an agronomically adapted crop. Petroselenic acid is produced as an offshoot from the usual pathways of fatty acid triacylglycerol as petroselenic acid biosynthesis. The process involves desaturation of a 16-carbon fatty acid (palmitic acid bound to a protein) at C-4 by a specific desaturase, elongation by the usual fatty acid elongation reactions (although there may be additional requirements for a specific elongase activity), and cleavage from the protein by a thioesterase before incorporation into triacylglycerol as petroselenic acid.

The key desaturase gene has been cloned and introduced into tobacco plants. Up to 5 percent of the total fatty acids in the modified tobacco's cells was petroselenic acid. Introducing the thioesterase and elongase genes along with the desaturation gene might yield greater quantities of petroselenic acid. Transgenic technologies might eventually produce seed oils with petroselenic acid levels rivaling that of coriander (up to 90 percent). If accomplished in a high-yielding oilseed crop, such as sunflower or rapeseed, the result would be a very high-yielding source for petroselenic acid that is stable, agronomically suited to the United States, and supported by a large agricultural and genetic infrastructure.

SOURCE: Based on Dormann et al. (1994), Cahoon and Ohlrogge (1994a,b), Kleiman and Spencer (1982), Meier zu Beerentrup and Roebbelen (1987), and Ohlrogge (1994).

neered microorganisms will eventually play a key role in the direct conversion of cellulose oligomers and 5- and 6-carbon sugars to ethanol.

To avoid destruction of sugars from lignocellulosic materials by acid treatment, enzymatic hydrolysis using mixtures of enzymes (cellulases and hemicellulases) is used. These enzymes, when combined with effec-

tive pretreatments of lignocellulosics, provide high yields of glucose, xylose, and other fermentable sugars with minimal sugar losses. However, these enzymes are currently too costly to use in large-scale conversion of lignocellulosic materials to fermentation substrates.

Improving Plant Raw Materials

The discoveries occurring in plant and microbial genomics will advance the fundamental biological research needed to support a biobased industry. Scientific investigations are under way to decipher the genetic code of a flowering plant, *Arabidopsis thaliana*; the genetic map is complete for the microbial organisms *Saccharomyces cerevisiae* (common yeast), *Bacillus subtilis* (gram-positive bacteria), *Escherichia coli* (gram-negative bacteria), and *Caenorhabditis elegans* (nematode). In addition, it is expected that complete genomic sequences of Drosophila, humans, and several other eukaryotic species will become available in the foreseeable future. The genetic information collected on these organisms will provide researchers with insights on the genes that control plant traits and cellular processes (NRC, 1997). For example, understanding of the functions of the *Arabidopsis* genes will permit identification of a desirable gene, which on transfer to a different plant will have the gene's functions expressed there (e.g., the manufacture of a particular chemical).

Genetic engineering is perhaps the most significant development in plant biology in the past two decades. It has profound implications for understanding the fundamental processes of plant growth, development, and metabolism and for generating new agricultural and forest products. It is now possible to modify an organism genetically so that the modified plant or microbe produces greater quantities of a particular polymer. It is also possible to transfer an entire biological process into new organisms.

Biomass production can be improved by development of new cultivars and crops with enhanced agronomic traits. Researchers will need to use both traditional plant breeding and genetic engineering techniques to improve yield and pest resistance of traditional crops and new crops. Techniques like genetic fingerprinting and markers can be used to facilitate classical plant breeding. The identification of crop strains and the durability of genes that confer resistance to pests and environmental stress will contribute to enhanced productivity, as it already does with food and feed crops.

Significant screening efforts are needed to identify carbohydrates, lipids, and proteins with industrial potential. Development of commercial plant sources for these compounds through breeding and genetic engineering will require elucidation of the genes and enzymes responsible for the production of compounds in the source organism (plant,

BOX 2-3
Genetic Engineering Methods

Traditional breeding is restricted to mobilization of genes within related plant species. In contrast, genetic engineering, through the process called transformation, allows scientists to transfer genes between not only unrelated species but also the kingdoms of living organisms. Transformation involves the introduction of DNA into plant cells and tissues. It changes the hereditary material in each cell of the altered plant, as well as the plant's biochemical reactions. Newly introduced traits might affect plant growth, development, nutrition requirements, nutrient content, or composition of harvested plant parts.

Plant transformation is one of the fundamental tools by which genetic engineers modify plants. However, the techniques have only been developed over the past two decades. In the late 1970s, scientists discovered that the common bacterium *Agrobacterium tumefaciens* causes plant tumors when oncogenes are transferred from the bacterial TI plasmid into plant chromosomes. Scientists at Monsanto Company and Washington State University, St. Louis, developed methods to delete the oncogenes and replace them with different genes of interest, thereby using the Ti plasmid as a vehicle to transfer desired genes into plant chromosomes. To ensure that all plant cells in an experimental mixture were transformed, they added a selectable marker gene (e.g., kanamycin resistance) to the transferred DNA (T-DNA). Exposure to selective growth conditions (e.g., a medium containing kanamycin) would then kill all of the nontransformed cells. Many plant cells are totipotent—an individual cell can grow into a whole plant. Thus, researchers could grow whole plants from individual transformed plant cells and select the plants that passed T-DNA to their progeny in a Mendelian-dominant manner. Various dicotyledon plants have been transformed using the *Agrobacterium* technology, including tomato, hybrid poplar, potato, soybean, cotton, rape, and sunflower.

Agrobacterium-mediated transformation initially did not work for most monocotyledon plants, including the majority of grain crops (the exceptions are rice and banana). Various academic and industrial laboratories developed new technologies for monocotyledon transformation based on particle acceleration. "Biolistic" guns shoot DNA into plant cells; the cells incorporate the DNA into their chromosomes and recover. Electroporation involves putting cells into an electric field.

animal, or microbe). As our understanding of plant metabolism continues to improve, scientists will be able to manage more sophisticated manipulations of these systems to produce the desired biochemicals in the desired quantities. Separations of plant components for industrial uses can also be improved by genetic engineering.

Biochemical pathways and genes can be mobilized within plants to create new products based on molecules that originate from nonplant sources such as microorganisms. Further, biomolecules often can be modified to facilitate purification. Such capabilities have no parallel in

When a current is passed through a plant cell, DNA (a charged molecule) can enter and be incorporated into the chromosome. "Biolistic" gun and electroporation methods have now been used to transform various monocot plants, such as corn, wheat, barley, rice, banana, and oats. More recently, even *Agrobacterium*-mediated transformation has been successful in monocot transformation. Most genetic engineering work has focused on introducing genes to the plant cell nucleus because chromosomes found there are passed on to progeny. However, technologies for transforming the DNA of plant cell organelles, such as chloroplasts and mitochondria, have recently been developed. The technologies are most advanced for tobacco, where an excellent chloroplast transformation system now exists. Chloroplast transformation has several advantages: because chloroplasts are maternally inherited, the T-DNA cannot be transferred to wild relatives via pollen; gene expression is high; and introduction of genes is site specific. The technology also makes possible the efficient expression of bacterial genes and operons in plants.

Although transformation introduces genes into plant cells, gene expression determines whether and how the introduced genes alter the plant's traits. Genes contain three distinct elements. First, the *promoter region* is the molecular switch. It determines the timing, the tissue in which the gene is expressed, and the quantity of a gene's product. Second, the *coding region* produces messenger RNA that usually provides a template for protein production. It determines the nature of the trait, such as increased starch or resistance to pests. Finally, the *termination region* or *polyadenylation signal* terminates RNA production and increases RNA stability by adding polyadenylate residues. Failure of any of the three regions can impair gene expression. Moreover, it can be difficult to obtain expression of certain coding sequences, even with appropriate promoter and termination regions. The genes for Bt (*Bacillus thuringiensis*) toxins that confer insect resistance are an example: it was necessary to design synthetic genes to circumvent the problems with poor Bt gene expression in order to develop useful insect-resistant crops. Similarly, transcription of plant DNA and the processing and stability of mRNA in various plant species are far from being well understood. Research in this area will be critical for harnessing the full power of biotechnology.

Source: Horsch et al. (1984; 1985); Jenes et al. (1993); McBride et al. (1993); and Perlak et al. (1990).

coal and petroleum-based feedstock systems and are a major potential advantage for biobased products.

Well-developed technologies do exist for transferring genes into plants using bacterial plasmids or particle acceleration (see Box 2-3). Many plant species have thus far been transformed. Nevertheless, more efficient methods for transformation and regeneration would improve development of commercial products. Most current transformation methods rely on the time- and resource-intensive process of producing large numbers of transgenics that are subsequently screened for gene activity and "true-to-type" plants. The pace of discovery and product introduction could be

accelerated if transgenes could be inserted at specific sites in the plant genome that consistently yield the desired gene activity level and that have little or no effect on the plant's agronomic fitness.

Transformation of "elite" germplasm is proving to be another methodological difficulty in genetic engineering. The present approach is to transform varieties most amenable to the process and then transfer the transgenes to elite germplasm by plant breeding. This is an inefficient process because evaluation and selection phases of research and development are prolonged, creating delays in the commercialization phase. Direct transformation and site-specific gene insertion might be especially useful for perennial plants because cycle times for evaluation and breeding of these plants are long, laborious, and impractical.

The current battery of promoters for gene expression (see Box 2-3) will be insufficient to meet the sophisticated expression profiles of the future. For example, redirection of carbon from starches and oils to other biopolymer (e.g., polyhydroxy alkanoate) production may be desired in specific cells of seed tissue during specific stages of seed formation. Further, subsequent degradation of the biopolymer during germination may be essential to permit carbon use for seedling growth. Such "fine tuning" of plant metabolism will require an extensive set of promoters from which appropriate selections can be made.

Enhanced Productivity

Biobased research should support improvement of plant productivity. First, any advances in research to improve crops will be relevant to biobased industrial crops. Second, the modern techniques used here for dealing with insect pests, pathogens, weeds, and stress are mostly transferable to plant design. High-level plant productivity on a consistent basis will be essential for supplying biomaterials for industrial production. Genetic engineering can enhance plant productivity by the introduction of traits that reduce farm inputs (e.g., pesticides, fertilizers, water), increase farm productivity, or modify biochemical content. Plant biotechnology products such as NewLeaf potato and Roundup Ready soybean are the precursors of a new generation of products to be commercialized over the next decade. Most of these products have been designed to improve agricultural crop productivity for food and feed uses. It is anticipated that the next generation of crops will be introduced to a broader range of markets, including biobased industries.

Environmental impacts from the release of genetically modified organisms continues to be an area of concern. An earlier report of the National Research Council concluded that crops modified by molecular and cellular methods should pose no risks different from those modified

by classical genetic methods for similar traits (NRC, 1989). A finding of relevance is that established confinement options are as applicable to field introductions of plants modified by molecular and cellular methods as to introductions of plants modified by classical genetic methods.

Resistance to Insect Pests

Biotechnology companies are commercializing transgenic seed with resistance to insects in some major agricultural crops. Current approaches to insect resistance are based on expression of genes from the bacterium *Bacillus thuringiensis* (Bt) in plants. Bt genes produce toxins that can control caterpillars and certain families of beetles and mosquitoes. Bt genes are target specific and not efficacious against other major pests that cause significant yield losses. Additional genes will therefore be required to successfully manage the range of insects that are crop pests or that transmit plant viruses (e.g., whitefly, aphids). Moreover, pests continuously evolve, and the current generation of genes cannot provide the spectrum and durability of resistance required over the long term (NRC, 1996a).

Resistance to Plant Pathogens

Plant breeding will continue to be a predominant tool of defense against many plant diseases. While classical breeding techniques are fundamental to disease management, genetic engineering is becoming increasingly important. The first example of genetic engineering for disease resistance involved a gene encoding the coat protein of the tobacco mosaic virus (TMV) that was introduced into tobacco plants through an Agrobacterium vector (Abel et al., 1986). These plants were resistant to TMV as well as other closely related viruses. Expression of coat protein genes has become increasingly important in developing new varieties of resistant crops. Research is under way to transfer viral-coat proteins into other horticultural and field crops (Fitchen and Beachy, 1993). In 1995 a transgenic squash seed variety conferring this resistance characteristic was commercialized, and virus-resistant papaya, melon, tomato, potato, and other crops are in advanced stages of development. It is anticipated that transgenic techniques will enlarge the pool of disease-resistant genes that can be introduced into susceptible crop varieties (NRC, 1996a).

Managing plant fungal disease has attracted significant attention from biotechnologists, but progress has been slow. Bacterial chitinase reportedly confers resistance to *Botrytis*, a major fruit tree pathogen. Researchers claim that introduction of the resveratrol pathway into tobacco confers *Rhizoctonia* and *Botrytis* resistance (Hain et al., 1993). Several antifungal proteins have been identified in plant roots and seeds (AFP1

and AFP2 from radish and osmotin from tobacco), and their expression appears to delay infection by fungi (Terras et al., 1992). Whether the resistance demonstrated experimentally will be commercially useful remains to be seen. Based on the successes with insect and viral-vector control, the major missing link is identifying several efficacious antifungal genes (either proteinaceous or nonprotein) that can be used concurrently.

Researchers have identified some genes responsible for disease resistance in plants (Staskawicz et al., 1995). Natural disease-resistant genes generally produce proteins that recognize a ligand produced by an invading pathogen, causing cells exposed to the pathogen to undergo "hypersensitive response" (HR) and cell death. This process physically contains the pathogens and also produces one or more signals that activate the whole plant's defense system. This "systematic acquired resistance" (SAR) apparently confers broad protection against diverse pathogens. The mechanisms of HR and SAR are viable targets for engineering disease resistance into plants. The specificity of R genes to specific ligands, however, makes it difficult to confer multirace resistance to pathogens using these genes unless R genes can be identified that recognize certain basic ligands present in and essential to the pathogen. An alternative approach would be to gain an understanding of the HR and SAR mechanisms and introduce these "activation" mechanisms by way of other systems. Sophisticated promoters that can turn the HR or SAR processes "on" might be essential in these efforts.

Weed Control

Weeds cause major losses in crop productivity and lower crop quality. Introduction of conservation-tillage, reduced-tillage, and no-tillage practices to lower soil erosion have been accompanied by use of broad-spectrum herbicides to control weeds previously managed by mechanical means. It is expected that in the near term weed management will be dominated by pesticides and by use of genetically engineered or classically bred crop varieties. Research leading to development of new herbicides and transgenic crops will involve efforts of scientists from industry and academe. Several herbicides with low risks to human health and the environment have been developed (e.g., glyphosate, sulfonylureas, imidazolinones, and glufosinate; Kishore and Shah, 1988).

Some low-toxicity herbicides can only be used safely on a narrow spectrum of crops, thus limiting their utility. Researchers have successfully introduced resistance genes into crop varieties to protect crops against herbicides. For example, sulfonylurea-tolerant soybean, imidazolinone-resistant corn, and glyphosate-resistant soybean varieties have been commercialized.

Resistance to Environmental Stress

In addition to pests, various abiotic agents lower plant productivity. Extremes in temperature, water, and salinity create plant stress and lead to declines in plant growth, productivity, and quality. In the 1980s, drought caused corn yield losses and led to contamination with aflatoxins in many parts of the United States. High soil moisture was responsible for major crop losses in the Midwest in 1993. Knowledge of the genes conferring resistance to abiotic stresses is improving, and further research can be expected to minimize environmentally induced yield losses. Recent research has identified certain genes that might mitigate these impacts, such as the biosynthetic genes for fatty acids, sugars, and amino acids (Yoshida et al., 1995; Tarczynski et al., 1993). Some genes may be introduced into plants via conventional or molecular techniques to increase a plant's tolerance to various stress conditions. Other genes might reduce costs associated with weather-related crop damage and result in yield gains. For example, fertilizer inputs may be reduced by growing plants that make more efficient use of nitrogen, phosphorus, and sulfur or that can fix atmospheric nitrogen. Development of such cultivars could potentially reduce nitrate contamination of groundwater caused by fertilizer seepage. A fundamental understanding of plant nitrogen metabolism will be essential to identify genes controlling plant utilization of nitrate and nitrogen. Researchers have recently cloned some important genes (e.g., glutamine synthetase, asparagine synthetase, glutamate-oxoglutarate aminotransferase, nitrate reductase, nitrate and ammonia carriers) and are now beginning to elucidate their ectopic expression on plant growth and metabolism (Tsai and Corruzzi, 1993).

Plant Design

Research to enhance productivity, improve processing characteristics, and reduce the time required for harvest could lead to crops designed specifically for industrial applications. For example, a large quantity of biomass is left on the field in the form of plant residues. In the future it may be desirable to redesign plants to maximize harvested plant biomass for industrial processing. If dwarf corn plants could produce two to three ears instead of one or two, harvested biomass and grain yields might double from 150 to 250 bushels per acre up to 300 to 500 bushels per acre. While this scenario is unlikely in the near term, such yield enhancement could lower the costs of biobased production and enhance the competitiveness of biobased industrial raw materials. Conversely, corn fiber might be improved for industrial uses, in which case larger plants, rather than dwarf plants, might be desired.