

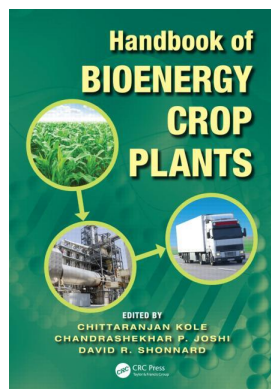
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## **Handbook of Bioenergy Crop Plants**

Chittaranjan kole, Chandrashekhar P. Joshi, David R. Shonnard

## **Genetic Engineering for Bioenergy Crops**

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# 3 Genetic Engineering for Bioenergy Crops

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### 3.1 INTRODUCTION

Fossil fuels (coal, petroleum, and natural gas) are neither sustainable nor ecofriendly because the source is finite and their use cause considerable pollution. (Naik et al. 2010). There has been a dramatic increase in the price of oil in 2008, and more drastic price increases nicknamed “peak oil” in the coming years are predicted (Goldemberg 2007; Potters et al. 2010). Dwindling oil reserves, less than adequate investments into oil exploration and production, and rising demand for oil are major reasons for the anticipated oil price increase (Lloyd’s 2011). Most greenhouse gas emissions are the result of electricity production and heating (27%) using fossil fuels. Other causes of greenhouse gas emissions include land use/change and forestry (18%), agriculture (13%), other energy sectors (13%), transportation (12%), manufacturing and construction (11%), and industrial process (3%) (World Resource Institute 2011). The concerns about dwindling fossil fuel reserves and oil price increases, and the relationship between fossil fuels and global climate change have generated great interest in bioenergy/biofuels.

Unlike fossil fuels, biofuels are renewable because they can be grown repeatedly. Also, biofuels are carbon neutral and essentially reduce the carbon emissions (Naik et al. 2010). Using biofuels to produce bioenergy is one part of the solution to curb global climate change and allow the United States to become energy independent.

Bioenergy is broadly defined as renewable energy derived from biological materials that are used to produce heat, generate electricity, and provide energy for transportation (Yuan et al. 2008). Generally, biofuels are broadly categorized into four categories—first-generation, second-generation, third-generation, and fourth-generation biofuels—depending on the type of feedstock being used. First-generation biofuels are based primarily on corn and soybean and other edible food crops that compete for agricultural croplands, natural fresh water resources, and fertilizers. These fuels are used primarily as small blends, and the energy input and output ratios do not meet the large-scale commercial use. Second-generation biofuels are derived from cellulosic biomass (e.g., miscanthus, switchgrass, sweet sorghum). Biofuels derived from nonedible plant resources are also considered to be second-generation biofuels. Algae are the source of third-generation biofuels, but fourth-generation biofuels are chemically created with the help of petroleum-like hydroprocessing and revolutionary processes such as Joule’s “solar-to-fuel” method (Zarrilli 2007; Yuan et al. 2008). Biodiesel is derived from animal fat and vegetable oil (Jena et al. 2010).

Biomass is a general term used to describe materials of biological origin, including all living matter derived from plants and animals. Energy from biomass is gained from several sources, such as wood, grasses, and animal materials (Babu 2008). Traditionally, biomass is similar to fossil fuels in that it is burned to heat water or produce steam and generate electricity. There are four categories

of biomass conversion: direct combustion, thermochemical, biochemical, and agrochemical. These aspects have been described in detail in Rooney et al. (2007).

In 2007, the United States used roughly  $542 \times 10^9$  L of gasoline, approximately one quarter of the global oil consumption (Vermerris 2008). The United States has been importing more fossil fuels than it produces within the country (EIA 2011). This dependence on imported oil, combined with the political instability and ongoing conflicts of the major oil-producing nations has made the energy crisis a political priority. As a result, the Energy Independence and Security Act (EISA) was enacted in 2007 to help stimulate biofuel production in the United States. According to the EISA, by 2022, a minimum of 16 billion gal of cellulosic ethanol per year must be produced (Leistriz and Hodur 2008). Biofuels will play a major role in the future economics of energy production (McLaughlin et al. 2011).

Not only will the U.S. economy benefit from an increase in biofuels production, but there are also large economic benefits that developing countries can gain by joining the biofuel industry. Conventional biomass products (e.g., wood) are used to provide household energy for people of the developing world. Here, biomass fuels meet the energy needs of households; however, the combustion of biofuels pollutes the air, causing serious health problems. Reducing these emissions using improved stoves and better fuels can reduce respiratory illnesses and greenhouse gas emissions (Kammen 2006). Developing countries would be able to replace a higher percentage of their oil use because of their smaller consumption level, thus decreasing these emissions. Additionally, the biofuel industry is labor-intensive and has the potential to create many new jobs. For example, the bioethanol industry offers 4.2 million jobs in Brazil. In the short term, until higher grain prices stimulate a renewed emphasis on agricultural development, areas that face food shortages or import most of their food could experience higher food insecurity challenges (Cassman and Liska 2007).

However, bioenergy is not without drawbacks. First-generation biofuels received criticism because they are produced from food crops. Crops with dual use as food and fuel get rated by their comparative value as food and biofuel feedstock, leading to an increase in food costs (Cassman and Liska 2007). For example, corn prices have seen an abrupt increase because of corn's use in ethanol production. Another criticism is about the replacement of arable land with biofuel crops in place of food crops, which threatens the sustainability of food production. Other criticisms surrounding first-generation biofuels include poor water use efficiency of the feedstocks, inability to meet large volume requirements (except for sugarcane), and the large carbon footprint of ethanol (Fargione et al. 2008; Searchinger et al. 2008; Stoeglehner and Narodslawsky 2009; Rathmann et al. 2010). Because, at the moment, ethanol is only profitable or competitive around \$50 per barrel, subsidies offered by the government play a great role in the expansion of the biofuel market and its increased production (Ruth 2008).

Second- through fourth-generation biofuels attract less criticism regarding water use efficiency of the crops and the carbon footprint of the fuel. With the aid of other sources of renewable energy, problems associated with first-generation biofuels can be reduced in second-generation biofuels. Using the cellulosic biomass crops of second-generation biofuels instead of food crops of the first generation would alleviate the food versus fuel competition. Biofuel crops such as sweet sorghum and jatropha could be grown on marginal lands and contaminated croplands that are deemed unsuitable for crop production. This approach will offer the greater environmental benefits of reduced soil erosion, carbon sequestration, and better utilization of land resources. Former wastelands could then be used in a profitable way, thus leading to true economic growth and development.

### 3.2 BIOFUEL CONVERSION

A first-generation biofuel, corn grain, is the most important biomass for bioethanol production in the United States (~97%) followed by sorghum (2%) and 1% from other crops, beverage/juice waste, and food processing waste (Nichols and Bothast 2008). Corn and sorghum used for ethanol

production account for approximately 20% of the U.S. corn crop and 15% of the sorghum crop. The fermentation process of starch to ethanol is similar for all grains. Essentially, starch (a polymer of glucose) is enzymatically converted to sugar, which is then fermented to produce ethanol. There are two methods of ethanol production from corn, namely, wet mill and dry grind processes (Nichols and Bothast 2008). In the dry grind process, saccharification and fermentation occur simultaneously after the dry grinding of corn. In the wet mill process, saccharification and fermentation are carried out in separate steps. Starch in the wet mill process is fairly pure, allowing for the separation of other components such as protein, lipids, vitamins, and fiber. Although ethanol production from starchy grains works well, it could result in competition between energy and food production and may not be sustainable in the long run (Yuan et al. 2008; Potters et al. 2010), hence, the drive to produce second-generation biofuels.

Plant cell walls are mostly composed of cellulose, lignin, hemicelluloses, and pectin. These compounds are integrated together to form a strong backbone of the cell wall that maintains the structural and physiological integrity of the cell. Because cellulose and hemicellulose are polysaccharides, they can be broken down into simple sugars and used for the fermentation of alcohol. However, cellulose microfibrils are embedded in a matrix where lignin is a part, which resists degradation. Lignin is composed of different subunits, namely, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). The bonds among the polymers are less reactive; therefore, a single enzyme cannot degrade them all. Lignification is correlated with secondary wall thickening (Weng et al. 2008). Energy from this source is appealing in that it is portable and compatible with the current fuel infrastructure (Rubin 2008). Pretreatment of the complex cell wall is essential to achieve enzymatic removal of sugars from the cell wall.

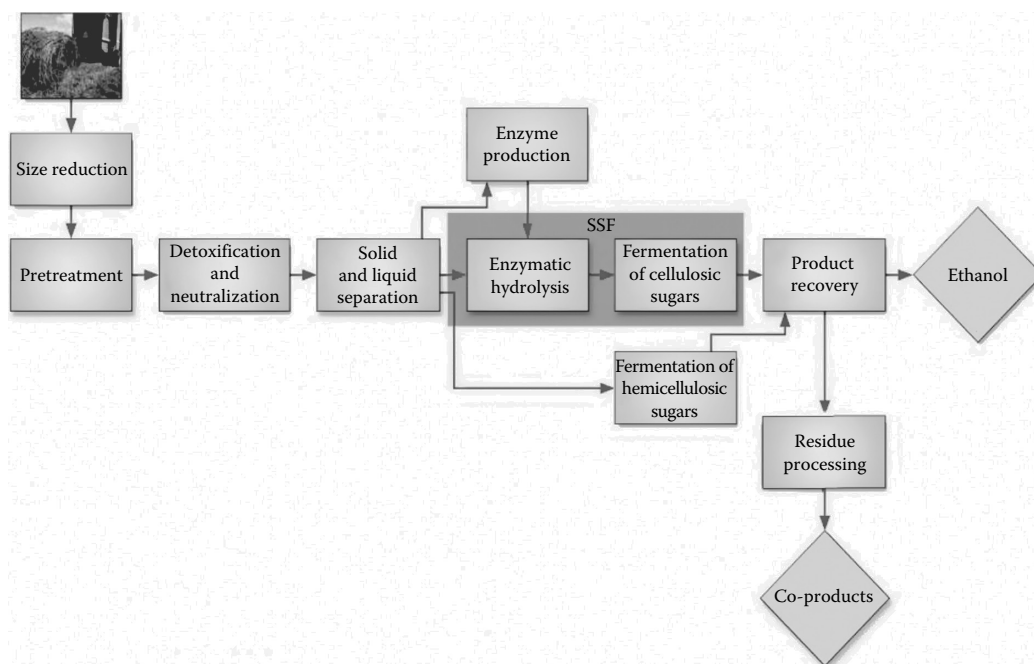
The general process of cellulosic ethanol production includes pretreatment, enzymatic hydrolysis, fermentation, and distillation and has been described in detail in Mosier et al. (2004).

### 3.3 BIOETHANOL

The steps in the process of cellulosic ethanol production are presented in Figure 3.1. There are various pretreatment methods, including steam explosion, liquid hot water, pH, dilute acid, concentrated acid, alkaline-based treatments of lime, and ammonia fiber expansion used in cellulosic ethanol production (Yang and Wyman 2008; Zhu and Pan 2010). In the steam explosion method, superheated steam approximately 160–260°C kept under high pressure (100–700 psi) is exposed to lignocellulose for a short period of time followed by a flashing process to release the steam in an explosion. This causes the lignocellulose to open and expose the cellulose, allowing for increased digestibility. The sugars are found in the liquid stream, but because of the nature of this process, several compounds (furfural, 5-hydroxymethylfurfural) that inhibit fermentation are formed (Abogbo and Coward-Kelly 2008; Lu and Mosier 2008; Brethauer and Wyman 2010).

In a hot water pretreatment, the explosive decompression of the steam explosion is replaced by controlled cooling to keep the water in the liquid phase throughout (Lu and Mosier 2008; Yang and Wyman 2008). Advantages of this method include making complete hydrolysis of hemicelluloses possible and making treated material highly digestible during enzymatic saccharification. The controlled pH liquid hot water treatment is a modified version of the hot water (140–220°C, for 10–30 min) pretreatment and provides greater control of the chemical reactions that occur during pretreatment. An advantage of this procedure is that it minimizes the formation of degradation products (Lu and Mosier 2008; Yang and Wyman 2008).

Dilute mineral acids such as hydrochloric acid, phosphoric acid, nitric acid, and sulfuric acid have been studied for their efficacy as a pretreatment in cellulosic ethanol production. Sulfuric acid hydrolysis seems promising because of its low cost and effectiveness (Lu and Mosier 2008; Yang and Wyman 2008). For effective acid hydrolysis, ideal operation conditions may be 0.5–1.4% (w/w) sulfuric acid treatment, 100–250 g/L biomass solid loading, and a residence time between 3 and 12 min at 165–195°C. A downside of this pretreatment is that it produces compounds that can inhibit



**FIGURE 3.1** Traditional cellulosic biomass conversion to ethanol on the basis of concentrated acid pretreatment followed by hydrolysis and fermentation. (From U.S. DOE, *Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda*, DOE/SC/EE-0095, U.S. Department of Energy Office of Science and Office of Energy Efficiency and Renewable Energy, 2006, available at <http://genomics.energy.gov/gallery/>.)

the fermentation process. Recycling and disposing of the large quantity of sulfuric acid used in this process also make it expensive (Lu and Mosier 2008; Yang and Wyman 2008).

Lime removes lignin under various conditions depending on the type of the lignocellulosic feedstock used (e.g., 100°C for 13 h for corn stover, 100°C for 2 h for switchgrass and 150°C for 6 h at 14 atm for poplar wood). When pretreating woody biomass containing a lot of lignin (e.g., poplar), adding oxygen can improve the process (Lu and Mosier 2008; Yang and Wyman 2008).

There are two pretreatments that use ammonia: ammonia fiber expansion (AFEX) pretreatment and ammonia recycle percolation (ARP) pretreatment. The AFEX process is widely applicable to pretreating biomass from grasses including sugarcane bagasse. This process permits nearly a complete conversion of cellulose and hemicelluloses to fermentable sugars at very low enzyme loadings. Being an efficient procedure, it is also less expensive than other methods (Holtzapple et al. 1991; Delarosa et al. 1994; Reshamwalla et al. 1995; Dale et al. 1996; Moniruzzaman et al. 1997). In the ARP process, aqueous ammonia solution, 15% of weight, is passed through the lignocellulosic biomass in a reactor at 80–180°C and then the ammonia is separated and recycled. This process is very efficient in separating fermentable sugars from the lignin component and also in recycling the ammonia (Iyer et al. 1996; Lee et al. 1996; Wu and Lee 1997; Lu and Mosier 2008).

Enzymatic hydrolysis is performed by cellulase and hemicellulase enzymes, either individually or in tandem (cellulosomes). These enzymes break down polymeric substrates into glucose. The best understood system is from *Trichoderma reesei*, a fungus that contains several enzymes (Lu and Mosier 2008; Nakagame et al. 2010). These enzymes include endoglucanases that “decrease the degree of polymerization of macromolecular cellulose by attacking accessible sites and breaking the linear cellulose chain” (Mousdale 2008), cellobiohydrolases that “attack the chain ends of the cellulose polymers, liberating the disaccharide cellobiose” (Mousdale 2008), and  $\beta$ -glycosidases that “hydrolyze soluble cellodextrins and cellobiose to glucose” (Mousdale 2008). Additionally,

there is a fourth enzyme, cellulodextrinase, which “attacks the chain ends of the cellulose polymers, liberating glucose” (Mousdale 2008). For the best productivity (converting all of the sugars at high rates), microorganisms that bring about fermentation should be able to withstand ethanol and inhibitory compounds as well as be resistant to contamination. The following microbes were improved to impart these properties: yeast (*Saccharomyces cerevisiae*), *Zymomonas mobilis*, and *Escherichia coli* (Lu and Mosier 2008; Kim et al. 2010a,b; Sun and Cheng 2002).

Yeast can tolerate ethanol and exists at a low pH, reducing the risk of contamination of other microorganisms. Various strains of yeast can ferment an array of sugars such as monosaccharides (glucose, mannose) and also disaccharides such as sucrose and maltose to ethanol. *Z. mobilis* is a Gram-negative bacterium that uses the Entner–Doudoroff pathway anaerobically to produce 5–10% more ethanol than yeast. This strain also cannot ferment pentoses effectively. *E. coli* can generate ethanol in small quantities because it can ferment sugars into lactic acid, formic acid, and acetic acid (Lu and Mosier 2008). However, most strains of yeast are unable to ferment pentose sugars such as xylose and arabinose that represent up to 40% of total biomass carbohydrates. *S. cerevisiae*, *E. coli*, and *Z. mobilis* have been genetically modified to enable them to ferment pentose sugars (Lu and Mosier 2008; Kim et al. 2010a, 2010b; Weber et al. 2010). Current trends indicate that corn grain ethanol will be displaced by cellulosic ethanol, ethanol production from sugarcane will include production from sugar and cellulosic biomass, considerable quantities of biodiesel will be produced from nonedible oil and cellulosic feedstock, and ethanol may be replaced by higher energy (more reduced) compounds, as biofuel discovery and practical applications of synthetic catalysts become game-changers in the biofuel production scenario.

The future of genomics-based biotechnology research for bioenergy crops has been detailed in a recent review by Yuan et al. (2008). Genetic improvement of biofuel crops through biotechnology will play an important role in improving biofuel production and making biofuels more sustainable (Gressel 2008; Vega-Sánchez and Ronald 2010; Harfouche et al. 2011). Incorporating new genes into plants uses various techniques for delivery. These genes are then made part of the plant’s chromosomal DNA through recombination. Particle bombardment (gene gun) forces the genes into the cell through pressure. To gain specificity in the plant cell requires the use of *Agrobacterium tumefaciens*, which allows the genes to enter the nucleus and combine with the host DNA. Although *A. tumefaciens* is only found naturally among dicot species, certain strains are able to infect monocot plants, such as corn, sorghum, and switchgrass (Sticklen 2008). The gene of interest is placed under the control of a promoter, allowing for tissue-specific inducible expression. Adding a selectable or screenable marker to the vector simplifies “the identification of transformed plants and increases the efficiency of recovery of transgenic plants” (Skinner et al. 2004). The selectable markers typically used are *gus* and *gfp* or those that confer antibiotic resistance (screenable): kanamycin, hygromycin, glyphosate, etc. Being able to observe the level of gene expression of a species is very useful in determining phenotypes that are desirable, leading to a better understanding of the specific genes that affect important features (Heaton et al. 2008). For the confirmation of a successful transformation, PCR, Southern blotting, and progeny analysis can be used (Skinner et al. 2004). Using the techniques of genomics allows for faster selection of desirable characteristics than traditional cross-breeding. Biotechnology using tissue culture techniques is also an important area of research. One method useful to researchers is somaclonal variation (spontaneous changes in plants), in which the opportunity arises to “develop new germplasm, better adapted to end-user demands” (Schroder et al. 2008). These variations could lead to better adaptations of plants to unfavorable conditions.

In addition to the use of genetics to improve bioenergy crops, it is equally important to use genetically improved organisms that will lower the operating costs and allow for faster and more efficient ethanol production. A recombinant strain of *S. cerevisiae* allows for the co-fermentation of glucose and xylose. The transgenic *Z. mobilis* strain (*Z. mobilis* CP4), producing up to 95% ethanol, can grow on a mixture of glucose and xylose to produce 95% ethanol. In *E. coli*, overexpressing pyruvate decarboxylase (*PDC*) and alcohol dehydrogenase (*ADH*) genes can produce a high percentage of alcohol (Lu and Mosier 2008). The ATCC11303 (*E. coli*) strain B seems to be the best

host for incorporating the PET vector, “producing more than 1000 mM ethanol from hemicellulose hydrolysate sugars” (Lu and Mosier 2008).

In addition to having improved microorganisms, incorporating these enzymes (namely cellulases) directly into plants gives them a distinct advantage over microorganisms, namely, a lower energy input than microbial production (Sticklen 2008). These hydrolytic enzymes are mainly microbial in nature and require codon alterations to become suitable for plant expression. Also, for the correct expression of these enzymes, proper protein folding is necessary. This problem can be fixed if the enzymes accrue in subcellular components rather than in the cytosol. Extraction from the plants will enable researchers to apply these enzymes as “part of the plant total soluble protein (TSP)” (Sticklen 2008) to biomass for the conversion into sugars. This process is relatively simple and can become incorporated into traditional cellulosic ethanol production. More research is needed to increase the levels of production and the biological activity of the heterologous enzymes (Sticklen 2008). An additional area of research is incorporating heat-induced enzymes into the plant to help increase its biomass conversion efficiency. One such enzyme is the *Acidothermus cellulolyticus* cellulase E1 (Yuan et al. 2008).

Lignin plays an important role in structural support, so the crop does not lodge, making harvesting difficult (Tew and Cobill 2008). Also, lignin is physically important in protecting against pathogens and helps water transport through the xylem (Torney et al. 2007). Downregulating the biosynthesis of lignin is an important part of reducing the amount of lignin present in plant cell walls, thereby reducing the cost of pretreatment (Sticklen 2008; Yuan et al. 2008). Cinnamyl alcohol dehydrogenase (CAD) downregulation in poplar resulted in improved lignin solubility in an alkaline medium, leading to more efficient delignification (Abramson et al. 2010; Harfouche et al. 2011). Having a cell wall more uniform in nature could also help in lowering the amount of pretreatment needed. Another method is to redirect carbon flux from lignin biosynthesis to overall biomass built up, helping to increase sugar release during enzymatic hydrolysis. This was seen in aspen when 4-coumarate CoA ligase (4CL) was downregulated. Although more research is needed in bioenergy crops and the downregulation of lignin biosynthesis, this is an important alternative in reducing pretreatment costs (Sticklen 2008).

Use of genetically modified (GM) feedstocks for the production of bioenergy has been complicated by public acceptance of these crops. The public (environmental organizations, consumer advocacy groups, and scientific community) scrutinizes these crops because of their concerns over “health and environmental safety and socioeconomic considerations” (Chapotin and Wolt 2007). Because biofuels are considered a greener technology and are intended to replace petroleum usage, this technology could be held to a higher environmental standard. Concerns regarding the use of genetic engineering have the potential to slow down the adoption of GM crops. General public perception of GM crops has been affected based on incidents of agricultural biotechnology crops such as the StarLink™ corn, co-mingling of pharmaceutical plants and food crops, unapproved GM rice, and *Bt* corn effects on monarch butterflies.

### 3.4 BIOENERGY CROPS

#### 3.4.1 CORN

Corn is an important cereal grain and a staple food. Corn is more productive as a biofuel crop than wheat because of its more efficient C<sub>4</sub> photosynthetic pathway compared with the C<sub>3</sub> pathway of wheat. C<sub>4</sub> plants are generally more efficient users of water and nitrogen compared with C<sub>3</sub> plants (Karp and Shield 2008). Corn is adapted to various soils and environmental conditions. Because corn is a widely available starchy feedstock, it is the principal source of ethanol production in the United States. Corn is in high demand as a food and feed, therefore criticism for its use as a biofuel feedstock is mounting (Fargione et al. 2008; Stoeglehner and Narodslawsky 2009; Ajanovic 2010). One way to help reduce competition between food and fuel is the use of corn stover, the remaining



vegetation after grain harvesting, for cellulosic ethanol. Traditionally, corn stover is left remaining on the fields to help reduce soil erosion and replenish nutrients. It is estimated that in 2030, 256 million dry tons per year of corn stover (de Leon and Coors 2008) will become available in the United States. The use of corn stover depends on its yield potential and carbohydrate composition of the cell wall. Carbohydrate composition of corn stover is 37% cellulose, 28% hemicellulose, and 18% lignin. Corn will be very important in the immediate production of cellulosic ethanol (de Leon and Coors 2008).

Cell wall composition in corn stover is being manipulated using various traits. The *brown midrib* mutations (*bm1*, *bm2*, *bm3*, *bm4*) are naturally occurring mutations found in corn that are known to alter the “lignin concentration and/or composition of the plant” (de Leon and Coors 2008). Maize *bm1* is found to affect the expression of *CAD*, and *bm2* plants have lower lignin contents and significantly diminished levels of Ferulic acid (FA) ether (Barriere et al. 2004). The *bm3* allele is the most efficient at enhancing cell wall digestibility. Expressing the gene trait of *Lfy1* allows corn hybrids to generate more forage yields than other normal corn hybrids. These plants tend to have more nodes and leaves on their main stalk. It is also thought that, through altered lateral branch formation, corn could have increased biomass. The *grassy tiller 1 (gt1)* and *teosinte branched1 (tb1)* genes are connected with activation of “lateral meristems and reduced apical dominance” (de Leon and Coors 2008). Expansins (proteins) play a part in relaxing cell walls for growth and expansion. Although these proteins have been found in corn stover, more research needs to be done to determine their ability to reduce pretreatment cost through modifying cell walls (Sticklen 2008).

Enabling plants to survive in adverse environments would allow for the use of a broader range of land, resulting in more biofuel production. Resistance to biotic and abiotic stresses is a key part to making it possible. It has been suggested that reactive oxygen species (ROS) “act as intermediate signaling molecules to regulate gene expression ... a central component of plant adaption” (Schroder et al. 2008). ROS are very toxic in that they react with several cell components, such as lipids, proteins, and/or nucleic acids. Higher production of ROS is induced by stresses, but plants have some control by expressing different mechanisms, mainly “enzymatic and non-enzymatic reactions” (Schroder et al. 2008). Such enzymes/proteins include “SOD, APOD, CAT, GST, GPOD, enzymes of ascorbate-glutathione pathway dehydrin, actin, histone” (Schroder et al. 2008).

### 3.4.2 SUGARCANE

Sugarcane is a large perennial  $C_4$  grass found in tropical and subtropical regions. Mostly table sugar (~70%) is produced from sugarcane (Matsuoka et al. 2009). Because sugarcane is efficient at converting solar energy into chemical energy, this crop is important in ethanol fuel. The three types of energy canes include sugarcane primarily grown for sugar production; type I energy cane, which is grown for sugar and fiber production; and type II energy cane, which primarily yields lingo cellulosic fiber (Tew and Cobill 2008). Bagasse, the residue left over from sugar mills, can be used to generate heat and electricity. The separation of juice from the fiber is done through milling or diffusion. Brazil has the most advanced sugarcane-based ethanol industry, and ethanol replaces a significant proportion of transportation fuel in Brazil (Goldemberg et al. 2008; Balat and Balat 2009; Matsuoka et al. 2009).

Genetic engineering of sugarcane focuses on several agronomic traits (e.g., resistance to disease and pests, herbicide resistance, increased sucrose content) because it is difficult and time-consuming to develop sugarcane exhibiting such qualities through conventional breeding. Recent reviews (Hotta et al. 2010; Watt et al. 2010) have discussed various methods used for transformation of sugarcane and applications of genetic engineering for improvement of sugarcane to make it a better biomass crop. Biolistic transformation using microprojectiles dominates sugarcane transformation studies, but there are reports of *Agrobacterium*-mediated and electroporation-based transformation of sugarcane (Arencibia et al. 1995, 1999; Enriquez et al. 2000; Manickavasagam et al. 2004; Lakshmanan et al. 2005; Jain et al. 2007; Molinari et al. 2007; Wu and Birch 2007;

Hotta et al. 2010). As of 2005, sugarcane was genetically transformed with three genes conferring resistance to herbicides, six genes offering resistance to diseases, and five genes each for imparting resistance to pests and modifying the metabolomics of sugarcane. However, only a few of the transgenic sugarcane were field tested (Lakshmanan et al. 2005). In South Africa, herbicide-resistant sugarcanes (glufosinate ammonium and glyphosate resistant) have reached field trial stage. Transgenic sugarcane resistant to Sugarcane Mosaic Potty virus with heterologous expression in antisense and untranslatable form has reached field trial stage, whereas insect-resistant sugarcane [CryIA (c)] heterologous expression is still in the pot bioassay stage. Several transgenic crops with modifications to sucrose metabolism have completed glasshouse trials or are being studied in the field trial stage (Watt et al. 2010). Recent trends in the genetic engineering of sugarcane are directed at the modification of sucrose metabolism to enhance sucrose production and accumulation, as well as recovery of high-value products from sugarcane (McQualter et al. 2004; Petrasovits et al. 2007; Wu and Birch 2007, 2010). Also, the range of transgenic sugarcane, especially those with modification of sugar metabolism, has increased in recent years to enhance the sucrose content of sugarcane (Ma et al. 2000; Wu and Birch 2007).

### 3.4.3 SWEET SORGHUM

Sweet sorghum is the fifth most important cereal crop worldwide. Although it can grow in harsh environmental areas, sweet sorghum is mainly found in hot/dry tropical and subtropical areas. Several factors make sorghum a good choice for the biofuel industry, including “yield potential and composition, water-use efficiency and drought tolerance, established production systems, and potential for genetic improvement” (Rooney et al. 2007). Several traits have been found to be connected with drought tolerance, which have been enhanced by breeders to make sorghum variants highly drought tolerant. These traits include “heat tolerance, osmotic adjustment, transpiration efficiency, rooting depth, epicuticular wax, and stay green” (Rooney et al. 2007). Grain sorghum provides starch and sweet sorghum produces sugar, and both types provide cellulose for biofuel conversion. The average yields from grain sorghum are only slightly lower than those of corn, if not the same as corn grain, with the potential of genetic improvement to increase yield. Hybrids are being cultivated for regions where sugarcane production is limited. Biomass sorghum has the potential to become a dedicated energy crop on the basis of high yield capability and broad growth range.

One characteristic feature of sorghum useful in improving its yield is its height. There are four genes known to influence this characteristic, the *Dwarfing* (*dw*)1-4 genes. These genes impart partial dominance of the tallness trait to plants, the effects of which are additive in nature (meaning a plant with *dw*1, 2, and 3 would be taller than a plant with *dw*1 only). Adapting sorghum to long days, as found in temperate regions, led to the discovery of *Maturity* (*Ma*) genes. Specifically *Ma*1 gene has been shown to be involved in controlling the rate of maturity, making the plant carrying this gene in its recessive form react to long days as it normally would to short days (photoperiod insensitive). Increasing yield is highly dependent on changing the source/sink balance. Drought-resistant sorghum keeps a higher photosynthetic rate during conditions of low water. One gene locus (*Alt<sub>sb</sub>*) is known to provide aluminum tolerance. Aluminum is found in acidic soils. These soils make up about half of the possible arable land available. However, iron is the main problem for alkaline soils, and some iron-tolerant lines have been found in sorghum (Saballos 2008).

Sweet sorghum is highly recalcitrant to *in vitro* manipulations, such as tissue culture and transformation. Reports on tissue culture of sweet sorghum are limited to MacKinnon et al. (1986), Rao et al. (1995), and Raghuwanshi and Birch (2010). There is only one published report on transformation of sweet sorghum. Raghuwanshi and Birch (2010) reported transformation of sweet sorghum variety Ramada using microprojectile bombardment. They reported development of a transformation system for sweet sorghum and demonstrated production of transgenic sweet sorghum resistant to the antibiotic hygromycin. Luciferase was used as the reporter gene in this case. Although the

reported transformation frequency is low (0.09%) for hygromycin resistance, the coexpression frequency of the reporter gene was 62.5%. With the development of this transformation system, a platform is laid to make further improvements for transformation of sweet sorghum. This development may pave the way for metabolic engineering of sugar metabolism in sweet sorghum. Genetic modifications that will impart sweet sorghum resistance to biotic and abiotic stresses may also be achieved with further refining of this protocol.

#### 3.4.4 SWITCHGRASS

Switchgrass is a perennial high biomass grass native to the prairies of North America. It is identified by the U.S. Department of Energy (DOE) as one of the United States' promising energy crops for many reasons. It is drought tolerant, high yielding, perennial, enhances soil and wildlife, can be established from seed, and is adaptable to marginal lands (Bouton 2008). Several soil types are tolerated, ranging from sands to heavy clays and pH levels of 5–7. Yields are approximately 10–25 Mg/ha per year (Yuan et al. 2008). Switchgrass is taxonomically divided into two groups: lowland and upland. Lowland plants are considered coarse and tall with large biomass yields and are found in wet regions with milder winters; upland plants are shorter, have a lower biomass yield, and are found in drier and colder regions. Studies have shown two types of chloroplast DNA, U and L, which show differences that can determine if the plant is an upland or lowland plant (Bouton 2008).

There are a few published reports on genetic engineering of switchgrass (Richards et al. 2001; Somleva et al. 2002, 2008; Xi et al. 2009; Li and Qu 2010). Richards et al. (2001) reported production of transgenic switchgrass expressing *GFP* reporter gene. Somleva et al. (2002) generated transgenic switchgrass expressing the *bar* gene, which confers resistance to the herbicide Basta. More recently, Somleva et al. (2008) developed transgenic switchgrass producing polyhydroxybutyrate, a value-added co-product. Xi et al. (2009) demonstrated transformation of switchgrass with a chimeric hygromycin phosphor-transferase gene. Li and Qu (2010) have developed a high-throughput, *Agrobacterium*-mediated transformation system for switchgrass cv. Alamo. Using this modified protocol, they have been able to transform switchgrass cv. Performer with 90% efficiency and cv. Alamo and Colony with 50% efficiency. With the advancement in transformation protocols, it is anticipated that other genes of interest to cellulosic biofuel production (e.g., genes controlling cellulose metabolism, lignin metabolism) may be introduced to this important biofuel feedstock in the near future.

#### 3.4.5 MISCANTHUS

Miscanthus is a high biomass, cold-tolerant, perennial grass easily propagated by rhizomes and has the potential to be a contributor to ethanol production (Clifton-Brown et al. 2008). Originating in East Asia, its natural range stretches from northeastern Siberia to the temperate area of Polynesia, and toward central India. Needless to say, miscanthus thrives in a wide range of climates. Currently *Miscanthus* spp., specifically *Miscanthus* × *giganteus*, is being used in Europe as the main feedstock for biofuel. This hybrid species is sterile and requires vegetative propagation. Miscanthus has shown better cold tolerance than switchgrass, possibly allowing for growth at high latitudes; it can possibly use nitrogen better than switchgrass; and has a yield between 7 and 38 Mg/ha per year (Yuan et al. 2008).

Although there are a couple of published reports on tissue culture of miscanthus (Holmes and Petersen 1996; Kim et al. 2010b), there is only one report of genetic engineering of miscanthus. Callus initiated from immature spikelets or germinating seeds was transformed using *A. tumefaciens*. Selection was carried out using antibiotic G-418 with a *npt II* selectable marker and plants were regenerated from callus (Engler and Chen 2009). It is anticipated that, with further refining of transformation protocols, this important cellulosic ethanol biomass will be genetically

engineered to improve agronomic traits (growth, yield, and resistance to biotic and abiotic stress) and to make it more amenable to pretreatment and saccharification through modification of cell wall properties.

### 3.4.6 POPLAR

Poplar is discussed as the representative tree biofuel crop because it is a model crop. Poplar is the model tree biofuel feedstock because of its wide range of adaptation, available genome, fast growth, clonal propagation ease, sexual compatibility with other species, and available transformation techniques (Davis 2008; Yuan et al. 2008). These trees are found throughout the northern hemisphere, are shade intolerant (grow best with complete weed control), grow in moist areas, and have medium to short life spans. Woody biomass plants have several advantages, including flexible range of harvesting time and good environmental impacts (Davis 2008). Yields for poplar are between 5 and 20 Mg/yr or 10 and 30 Mg/yr based on genotype, site, region, etc. Poplar is used for cellulosic ethanol or for co-firing with coal, but the costs for harvesting and chipping are the major disadvantages to using poplar for bioenergy needs. Areas of genetic improvement would include genes influencing growth, branching, stem thickness, light response competition, plant height, and cell wall makeup (Rubin 2008).

Poplar is highly amenable to genetic transformation (Cseke et al. 2007). Therefore, poplar has been variously genetically engineered to improve its growth and wood properties (Park et al. 2004; Cseke et al. 2007; Baba et al. 2009). Expression of carbohydrate-binding modules (CBMs) has been shown to increase cell growth in poplars transformed with cell-wall-targeted *Clostridium cellulovorans* CBM (Shani et al. 1999). Xyloglucanase was overexpressed in poplar to enhance the growth and yield in poplar. The overexpression of xyloglucanase reduced crosslinks in their cell wall, which, in turn, increased the plasticity under turgor pressure during growth (Park et al. 2004). Similarly, the expression of *Arabidopsis* endo-(1-4)- $\beta$ -glucanase gene (*Cell*) in poplar trees resulted in longer internodes compared with their wild-type control (Shani et al. 2004). Accelerated growth of poplar expressing expansin has been reported recently (Gray-Mitsumune et al. 2008). Hu et al. (1999) produced lines of transgenic poplar (*Populus tremuloides* Michx), which exhibited a 45% reduction of lignin and an increase of 15% in cellulose. This altered cell wall composition enhanced leaf, root, and stem growth without affecting the structural integrity of the transgenic plant, one of the key requirements of successful modification. Introduction of tyrosine-rich peptides to poplar trees through genetic transformation resulted in the formation of wood that is more susceptible to protease digestion than wild-type plants. This genetic modification caused greater release of sugar from lingo-cellulose complex during saccharification (Liang et al. 2008). Transgenic poplar resistant to heavy metals such as mercury, cadmium, arsenic, and lead have been developed. Poplars were engineered with *merA*, *merB*, *gshI*, *CYP2E1*, *MnP*, *cysI*, and *PsMT<sub>A</sub>1* genes. The introduced bacterial genes *merA* and *merB* conferred resistance to organomercurial pollutants (Yadav et al. 2010). Genetic engineering of trees in relation to their application for forestry was recently reviewed (Harfouche et al. 2011). This review reported the issue of 25 permits for field trials of GM poplars in Europe since 1991. These GM poplars are being evaluated for altered wood composition, altered wood properties, sterility, lignin modification, herbicide tolerance, faster growth, and phytoremediation.

### 3.4.7 EUCALYPTUS

*Eucalyptus* is a large genus of trees and large shrubs of more than 700 species. Although many species of eucalyptus are naturalized across the globe, they originated in Australia. Eucalyptus comprises multipurpose trees used for timber and firewood or for the extraction of high-value chemicals (Eldridge 1993; Ladiges et al. 2003; Domingues et al. 2011). Many species of *Eucalyptus* grow fast and generate large biomass suitable for bioenergy production (Stricker et al. 2000). Eucalyptus hybrid (*E. grandis*  $\times$  *E. urophylla*) has proved to be ideal for forestry because of their growth

characteristics and wood properties. This hybrid is also amenable to in vitro manipulations including genetic transformation.

Recent reviews (Hinchee et al. 2009; Abramson et al. 2010; Harfouche et al. 2011) have considered genetic engineering of *Eucalyptus* along with other tree crops. Eucalyptus genome sequencing was also reviewed recently (Grattapaglia and Kirst 2008). Several microsatellite markers of *Eucalyptus* were developed and characterized by Brondani et al. (1998). Complete nucleotide sequence of the chloroplast genome from the Tasmanian *E. globulus* was reported by Steane in 2005. Harcourt et al. (2000) reported development of transgenic *Eucalyptus*. They used *Agrobacterium*-mediated transformation to transfer insecticidal *cry3A* gene to *E. camaldulensis*. Recently, the forestry biotechnology company has patented transgenic Eucalyptus hybrid (*E. grandis* × *E. urophylla*). These freeze-tolerant transgenic plants harbor transcription factor *CBF2* from *Arabidopsis* driven by the *Arabidopsis rd29a* stress-inducible promoter (Hinchee et al. 2009). According to a recent report from the Institute of Science in the Society (ISS), Arborgen's transgenic *Eucalyptus* were modified with genes conferring resistance to antibiotics and altering the lignin pathway in addition to the CBF transcription factor (<http://www.i-sis.org.uk/FTGEEEEASI.php>).

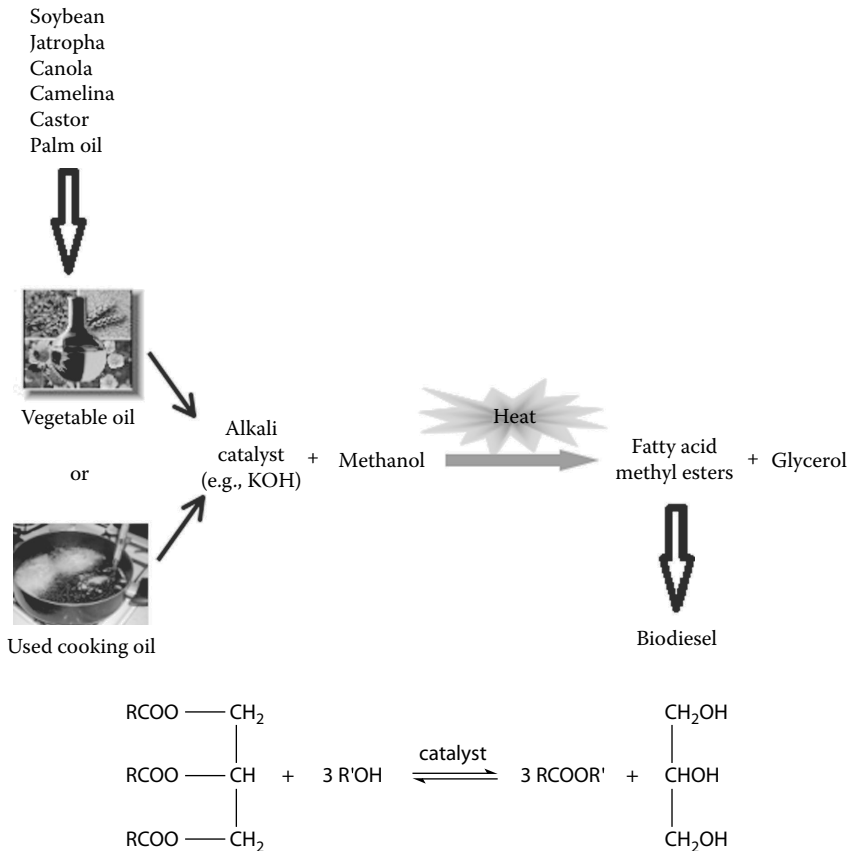
### 3.5 BIODIESEL

The diesel engine, made by Dr. Rudolph Diesel, was originally run on peanut oil at the Paris Exposition of 1900. Various vegetable oils were used to run these first engines, and Dr. Diesel stated “the diesel engine can be fed with vegetable oils and will help considerably in the development of the agriculture of the countries which use it” (Demirbas 2008). Transesterification is the process of making biodiesel from triglycerides (fatty acids). During this process, triglycerides are reacted with methanol or ethanol in the presence of a catalyst (that speeds up the reaction rate) to produce “biodiesel, (m)ethylesters, and glycerin” (Demirbas 2008) (Figure 3.2). Some of the benefits of biodiesel are availability, renewability, biodegradability, lower exhaust emissions, and it is nonflammable and nontoxic (Demirbas 2008).

Although the use of biodiesels can help stem the use of foreign oil, the present cost of commercial production outweighs its benefits, with a gallon of biodiesel (100%) costing from \$2.00 to \$2.50 plus taxes (Demirbas 2008). Also, “the competitiveness of biodiesel relies on the prices of biomass feedstock and costs, linked to the conversion technology” (Demirbas 2009). When blended with petrol (up to ~20%), there seems to be no problem incorporating the new fuel in engines or equipment, and little modification is required with higher percentage blends (Demirbas 2009). Production of biodiesel can lead to useful by-products that may help reduce the total cost of seed cake, fruit husks, and glycerin (Achten and Mathijs 2007). The use of vegetable oils is likely an inexhaustible source of renewable energy with several different sources: cottonseed, rapeseed, sunflower seed, soybean, jatropha, and palm. In the United States, soybean oil is the primary biodiesel source, palm oil is the source in Indonesia and Malaysia, rapeseed oil in Europe, and jatropha in India and Southeast Asia (Demirbas 2008).

#### 3.5.1 BIODIESEL PRODUCTION METHODS

A general process for biodiesel production is represented in Figure 3.2. Various methods of biodiesel production from different sources were reviewed recently (Andrade et al. 2011). This review described the transesterification of vegetable oils, animal fats, and oil from algae to produce biodiesel using homogeneous, heterogeneous, and enzyme catalysts along with ultrasound, microwave, and supercritical alcohol techniques. Transesterification (base, acid, or enzyme catalyzed) and noncatalytic transesterification are two general methods used for biodiesel production. During transesterification, methanol is preferred over the use of ethanol because of its relatively low cost, lower moisture sensitivity, miscibility with biodiesel, and ability to reduce the viscosity of biodiesel (Demirbas 2008).



**FIGURE 3.2 (See color insert)** The biodiesel production process. (Modified from Sustainable Green Technologies, [sgth2.com/bio-diesel\\_faq](http://sgth2.com/bio-diesel_faq))

### 3.5.1.1 Catalytic Transesterification

Base-catalyzed reactions involve sodium hydroxide or potassium hydroxide, with potassium hydroxide being preferred because of the fast reaction rates, cheap catalyst, and less corrosive reaction. One disadvantage of using a base catalyst is its reaction with free fatty acids to form soaps, producing alkaline water that requires energy-intensive waste treatment. Although acid catalyst transesterification using sulfuric acid and/or phosphoric acid can handle larger amounts of free fatty acids and water, its disadvantages are a longer reaction time and require higher temperatures (>100°C) (Demirbas 2008; Nag 2008; Andrade et al. 2011).

Enzymatic transesterification can be used instead of chemical catalysts for several reasons. The enzymes are generally more selective, allow for easier glycerol removal, convert free fatty acids, perform at lower temperatures, and have increased reusability of the catalyst. Lipases are derived from microbes or fungi. According to Al-Zuhair (2007), although the general lipase used is from *Candida antarctica* B, the *Pseudomonas fluorescens* lipase had the better enzymatic activity. Other factors such as water content, type of alcohol used, type of lipase, and temperature all affect the usefulness of the lipase in biodiesel production.

### 3.5.1.2 Noncatalytic Transesterification

Noncatalytic methods are supercritical methanol and BIOX. Supercritical methanol has a simpler procedure and shorter reaction time than catalytic methods as well as being environmentally

friendly, but under cost-intensive higher temperature and pressure (Al-Zuhair 2007; Demirbas 2008; Andrade et al. 2011). BIOX is a new method that “converts both triglycerides and free fatty acids in a two step, single phase, continuous process at atmospheric pressures and near-ambient temperatures, all in less than 90 minutes” (<http://www.bioxcorp.com/>). This process can use grain, cooking grease, and animal fats as feedstocks.

### 3.5.2 MAJOR BIODIESEL CROPS

#### 3.5.2.1 Soybean

Soybean is a biodiesel plant that has garnered a lot of interest. It is thought to have originated from China and is cultivated worldwide. It is a bushy, green legume with seeds typically consisting of approximately 20% oil and is the world’s main supply of vegetable oil. The oil has five fatty acids that make up its composition: palmitic, stearic, oleic, linoleic, and linolenic. There are many uses of soybean in areas such as livestock/poultry feed, plastics, paints, cosmetics, pharmaceuticals, and diesel fuel. In general, soybean is planted in spring/early summer and harvested in fall. Tropical areas allow for soybean growth year round. Lately, genetic improvement of this crop has been focused on several traits such as yield, pest resistance, and seed oil composition (Lee et al. 2007), but emphasis is geared more toward modifying oil content (fatty acid composition). There are numerous genetic maps of soybean that help in identifying potential target genes to modify, such as those affecting growth/flowering (*E1-5*), leaf form (*lf1*, *Lf2*, *Ln*, *Lo*, *Lnr*, *lw1*, *lw2*, *lb1* *lb2*), disease (*Hb*, *Hm*, *hsl-hs 3*, *CP4*, *Als1*), sterility (*st1-st8*, *ms1*, *ms6*, *fs1*, *fs2*, *msp*), and fatty acid composition (*fap2*, *Fas*, *St1*, *St2*, *Ol*, *fan1-fan3*). Currently, soybean is transformed through *Agrobacterium* or particle bombardment using one of three typical explants: cotyledonary node, embryonic axis, or somatic embryos (Lee et al. 2007). *Agrobacterium* relies on a biological method of transformation, whereas particle bombardment uses physical induction (Finer and Larkin 2008). One advantage of using soybean is its ability to grow without the addition of nitrogen as fertilizer. Yields in the United States for soybean are 2668 kg/ha, with an overall net negative energy return when producing biodiesel (Pimentel and Patzek 2005) with costs of \$0.70/L (\$2.64/gal) (Demirbas 2009). One of the main disadvantages to using soybean oil is that it is a major food crop in the United States, with similar problems to that of incorporating corn for bioethanol.

#### 3.5.2.2 Jatropha

Jatropha is an important nonfood crop adapted to environments of tropical and subtropical climates, semi-arid, and marginal lands. Jatropha can grow in various soils, but it is sensitive to frost and waterlogging (Achten and Mathijs 2007). Other attractive features of jatropha include drought hardiness, rapid growth, easy propagation, low cost of seeds, and small gestation period (Sujatha et al. 2008). Jatropha oil is odor and colorless, with its seed oil content variously reported to be between 30% and 50%. The main disadvantage of using jatropha oil for biodiesel is its high viscosity (Pramanik 2003). The high viscosity of jatropha oil reduces the efficiency of fuel injectors in the diesel engines (Demirbas 2008). Reducing the viscosity was done through dilution with diesel blends, with 70–80% diesel showing the greatest improvement (Pramanik 2003). In addition to being cultivated for its oil content, jatropha plants are being used in water and wind erosion control as a living fence to protect food crops and to make soap (Achten and Mathijs 2007; FAO 2010). Citing Bayer Crop Science AG as source, CSA News (2008) reported that Jatropha may provide up to 2270 L/ha oil and boasted that Jatropha oil is a high octane oil/Octane 60 oil (CSA News 2008). Because of this, jatropha is one of the very efficient biofuels and can be used with slight modifications to the diesel engine. Also, this oil is environmentally clean (Bayer Crop Science AG 2008). Overall, jatropha is expected to have a positive effect on land currently thought to be wasteland. Because jatropha harvesting is currently not mechanized, manual labor will be needed, potentially increasing the number of jobs available in rural environments (FAO 2010).

Genetic transformation of jatropha is still in the developmental stage because not many genes of significant value have been incorporated through genetic engineering. More research is needed in jatropha to make this crop more reliable as a bioenergy source. Currently, the germplasm available has many setbacks, including the lack of genetic information, poor yields, vulnerability to pests, and low genetic diversity. Areas of improvement should focus on improving yields, higher oil content, and achieving faster maturity and enhanced fuel properties (Sujatha et al. 2008). *Agrobacterium*-mediated transformation has been performed with the *SaDREBI* gene, with the *bar* gene for selection on phosphinothricin and *gus* as a reporter gene (Sujatha et al. 2008). Genes such as curcumin and stearoyl-acyl carrier protein denaturase and *JcERF* (demonstrating salt and frost tolerance) have been identified. Most recently, the jatropha genome (~400 million base pairs) has been sequenced by a collaborative effort between Synthetic Genomics, Inc. (SGI) and the Asiatic Centre for Genome Technology (ACGT) (<http://checkbiotech.org/node/26008>). A high-quality normalized cDNA library using developing jatropha seeds has been developed by Natarajan et al. (2010). Li et al. (2008) have demonstrated *Agrobacterium*-mediated transformation of jatropha, and Purkayastha et al. (2010) have demonstrated genetic transformation of jatropha using a particle bombardment method. These technologies will be useful in speeding up the genetic engineering process of jatropha.

### 3.5.2.3 Canola

The genus *Brassica* consists of approximately 100 species including *Brassica napus* (canola), which is believed to have originated in the Mediterranean region or in northern Europe. Through breeding, excellent varieties of canola lines have been developed in the Organisation for Economic Co-operation and Development (OECD) countries (OECD Paris 1997). Edible oil, low in erucic acid, was first extracted in Canada in 1956 (Colton and Potter 1999). Canola is currently grown for its seeds, which yield from 35% to more than 45% oil. Canola oil is an excellent cooking oil and can be used to manufacture biodiesel through enzymatic and chemical processes (Dizge and Keskinler 2008; Issariyakul et al. 2008; Cheng et al. 2010). The remaining by-product after seed oil extraction, canola seed meal, is used as a high-protein animal feed.

Canola is highly amenable to *in vitro* manipulations, including tissue culture and genetic engineering. Transformation efficiency of canola was improved to make a working protocol that suits multiple cultivars (Cardoza and Stewart 2004, 2007; Bhalla and Singh 2008). Canola has been genetically engineered using *Agrobacterium* to impart herbicide-resistance to imidazoline, glufosinate, glyphosate, sulfonyleurea, and bromoxynil (Blackshaw et al. 1994; Zhong et al. 1997; Cardoza and Stewart 2007). Canola became the number one crop of Canada because of the use of GM canola. The Canola Council of Canada provides a wide variety of information on the significance of GM canola ([http://www.canolacouncil.org/facts\\_gmo.aspx](http://www.canolacouncil.org/facts_gmo.aspx)). Oleic acid level in *B. napus* was increased by silencing the endogenous oleate desaturase gene (Stoutjesdijk et al. 2000). Canola that can produce high levels of  $\gamma$ -linolenic acid was achieved by introducing  $\delta$ 12-desaturase genes from the fungus *Mortierella alpina* (Liu et al. 2001). Transgenic canola with elevated levels of stearate content was obtained by the overexpression of the *Garm Fata1*, an acyl-carrier protein (ACP) thioesterase, isolated from *Garcinia mangostana* (Hawkins and Kridl 1998). The seed-specific mutants derived from engineering *Garm Fata1* gene resulted in transgenic plants that can accumulate 55–58% more stearate than the wild-type plants (Facciotti et al. 1999). In addition, these lines also showed an increase in laurate at the sn-2 position (Knutzon et al. 1999). Because there are increasing pest problems in canola cultivation, insect resistance is a target trait for genetic improvement. For example, canola is very susceptible to the diamond back moth. Halfhill et al. (2000) introduced *Bt* toxin through the *Bt cryIA(c)* gene to *B. napus* to develop insect-resistant canola. The *B. napus* genome has been sequenced recently, and a sequence-level comparative analysis at the scale of the complete bacterial artificial chromosome (BAC) clones was conducted (Cho et al. 2010).



#### 3.5.2.4 Camelina

Camelina was used for oil production long before World War II and grown all over Europe, but this practice declined after the war. The oil is mostly unsaturated, making it a good source of omega-3 fatty acids. Unfortunately, a large portion of the fats are polyunsaturated, making this oil difficult to work with for fuel production (Lu and Kang 2008). Camelina is adaptable to harsh environments such as “semiarid regions and in low-fertility or saline soils” (Budin et al. 1995), is resistant to pests, and is high in nutrient efficiency with a short vegetation period. The high amount C<sub>18</sub> fatty acids makes camelina a renewable source of oleochemicals, which are used in varnishes and in drying oils for paint. Camelina yields range from 2 to 3 t/ha, with its oil content between 28% and 42% (Gehring et al. 2006).

There are a few published reports of transformation of Camelina using *Agrobacterium* (Lu and Kang 2008; Kuvshinov et al. 2009; Nguyen et al. 2009). Also, there are three patent applications for transformation of Camelina and transgenic plants produced. This indicates the potential of Camelina as a dedicated biodiesel crop. Lu and Kang (2008) used DsRed as a fluorescent protein marker and also transformed Camelina with castor fatty acid hydroxylase, resulting in a change in the fatty acid profile of Camelina. With the availability of these protocols, it is anticipated that more value-added genes that will confer resistance to biotic and abiotic stresses, herbicide resistance, enhance oil yield, and increase quality of oil can be generated in the near future.

#### 3.5.2.5 Castor

Castor is a monotypic genus belonging to the family *Euphorbiaceae*. The center of origin of castor is believed to be Africa and India (Ramaprasad and Bandopadhyay 2010). Castor is widely used as a lubricant and has many other medicinal properties. Castor oil is widely used in India and several countries as a source of biodiesel (Ogunniyi 2006; Sujatha et al. 2008). Such diversity of use has led to a steady increase in the demand for castor oil on the world market.

Malathi et al. (2006) developed semilooper-resistant transgenic castor using *Agrobacterium*-mediated transformation. The transgenics contained *bar* and *cryIAb* genes, and 88% of the semilooper larvae that fed on these castor plants did not survive. Sujatha et al. (2008) generated stable transformants of castor using particle bombardment, although this study only used reporter gene *UdA* encoding  $\beta$ -galacturonidase (GUS) and selectable marker hygromycin-phosphotransferase (*hptII*). They standardized the conditions for transformation by particle bombardment, including the helium pressure (1100 psi), target distance (6.0 cm), and size of the gold particles (0.6  $\mu$ m). *Agrobacterium*-mediated transformation of castor with the *cryIEC* gene offered field resistance to tobacco caterpillar larva and semilooper larva (Sujatha et al. 2009). *Ricinus communis* genome has been sequenced quite recently and is estimated to be approximately 320 Mb in size (Chan et al. 2010). Approximately, 50% of this genome is made up of repetitive DNA. With these advances in genetic engineering and genomics, GM castor with many favorable traits could be developed in the near future, which would enhance the value of castor as a biodiesel crop.

#### 3.5.2.6 Oil Palm

Oil palm is known to have originated from West Africa (Hardon et al. 1985). It is currently a significant cash crop in Malaysia and Indonesia (Sambanthamurthi et al. 2000, 2002, 2009). Palm oil contributes to approximately 20% of world oil and fat production (Oil World Annual 2001). Current and rising demand for biodiesel will increase the demand for palm oil. Therefore, conventional breeding, genomics, and genetic engineering are being applied to achieve genetic improvement of oil palm (Parveez 1998).

Genetic engineering is applied to oil palm to reduce the time needed to develop improved varieties by accelerated breeding approaches, achieve precision gene transfer, and widen the genetic base of oil palm (Sambanthamurthi et al. 2009). Abdulla et al. (2005) reported genetic transformation of immature embryos of oil palm using gene gun and *Agrobacterium*-mediated transformation.

An immature embryo system enhanced the amenability of oil palm to in vitro manipulations. Oil palm was recently transformed with polyhydroxyvalerate gene using *Agrobacterium*-mediated gene transfer (Faud et al. 2008). The giant gene construct used in this experiment included pFA2 (binary vector, ~24.4 kb) and pFA3 (super binary vector, ~39.4 kb) that contained several genes (*bktB*, *phaB*, *phaC*, and *tdcB*) essential for the synthesis of polyhydroxyvalerate. This construct was effectively transferred to the immature embryos using *Agrobacterium*-mediated transformation.

### 3.6 CONCLUSIONS

Biofuel is the future fuel because it is a renewable, less polluting, and environment friendly sustainable fuel. Major challenges for the biofuel industry include but are not limited to (1) production of biofuel feedstocks at a reasonable cost and in sufficient quantities, (2) cost-effective transportation of the biomass to processing facilities, and (3) cost-effective processing of biomass into biofuels and developing methods for cost-effective, environment friendly utilization of by-products of the processing. A combination of biotechnologies such as genomics, marker-assisted breeding, and genetic engineering has the potential to accelerate breeding to develop biofuel crops that are more productive and highly adapted to abiotic and biotic stress. Availability of such novel biofuel crop cultivars may increase acreage and production of biofuel crops to meet the ever-increasing demand for biofuel feedstocks without affecting the global food security. However, it is not clear at this moment as to which crop will be the “winner” among several crops in terms of being cost-effective for large-scale production of biofuels.

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