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Review

Transgenics are imperative for biofuel crops

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Abstract

Petroleum dependency is a challenge that can potentially be partly offset by agricultural production of biofuels, while decreasing net, non-renewable carbon dioxide output. Plants have not been domesticated for modern biofuel production, and the quickest, most efficient, and often, the only way to convert plants to biofuel feedstocks is biotechnologically. First generation biofuel feedstock sources: sugarcane and cereal grains to produce bioethanol and biobutanol and oilseeds to produce biodiesel compete directly with needs for world food security. The heavy use of oilseed rape releases quantities of methyl bromide to the atmosphere, which can be prevented by gene suppression. Second generation bioethanolic/biobutanolic biofuels will come from cultivated lignocellulosic crops or straw wastes. These presently require heat and acid to remove lignin, which could be partially replaced by transgenically reducing or modifying lignin content and upregulating cellulose biosynthesis. Non-precipitable silicon emissions from burning could be reduced by transgenically modulating silicon content. The shrubby *Jatropha* and castor beans should have highly toxic protein components transgenically removed from their meal, cancer potentiating diterpenes removed from the oils, and allergens from the pollen, before extensive cultivation. Algae and cyanobacteria for third generation biodiesel need transgenic manipulation to deal with ''weeds'', light penetration, photoinhibition, carbon assimilation, etc. The possibilities of producing fourth generation biohydrogen and bioelectricity using photosynthetic mechanisms are being explored. There seem to be no health or environmental impact study requirements when the undomesticated biofuel crops are grown, yet there are illogically stringent requirements should they transgenically be rendered less toxic and more efficient as biofuel crops.

Keywords: Biofuels; Lignocellulosics; *Jatropha*; Switchgrass; Straw

Contents

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1. Introduction

The elevation of using ''renewable resources'' as almost romantic sources of energy has been in headlines and is a new target for investors, but a more jaundiced look is needed. If the pros and cons are not fully discussed, the research and development that needs to be done will not be done. Clearly it is naive, ignorant of history, or conceited to think that one can efficiently grow species as biofuel crops that have not been domesticated for that purpose, yet the cultivation of species that have not undergone years of breeding let alone millennia of selective domestication is being widely promoted. Biotechnology has the potential to assist in rapidly overcoming many of the short-comings of the species being promoted, especially those characters that are intractable to breeding, where exogenous genes are needed, or where tissue-specific or temporal expression or suppression of endogenous genes would be valuable.

Biofuels are not new. The use of biofuels began first when it was learnt how to ignite biological material in prehistoric times to cook food and allow early primate *Homo sapiens* to move from more tropical and subtropical climes, to more temperate climates by heating their caves and huts. Drying cow manure for use of fuel is still a traditional entry-level job of young brides into the realms of their mothers-in-law in rural India. Throughout the developing world firewood is gathered for cooking. Not to be left out, wood-burning home heating was a craze in parts of the developed world a few years ago as part of a romance with renewable resources. A century ago, 20% of arable land in temperate Europe and North-America was dedicated to oats, the biofuel that powered the horses, mules, and farm laborers of agriculture, as well as much of urban transport (with concomitant urban manure pollution). In all cases this is highly polluting, especially in the developing world when much of the cooking is performed in a chimney-free environment (with a high incidence of pulmonary diseases). The use of manure depletes the soil of potential minerals and organic matter, and uncontrolled gathering of firewood denudes vast areas. Food oils and animal tallow and even butter have been used for lighting for time immemorial. Thus, ''traditional'' biofuels are not new, have not always been good for health or for the environment and have competed with food production. Will the next generations be better? Not necessarily if we ignore the ''warts'' of some of the technologies coming on line. If we are cognizant of the problems, we will discover that the quickest and probably the best solutions will come from agricultural biotechnology. Most of our cultivated crops have been domesticated for over more than five millennia. Suddenly man has decided to cultivate a series of species that are basically wild, without any real domestication. Are we not being presumptuous? Is there not a certain naïveté or conceitedness to assume that one can immediately start plantations of biofuel crops with little or no domestication? This review covers mainly the environmental and fuel and residue quality issues. Putting wild species into intensive, large scale production is bound to lead to disease and insect problems that will not be covered.

Agro-forestry is being proposed to provide firewood (not further discussed), and the cultivation of special oil crops is being proposed for use in cook stoves and lighting. At least as far as the oils are concerned, they can be far more dangerous than previous technologies (see Section 3.1).

1.1. Food versus fuel

The earth has a limited area of arable land, and grain reserves have been limiting. There is typically a 30-day supply of wheat in storage at any given time. When the supply is 33 days, it is considered a glut and prices drop; at 27 days the prices skyrocket. The small amount of grain (mainly sugarcane, maize and oilseeds) now being used for biofuel has had a domino effect, causing all grain prices to double. This will soon trickle through the food chain and all food prices should soon double, and little grain will be available for emergency food aid. And then there is the bioethical issue of burning grain for fuel to run luxury automobiles when people are undernourished.

The developed world is near top yields that are economically achievable. It would be possible to slightly increase yields by increasing pesticide and fertilizer use, but this would not be overly cost effective and often environmentally undesirable. The long-term increases in yield to sustain (*senso stricto*) human nutritional needs will have to come from the parts of the developing world that practices subsistence agriculture with yield one third world averages (which they bring down). The doubled grain prices should allow these developing areas to produce competitively with subsidized Western grain that had been sold below production prices (''dumped'' in economic terminology). It is a good question as to how quickly this turnabout can come about: a key seems to be in developing country governments dealing with this directly, as recently was done in Malawi [1] instead of depending on foreign aid, which always has strings attached.

1.2. What are ''marginal'' lands for biofuel production?

Many proponents discuss how biofuels will be cultivated on ''marginal'' lands. They use the term correctly in the economic sense, but give it a non-economic spin. Marginal lands are those

with least (''marginal'') economic value, but are they truly marginal and what will be the implications of converting them to biofuel crops? Obviously something had been on the marginal land; if it was pasture, what will happen to meat and dairy prices? Was it wildlife habitat? Was it a watershed? Was it a wetland, or was it forest? Something must have been there despite it's being economically marginal, and the environmental, ecological and economic impacts should be more fully considered. The land may be less ''marginal'' after these other aspects are factored into the equation.

2. First generation "rediscovered" biofuels; ethanol and butanol from sugar and grains, and diesel from oilseeds

2.1. Bioethanol and biobutanol

Sugars (mainly from cane) and digested starchy grains are being fermented to ethanol and butanol as gasoline (petrol) replacements. Ethanol fermentation to fuel human joy is known from prehistoric times, but is harder to transport and has less caloric value as an automotive fuel than butanol. Butanol fermentation of grain was researched and commercialized during World War I by the founder of the author's institute, who isolated an efficient *Clostridium acetobutylicum* able to use a variety of starchy substances and to produce high yields of butanol and acetone, leading to a whole industry based on this fermentation [2].

It was only recently when sugar prices were low and petroleum expensive that Brazil pioneered conversion of cane sugar to ethanol, replacing foreign currency outlays and stabilizing higher sugar prices worldwide. It took over a decade for grain producers to realize that this could be done with grain. These are ready technologies, albeit slight improvements in the chemical engineering are continually being made, and there has been genetic engineering of the yeast used to higher overall efficiency. The enzymes used to digest starch have also been made more efficient and cheaper through genetic engineering.

The energy for distillation of the ethanol or butanol from sugar fermentation comes from burning the stem residues (bagasse) of the pressed sugarcane. This is highly polluting, causing high incidences of pulmonary problems over wide radii from the factories [3]. Part is due to the release of micro-silica particles that cannot be removed by the best precipitators. Biotechnological ways to reduce this silica are discussed in Section 3.2.3. The process would be more energy efficient if the cellulose in bagasse would be used to produce sugars and then alcohols, and the discussion on lignocelluosics in Section 3.2.4 is relevant to bagasse.

The grains used to make ethanol (mainly maize) have been subject to decades of intensive breeding, resulting in high yields of this most domesticated of crops. Changes in starch composition to increase the ease and rapidity of enzymatic conversion to sugars could be performed more quickly by genetic engineering than by breeding, and both approaches are being tested. As the author considers the use of human food/animal feed for production of fuel to be short-term feedstocks, both due to doubling economic values of the grain, and the needs of the grain to sustain human nutrition, there will be little further elaboration.

2.2. Increasing sugar content of sugar crops

Breeding for higher sugar content in sugarcane reached an asymptote years ago, and it was thus thought that a theoretical limit had been achieved. This assumption was shattered when it was recently reported that vacuolar targeting of a highly efficient sucrose isomerase , which converts vacuolar sucrose to isomaltulose (also called palatinose), by enzymatic rearrangement of the glycosidic linkage from a (1,2)-fructoside in sucrose to a (1,6)-fructoside. This allowed accumulation of 0.5M isomaltulose in sugarcane stems without a reduction in sucrose concentration, resulting in a doubling of the total sugar concentration in juice from selected transgenic lines relative to their elite parent cultivar [4]. This occurs even though the enzyme is highly unstable in the vacuole, and activity levels might be further increased by re-engineering the gene conferring isomerization to be stable to this proteolytic environment. There is no reason to doubt that such an enzyme might be useful in sweet-stemmed varieties of maize and sorghum that are being proposed for bio-ethanol production. The only reported enzymes the author could find that could degrade isomaltulose were mammalian [5], and they degraded isomaltulose slowly compared to sucrose. This suggests that either more appropriate enzymes/genes be found, or gene shuffling is required to increase the rate of metabolism. Such genes will have to be transformed into the organisms used to convert sugars to bioethanol or biobutanol.

2.3. Biodiesel

Biodiesel is presently made from palm oil, soybean oil and oilseed rape, with areas under cultivation rapidly increasing (at the expense of what?). The food grade oil is de-esterified by an alkali/methanol treatment that cleaves the glycerol from the fatty acids, with the fatty acids used as biodiesel. There are many places for biotechnological interventions. Palm oil biodiesel has a large proportion of long chain fatty acids, mainly unsaturated. In cool weather this congeals to a fat like texture clogging vehicle fuel lines, and does not meet fuel standards of temperate countries. The oil must be ''cracked'' to shorter chain lengths at a considerable loss of energy. Although not easy to transform, oil palm could be genetically engineered with antisense/RNAi to suppress the elongases that add acetyl CoA molecules to C_{14} to attain $C_{16/18}$ and with (sense) desaturases to increase double bonds (and fluidity) [6]. Not only would this make better biodiesel; the oil would be less cholesterolgenic and far more healthy for human consumption when the fad of using food for fuel is over [6].

2.4. Oilseed rape (canola) and enforcing the intent of the world ban on methyl bromide

There is a world ban on the use of methyl bromide as a soil fumigant to kill insects, pathogens, and weed seeds, because it

is a potent greenhouse gas. Despite the ban on the use of methyl bromide, the reduction in ozone layer depleting alkane halides has not decreased to near zero, as had been expected. This is due to the cultivation of oilseed rape, which can be questioned on environmental grounds. This is because of the release of such compounds into the atmosphere from mainly natural sources, from algae and fungi through to higher plants. Most of these natural sources cannot be controlled by man, except the release from crops [7]. Among the crops, *Brassica* species emit orders of magnitude more methyl bromide than all others [8], and the 1998 estimate was 7000 tonnes per year from oilseed rape [9], which translates to 9700 tonnes in 2005 due to the expansion of cultivation of this crop [10]. About 70,000 tonnes of methyl bromide gas were used in agriculture at its peak, but only about half would reach the air, the rest sequestered in the soil. It is telling that the so-called environmental groups that so vehemently campaigned against methyl bromide are mute as to the environmental impact of this major anthropomorphic source of pollution. Could it be because the ''fix'' to this problem may be transgenic?

It is clearly necessary to lower the levels of methyl bromide and its alkane halide analogs emitted by oilseed rape and its relatives. *Brassica oleracea* a progenitor of oilseed rape was shown to possess a bifunctional methyl transferase that uses *S*-adenosyl-L-methionine (SAM) to methylate halides to methyl halides and bisulfides to methanethiol [11]. Methanethiol is transformed to sulfuric acid, and comes down as acid rain. This bifunctional enzyme was purified to homogeneity and characterized [11], and the gene later cloned [12]. This was taken further in *Arabidopsis*, where an ortholog was found and euphemistically named HOL (harmless to ozone layer) when suppressed [13]. Suppression was achieved by a TDNA disruptive insertion into the gene, resulting in plants that produced less than 1% the amount of methyl halides as the wild type [13]. Many of the plants emitting methyl halides are salt tolerant. Cotton is among the crops that are salt tolerant and it possesses an ortholog of the halide methyl transferase [13], but has not been tested for methyl halide production. If one were a believer in conspiratory theories, or if one accepts some recent historical analyses about governmental interference in environmental research [14], one would wonder if the there are pressures not to find out more about crops producing methyl halides. The group that first ascertained that plants emit such large quantities of methyl halides in 1998 [9] has subsequently published many articles on agricultural replacements for synthetic methyl bromide as a fumigant, but nary a follow-up paper on natural methyl bromide production by crops. The results with the *Arabidopsis* mutant that does not produce these gasses suggest that there is little cost to not producing methyl halides, but this remains to be seen on the field scale. It clearly should not be hard to RNAi the orthologous gene in oilseed rape and in other methyl-halogenic crops to ascertain whether this can be easily done without detriment to the crop, but who has the incentive? The so-called environmentalists seem to be activists that hate transgenics more than they love the environment, and no environmental regulatory authority has taken up the issue. There could be an easy incentive to deal with this issue. If authorities demanded that within a fixed period of time, methyl halide emission by crops must be under a certain threshold, the development work would be performed.

3. The second generation—cropping solutions

More arable land than is available in the USA would be required for a 15% blend in fuels if maize or soybeans were to be used as the sources of biofuels for that country (Table 1). The situation is not quite that bad elsewhere, but is still imperative to develop feedstocks that will replace the use of foods to produce fuels. While the use of food for fuel can but replace a small proportion of the fossil/mineral fuel used, and thus cannot have any major effect on fuel prices, it has had a major effect on food and feed prices. Thus, new feedstocks are needed, and the next generation of feedstocks is being developed from agricultural wastes.

3.1. Special oilseeds; castor bean to black vomit nut—appropriate unmodified?

A group of oilseed bearing shrubs, from castor bean (*Ricinus communis*), to *Pongamia pinnata*, *Calophyllum inophyllum*, to black vomit nut = *Jatropha curcas* have been proposed, especially on websites of government panels, various NGOs, and commercial groups promoting their favorite species.

Table 1
Cropping area needed to replace 15% of transport fuels in the USA

Crop	Oil yield (l/ha)	Land area needed (million hectares)	Existing US cropping area (%)
Maize	172	462	178
Soybean	446	178	67
Oilseed rape	1,190	67	42
Jatropha	1,892	42	13
Oil palm	5,950	13	7.2
Algae/cyanobacteria[a]	59,000	1.3	1.3
Algae/cyanobacteria[b]	137,000	0.6	0.6

Source: Calculated from data in Chisti [15].
[a] Containing 30% oil.
[b] Containing 70% oil.

3.1.1. Jatropha

J. curcas is probably the most highly promoted oilseed crop at present [16]. Much of the hyperbole is reminiscent of that a few decades ago when many naive investors were separated from their capital by investing in plantations of jojoba, trumpeted as a crop with an oil that could replace sperm (whale) oil, and grow in the desert without water and fertilizer. Indeed both jojoba and Jatropha can grow in the desert without water and fertilizer, but without commercial yield. Only with inputs of fertilizer and water are there high yields. The fuel properties of *Jatropha* biodiesel are comparable to those of diesel and conforming to the American and European standards [17]. Because it is highly unsaturated, *Jatropha* oil would complement palm oil to give a mixed product that will not congeal at cool temperatures.

There is very little information about this perennial shrub and its oil-bearing seed plant in peer-reviewed literature. The not quite mature *Jatropha* fruits are harvested by hand in dry season (winter) when the leaves have fallen, and dried in the shade, and the seeds removed by hand. Many of the websites promoting it suggest crushing using primitive machinery to develop an industry reminiscent of the backyard steel mills promoted in China during the Cultural Revolution.

None of the 60 hype websites visited by the author gave any of the common names of *J. curcas* which are; black vomit nut, purge nut, physic nut, etc., nor do they tell the names of its oil; hell oil, oil infernale. This information is only available in older literature and on the official intergovernmental poison website [18]. One can presume that if such common names were to appear on the websites promoting this species it might raise some red flags. Even the family name Euphorbiaceae is rarely mentioned, perhaps because the family is renowned for having species that contravene the Geneva conventions on chemical warfare, and are highly poisonous to herbivores. The fruits contain irritants affecting pickers and those who remove the seeds by hand. The seeds contain alkaloids as well as curcin, a toxalbumin similar in structure and effect to ricin. The oil was claimed to contain a fatty acid ''curcanoleic acid'', structurally and functionally related to ricinoleic and crotonoleic acids, and like them, is a potentiator of skin tumors [18,19] hirota. The chemical information was incorrect, and curcanoleic acid seems not to exist. The irritant/cancer potentiator/synergist seed oil contains curcusones, diterpenoids of the tiglian (phorbol) type with levels between 0.03 and 3.4% of phorbol esters ref Hirota. The best extraction procedures available for the removal of the phorbol esters remove about half [20], which is unacceptable toxicologically in accessions with high initial content, yet does not seem to stop the development of the use of all *Jatropha* accessions. This author could not find any toxicological information about the effects of burning this oil in closed quarters, which is important as it is proposed as a cooking fuel as well as a feedstock for biodiesel production. The ecological life cycle and the socioeconomic aspects of cultivating this crop have been described at length [21].

As *Jatropha* seeds have a pleasant taste, the plants are particularly attractive to children [18], possibly because the seeds contain dulcitol and sucrose [22]. Toxicoses are reported in the medical literature and ingesting four seeds can be toxic to a child, with symptoms resembling organophosphate insecticide intoxication, yet with no known antidote for the lethal mixture [18,23]. None of the websites describe uses for the protein residue after crushing except for spreading on fields (which in developed country jurisdictions might be illegal, considering the toxicity). This is in contrast with soybeans whose meal after pressing is high quality animal feed and also used in human food, yet the amino acid content of *Jatropha* meal is exceptional, except for low lysine [22]. If the seed cake were rendered non-toxic and could be used as animal feed, the profitability of cultivating Jatropha, which was more expensive than diesel in India in 2005, would be ''dramatically increased'' [24].

Jatropha indeed might be an excellent industrial crop, and if it is to be grown using manual labor, then it should be domesticated to a point of human safety. Even then, it is more likely to perpetuate rural poverty than to alleviate it if it is to be cultivated manually. The websites claim that farmers should get an equivalent of $0.14/kg seed (e.g. http://www.jatrophaworld.org/47.html). How many hours go to picking, drying, shucking the seeds, and the variable input costs are near the harvest costs? As the seeds contain about 30% oil, the non-extracted, unesterified farmgate price of oil would be near $0.50/l, close to the retail USA pump price for processed diesel. Thus, $0.14/kg seed may be overly optimistic for an oilseed crop such as *Jatropha*, which has a meal with negative economic value. For comparison, at the time of submission, the price of soybeans, where most of the value is in the meal, is $0.37/kg, and soybeans are not picked, dried and threshed by hand. This illustrates what the value of *Jatropha* might be if the meal could be used. The economics of cultivating *Jatropha* in India have been analyzed at length [24].

Even perennials can be domesticated to be cultivated mechanically and *Jatropha* could be modified to fit mechanical cultivation. This would require a considerable amount of basic background research to ascertain genes for modification. Some selections have been performed to find accessions that are less poisonous. The results were still quite poisonous, probably because the screening was performed to assay amounts of a single poisonous component, forgetting that *Jatropha* contains a suite of toxic compounds. For example, a ''non-toxic'' Mexican variety has 5% the amount phorbol esters, but still has half the amount of toxic curcin lectins as the toxic varieties, and about 25% more trypsin inhibitors and 50% more saponins [25]. If all the poisons could be removed, the meal would be appropriate for animal feed, a useful byproduct that can be more valuable than the oil (as with soybeans). Some toxins are already known or could be quickly isolated and the relevant genes cloned by analogy or probable homology to known genes. It is proposed below to genetically engineer this species, and there is some literature on regenerating *Jatropha* [26,27] and claim that transformation and callus formation has been achieved [28,29]. Some possible examples of potentially useful genetic engineering are listed below. A few groups in India and China have published over 70 *Jatropha* nucleotide sequences in GenBank, a few corresponding to known genes, a small

beginning in knowing this species. *Jatropha* is used as an example, but the messages can be used for other perennial, bushy oilseed crops such as castor beans:

(a) Dwarf the stalks for easier harvesting as well as to increase the harvest index (seed yield divided by biomass). Many of the genes controlling dwarfism seem to have an unknown function. Still many other genes are known that control height.

It can be determined whether height is determined by gibberellins by spraying actively growing plants with anti-gibberellins such as chlormequat and paclobutrazol [30]. Such chemicals should dwarf the plants as they do so with another euphorb [31] and they should increase the harvest index. If so the plants can be transgenically dwarfed by RNAi or antisense to achieve the same effect. The three enzymes and genes controlling various steps in gibberellin biosyntheses are known and cloned [32,33]. Mutations in any one of these genes are dwarfed, and the dwarfing is reversible by gibberellin treatment. Overexpression of a gene coding for ent-kaurene synthase, causing co-suppression also mimicked the mutant phenotype. Additionally, a defective GA receptor gene has recently been isolated that confers gibberellin insensitivity when transformed into grains (ΔGAI) by competing with the native receptor; thereby inducing dwarfing [34].

Brassinosteroid hormones also cause elongation of stems in many plant species, and their absence results in dwarf plants. A 22 Δ-hydroxylase cytochrome P_{450} controls a series of these steps in brassinosteroid biosynthesis, and plants lacking the enzyme are dwarfed [35]. Plants are also dwarfed when they produce normal levels of these growth regulators but are mutated in the bri1 gene coding for the receptor [36]. Additionally, suppressive overexpression of a sterol C24-methyl transferase in the pathway also causes dwarfing [37].

Various forms of the pigment phytochrome interact to detect whether a plant is being shaded [38]. It is advantageous for a crop plant to grow taller when shaded by a weed, but not so when shaded by cohorts, as less grain is produced on the latter, taller stalks. The engineering of suppressive overexpression constructs of one of these phytochromes led to plants that did not elongate in response to shading [39].

(b) *Suppress branching*. Rendering the plants to have single or less branched stalks can facilitate mechanical harvesting. This can often be obtained by transforming the plants with genes for auxin (IAA) biosynthesis. This can often be best done in higher plants by engineering the genes for the microbial IAA biosynthesis pathway, which is different from the plant pathway, using the fungal genes iaaH, and iaaM encoding the two steps from tryptophan to IAA [40]. If one were to over express the plant pathway there would be a likelihood of getting co-suppression of IAA biosynthesis instead of overexpression.

(c) *Anti-shattering genes*. The fruits are picked green, because if they were allowed to dry on the crop, the fruits would open, and drop the seeds to the ground. Inserting genes that will allow the fruits to dry on the plants so that the mechanical harvester can pick the fruits when fully mature and dry, and simultaneously thresh the fruits to remove the seeds with a combine harvester. Anti-shattering genes are quite variable among species; an in depth survey will show that one size (gene) does not fit all cases.

Physiologically, one way to avoid premature seed shattering is to have uniform ripening. Early maturing seeds of oilseed rape on indeterminate, continuously flowering varieties typically shatter. Determinacy, with its single uniform flush of flowering is one method to prevent shattering, but this often shortens the season, reducing yield. Hormones often affect abscission and control when/whether shattering will occur, and it is possible that if cytokinins are overproduced, then shattering will be delayed. The cytokinin pathway is well documented and there are genes that could be put in constructs for cytokinin overproduction [41].

A SHATTERPROOF gene has been isolated from *Arabidopsis* that prevents seed shatter by preventing seed dehiscence [42] by delaying valve opening on the silique. This may be the ideal gene for closely related oilseed rape. Many other genes control flowering, including: TERMINAL FOWER1 or (TFL1 and TLP2) from *Arabidopsis*, which has orthologs among fruit trees [43]. Grasses have multiple pathways for seed shattering, relating to the different mechanisms used even in the same species to shatter [44].

Thus, basic developmental molecular biology will probably be required to deal with shatter. If the shattering is not due to a cracking of the fruit but to an abscission zone forming on the fruit stalk or near the joint with the fruit, antisense/RNAi can be used to prevent the endogenous hormone control of abscission. A plant hormone can then be applied to loosen the fruits when they are dry, and fruits easily removed by a combine or by mechanically shaking the bushes over collecting tarpaulins, as is commercially done with olives [45] and nuts.

(d) *Omit curcin*. This toxalbumin has been purified, sequenced and the gene cloned [46]. Curcin production could be suppressed by partial gene deletion by chimeraplastic surgery [47], or could be performed by RNAi or antisense. Chimeraplasty would be preferable on two conditions; (1) if curcin in leaves, stems and roots is not utilized to prevent attack by herbivores and (2) that native pollen from plantations that are not curcin suppressed will not introgress into suppressed plantations, causing production of hybrid seed. If there is a possibility of either of the latter two scenarios, the RNAi/antisense with a fruit specific promoter should be a better option, as antisense RNAi suppression is functionally dominant whereas chimeraplastic deletion is functionally recessively inherited.

(e) *Suppress phorbol ester production*. As discussed above strains are available with 5% the phorbol ester levels found in most varieties [25]. Whether there is a yield drag pleiotropic with this trait and whether it is controlled by a

single gene has not been published. If the 95% reduction is not sufficient, if the trait is polygenic or if there is a yield drag, then there would be a need to antisense or RNAi suppress genes in the phorbol ester biosynthesis pathway. Basic pathway research will be required as none of the genes are known in *Jatropha*. There are many genes for late diterpene biosynthesis isolated from several plants. They share little similarity amongst themselves, so the specific genes of *Jatropha* may have to be found for an RNAi approach to work using conserved motifs of all terpene synthases, and they may have to be targeted with a seed specific promoter to limit damage to the rest of the plant. Thus, the best approach would be to isolate the phorbol diterpene synthase gene from *Jatropha* and then specifically antisense/RNAi it. Antisense/RNAi inhibiting the acyltransferases that form the phorbol esters might kill the plant due to phorbol accumulation. If the GGDP synthase early in terpene synthesis is compartmented differently than the GGDP necessary for carotenoid and chlorophyll biosyntheses, there may be another possible target for interference.

Any new transgenic insertions would have to be coupled with backcross breeding into locally adapted varieties, unless the regulatory climate of ''event based'' regulation (Section 4.1. in Ref. [6]), where each transformant to be released, no matter if the gene is the same is to be overcome. It is sad that there is no regulatory approval needed to release toxic, wild type *Jatropha*, but lines rendered non-toxic will have to go through expensive regulatory hurdles to prove the scientifically impossible, that they are 100% risk free, whereas the risk of cultivating the non-transgenic material is well known. Of course to cultivate these toxic crops without genetic engineering has no regulatory hurdles, which makes no sense to those looking for regulations to be attuned to protecting the populace as well as to be logical.

3.1.2. Castor bean

Over half a century ago castor bean was promoted, because of an oil composition for specialty lubrication uses and for plastics, and now for biofuels. There was an interest in male sterility for hybrid production [48], and high yielding hybrid varieties were released [49]. It was quickly ascertained that castor bean could not develop into a commercially cultivatable crop without considerable use of hand labor. It is being reintroduced as a biodiesel crop, especially in Brazil, presumably with varieties that can be machine harvested.

Nearly 8% of the population is allergic to castor bean pollen [50], which contains many different allergens [51] including some that are very common (e.g. latex allergens) and some that are also found in the poisonous seeds [52,53]. The material is so toxic that a third of a flock of sheep browsing on garden waste containing castor-bean trash died [54], which was probably due to some residual seed material, as the leaves are considered edible [49]. The pretty seeds are often used in necklaces and severe allergies have been reported from wearing or handling such necklaces or other trinkets made from castor bean. Ricin, a toxalbumin is the major toxic protein of the seeds, which contain 0.2–3% ricin [55]. The estimated oral lethal dose of ricin to man is 1 mg/kg [56]. Two to four seeds may cause severe poisoning in an adult, and eight are generally fatal, but one to three seeds can be fatal to a child [57]. The seeds also contain an agglutinin that is closely related to ricin, but less toxic. The amount of ricin in the residue from manufacturing 50 l of biodiesel (a typical small vehicle fueling) would have sufficient ricin to kill about two average size people at the lowest ricin levels, and 30 at the highest levels. Ricin has been a tool in the hands of terrorists, as inhalation of small amounts is lethal, and it has been used by injection for assassination. Currently, no antidote, vaccine, or other specific effective therapy is available for ricin poisoning [58], although attempts have been made to develop a vaccine [59].

Large-scale cultivation of castor bean for the oil would give rise to large amounts of toxic meal, useless as a feed unless treated by high temperatures that denature the proteins, and useful to bioterrorists. Varieties have been bred that have low ricin and the related castor bean agglutinin [60], but they are still sufficiently toxic to be unusable as feed. It has been proposed to use castor bean cake as a manure, even though ricin can be immunologically detected in soil samples two years after application [61]. No environmental impact studies of ricin in soil could be found in the literature. Thus, it would seem imperative that an RNAi technology be used to suppress ricin production, even if that necessitates using pesticides to limit attack by insects and pathogens. The ricin gene has been sequenced [62] and the species has been transformed and regenerated [28,63,64]. Organisms can evolve resistance to ricin, and one group included a Bt gene in the transformation cassette to control the castor semilooper [63].

Castor oil contains about 90% of the triglyceride as ricinoleic acid, a C_{18} monounsaturated and monohydroxylated fatty acid, without acute toxicity [65], i.e. a C_{12} hydroxylated derivative of oleic acid. The gene encoding the hydroxylating enzyme from *Ricinus* has been sequenced [66], and the oil would have a higher energy value if the hydroxylase was suppressed by RNAi or antisense.

Over half a century ago it was proposed to breed dwarf castor beans that could be combine harvested [49]. This concept that has recently been resuscitated [67] with suggestions to find mutants with restricted cambial growth so that the plants will be annuals, and finding auxin overproducing mutants that would have such apical dominance that they would be single stalked, but it would probably be easier to do this transgenically, as described above for *Jatropha*.

3.1.3. Pongamia pinnata and Calophyllum inophyllum and other shrubs

Various other perennial shrubs bearing seeds with high oil content are being promoted, especially in India [68] with very little preliminary genetic screening or agronomic data. *Pongamia pinnata* is high on the list, having an oil with excellent combustion characteristics after de-esterification [69,70]. Still, the pods are collected and shells removed by hand, with oil yields of 200–2000 kg/ha. The byproduct cake though is bitter and poisonous due to furano-flavonoids, tannins

and trypsin inhibitor, that are hard to remove [71], precluding the use of the meal as animal feed [72].

Calophyllum inophyllum is another shrub from which oil and a non-edible meal are produced. It yields about twice as much oil per hectare as *Jatropha* [68]. The esterified oil is not as efficient as diesel oil, but emissions are lower [73]. The meal contains many cytotoxic compounds, interesting from a pharmaceutical point of view [74], but rendering the meal unusable as feed. The above two species seem to have so many toxic elements that there would seem to be little value in trying to render them less toxic transgenically.

Simarouba glauca with 60–75% oil with claimed yields of 1000–1500 kg oil/ha and valuable byproducts [75]. It has 36% saturated palmitic and stearic and the oil would need cracking in cool climates. Indeed, before de-esterification the oil congeals into a buttery material. Unlike the others, its pulp (and oil) are edible and can be consumed by humans. This added value of the species might render it worthwhile to consider engineering fatty acid desaturases and anti-sensing a fatty acid elongase into this species, so that an oil can be obtained that can be used without cracking.

Many other species being tested are described and their oils compared in the extensive review by Azam et al. [68].

3.2. Lignocellulosic materials as a substrate for producing biofuels

Two types of cellulosic substrates are being considered for biofuel production: straws, and specially cultivated perennial materials. The present situation of their use, and future research needs have been recently summarized by the US Department of Energy [76]. The use of cellulosics will have a much higher net energy gain than seed grains/oilseeds [77].

3.2.1. Straws

Straws are basically free for the baling. About half of the above ground biomass of grain crops is wasted: the straw that bore the grain. Most of the nearly 2 billion tonnes of cereal straw produced annually in the world has a negative economic/environmental value. The thick straw of maize and sorghum is often termed ‘‘stover’’, but will be referred to here as straw. In years past, much of the cereal straw had been burnt after harvest to kill crop pathogens, and since the banning of straw burning, fungicide use has increased. Plowed-under straw temporarily binds mineral nutrients while being degraded by soil microorganisms, often requiring additional fertilizer in the following crop, with negative economic and environmental consequences. Small amounts of straw are fed to ruminants as roughage or as an extender to animal feeds, but very little caloric value is derived from it. At grain harvest straw contains polymeric hemicelluloses (mainly xylans) and cellulose, but their biodegradation by carbohydrases, i.e. the cellulases used in commercial bioreactors for ethanol production, is heavily prevented by a smaller component (12–15%) of lignin. Very small amounts of lignin intercalate into and around as well as bind to the cellulose and prevent biodegradation due a steric hindrance to the cellulolytic enzymes [78]. Thus, companies developing bioethanol from straw achieve only a 20% efficiency of conversion (250 l of ethanol, equaling 200 kg of ethanol per tonne of straw) [79], despite all but a few percent of straw being organic carbon compounds that theoretically can be metabolized.

One of the few advantages of not burning straw is an increase in soil organic matter, which would be abrogated by straw removal as a biofuel feedstock. Various soil scientists have a consensus view, which must be clarified under local conditions, that if straws are cut higher, removing only about 80% for biofuels, the extent of the damage to the soil will be negligible, at least in the temperate zone.

3.2.2. Specially cultivated grasses: “switchcanthus”

There is considerable discussion of cultivating perennial grasses as crops such as switchgrass (*Panicum virgatum*) and *Miscanthus* for production of biofuels or to be burnt in power plants [80], or to be pyrolyzed to gas, with a preference of *Miscanthus* in Europe. A meta-analysis suggests that the latter has more potential in temperate climates [81]. Both have the same problems as straw; lignin limits digestibility. The cost of producing a tonne of specially cultivated grasses is infinitely more than producing straw, as straw is a by-product with negative value, while inputs must be invested to cultivate, fertilize, and harvest specially cultivated grasses. Thus, the cost of a ton of baled straw or stover will always be considerably less than a tonne of baled specially cultivated grasses. The lignin content of mature switchgrass is close to 17%, compared to about 12–14% in wheat straw and maize stover. Without modification of lignin, switchgrass will be far more appropriate for direct burning or direct pyrolytic production of gas than for bioethanol, unless the lignin content is modified, as far less cellulose will be available. The simultaneous saccharification and fermentation of switchgrass to fuel ethanol was less efficient than maize cobs and stover, wheat straw and even wood residues [82]. Saccharification necessitates pretreatments with either hot dilute acid [83] or ammonia explosion [84]. The latter technique raised the ethanol yield two and a half times to 20%[84], the same as with wheat straw without such treatments [79]. Clearly considerable research will be needed to make these specially cultivated grasses a viable proposition for bioethanol or biobutanol. The problems of getting ethanol from these grasses have some calling for their use by direct combustion [80]. Even when used for ethanol production, the residues are burnt, but this requires water removal to be efficient, a process yet to be optimized.

The commercial proponents of specially cultivated grasses make it sound simple; these are perennial grasses that are drought tolerant and require few inputs, even on poor lands with low rainfall, and low inputs of the farmers’ time and energy. Stand establishment is not easy, and frequencies of 25% establishment are not uncommon [85], and harvest is delayed a few years. After establishment yields can vary more than fourfold from less than two to more than nine tonnes per hectare and 90% of this yield variability could be explained by the amount of rainfall if there is sufficient mineral nitrogen available [86]. Switchgrass consumes more water than

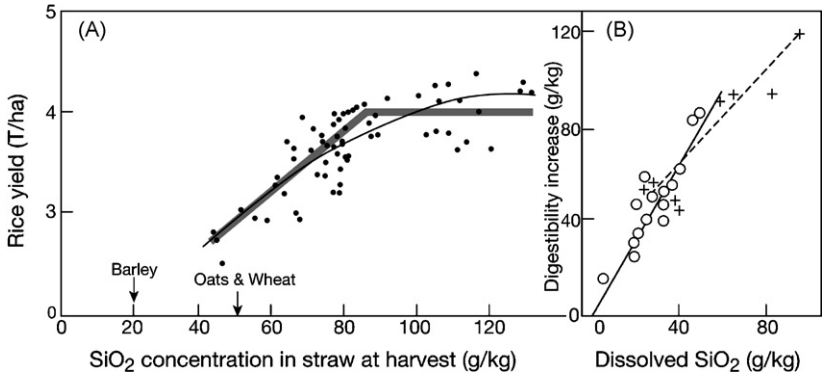

Fig. 1. Silica: relationship with yield and digestibility. (A) Is there a linear relationship between silicon in straw and rice yield? The conventional interpretation is a linear regression (narrow line), and this author's interpretation suggests a threshold (thick line). Data of [93] reproduced in [92], and modified herein with further data added from Ref. [92]. (B) The less silica, the more the straw is enzymatically digestible. Silicon, in this case was partially removed from the straws of various grasses with a neutral detergent, and the amounts removed correlated with an increase in digestibility. Redrawn from [92].

traditional crops under all climatic conditions (but is also better at preventing erosion than many others except winter wheat) [87]. Switchgrass is highly responsive to nitrogen fertilizer with yields increasing linearly to over 20 tonnes per hectare at rates from a quarter tonne per hectare in a multi-year, multi-site study. Lodging becomes a problem at high nitrogen rates with this 2 m tall species [88] and dwarfing could overcome lodging. Stem tissue is more lignified than leaf tissue, so dwarfed material might give higher biofuel yields or require less acid and heat pretreatment.

3.2.3. The ash problem

Whether straws and specially cultivated grasses are pyrolyzed, or first treated with acid and heat and then digested with cellulases for production of bioethanol, it is envisaged that residues will be used for the production of energy by burning or pyrolysis. Considerable ash is then produced, as with all plant material, but part of the particulate material is very potentially dangerous silicon particles [89]. Straws of crop species (except rice) generally can have as low as 2% ash, but switchgrass has almost 5% ash [89], and in some straws up to 10% [90]. This ash contains over 60% silica. Fifty percent more of this potentially dangerous compound (when in the form of microscopic particles) is emitted on burning than by coal [89]. When switchgrass was mixed with coal, the fine particle concentrations were much higher than with dedicated coal combustion [89]. These fine particles are a concern because they are not captured by electrostatic precipitators or by other devices used to lower particulate emissions. The micro-particles of switchgrass ash should be captured, as besides the silica they contain large amounts of phosphorus and potassium salts, which should be recycled back to the field by farmers, not by wind. This is far more important than with coal, where most of the particulates are aluminates, of no agronomic value.

Silicon is not an essential element for plant growth (although small amounts might be of some value [91], especially for rice [92]). As there is less silica in leaves, dwarf rice, with less stems and just as leafy is proportionately more digestible by cellulases [92], but good correlations between silicon needs within height ranges, and between varieties are yet not available. The amount of silica in rice straw when there are high yields is many fold greater than the maximum amounts found in barley, oat, and wheat straws (Fig. 1A). Chemical dissolution of silica in straw leads to greater enzymatic digestibility (Fig. 1B), so it is clear that silica acts like lignin by somehow decreasing enzymatic digestibility. Can the amount of silica in rice straw be reduced without affecting yield? This depends on how the data are interpreted, and if the causes are understood. Old data [93] are still continually being interpreted as evidence that the higher the silica content in rice straw, the higher the yield (Fig. 1A, narrow line), but I interpret the same data as indicating that above 90 g Si/kg straw, the rest is superfluous (Fig. 1A, thick line). Silicon is a known deterrent to rabbit feeding [94], to a sugar cane moth [95], and to various pathogens [96], but these pests can be controlled by other means. Fungicides and insecticides may well be cheaper than the applications of silica described. If the yield increases up to the threshold of 9% silicon are due to enhanced resistance to insects and pathogens, one must ascertain whether there are genes for resistance that can replace the silicon, lowering or obliterating the threshold requirement. Biotechnological solutions to this excess silicon problem are just becoming imaginable, as the genes for rice silicon uptake have recently been found [97], and could be the basis for future transgenic reduction where there is more than ample silicon in soil and in rice plants. The same should be true for other straws [98].

3.2.4. Biotechnologies for increasing efficiency of lignocellulose utilization

3.2.4.1. The enzymes for biofuels. At present there is a large and expanding effort to increase the efficiency of the cellulolytic enzymes [76]. There has already been an increase in efficiency of production, with the price per unit activity decreasing 20-fold over the past decade. There is now competition from transgenic maize expressing a bacterial endo-cellulase and a fungal exo-cellulase, which are targeted to different compartments in the embryo. Yields of 16% of the soluble proteins being these enzymes have been achieved, but this represents only 0.04% of the dry matter [99]. The endosperm starch could be used to produce bioethanol, and the

"germ" extract used to digest the straw for bioethanol production. Gene shuffling could further increase activity, stability, and temperature optima, of the cellulases used. Besides cellulose, straws and specially cultivated grasses contain large amounts of hemicelluloses, which have a high content of five carbon sugars that are not well degraded by present technologies, and there is ongoing research to develop better enzymes. Yeast does not naturally use five carbon sugars, and yeasts are being engineered with the enzymes to do so.

The major problem that cannot be overcome by enzyme production is the steric hindrance where lignin limits access by cellulases. Although the lignin degrading peroxidases are known, they have high energy requirements, and only work in living microorganisms in direct contact with lignocellulose, and cannot be used efficiently as process enzymes. Pretreatments of wastes could be envisaged by specific microorganisms, as been envisaged, but they have not even been perfected for the reduction in lignin prior to manufacturing of paper from wood pulp (bio pulping), despite decades of research.

3.2.4.2. Modifying biofuel crops for digestibility by industrial carbohydrases by breeding. Breeders are renewing an interest in sweet (stemmed) sorghums and maize, which are similar in sugar content (but not composition) as sugar cane, as specialty crops for bioethanol production. The sugar and mineral content of the syrups rendered these not amenable to production of crystalline sugar (sucrose), but this is inconsequential for bioethanol production.

Breeders have endeavored to breed higher straw digestibility within the limited variability of the genomes of the various crops. Brown mid-rib (*bmr*) mutations in maize and sorghum have been isolated that have a lower lignin content and much higher digestibility. They have been used to breed forage (silage) maize and sorghum, invariably with somewhat lower yields, which can be economically compensated for by the greater digestibility [100,101]. The brown midribs are due to mutations in lignin biosynthesis, which lead to slightly less lignin as well as a modified lignin sub-unit composition. There may well be mutations in lignin composition/quantity that do not have this brown midrib signature, but they would be too hard to discern as there would be no visible phenotype to be detected. Mutations similar to the brown mid-rib in sorghum and maize that would allow breeding decreased lignin have not been identified in small grains such as rice, wheat, and barley, probably because the genes for lignin biosynthesis are in multigene families in grains, which are not amenable to single mutations. Most sources of variability would probably be quantitative, where more than one isozyme may have to be suppressed, requiring extensive breeding to modify lignin without modifying other grain quality characters. Thus transgenic lignin reduction/modification would be simpler.

Breeding switchgrass and *Miscanthus* for anything but dominant traits is not easy, as switchgrass is an autotetraploid, with a high degree of preferential pairing [102], and *Miscanthus* is actually an interspecific hybrid [103]. Still, there has been some success in varietal selection from amongst the natural variability of switchgrass [104]. Any transgenic improvements of switchgrass will have to consider the necessity of curtailing gene flow to remnant wild populations and to interbreeding related species [105].

3.2.4.3. Possible biotechnological solutions to steric hindrance by lignin. The solutions to increasing digestibility without affecting important varietal traits of crops are to transform elite material to have modified lignin and cellulose contents. Plant material containing more cellulose or less lignin, or with modified lignin composition is more digestible by the carbohydrases in biofuel bioreactors [6,101,106]. For each percent less lignin, two to four times more cellulose is available to bioreactor carbohydrases when acid/heat pretreatment are not used, or require less acid and heat to release the same amount of available carbohydrate. Wheat and rice have very little genetic variability in straw composition, so it is doubted that classical breeding can provide a solution, especially in hexaploid wheat where recessive mutations are hard to find.

Increasing cellulase digestible material by 20% would upgrade the immediate economy of liquid biofuel production.

Table 2
Most cereal phenylpropanoid pathway enzymes are encoded by small gene families

Rice gene[a]	No. copies identified	Sequence identity using MegaBLAST (%)		
		Barley	Wheat	Maize
Phenylalanine ammonia-lyase (Oso4g0518400)	At least 5[b]	86	85	89
Cinnamate 4-hydroxylase (Os05g0320700)	3	86	89	87
p-Coumarate 3-hydroxylase (Os05g0494000)	2[c]	ni	88	79
4-Hydroxycinnamoyl CoA ligase (Os02g0697400)	At least 2	83	ni	78
Caffeoyl CoA *O* methyl transferase (Os06g0165800)	1	ni	93	90
Ferulate 5 hydrolase (Os10g0512400)	2	ni	ni	ni
Caffeate-*O*-methyl transferase (Os08g0157500)	1	71	86	87
p-Coumaryl CoA reductase (Os08g0441500)	3	90	90	92
Cinnamyl alcohol dehydrogenase (Os09g0400400)	4[d]	ni	82	83

Source: Updated and modified from Ref. [106] by Prof. Aviah Zilberstein. ni = no information

[a] Rice gene reference for homology comparisons—numbers based only on full length sequences.

[b] Number of copies as of early 2007.

[c] One may be a pseudogene (to be confirmed, as transcripts are detected).

[d] Additionally, eight less homologous genes were identified by Tobias and Chow [114].

This can potentially be achieved transgenically by creating transformants with increased cellulose having a more open (biodegradable) structure using the *CBD* gene, and separately or together using RNAi techniques to modulate the lignin content.

Considerable efforts have been invested in decreasing chemical wastes during paper pulping by transgenically reducing the lignin content or composition of trees by affecting the genes controlling biosynthesis of lignin monomers. This led to a beginning of understanding of lignin biosynthesis and its relationship to cellulose availability [107]. Still, there are many basic compositional differences between tree and other dicot lignins and those of grasses, and it is thus not clear how much one can extrapolate from dicots to grasses.

Partial silencing of the phenylpropanoid pathway enzymes leading to lignin, encoded by whole gene families (Table 2) can be achieved by antisensing or other RNAi strategies using small interfering RNAs (siRNAs) that conform to consensus sequences of the gene family. Most of these genes have already been partially silenced in dicots [108], changing monolignol levels, increasing cellulose levels and digestibility [101,106]. The rice genome sequence and additional maize, wheat EST and other genomic data of cereals and grasses [109] have allowed identification of cereal orthologs of genes encoding enzymes involved in producing monolignols. Whether one needs to suppress the whole family, or just particular members is a subject for research. Decreasing transcript levels of gene families may suffice, but inhibiting more than one gene type may be necessary because of biochemical compensation by parallel pathways producing monolignols. A decrease in function of a single gene provided sufficient down-regulation and modification of lignin structure and enhanced the digestibility in maize (cf. [110]), sorghum and pearl millet [111], poplar [112] and pine [113], but there has been little published evidence that affecting more genes each to a lesser extent, can increase digestibility with fewer side effects.

3.2.4.4. The reduced lignin causes lodging myth?. Partially suppressing shoot lignification by antisense based on the desired phenotype is unlikely to affect mechanical strength. The compressed internodes of semi-dwarf and dwarf wheat and rice should maintain structural integrity with somewhat less lignin. It is unlikely that selected modulation of lignification would affect defense mechanisms deriving from phenylpropanoid intermediates, as the gene encoding (at least one) isozyme involved in defense lignification had a quite different sequence from the isozyme for xylem lignification [115]. Still, the task will not be easy as it is still unclear which lignin modifications/reductions will do so without affecting yield [116].

Partial (but not major) reduction or change in lignin composition should leave dwarf and semi-dwarf wheat and rice and specialty biofuel crops with sufficient strength to resist lodging. It is a general misconception, without proof, that all the lignin present in a stem lignin is singularly responsible for the structural stability that prevents lodging, the propensity to keel over in windstorms, which is a major impediment to mechanical harvesting. A comparison of lignin content in straw and the susceptibility to lodging showed no significant correlation [117]. Lodging is typically precipitated by wind and may result from buckling or partial breaking of the lower stem, or from the roots twisting out of the soil ''dislodging'' [118] due to wind drag exerted mainly on the grain head in crops. Considerable research is needed on how to balance the competing constraints imposed by stem rigidity and flexibility to select the best varieties to deal with lodging. Physicists and biologists are just recently beginning to follow the effects of artificial wind gusts on wheat plants by video photography [119]. Another group built a portable wind tunnel, which they took to the field [118], but did not compare materials for the same reasons. The large-scale work in comparing varieties with different lignin compositions and contents is not yet under consideration due to a lack of perceived priority [118].

Stem-borer damage to a stem renders the stems more susceptible to lodging, which may correlate with low/modified lignin in some maize varieties. An analysis of QTLs controlling lignification shows that many co-localize with those of natural resistance to corn borers [120]. Root worm damage is also highly correlated with susceptibility to root lodging, especially under environmental stress [121] but this was not correlated with lignin content or composition. The problems of stem-borers and root worms can only ignored when modifying lignin at the risk of not coming up with a useful, highly digestible crop. Transgenic Bt crops have been highly successful in controlling stemborers and rootworms, and it might be foolish to put modified/reduced lignin transgenes in a background that does not have these traits.

3.2.4.5. Need for integrated systems biology research to define ideotypes. A courageous attempt has been made with maize to use systems biology to define the ideal ideotype with optimal straw digestibility, which should be of use to both the breeder and the genetic engineer, as well as to facilitate their interactions [122]. Various combinations of the results could then genetically define 90% of the results. The modeled ideal ideotype contained less lignin with a higher ratio of syringyl to guaicyl sub-units, which were preferentially located in the cortex and not the pith tissues of the maize stems [122]. They modeled in vitro digestibility based on two histological and two biochemical variables and computer generated a highly significant regression correlation with observed digestibility of 13 inbreds. The present model is most inadequate in predicting what will happen with mutants and transformants. Brown midrib *bm3* mutant maize was actually far more digestible than the model predicted [122]. Such results must yet be compared in the type of wind tunnel described above to add a correlation with lodging. The results should also be econometrically correlated with added/reduced yield and the added value of the digestible straw and stover.

The ''switchcanthus'' type grass species envisaged for biofuel production have additional problems that might be addressed by additional transgenic approaches to the ones above. The problem of too much lignin, and the problem of too much lodging at high nitrogen fertilization rates might be

partially solved in a single step by transgenic dwarfing. Dwarfing has continuously worked in the past to prevent lodging, with more of the biomass in leaves, and leaves of switchgrass contain a lower proportion of lignin than stems [123]. Another approach is to delay flowering, as a switchgrass has less lignin during the vegetative phase [123], but also less cellulose, as it has starch and protein, both of which can be utilized by the yeast making bioethanol. These approaches might be quicker to lower the lignin content, than the direct anti-lignin approaches described above, and of course successes from both approaches could be stacked.

A challenge has been raised to the molecular biologists from a study of 12 lodging resistant and susceptible varieties of wheat. In the analysis of all their data, it appears that a higher fiber (including lignin) content in the second and third internodes correlates with resistance to lodging (with a correlation coefficient of ca. 0.6) [124]. Breeding to increase the lignin contents of these two internodes and lower it in all other ones is nigh impossible, but comparatively simple by using tissue-specific promoters for sense and antisense to raise and lower lignin contents. Having high lignin content in the lower internodes and less elsewhere may not affect biofuel production. As noted above, it is considered advisable in many cases to leave the lower 20% of straw in the field to maintain soil organic matter. There have been no reported lodging problems with decreased lignin transgenic poplar trees, except for the dislodging of trees by eco-terrorists in a European field trial.

3.2.4.6. Enhancing cellulose. Increasing the amount of cellulose (especially at the expense of lignin), or modifying its structure such that more is available to cellulases could also increase the bioethanol yield of straws and specialty grasses. Transgenic poplars overexpressing *CEL*1 (an endo-1,4-glucanase gene) were taller, had larger leaves, increased stem diameter, wood volume index, dry weight and a higher percentage of cellulose and hemicellulose than control plants [125]. Transgenic poplars overexpressing the poplar endo-1,4-β-glucanase gene also produced more cellulose [126]. Transgenic plants over expressing *CBD* encoding the cellulose-binding domain of cellulase on cellulose grew faster with more biomass accumulation [127]. There are yet no extant published reports of modulating these genes in straws.

3.2.4.7. Transgene flow issues. The problem of transgene flow from switchgrass to related *Panicum* species [105] could be obviated by many of the transgene mitigation strategies discussed in depth elsewhere [6].

3.2.4.8. Integrated basic and applied research needs for cellulosic biofuel substrates. A mixture of these transgenic with other technologies could yield nearly 2 billion tonnes of inexpensive, high quality substrate for biofuels from straws, without putting more land into cultivation. As less straw is incorporated into the soil, there would be fewer of the mineral binding problems during the initial microbial degradation of fast-degrading components, which requires additional fertilization in spring. As plant disease carrying straw is removed from the field, there could be less need for fungicide application the following season. Specially cultivated grasses will mainly be useful when they are not grown on land taken from cereal production.

It will require a considerable duration to isolate the genes, transform the plants, analyze each series of transformants, and fine-tune the levels of expression such that sturdy, high yielding cereals will result, with more digestible cellulose. It will take years more to either cross and backcross the genes into more varieties of the crop, or to transform each variety. The subsidiary technologies of processing the straw to fuel or feeding the straw will also have to be developed.

3.2.5. Is biocontrol of water hyacinth a mistake?

Water hyacinth (*Eichhornia crassipes*) is a major tropical waterweed, clogging fresh waterways, canals, rivers, and lakes. It can quickly get so thick that it rots, causing massive anaerobiosis, euthrophication, and fish death, laying havoc to fisheries and shipping. It grows best in polluted waters, using the organic matter, supplementing photosynthesis, as well as the minerals [128], and thus can be used for sewage purification if carefully managed [129]. Otherwise it itself becomes a eutrophied pollutant as so often happens. Cutting and removal was very expensive, as there was little use for the removed material, and the water hyacinth rapidly grows back. New and more efficient kinds of harvest equipment are continually being constructed [130]. The 90% water content requires that shipping distances be minimal. There have been suggestions of using it as compost, as animal feed, or for biogas production on a village scale [131], but not as a larger scale feedstock for ethanol production, using mechanical cutting, chopping and conveyance.

Biologists have done an excellent job in finding and propagating insect and sometimes fungal biocontrol agents that rapidly and continually decimate the water hyacinth populations, e.g. [132,133]. Thus, with successful biocontrol, the pollutant remediation potential of water hyacinth was lost, and it may be expected that there will be algal blooms that will replace water hyacinth as a cause of eutrophication in polluted waters. The movement of biocontrol agents around the world to facilitate biocontrol results in a lower growth rate and lower yields.

Water hyacinth contains 90% water, and as such was never found to be appropriate as an animal feed. Still, it has a low lignin content (<10%) [131], and as such could be an excellent feedstock for bioethanol/biobutanol production. Water hyacinth has a slightly higher water content than would be used directly for loading in a bioreactor. A slurry of homogenized water hyacinth could be ‘‘thickened’’ with ground air-dried straw, preferably engineered to reduced/modified lignin content. The initial heating of the fermentation broth to distill off the bioethanol to distill the bioethanol from the fermentation broth after fermentation should coagulate/precipitate proteins, and the precipitate should make an excellent animal feed.

The naturally low lignin (compared to straw) should render the water hyacinth cellulose readily available to cellulolytic

enzymes without acid/heat pretreatment, perhaps more so than other feedstocks. If total separation between lignin and carbohydrate is desired, then less acid would be required than with straw. It is possible that if the material is continually re-cut during logarithmic growth, the lignin content may be lower yet. The value of water hyacinth as a pollutant remediator, and its value as biofuel substrate should offset the cost of repeated harvesting that will keep the waterways open, the fisheries healthy. The cutting should reduce human health problems as water hyacinth mats are breeding ground for the vectors of malaria, bilharziosis and river blindness. The primary productivity of water hyacinth can be as high as 140 tonnes dry matter per ha/year with a doubling time of week [134]. Yields of more than double that have been reported when there was continuous harvesting, such that there could be sufficient material to supply biofuel facilities along the banks of the waterway, especially if there is ample straw available for co-fermentation, e.g. in rice growing areas where water hyacinth has been a major pest. The liquid effluent from a biofuel facility should provide nutrient rich irrigation water, and the sludge compost. If the yeast and the proteins can be separated, there should be good animal feed, as long as the calcium oxalate present does not remain. There are enzymes that degrade oxalate, and the genes encoding them could be engineered into the yeast used in fermentation. As the water hyacinth has a very high nitrogen and mineral content, there will probably be no need to add nutrients to an ethanol bioreactor, even if straw is added.

Will one now have to consider the biocontrol insects as pests to be controlled? Will the water hyacinth, developed as a feedstock for biofuels (if this comes about) have to be genetically engineered with a Bt or other gene to control the biocontrol insects? Time will tell. No extant literature could be found stating that water hyacinth has been transformed, as until there is a use for this noxious weed, one would be loathe to put in genes that might make it weedier. This could change and genes such as lower lignin might be useful, as would genes to ward off predators.

4. Third generation technologies: algae and cyanobacteria for biofuel production

Algae in ponds can be far more efficient than higher plants in capturing solar energy, more so in bioreactors. The US Department of Energy funded a large international project on microalgae for biofuel production that ceased operations a decade ago [135]. The project achieved sporadic maximum yields of more than one hundred times greater than oil palm (per unit area) and oil palm is among the most efficient of conventional crops (see Table 1). If algal production could be scaled up industrially, less than 6 million hectares would be necessary worldwide to meet current fuel demands, amounting to less than 0.4% of arable land, an achievable goal for global agriculture [135]. Additionally, many of the very efficient oil producer organisms are marine. Thus ponds near the world's seas could conceivably produce sufficient biofuels, without needing or affecting fresh water supplies, which are becoming limiting.

Low petroleum prices and a lack of a full understanding of the biological parameters led to the cessation of the USDOE project. In 1996 when the project was terminated, it was considered that the production of algae biodiesel would be double the cost of petrodiesel. Given the increase in the cost of fossil/mineral diesel fuel at present, before biotechnological optimizations, the use of algae may already provide a margin of profitability. If the limitations of algal production systems can be overcome (see below) such a crop would be highly competitive with diesel derived from fossil fuel. There is much to learn from the DOE project as they had screened a large number of algae and cyanobacteria for growth rate, and oil production as well as composition. These cultures have been maintained and remain available to researchers.

A few continue their interest in producing biodiesel using unmodified organisms, engendering skepticism by others [136]. One company claims to be using fiber optics to enhance light penetration to expensive bioreactors. This is unlikely to become a large-scale solution for a commodity such as biodiesel, but could be useful for specialty products such as beta-carotene and omega-3 oils. The known groups dealing with production of biodiesel from algal crops predominantly consist of engineers and physicists who have not carefully considered the DOE summary: ''the factors that limit cost are biological, and are not engineering related, if low cost ponds are used'' [135].

The DOE [135] report (and other sources) summarize these biological limitations, all of which this author perceives could be overcome by transgenics:

(a) *Organism survival*: Current systems have been unable to maintain the best laboratory organisms under field conditions. They become contaminated and taken over by indigenous local organisms. This is the equivalent of weed problems in agriculture, where indigenous organisms compete with introduced ones. Transgenes conferring herbicide resistance might overcome this problem.
(b) *Growth and lipid content*: Most algae either grow *or* alternatively they produce lipid (fat) bodies, but not both simultaneously. This requires either batch culture or separate growing ponds and lipid producing ponds, rendering substantially higher production costs. The microbial and algal pathways and genes for production of the lipids best for biodiesel are becoming known [137]. One solution has been fermenter type photo-bioreactors for rapid cell division feeding into open ponds for oil production that begins upon nutrient depletion [138];
(c) *Carbon dioxide enrichment*: Carbon dioxide levels above 5% slow the growth of higher plants and animals, yet many algae and cyanobacteria grow happily when ''aerated'' with 100% carbon dioxide or directly with ca. 14% CO_2 flue gas from power generators [139,140]. Still, the response of algae to added carbon dioxide is not as good as it could be, and there is a place for improvement of carbon assimilation. Transforming higher plants with cyanobacterial fructose-1,6-bisphosphate aldolase (ALD) and triose phosphate

isomerase (TPI) enhances carbon fixation. The reverse was also effective, at least with a nitrogen-fixing cyanobacterium. Co-engineering rice cytosolic fructose-1,6-bisphosphate aldolase and a spinach chloroplast triosephosphate isomerase in cells of doubled these enzyme activities, as well as photosynthetic and biomass yields [141,142]. The effect was strongest when the cells were heavily enriched with carbon dioxide [142].

It is necessary to ascertain what other steps in the dark reactions might be rate limiting under high carbon dioxide enrichment. Conversely, most of the soluble protein in chloroplasts is RUBISCO, the low affinity protein that is in huge amounts necessary to scavenge rare carbon dioxide dissolved in water or present in air. If the algae or cyanobacteria are cultivated at very high carbon dioxide concentrations there may be an overabundance of this enzyme, at the expense of more-needed enzymes. If the RUBISCO levels in organisms are under self regulation, transgenically lowering RUBISCO levels to nearer the amounts needed may ''make room'' for other needed enzymes that are rate limiting.

(d) *Light penetration*: Ongoing molecular research in photosynthesis will have much to offer this area, as it has the potential to increase yield, while sequestering fossil fuel generated carbon dioxide. Inserting baffles in raceway ponds causes turbulence and mixing the efficiency of the pond went up with greater densities of algae, bringing cells in and out of light [143–145]. To achieve higher photobioreactor efficiency by using intense penetrating light, one group reasoned and demonstrated that rapidly circulating the algae would quickly put some in the shade of the others and thereby prevent photoinhibition [144]. With the cells they were using, they quickly reached a point where the rapid mixing was shearing the cells. The energy required for this rapid mixing of large volumes of viscous liquid is not inconsequential, greatly reducing the utility of this concept. Another approach to dealing with the problem of reactor depth and photoinhibitions that is central to one microalgae program has been to diffuse light throughout the depth of the culture, using optical fibers, thus avoiding high a surface irradiance. This approach is not practical at present for biodiesel production because of the very high cost of the system [135].

Light can penetrate deeper and the photoefficiency considerably decreased by lowering the number of chlorophyll molecules in the reaction center (=reduce antenna size), which can allow very great efficiency at high light intensities [146,147]. The culture of some algae under continuous high light ''adapts'' cells to this. This works well with continuous light in the laboratory, but not with solar ponds, as the cells ''de-adapt'' at night [146,147]. When cells can be rendered to retain this trait, it could further increase pond and photo-bioreactor efficiency. Attempts to do so were made by performing insertional mutatgenesis on the *tla1* (truncated light harvesting chlorophyll antenna) gene into cell wall-less *Chlamydomonas*. This gene is part of a signal pathway regulating antenna size [148]. Mutants were isolated with half the antenna size, i.e. with half the chlorophyll, resulting in greater solar energy conversion, with photosynthesis per unit chlorophyll increased over 80%, but photosynthesis per cell was 30% less [149]. The mutant cells could withstand full maximum noon sunlight (2500 μEin m^{-2} s^{-1}) whereas the wild type saturated at 1000 μEin m^{-2} s^{-1}. The gene pleiotropically controls other aspects of the photosynthetic mechanisms as well [149]. Engineering the wild type gene into mutant cells restored the large antenna [150].

(e) Seasonality: The high yields attained in ponds are usually seasonal. Algal growth is a function of temperature—when it is too cold they grow less, and most do not do well at high summer temperatures, requiring expensive cooling. Recent [151] and future research with plants will have much to offer to overcome this problem.

(f) Harvest: Harvesting by centrifugation is a cost-prohibitive engineering factor. Various chemical flocculation technologies have been tested. This problem too may be eventually solvable through biological means, introducing inducible transgenes that will cause flocculation or apoptosis.

(g) Biosafety of transgenics: One major advantage of generating transgenic algae/cyanobacteria is that the products become so domesticated that the organisms become totally unfit to exist in the wild. Thus, if there is an inadvertent spill, the transgenic organisms will quickly dissipate from the wild ecosystem.

5. Fourth generation technologies—producing biohydrogen and bioelectricity

Biophysicists have seen it as an intellectual and practical challenge to harvest solar energy for hydrogen or electricity [152–154] using nature's photosynthetic mechanisms, directly, or by embedding parts of the photosynthetic apparatus in artificial membranes, or using algae to produce sugars, and yeast or bacterial enzymes to produce electrochemical energy.

One method of producing hydrogen typically consisted of substrate cycling systems using anaerobic photosynthetic bacteria to capture the near infrared part of sunlight to produce hydrogen (while not emitting oxygen in photosynthesis) while consuming small organic acids, which they convert to carbohydrates.

Dark anaerobic fermentative bacteria consume carbohydrates, thus generating hydrogen and small organic acids. Recently, an integrated biological hydrogen generation system was achieved by co-cultivating a unicellular green alga whose photosynthesis is driven by the visible light, together with a purple photosynthetic bacterium adsorbing the near infrared light [155]. The system works only at low light intensities so far because of the emission of oxygen by the green alga used, which suppresses hydrogen production [155]. The newest approach is to antisense the *sulP* gene (encoding a chloroplast sulfate permease), as it would reduce sulfate availability to the chloroplast, downregulating the rate of oxygenic photosynthesis.

All approaches will necessitate considerable long-term multidisciplinary efforts to become more than a laboratory curiosity, but the informational gains about basic biophysical processes are bound to be exceedingly important.

6. Concluding remarks–predictions

Only second generation and beyond biofuels will make a real dent in the amount fossil of petroleum used. The biofuel crops will only be cost-effective in the long run if they are further domesticated transgenically to remove toxins and environmental contaminants, and to be more productive and have the right properties as fuels, as well as have residues that have value. Large-scale planting of many of these species before they are domesticated is of questionable value, unless the presently being planted material can serve as rootstocks on which newer and better transgenic varieties can be grafted. Unfortunately, it is unlikely that it will be possible to graft euphorbs such as castor bean and *Jatropha*, as they all have mature stems that are hollow. Crops that cannot be wholly used will lose even more value after transgenic algae and cyanobacteria come on line, as every bit of value must be derived from such crops. The plantations of perennial lignocellulosics and straws will then only be of value if they have been engineered to have lower/modified lignin, such that they can be used as fodder for ruminants.

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References

[1] B. Chinsinga, Reclaiming policy space: lessons from Malawi's fertiliser subsidy programme, briefing, future agricultures, available online: http://www.future-agricultures.org/WDR/Briefing_Malawi_fertiliser.pdf, Institute of Development Studies, Brighton, 2007.
[2] D.T. Jones, D.R. Woods, Acetone–butanol fermentation revisited, Microbiol. Rev. 50 (1986) 484–524.
[3] C.Y.M. dos Santos, D.D. Azevedo, F.R.D. Neto, Selected organic compounds from biomass burning found in the atmospheric particulate matter over sugarcane plantation areas, Atmos. Environ. 36 (2002) 3009–3019.
[4] L.G. Wu, R.G. Birch, Doubled sugar content in sugarcane plants modified to produce a sucrose isomer, Plant Biotech. J. 5 (2007) 109–117.
[5] B.A.R. Lina, D. Jonker, G. Kozianowski, Isomaltulose (Palatinose (R)): a review of biological and toxicological studies, Food Chem. Toxicol. 40 (2002) 1375–1381.
[6] J. Gressel, Genetic Glass Ceilings: Transgenics for Crop Biodiversity, Johns Hopkins University Press, Baltimore, 2008.
[7] D.B. Harper, J.T.G. Hamilton, The global cycles of naturally occurring monohalomethanes, in: G. Gribble (Ed.), Natural Production of Organohalogen Compounds, vol. l, Springer, Berlin, 2003, pp. 17–41.
[8] H.S. Saini, J.M. Attieh, A.D. Hanson, Biosynthesis of halomethanes and methanethiol by higher-plants via a novel methyltransferase reaction, Plant Cell Environ. 18 (1995) 1027–1033.
[9] J. Gan, S.R. Yates, H.D. Ohr, J.J. Sims, Production of methyl bromide by terrestrial higher plants, Geophys. Res. Lett. 25 (1998) 3595–3598.
[10] FAOStat, FAO statistical database, http://faostat.fao.org/site/340/DesktopDefault.aspx?PageID=340, 2007.
[11] J.M. Attieh, A.D. Hanson, H.S. Saini, Purification and characterization of a novel methyltransferase responsible for biosynthesis of halomethanes and methanethiol in *Brassica oleracea*, J. Biol. Chem. 270 (1995) 9250–9257.
[12] J.M. Attieh, et al., Cloning and functional expression of two plant thiolmethyltransferases; a new class of enzymes involved in the biosynthesis of sulfur volatiles, Plant Mol. Biol. 50 (2002) 511–521.
[13] R.C. Rhew, L. Ostergaard, E.S. Saltzman, M.F. Yanofsky, Genetic control of methyl halide production in *Arabidopsis*, Curr. Biol. 13 (2003) 1809–1813.
[14] C. Mooney, The republican war on science, Basic Books, New York, 2005, 342 pp.
[15] Y. Chisti, Biodiesel from microalgae, Biotech. Adv. 25 (2007) 294–306.
[16] D. Fairless, Biofuel: the little shrub that could—maybe, Nature 449 (2007) 652–655.
[17] A.K. Tiwari, A. Kumar, H. Raheman, Biodiesel production from jatropha (*Jatropha curcas*) with high free fatty acids: an optimized process, Biomass Bioenergy 31 (2007) 569–575.
[18] INCHEM, *Jatropha curcas* L., Intl. Programme Chem. Safety, http://www.inchem.org/documents/pims/plant/jcurc.htm, 1994.
[19] M. Hirota, et al., A new tumor promoter from the seed oil of *Jatropha curcas* L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol, Cancer Res. 48 (1988) 5800–5804.
[20] W. Haas, M. Mittelbach, Detoxification experiments with the seed oil from *Jatropha curcas* L., Ind. Crops Prod. 12 (2000) 111–118.
[21] W.M.J. Achten, et al., *Jatropha* biodiesel fueling sustainability? Biofuels Bioprod. Biorefin. 1 (2007) 283–291.
[22] G.M. Gübitz, M. Mittelbach, M. Trabi, Exploitation of the tropical oil seed plant *Jatropha curcas* L., Bioresource Technol. 67 (1999) 73–82.
[23] P.H. Joubert, J.M.M. Brown, I.T. Hay, P.D.B. Sebata, Acute poisoning with *Jatropha curcas* (purging nut tree) in children, S. Afr. Med. J. 65 (1984) 729–730.
[24] G. Francis, R. Edinger, K. Becker, A concept for simultaneous wasteland reclamation, fuel production, and socio-economic development in degraded areas in India: need, potential and perspectives of *Jatropha* plantations, Natural Resource Forum 29 (2005) 12–24.
[25] H.P.S. Makkar, A.O. Aderibigbe, K. Becker, Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors, Food Chem. 62 (1998) 207–215.
[26] M. Sujatha, H.P.S. Makkar, K. Becker, Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L., Plant Growth Reg. 47 (2005) 83–90.
[27] S. Rajore, A. Batra, Efficient plant regeneration via shoot tip explant in *Jatropha curcas* L., J. Plant Biochem. Biotechnol. 14 (2005) 73–75.
[28] M. Sujatha, M. Sailaja, Stable genetic transformation of castor (*Ricinus communis* L.) via *Agrobacterium tumefaciens*-mediated gene transfer using embryo axes from mature seeds, Plant Cell Rep. 23 (2005) 803–810.
[29] M.R. Li, H.Q. Li, G.J. Wu, Study on factors influencing *Agrobacterium*-mediated transformation of *Jatropha curcas* (in Chinese), J. Mol. Cell Biol. 39 (2006) 83–89.
[30] E.L. Armstrong, H.I. Nicol, Reducing the height in rapeseed with growth regulators, Aust. J. Exp. Agric. 31 (1991) 245–250.
[31] G. Niu, R. Heins, W. Carlson, Using paclobutrazol to control height of poinsettia 'Freedom', Horttechnology 12 (2002) 232–236.
[32] S. Yamaguchi, T.P. Sun, H. Kawaide, Y. Kamiya, The GA2 locus of *Arabidopsis thaliana* encodes ent-kaurene synthase of gibberellin, Plant Physiol. 116 (1998) 1271–1278.
[33] C.A. Helliwell, et al., Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 9019–9024.
[34] J. Peng, et al., 'Green revolution' genes encode mutant gibberellin response modulators, Nature 400 (1999) 256–261.
[35] R. Azpiroz, Y. Wu, J.C. LoCascio, K.A. Feldmann, An *Arabidopsis* brassinosteroid-dependent mutant is blocked in cell elongation, Plant Cell 10 (1998) 219–230.

[36] T. Noguchi, et al., Brassinosteroid-insensitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids, Plant Physiol. 121 (1999) 743–752.
[37] H. Schaller, P. Bouvier-Navé, P. Benveniste, Overexpression of an *Arabidopsis* cDNA encoding a sterol-C24-methyltransferase in tobacco modifies the ratio of 24-methyl cholesterol to sitosterol and is associated with growth reduction, Plant Physiol. 18 (1998) 461–469.
[38] P.F. Devlin, S.R. Patel, G.C. Whitelam, Phytochrome, E influences internode elongation and flowering time in *Arabidopsis*, Plant Cell 10 (1998) 1479–1487.
[39] P.R.H. Robson, A.C. McCormac, A.S. Irvine, H. Smith, Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene, Nat. Biotechnol. 14 (1996) 995–998.
[40] V. Gaudin, T. Vrain, L. Jouanin, Bacterial genes modifying hormonal balances in plants, Plant Physiol. Biochem. 32 (1994) 11–29.
[41] S. Kusaba, et al., Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene *OSH*1, Plant Physiol. 116 (1998) 471–476.
[42] S.J. Liljegren, et al., *SHATTERPROOF MADS*-box genes control seed dispersal in *Arabidopsis*, Nature 404 (2000) 766–770.
[43] T. Esumi, R. Tao, K. Yonemori, Isolation of *LEAFY* and *TERMINAL FLOWER* 1 homologues from six fruit tree species in the subfamily Maloideae of the Rosaceae, Sex. Plant Reprod. 17 (2005) 277–287.
[44] W. Li, B.S. Gill, Multiple genetic pathways for seed shattering in the grasses, Funct. Integr. Genom. 6 (2006) 300–309.
[45] Y. Bental, Quantification of ethephon requirements for abscission in olive fruits, Plant Growth Reg. 11 (1992) 397–403.
[46] J. Lin, et al., Cloning and expression of curcin, a ribosome-inactivating protein from the seeds of *Jatropha curcas*, Acta Bot. Sin. 45 (2003) 858–863.
[47] T. Zhu, K. Mettenburg, D.J. Peterson, L. Tagliani, C.L. Baszczynski, Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides, Nat. Biotechnol. 18 (2000) 555–558.
[48] K.M. Jakob, D. Atsmon, Sex inheritance in *Ricinus communis* L.—evidence for a genetic change during ontogeny of female sex reversals, Genetica 36 (1965), 251-259.
[49] D. Atsmon, Castor, in: G. Roebbelen, R.K. Downey, A. Ashri (Eds.), Oil crops of the world, vol. 1, McGraw-Hill, New York, 1989, pp. 438–447.
[50] J.J. Garcia-Gonzalez, et al., Pollinosis to *Ricinus communis* (castor bean): an aerobiological, clinical and immunochemical study, Clin. Exp. Allergy 29 (1999) 1265–1275.
[51] S. Parui, A.K. Mondal, S. Mandal, Identification and partial characterization of the allergenic proteins of *Ricinus communis* L. pollen—a new approach, Grana 38 (1999) 311–315.
[52] A.B. Singh, P. Kumar, Aeroallergens in clinical practice of allergy in India. An overview, Ann. Agric. Environ. Med. 10 (2003) 131–136.
[53] T. Palosuo, et al., Allergen cross-reactivity between proteins of the latex from *Hevea brasiliensis*, seeds and pollen of *Ricinus communis*, and pollen of *Mercurialis annua*, members of the Euphorbiaceae family, Allergy Asthma Proc. 23 (2002) 141–147.
[54] M.R. Aslani, et al., Castor bean (*Ricinus communis*) toxicosis in a sheep flock, Toxicon 49 (2007) 400–406.
[55] INCHEM, *Ricinus communis* L., Intl. Programme Chem. Safety, http://www.inchem.org/documents/pims/plant/ricinus.htm, 1990.
[56] J. Kopferschmitt, et al., Acute voluntary intoxication by ricin, Hum. Toxicol. 2 (1983) 239–242.
[57] G.P. Wedin, S.N. Jeffrey, G.W. Everson, E.P. Krenzelok, Castor bean poisoning, Am. J. Emerg. Med. 4 (1986) 259–261.
[58] J. Audi, M. Belson, M. Patel, J. Schier, J. Osterloh, Ricin poisoning—a comprehensive review, JAMA-J. Am. Med. Assoc. 294 (2005) 2342–2351.
[59] G.D. Griffiths, G.J. Phillips, J. Holley, Inhalation toxicology of ricin preparations: Animal models, prophylactic and therapeutic approaches to protection, Inhal. Toxicol. 19 (2007) 873–887.
[60] D.L. Auld, et al., Registration of TTU-LRC castor germplasm with reduced levels of ricin and RCA(120), Crop Sci. 43 (2003) 746–747.
[61] E. Boroda, et al., Enzyme-Linked ImmunoSorbent Assay measurement of castor toxin in soils, Comm. Soil Sci. Plant Anal. 35 (2004) 1185–1195.
[62] J.W. Tregear, L.M. Roberts, The lectin gene family of *Ricinus communis*: cloning of a functional ricin gene and three lectin pseudogenes, Plant Mol. Biol. 18 (1992) 515–525.
[63] B. Malathi, S. Ramesh, K.V. Rao, V.D. Reddy, *Agrobacterium*-mediated genetic transformation and production of semilooper resistant transgenic castor (*Ricinus communis* L.), Euphytica 147 (2006) 441–449.
[64] T.A. McKeon, G.Q. Chen, Transformation of *Ricinus communis*, the castor plant, US Patent 6,620,986 (2003).
[65] G.A. Burdock, I.G. Carabin, J.C. Griffiths, Toxicology and pharmacology of sodium ricinoleate, Food Chem. Toxicol. 44 (2006) 1689–1698.
[66] F.J. van de Loo, P.T. Broun, S.C. Somerville, An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 6743–6747.
[67] M. Baldanzi, M. Fambrini, C. Pugliesi, Redesign of the castorbean plant body plan for optimal combine harvesting, Ann. Appl. Biol. 142 (2003) 299–306.
[68] M.M. Azam, A. Waris, N.M. Nahar, Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India, Biomass Bioenergy 29 (2005) 293–302.
[69] P. Mahanta, S.C. Mishra, Y.S. Kushwah, An experimental study of *Pongamia pinnata* L. oil as a diesel substitute, Proc. Inst. Mech. Eng. Part A-J. Power Energy 220 (2006) 803–808.
[70] H. Raheman, A.G. Phadatare, Diesel engine emissions and performance from blends of karanja methyl ester and diesel, Biomass Bioenergy 27 (2004) 393–397.
[71] P. Singh, et al., Effect of long term feeding of expeller pressed and solvent extracted karanj (*Pongamia pinnata*) seed cake on the performance of lambs, Animal Feed Sci. Tech. 126 (2006) 157–167.
[72] A.K. Panda, V.R.B. Sastry, A. Kumar, S.K. Saha, Quantification of karanjin, tannin and trypsin inhibitors in raw and detoxified expeller and solvent extracted Karanj (*Pongamia glabra*) cake, Asian–Australas. J. Anim. Sci. 19 (2006) 1776–1783.
[73] P.K. Sahoo, L.M. Das, M.K.G. Babu, S.N. Naik, Biodiesel development from high acid value polanga seed oil and performance evaluation in a CI engine, Fuel 86 (2007) 448–454.
[74] M.C. Yimdjo, et al., Antimicrobial and cytotoxic agents from *Calophyllum inophyllum*, Phytochemistry 65 (2004) 2789–2796.
[75] S. Joshi, S. Joshi, Simarouba Paradise Tree, University of Agricultural Sciences, Bangalore, 2007, 36 pp..
[76] DOE, Breaking the biological barriers to cellulosic ethanol: a joint research agenda DOE/SC-0095, available online at www.genomicstolife.org.biofuels, US Department of Energy, Washington, DC, 2006.
[77] A.E. Farrell, et al., Ethanol can contribute to energy and environment goals, Science 311 (2006) 506–508.
[78] J. Gressel, Y. Vered, S. Bar-Lev, O. Milstein, H.M. Flowers, Partial suppression of cellulase action by artificial lignification of cellulose, Plant Sci. Lett. 32 (1983) 349–353.
[79] Iogen, Cellulose ethanol brochure, http://www.iogen.ca/cellulose_ethanol/what_is_ethanol/cellulose_ethanol.pdf, 2006.
[80] R. Samson, et al., The potential of C_4 perennial grasses for developing global BIOHEAT industry, Crit. Rev. Plant Sci. 24 (2005) 461–495.
[81] E. Heaton, T. Voigt, S.P. Long, A quantitative review comparing the yields of two candidate C-4 perennial biomass crops in relation to nitrogen, temperature and water, Biomass Bioenergy 27 (2004) 21–30.
[82] C.E. Wyman, D.D. Spindler, K. Grohmann, Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol, Biomass Bioenergy 3 (1992) 301–307.
[83] Y.C. Chung, A. Bakalinsky, M.H. Penner, Enzymatic saccharification and fermentation of xylose-optimized dilute acid-treated lignocellulosics, Appl. Biochem. Biotechnol. 121 (2005) 947–961.
[84] H. Alizadeh, F. Teymouri, T.I. Gilbert, B.E. Dale, Pretreatment of switchgrass by ammonia fiber explosion (AFEX), Appl. Biochem. Biotechnol. 121 (2005) 1133–1141.
[85] M.R. Schmer, et al., Establishment stand thresholds for switchgrass grown as a bioenergy crop, Crop Sci. 46 (2006) 157–161.
[86] D.K. Lee, A. Boe, Biomass production of switchgrass in central South Dakota, Crop Sci. 45 (2005) 2583–2590.

[87] R.A. Brown, N.J. Rosenberg, C.J. Hays, W.E. Easterling, L.O. Mearns, Potential production and environmental effects of switchgrass and traditional crops under current and greenhouse-altered climate in the central United States: a simulation study, Agric. Ecosyst. Environ. 78 (2000) 31–47.
[88] J.P. Muir, M.A. Sanderson, W.R. Ocumpaugh, R.M. Jones, R.L. Reed, Biomass production of 'Alamo' switchgrass in response to nitrogen, phosphorus, and row spacing, Agron. J. 93 (2001) 896–901.
[89] L.G. Blevins, T.H. Cauley, Fine particulate formation during switchgrass/coal cofiring, J. Eng. Gas Turbines Power-Trans. ASME 127 (2005) 457–463.
[90] P.G. Jefferson, W.P. McCaughey, K. May, J. Woosaree, L. McFarlane, Potential utilization of native prairie grasses from western Canada as ethanol feedstock, Can. J. Plant Sci. 84 (2004) 1067–1075.
[91] Y.C. Liang, et al., Importance of plant species and external silicon concentration to active silicon uptake and transport, New Phytol. 172 (2006) 63–72.
[92] P.J. Van Soest, Rice straw, the role of silica and treatments to improve quality, Anim. Feed Sci. Technol. 130 (2006) 137–171.
[93] C.S. Park, The micronutrient problem in Korean agriculture, in: Proceedings of the Symposium Commemorating 30th Anniversary of Korean Liberation, Natl. Acad. Sci., Seoul, 1975, pp. 847–862.
[94] J.V. Cotterill, R.W. Watkins, C.B. Brennon, D.P. Cowan, Boosting silica levels in wheat leaves reduces grazing by rabbits, Pest Manage. Sci. 63 (2007) 247–253.
[95] O.L. Kvedaras, M.G. Keeping, Silicon impedes stalk penetration by the borer *Eldana saccharina* in sugarcane, Entomol. Exp. Appl. 125 (2007) 103–110.
[96] S. Ranganathan, et al., Effects of silicon sources on its deposition, chlorophyll content, and disease and pest resistance in rice, Biol. Plantarum 50 (2006) 713–716.
[97] J.F. Ma, et al., A silicon transporter in rice, Nature 440 (2006) 688–691.
[98] D.W. Rains, E. Epstein, R.J. Zasoski, M. Aslam, Active silicon uptake by wheat, Plant Soil 280 (2006) 223–228.
[99] E.E. Hood, et al., Subcellular targetting is a key condition for high level accumulation of cellulase protein in transgenic maize seed, Plant Biotechnol. J. 5 (2007) 709–719.
[100] J. Miron, et al., Yield, composition and in vitro digestibility of new forage sorghum varieties and their ensilage characteristics, Anim. Feed Sci. Technol. 120 (2005) 17–32.
[101] Y. Barriere, et al., Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from brown-midrib mutants, Comptes Rendus Biol. 327 (2004) 847–860.
[102] A.M. Missaoui, A.H. Paterson, J.H. Bouton, Investigation of genomic organization in switchgrass (*Panicum virgatum* L.) using DNA markers, Theor. Appl. Gen. 110 (2005) 1372–1383.
[103] E. Heaton, T. Voigt, S.P. Long, A quantitative review comparing the yields of two candidate C_4 perennial biomass crops in relation to nitrogen, temperature and water, Biomass Bioenergy 27 (2004) 21–30.
[104] S.B. McLaughlin, L.A. Kszos, Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States, Biomass Bioenergy 28 (2005) 515–535.
[105] K.P. Vogel, H.J.G. Jung, Genetic modification of herbaceous plants for feed and fuel, Crit. Rev. Plant Sc. 20 (2001) 15–49.
[106] J. Gressel, A. Zilberstein, Let them eat (GM) straw, Trends Biotechnol. 21 (2003) 525–530.
[107] N. Morohoshi, S. Kajita, Formation of a tree having a low lignin content, J. Plant Res. 114 (2001) 517–523.
[108] A.M. Anterola, N.G. Lewis, Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity, Phytochemistry 61 (2002) 221–294.
[109] Gramene, http://www.gramene.org, 2007.
[110] C. Halpin, Investigating and manipulating lignin biosynthesis in the postgenomic era, Adv. Bot. Res. 41 (2004) 63–106.
[111] T.B.T. Lam, K. Iiyama, B.A. Stone, Lignin and hydroxycinnamic acids in walls of brown midrib mutants of sorghum, pearl millet, and maize stems, J. Sci. Food Agric. 71 (1996) 174–178.
[112] C.-J. Tsai, et al., Suppression of *O*-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brown wood phenotypes, Plant Physiol. 117 (1998) 101–112.
[113] J.J. MacKay, et al., Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase, Proc. Natl. Acad. Sci. U.S.A. 94 (1998) 8255–8260.
[114] C.M. Tobias, E.K. Chow, Structure of the cinnamyl alcohol dehydrogenase gene family in rice and promoter activity of a member associated with lignification, Planta 220 (2005) 678–688.
[115] W.-J. Hu, et al., Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees, Nat. Biotechnol. 17 (1999) 808–812.
[116] J.F. Pedersen, K.P. Vogel, D.L. Funnell, Impact of reduced lignin on plant fitness, Crop. Sci. 45 (2005) 812–819.
[117] A.J. Travis, S.D. Murison, D.J. Hirst, K.C. Walker, A.C. Chesson, Comparison of the anatomy and degradability of straw from varieties of wheat and barley that differ in susceptibility to lodging, J. Agric. Sci. 127 (1996) 1–10.
[118] A. Sterling, C.J. Baker, P.M. Berry, A. Wade, An experimental investigation of the lodging of wheat, Agric. Forest Meteor. 119 (2003) 149–165.
[119] T. Farquhar, J. Zhou, W.H. Wood, Competing effects of buckling and anchorage strength on optimal wheat stalk geometry, J. Biomech. Eng.-Trans. ASME 124 (2002) 441–449.
[120] J. Ralph, S. Guillaumie, J.H. Grabber, C. Lapierre, Y. Barriere, Genetic and molecular basis of grass cell-wall biosynthesis and degradability. III. Towards a forage grass ideotype, Comptes Rendus Biol. 327 (2004) 467–479.
[121] J.D. Oleson, Y.L. Park, T.M. Nowatzki, J.J. Tollefson, Node-injury scale to evaluate root injury by corn rootworms (Coleoptera: Chrysomelidae), J. Econ. Entomol. 98 (2005) 1–8.
[122] V. Mechin, et al., In search of a maize ideotype for cell wall enzymatic degradability using histological and biochemical lignin characterization, J. Agric. Food Chem. 53 (2005) 5872–5881.
[123] H.J.G. Jung, K.P. Vogel, Lignification of switchgrass (*Panicum virgatum*) and big bluestem (*Andropogon gerardii*) plant-parts during maturation and its effect on fiber degradability, J. Sci. Food Agric. 59 (1992) 169–176.
[124] S.C. Tripathi, K.D. Sayre, J.N. Kaul, Fibre analysis of wheat genotypes and its association with lodging: effects of nitrogen levels and ethephon, Cereal Res. Commun. 31 (2003) 429–436.
[125] Z. Shani, M. Dekel, T. Tzbary, R. Goren, O. Shoseyov, Growth enhancement of transgenic poplar plants by overexpression of *Arabidopsis thaliana* endo-1,4-beta-glucanase (*cel1*), Mol. Breeding 14 (2004) 321–330.
[126] Y. Ohmiya, et al., The role of PopCel1 and PopCel2 in poplar leaf growth and cellulose biosynthesis, Plant J. 33 (2003) 1087–1097.
[127] Z. Shani, et al., Cellulose binding domain, increases cellulose synthase activity in *Acetobacter xylinum*, and biomass of transgenic plants, in: A. Altman, M. Ziv, S. Izhar (Eds.), Plant Biotechnology and In Vitro Biology in the 21st Century, vol. l, Kluwer, Dordrecht, 1999, pp. 213–218.
[128] Y.H. Xie, M.Z. Wen, D. Yu, Y.K. Li, Growth and resource allocation of water hyacinth as affected by gradually increasing nutrient concentrations, Aquat. Bot. 79 (2004) 257–266.
[129] Y. Kim, D.L. Giokas, J.W. Lee, P.A. Paraskevas, Potential of natural treatment systems for the reclamation of domestic sewage in irrigated agriculture, Desalination 189 (2006) 229–242.
[130] S.M. Mathur, P. Singh, A cylindrical chopper with crusher for water hyacinth volume and biomass reduction, J. Aquat Plant Manage. 42 (2004) 95–99.
[131] C.C. Gunnarsson, C.M. Petersen, Water hyacinths as a resource in agriculture and energy production: a literature review, Waste Manage. 27 (2007) 117–129.
[132] M. Martinez Jimenez, M.A. Gomez Balandra, Integrated control of *Eichhornia crassipes* by using insects and plant pathogens in Mexico, Crop Protect. 26 (2007) 1234–1238.
[133] B.S. Ripley, E. Muller, M. Behenna, G.M. Whittington-Jones, M.P. Hill, Biomass and photosynthetic productivity of water hyacinth (*Eichhornia*

crassipes) as affected by nutrient supply and mirid (*Eccritotarus catarinensis*) biocontrol, Biol. Control 39 (2006) 392–400.
[134] J.R. Wilson, N. Holst, M. Rees, Determinants and patterns of population growth in water hyacinth, Aquat. Bot. 81 (2005) 51–67.
[135] J. Sheehan, T. Dunahay, J. Benemann, P. Roessler, A Look Back at the U.S. Department of Energy's Aquatic Species Program—Biodiesel from Algae. Prepared for the U.S. Department of Energy's Office of Fuels Development, National Renewable Energy Laboratory, Golden, Colorado, 1998.
[136] D. Schneider, Grow your own? Would the widespread adoption of biomass-derived transportation fuels really help the environment?, Am. Sci. 94 (2006) 408–409.
[137] N. Ladygina, E.G. Dedyukhina, M.B. Vainshtein, A review on microbial synthesis of hydrocarbons, Proc. Biochem. 41 (2006) 1001–1014.
[138] M.E. Huntley, D.G. Redalje, Carbon dioxide mitigation and renewable oil from photosynthetic microbes: A new appraisal, Mitigation Adapt. Strateg. Global Change 12 (2007) 573–608.
[139] M. Murakami, M. Ikenouchi, The biological CO_2 fixation and utilization project by RITE (2)—screening and breeding microalgae with high capability in fixing CO_2, Energy Conserv. Manage. 38 (1997) S493–S497.
[140] M. Negoro, et al., Carbon dioxide fixation by microalgae photosynthesis using actual flue gas discharged from a boiler, Appl. Biochem. Biotechnol. 39–40 (1993) 643–653.
[141] W.M. Ma, L.Z. Wei, Q.X. Wang, D.J. Shi, H.B. Chen, Increased activity of the non-regulated enzymes fructose-1,6-bisphosphate aldolase and triosephosphate isomerase in *Anabaena* sp strain PCC 7120 increases photosynthetic yield, J. Appl. Phycol. 19 (2007) 207–213.
[142] R.J. Kang, et al., Effects of co-expression of two higher plants genes ALD and TPI in *Anabaena* sp. PCC7120 on photosynthetic CO_2 fixation, Enzym. Microb. Technol. 36 (2005) 600–604.
[143] L.P. Raymond, Mass algal culture system, US Patent 4,253,271 (1981).
[144] A.B.P. Leonard, M.E. Huntley, P.P. Niiler, D. Redalje, Method of control of *Haematococcus* spp. growth process, US Patent 5,882,849 (1999).
[145] K.L. Terry, Photosynthesis in modulated light: quantitative dependence of photosynthetic enhancement on flashing rate, Biotechnol. Bioeng. 28 (1986) 988–995.
[146] A. Melis, J. Neidhardt, J.R. Benemann, *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells, J. Appl. Phycol. 10 (1998) 515–525.
[147] J. Neidhardt, J.R. Benemann, L.P. Zhang, A. Melis, Photosystem-II repair and chloroplast recovery from irradiance stress: relationship between chronic photoinhibition, light-harvesting chlorophyll antenna size and photosynthetic productivity in *Dunaliella salina* (green algae), Photosyn. Res. 56 (1998) 175–184.
[148] T. Masuda, A. Tanaka, A. Melis, Chlorophyll antenna size adjustments by irradiance in *Dunaliella salina* involve coordinate regulation of chlorophyll a oxygenase (*CAO*) and *Lhcb* gene expression, Plant Mol. Biol. 51 (2003) 757–771.
[149] J.E.W. Polle, S.D. Kanakagiri, A. Melis, tla1, a DNA insertional transformant of the green alga *Chlamydomonas reinhardtii* with a truncated light-harvesting chlorophyll antenna size, Planta 217 (2003) 49–59.
[150] S.D. Tetali, M. Mitra, A. Melis, Development of the light-harvesting chlorophyll antenna in the green alga *Chlamydomonas reinhardtii* is regulated by the novel *Tla*1 gene, Planta 225 (2007) 813–829.
[151] O. Shlyk-Kerner, et al., Protein flexibility acclimatizes photosynthetic energy conversion to the ambient temperature, Nature 442 (2006) 827–830.
[152] S. Tsujimura, A. Wadano, K. Kano, T. Ikeda, Photosynthetic bioelectrochemical cell utilizing cyanobacteria and water-generating oxidase, Enzyme Microb. Technol. 29 (2001) 225–231.
[153] B.E. Logan, J.M. Regan, Electricity-producing bacterial communities in microbial fuel cells, Trends Microbiol. 14 (2006) 512–518.
[154] M. Chiao, K.B. Lam, L.W. Lin, Micromachined microbial and photosynthetic fuel cells, J. Micromech. Microeng. 16 (2006) 2547–2553.
[155] A. Melis, M.R. Melnicki, Integrated biological hydrogen production, Intl. J. Hydrogen Energy 31 (2006) 1563–1573.